CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Department of Forest Genetics and Physiology

Molecular Underpinnings of Bark Beetle Resistance against Conifer Chemical Defences

A dissertation thesis submitted for the degree of Doctor of Philosophy

October 2024

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Field of Study: Forest Biology

Supervisor: Amit Roy, Ph.D.

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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Ph.D. THESIS ASSIGNMENT

Aisha Naseer

Forestry Engineering
Forest Biology

Thesis title

Molecular Underpinnings of Bark Beetle Resistance against Conifer Chemical Defences.

Objectives of thesis

To investigate the molecular mechanisms of bark beetle (Ips typographus) resistance to host chemical defenses by identifying key genes involved in detoxifying or adapting to host metabolites. This study aims to enhance the understanding of host-beetle interactions and contribute to pest management strategies.

Specific objectives:

1- To identify the detoxification machinery genes in Ips typographus and elucidate their roles in overcoming host plant toxins during feeding.

2- To identify the key genes in Ips typographus that are responsible for the breakdown or modification (detoxification) of specific allelochemicals present in host plants.

Methodology

Bark beetles will be collected from the infested spruce logs at Kostelec nad Černými lesy, specifically from a research plot established by School Forest Enterprise. A new generation of beetles will be reared on fresh spruce logs in a controlled rearing chamber to maintain a continuous and homogeneous culture. The beetles will then be exposed to natural host wood or treated with host allelochemicals through fumigation bioassays. The treated beetles will subsequently be utilized in the follow-up pipelines.

i) testing and evaluating the effect of individual allelochemical toxicity

ii) expression analysis of key genes involved in detoxification extracting total RNA for transcriptome profiling (RNA-seq) and quantitative gene expression (RT-qPCR).

1) Fumigation Bioassay

Bioassay will be performed using monoterpene applied to beetles at different concentrations to calculate the Lethal Concentration (LC) following the protocols of Chui et al., 2017. Briefly, A 1.5 cm × 1.5 cm piece of Whatman filter paper will be placed in a 20 mL scintillation vial, onto which defined volumes of undiluted monoterpenes will be applied, a moist filter paper will be placed for humidity, a single beetle will be placed into the vial and vial will be sealed. Mortality will be recorded for 72 hours with a 12-hour interval. Lethal concentration will be calculated using probit analysis.

2) Identification of detoxification genes

RNA-seq protocol- the whole body will be homogenized in liq. Nitrogen and total RNA will be extracted using TRIzol reagent or PureLink™ RNA Kit from Ambion (Invitrogen, Carlsbad, CA, USA) followed by purification DNase I (TURBO DNase Kit, Ambion, Austin, TX, USA). One according to the manufacturer's protocol. The integrity of the RNA will be verified, and the quantity of RNA will be determined. Total RNA will be outsourced for sequencing on the Illumina HiSeq4000 Genome Analyser platform using paired-end read technology. Quality control measures, including the filtering of high-quality reads based on the quality score given in fastq files, removal of reads containing primers/ adaptor sequences, and trimming of read length, will be carried out using CLC Genomics Workbench v10.1 (http://www.clcbio.com). The reads will be then mapped back to the Ips typographus genome for differential gene expression analysis.

RT-qPCR validation- RNA-seq results will be validated using RT-qPCR for the identified detoxification genes. One µg of RNA will be used to synthesise cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Waltham, MA, USA). Primers will be designed using IDT PrimerQuest software (IDT, Leuven, Belgium). Primers will be tested by PCR followed by gel electrophoresis for correct product amplification. RT-qPCR will be performed using SYBR® Green PCRMaster Mix (Applied Biosystems, Waltham, MA, USA) following the existing lab protocol (Roy et al., 2017). Melt curves would be generated to ensure single-product amplification. The target gene expression levels will be calculated using the $2-\Delta\Delta CT$ method with a ribosomal protein L7 as a reference gene.

The proposed extent of the thesis

30 SP/100 SP

Keywords

Ips typographus, detoxification, RNA seq, Norway spruce, monoterpenes, bark beetles

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Recommended information sources

1. Celorio-Mancera, Maria de la Paz, et al. "Transcriptional responses underlying the hormetic and detrimental effects of the plant secondary metabolite gossypol on the generalist herbivore Helicoverpa armigera." BMC Genomics 12 (2011): 1-16
2. Lindgren, B. S., and K. F. Raffa. "Evolution of tree-killing in bark beetles (Coleoptera: Curculionidae): trade-offs between the maddening crowds and a sticky situation." The Canadian Entomologist 145.5 (2013): 471-495
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7. Biedermann, Peter HW, et al. "Bark beetle population dynamics in the Anthropocene: challenges and solutions." Trends in ecology & evolution 34.10 (2019): 914-924

Expected date

2024/25 WS – FFWS – Doctoral Thesis Defense

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Electronic approval: 07. 06. 2023

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Electronic approval: 08. 10. 2024

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Dean

Prague on 08. 10. 2024

Declaration

I declare that the Ph.D. thesis “Molecular Underpinnings of Bark Beetle Resistance against Conifer Chemical Defences” was developed independently under the guidance of my supervisor. Additionally, I declare that all the sources of information used in this work were cited accordingly. I agree with publishing this Ph.D. thesis according to the Act no. 111/1998 Coll., in the universities in its current valid wording. This agreement is independent from the results of defence.

October 15th, 2024, Prague

Aisha Naseer

Acknowledgment

I would like to express my gratitude to my supervisor, Dr. Amit Roy, for his guidance, the postdocs Dr. Gothandapani Sellamuthu and Dr. Kanakachari Mogilicherla for their insightful discussions. I am grateful to my office mates Vivek, Rajarajan, Alex, Souleymane, and Shan for keeping the bar for scientific discussions and fun very high and the many moments of laughter that brightened me even on the darkest days. Thanks to the administrative staff, Ms. Hana Ayad and Ms. Andrea Lepiešová, for their constant availability and readiness to help in any issue whatsoever.

My heartfelt thanks to my family, whose unwavering love, encouragement, and patience gave me the strength to keep pushing forward. And I truly apologise to them for not being able to be there when they needed me the most. To my friends, both near and far, your emotional support and understanding have meant the world to me. And, my cat, Goldie, for giving me trouble that kept me distracted away from many stresses.

I would also like to acknowledge the generous financial support from the Faculty of Forestry and Wood Sciences, Internal Grand Agency (IGA FLD), Excellent Team Grant-FLD, project EXTEMIT-K, and the ERASMUS+ program, which made this research and my survival in Prague possible. I am thankful to my collaborators for their valuable contributions and to my host, Dr. Fredrik Levander for providing me with a welcoming and inspiring environment to carry out my research.

To my guide and mentors, Dr. Arun Salic Moses and Dr Sanjay Mishra from Ewing Christian College, India, Dr. Jan Bílý from CZU, and Dr. Shuvolina Mukherjee from Lund University, thank you for seeing the potential and making me believe that I am worth something.

And, to the city of Prague, which broke me and built me again, gave me my darkest and some of the brightest days, I will always have a love-hate relationship with you, and you'll forever be a home no matter where I go next.

Lastly, I wish I could tell my 15-year-old self, that the world is beautiful, and no dream is too big.

All thanks to Allah for never giving me up and walking by my side all the way and always.

Dedication

To the two most kind and strong women I've known, my Grandma and my Mother.

”إِنَّ اللّهَ مَعَ الصَّابِرِينَ“

“Inn Allah M'a As-Saabireen”

“Indeed, The God (Allah) is with those who have patience”.

-The Holy Quran (2:153)

Table of Content

List of Original Publications

Publications included in the thesis:

1. Singh, V.V., **Naseer, A.,** Mogilicherla, K., Trubin, A., Zabihi, K., Roy, A., Jakuš, R. and Erbilgin, N., 2024a. Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management. Reviews in Environmental Science and Bio/Technology, pp.1-34. https://doi.org/10.1007/s11157-024-09692-5

2. **Naseer, A**., Mogilicherla, K., Sellamuthu, G. and Roy, A., 2023. Age matters: Life-stage, tissue, and sex-specific gene expression dynamics in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae). Frontiers in Forests and Global Change, 6, p.1124754. https://doi.org/10.3389/ffgc.2023.1124754

3. **Naseer, A.**, Singh, V.V., Sellamuthu, G., Synek, J., Mogilicherla, K., Kokoska, L. and Roy, A., 2024. Insights into the detoxification of spruce monoterpenes by the Eurasian spruce bark beetle. International Journal of Molecular Sciences, 25(18), p.10209. https://doi.org/10.3390/ijms251810209

4. Sellamuthu, G., **Naseer, A.**, Hradecký, J., Chakraborty, A., Synek, J., Modlinger, R. and Roy, A., 2024. Gene expression plasticity facilitates different host feeding in *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae). Insect Biochemistry and Molecular Biology, 165, p.104061. https://doi.org/10.1016/j.ibmb.2023.104061

Publications not included in the thesis:

1. Singh, V.V., **Naseer, A.,** Sellamuthu, G. and Jakuš, R., 2024b. An optimized and cost-effective RNA extraction method for secondary metabolite-enriched tissues of Norway spruce (*Picea abies*). Plants, 13, 389. https://doi.org/10.3390/plants13030389

2. Singh, V.V., **Naseer, A.,** Sellamuthu, G., Mogilicherla, K., Gebauer, R., Roy, A. and Jakuš, R., 2024c. Robust reference gene selection in Norway spruce: essential for real-time quantitative PCR across different tissue, stress, and developmental conditions. Frontiers in Forests and Global Change, *in press.*

List of Abbreviation and Symbols

ABC	ATP-binding cassette
AChE	Acetylcholine esterase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
C.I.	Confidence interval
CCE	Carboxy-choline esterase
EGT	Ecdysteroid UDP glucosyltransferase
ESBB	European/Eurasian spruce bark beetle
EST	Esterase
GST	Glutathione S-transferase
ISx	*Ips sexdentatus*
LC_{70}	Lethal concentration for 70% mortality
OPLS-DA	Orthogonal partial least squares discrimination analysis
P450/ CYP	Cytochrome P450/ Cytochrome P450 monooxygenase
PCA	Principal component analysis
RQ	Relation Quotient
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
UDP	Uridine diphosphate
UGT	UDP-glucuronosyl transferase

List of Figures and Table

Abstract

Eurasian Spruce Bark Beetle (ESBB), *Ips typographus* (Coleoptera: Scolytinae), is one of the primary forest pests around Europe. They attack the bark and inter-cortical regions of the host, *Picea abies*, generally, the phloem and growing cambial zone, where the adults feed and reproduce, and the larvae develop. To protect themselves from colonisation, plants use anatomical and chemical defences. However, bark beetles have defence mechanisms to tolerate this harsh environment in their host, which can be attributed to one or more biochemical co-evolutions that allow them to survive in a host defence-rich environment. Such biochemical tolerance mechanisms may be achieved by detoxification of toxic plant compounds or their digestion in the midgut of the beetle by the production of certain enzymes. In invertebrates, many genes, and their enzymes (like P450s, UGTs, GST, AChE, and ABC transporter genes) play a vital role in defending the organism against oxidative stress by regulating many stress-responsive genes and are responsible for the detoxification of plant defence chemicals. Hence, greater knowledge of the function of these genes mentioned above is required to breakdown the molecular mechanism underlying beetle resistance against conifer defences. The presented study focused on how *I. typographus* efficiently conquered the spruce chemical defences using an *omics*-based approach. We reported that the genes related to detoxification, digestion, and resistance are critically expressed during host feeding or overexposure to host allelochemicals. We identified key genes, such as, CYP6A2, UGT2B17, ST1B1, GSTT1, and members of ABC gene families and multidrug resistance-associated proteins involved in the detoxification of host chemicals. The presented study provides a catalogue of genes that can be targeted for future *I. typographus* management that can subsequently be functionally validated by gene silencing via RNA interference (RNAi) or CRISPR/Cas9 mechanism.

Keywords: *Ips typographus*, *Picea abies*, Detoxification, RNA-seq, RT-qPCR, Enzyme assay, Monoterpene bioassay.

1. Introduction

Global climate change has led to severe bark beetle attacks in the last decade, especially in monoculture boreal forests, as it accelerates the development of bark beetles, reduces tree defences, and facilitates the beetles' expansion to new territories (Biedermann et al., 2019; Singh et al., 2024). Pine and spruce are the most economically important conifers in Europe. The pine and spruce timber damage by bark beetles has increased by 700% in the last four decades (1971–2010) and is expected to increase six-fold until 2030, reaching up to 17.9 million cubic meters per year (Seidl et al., 2014). In the Czech Republic, timber prices decreased due to massive outbreaks in 2018, from 56–64 euro per m^3 (2011–2017) to 14–16 euro per m^3, causing a substantial economic loss to the country. The economic and ecological impacts of such attacks are undeniable as they reset forest succession, disturb nutrient cycles, and turn the forest from carbon sinks to carbon sources. Several conventional approaches have been applied to manage and control such outbreaks in the past, like sanitation felling, debarking, salvage logging, fumigation, and use of pheromone-baits and poisoned tripod, but none of them has been entirely successful in mitigating the problem so far (Dobor et al., 2020; Singh et al., 2024)**.** The need of the hour calls for novel -omics-based approaches for targeted-management of these pests.

The Eurasian Spruce bark beetle (ESBB; *Ips typographus*) is one of the most eruptive herbivorous polyphagous economical forest pests (*Picea abies* being its primary host) in Europe. In their endemic stage, they attack stressed trees such as wind-felled or drought-prone, as they are more welcoming to attackers due to their weakened defence. The pioneer beetles are males that, after colonising the tree, release aggregation pheromones that cause a mass attack, leading to an epidemic where they infest the healthy trees in the forest. Most such attacks are lethal to the plant, and the virulence of the attack can be overwhelmed by some microbial associates (Krokene and Solheim, 1998; Fäldt et al., 2006; Davis, 2015; Chakraborty et al., 2020a; Chakraborty et al., 2020b; Gupta et al., 2023; Kandasamy et al., 2023; Singh et al., 2024)**.** Host

plants produce secondary metabolites, such as mono, di, and sesqui-terpenes, as part of their defence when attacked by insects, which the beetles counteract by producing detoxifying enzymes during feeding. Through the course of evolutionary interaction with hosts, insects have acquired the ability to detoxify plant allelochemicals in their gut, allowing them to bypass plant defences (Roy et al., 2016). This detoxification is performed by enzymes such as cytochrome P450s (P450s), glutathione S-transferases (GSTs), Carboxyl/choline esterases (CCE), UDP-glycosyltransferases (UGTs), and ATP-binding cassette (ABC) transporters (Kshatriya and Gershenzon, 2024). However, most of these enzymes and their gene complements have not yet been identified in ESBB, and the mechanism remains poorly understood.

For studying such mechanisms, the current study aims to identify the detoxification machinery genes in different life stages and tissues of ESBB using the RNA-seq approach. Further, to understand the genes involved in detoxifying the spruce monoterpenes, this study employed fumigation bioassay and diet-switch response analysis that effectively unravel the molecular mechanism underlying the beetle adaptation to conifer secondary metabolite defences. This approach of toxin overexposure had also been previously used in other bark beetle species, such as *Dendroctonus* (Smith, 1961; 1963; 1965; Everaerts et al., 1988; Chiu et al., 2017), and agricultural pests like *Helicoverpa* (Celorio-Mancera et al., 2011; Wu et al., 2011; Fabrick et al., 2022). Further studies deployed RNA-seq based approaches to study differential gene expression. This work aims to identify the key genes responsible for catering such defences to beetles, which can be further validated in the future using gene silencing techniques like RNAi, that had already been applied to control various agricultural pests and has been found efficacious in forest pests, especially those belonging to Coleoptera (Rodrigues et al., 2017a; Rodrigues et al., 2017b; Rodrigues et al., 2018; Kyre et al., 2019; Dhandapani et al., 2020a; Dhandapani et al., 2020b; Kyre et al., 2020; Kyre and Rieske, 2022). A study conducted by Yoon et al. (2018) has confirmed the excellent performance of RNAi against coleopteran insects, including beetles. It has also elucidated the possibility of using RNAi against ESBB to develop eco-friendly species-specific biopesticides to control forest pests in the future (Joga et al., 2021; Mogilicherla and Roy, 2023).

3. Aims and Objectives

Even though the chemical defences of the tree are strong enough to fend off the pests, the ESBB has co-evolved to overcome the host allelochemical defence. We hypothesised that the detoxification machinery in ESBB helps the beetles to overcome this conifer allelochemical barrier and thrive. However, the specific knowledge of the putative genes involved in such mechanisms is unknown.

To fill this knowledge gap, this study follows the overall objective to investigate the molecular mechanisms of *I. typographus* resistance to host chemical defences by identifying key genes involved in detoxifying or adapting to host metabolites. This study aims to enhance the understanding of host-beetle interactions and contribute to pest management strategies (Chapters 5.1 and 5.4).

Specific objectives:

1. To identify the detoxification machinery genes in *I. typographus* and elucidate their roles in overcoming host plant toxins during feeding (Chapter 5.2).
2. To identify the key genes in *I. typographus* that are responsible for the breakdown or modification (detoxification) of specific allelochemicals present in host plants (5.3).

3. Literature review

3.1. Climate Change and Bark Beetle

Forests play a main role in the carbon balance on the earth as they are the main carbon sink (Bastrup-Birk et al., 2016; Hlásny et al., 2019). However, due to rising temperatures and climate change, the pest outbreaks in the forest, especially the bark beetle outbreak, have intensified, leading to the conversion of forests from carbon sink to carbon sources (Ghimire et al., 2015; Hlásny et al., 2019). Various studies propound climate change being the driving factor in intensifying the bark beetle attacks in Europe and all across the world by reducing tree defences due to heat and drought, accelerating bark beetle life cycle and reproduction, increasing the number of generations, and facilitating its expansion to new territories (Schlyter et al., 2006; Ghimire et al., 2015; Raffa et al., 2015; Bastrup-Birk et al., 2016; Marini et al., 2017; Holopainen et al., 2018; Biedermann et al., 2019; Hlásny et al., 2019; Netherer et al., 2022). Due to climate change, the uneven drought and frost patterns have increased, which serves as preconditioning factors for decreasing the tree vitality and, in turn, rendering the plant more susceptible to windstorm felling and triggering bark beetle outbreaks as such trees serve as breeding grounds for these insects (Figure 1) (Schlyter et al., 2006; Marini et al., 2012).

Figure 1. The interconnected cycle of bark beetle attack and weakened spruce trees. Source- original, Aisha Naseer.

3.2.1. Norway Spruce

Norway spruce, *Picea abies* (L.) H. Karst., constitute the most economically important forest species. Due to its good performance growth and economic value to the wood industry, the extensive planting of the Norway spruce within and outside its native range gave rise to what is termed as monoculture or secondary forests at the expense of the broad-leave plants. These monoculture forests have less vitality and are more susceptible to biotic and abiotic stresses due to resource competition and providing large-scale breeding species breeding grounds to pests like bark beetle (Figure 2) (Singh et al., 2024).

Although spruce have specialized and sophisticated mechanisms to overcome the beetle attack via- i) morphological and anatomical structures- such as necrotic lesions, tough bark, stone cells and ii) chemical defences- production of toxic chemicals like terpenoid oleoresins in the resin

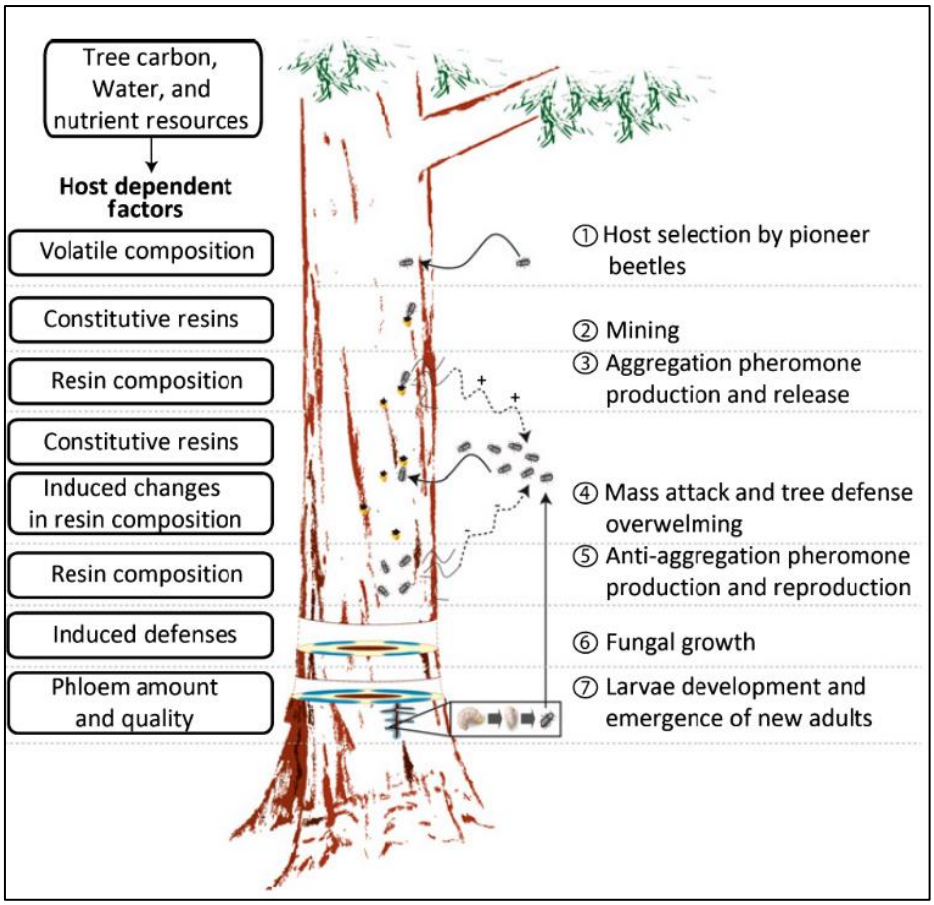

Figure 2. Effects of severe drought and stand density on tree host factors influencing and interacting with bark beetle life cycle stages. The + and - for stand density and severe drought show the potential correlations of tree host factors with the comp component that interacts with the bark beetles. Figure source- Ryan et al. (2015).

ducts or stored phenolics compounds in the parenchyma cells (Krokene, 2015; Biedermann et al., 2019). The chemical defences of the conifers consist of terpenes (mono- (C_{10}), sesqui- (C_{15}), and di-terpenes (C_{20})) and phenolic (stilbenes and flavonoids). Phenolics, mainly stilbenes and flavonoids, provide defence against bark beetles and their associated fungi (Martin et al., 2002; Celedon and Bohlmann, 2019; Hammerbacher et al., 2019). Such defences can be present as constitutive (already present) or induced upon stress (elicitation of induced defence system or defence priming), for example, by the beetle attack, fungal inoculation, methyl jasmonate (MeJA) treatment, or mechanical wounding (Nagy et al., 2000; Martin et al., 2002; Zhao et al., 2011a; Zhao et al., 2011b; Celedon and Bohlmann, 2019; Chen et al., 2021).

The levels of induced mono-, sesqui-, and diterpenes were reported to increase 240 folds in some trees within 35 days of inoculation of blue stain fungi *Ceratocystis polonica* (Siem.), a virulent fungal associate of the *I. typographus* and such trees witness only five percent of the beetle attack as compared to the non-induced tree (Zhao et al., 2011b). Similar kinds of studies were reported in the case of Scots Pine, *Pinus sylvestris,* in which the monoterpene level increased 800 folds and 30 folds within 28 days of inoculation with *Leptographium wingfieldii* and *Ophiostoma canum,* respectively (Fäldt et al., 2006). In an analogous study on the Norway spruce by Martin et al. (2002, 2003) showed the high accumulation of monoterpenes in the wood and bark after MeJA treatment. The highest rise and amount present in the treatment were reported in the case of monoterpene, depicting their role as the main compounds of chemical defences in Norway Spruce. Despite the elaborated plant defences of the conifers, the beetles, along with their associates, can colonise the spruce. Generally, two strategies are used: i) entering dead or stressed trees and ii) mass-attacking a healthy tree. The latter is the key adaptation that facilitates a bark beetle outbreak, which is also fatal to healthy trees.

3.2.2. Bark Beetle Complex

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are the most diverse and economically important forest pests of the conifers. More than 6,000 species of bark beetles are

found worldwide, most of which specialize in attacking dead or weak trees and play a role in nutrient cycling. However, some of them have specialized to carry mass attacks on healthy trees, which causes the most damage (Raffa et al., 2015; Valeria et al., 2016). These weevil family members specialise in living inside the host plant tissue (Raffa et al., 2015). They can be monogamous like *Dendroctonus*, where the female initiates the attack and mate with one male, or polygamous, where the male initiates the attack and may mate with more than one female as in the case of *Ips* species (Bleiker et al., 2013; Biedermann et al., 2019; Singh et al., 2024). To attack the appropriate host, these beetles use kairomones to locate the appropriate host. These beetles attack in mass numbers and enter the stem of the conifers, where they breed in the intercortical regions. A single egg is laid in tunnels forming galleries where larvae develop and start feeding on the phloem tissues, radiating away from the parent gallery to develop into adults. This way, characteristic gallery shapes are formed, which can be attributed to each species and used in their identification. The adults emerge from the holes in the bark and prepare to infest a new host. Most of these beetles are associated with bacteria, fungi, and/or nematodes, facilitating their entry into the host and helping them metabolize the conifer chemical defences (Zhao et al., 2011b; Krokene, 2015; Zhao et al., 2019).

3.2.3. Eurasian Spruce Bark Beetle (ESBB)

The Eurasian spruce bark beetle (ESBB), *Ips typographus* (L.), is one of Europe's most important spruce bark beetles, causing enormous economic wood loss of the Norway Spruce, (*Picea abies*). ESBBs are small-sized beetles, generally 4.5 to 5 mm in size, and show sexual dimorphism (Figure 3). The species is generally univoltine (completing one generation per year) but may increase to polyvoltine (multiple generations per year) due to an increase in temperature, i.e. warmer temperature, and with altitude due to faster development time. Climate change also enhances the performance of the beetle due to droughts, frost, and severe storms (Schlyter et al., 2006; Marini et al., 2017; Singh et al., 2024)**.** The wind caused tree mortality plays an essential role in expediting the outbreak by increasing the availability of food and brood trees and colonising density of the standing trees (Schlyter et al., 2006; Komonen et

al., 2011; Mezei et al., 2014). ESBB is also associated with their microbial partners who play an essential role in blocking the sapwood and in the flavonoid metabolism (Cale et al., 2019; Chakraborty et al., 2020a; Chakraborty et al., 2020b; Kandasamy et al., 2023). Certain naturally occurring competitors and predators of *Ips,* such as *Medetera, Monochamus,* and *Thanasamus,* predate on various life stages of beetles and keep the population in check (Vega and Hofstetter, 2014; Sousa et al., 2023) (elaborated in Chapter 5.1).

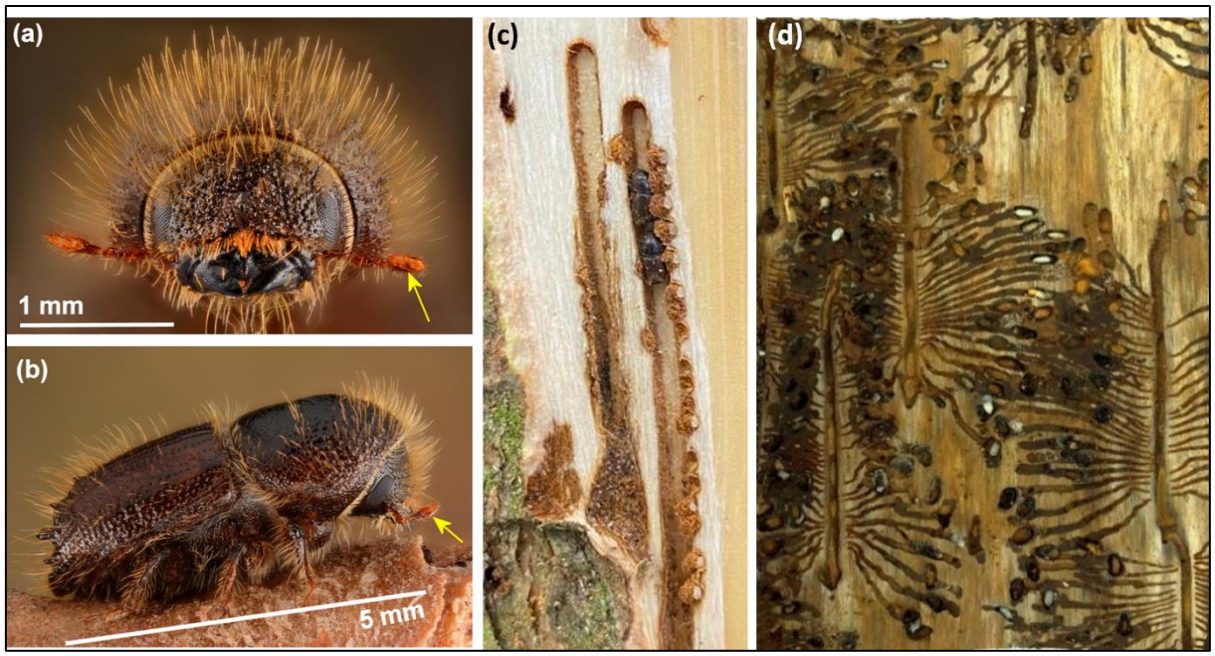

Figure 3. The Eurasian spruce bark beetle. Adult *Ips typographus* (a) front and (b) side view with sensory array on antennae indicated by arrows. (c) Female in mating chamber with eggs, (d) Larvae and pupae in radial galleries. Figure source (a) and (b) – Powell et al. (2021), (c)- original, Aisha Naseer, and (d)- Hlásny et al. (2019).

To initiate the colonisation of the host, ESBB, after overwintering, emerges from the litter or Norway spruce stems and disperses 100 meters to tens of kilometres (Biedermann et al., 2019) while locating a suitable host. Such host selection strategies are based on the plant volatiles in the forest, which may play a role as attractants or repellents (Raffa et al., 2015). During initial outbreaks, called green attacks or endemic stages, the beetles tend to infest the matured trees, which are either dead or are stressed and weakened due to high temperature, drought, disease,

or physical damage by wind or other factors. During the endemic stage, the male initiates the colonisation of the host phloem tissues of the dead/weakened host and releases aggregation pheromones to attract the male and the female conspecifics. The adults enter the host and make small chambers where they mate. If many beetles have entered the same tree, the mated females may re-emerge to infest the sister broods. The females lay eggs in the galleries below the bark, where they hatch into larvae. Also, anti-aggregation pheromones can be deployed to reduce intraspecific competition and mass colonisation of the same tree (Figure 4) (Biedermann et al., 2019; Ramakrishnan et al., 2022a; Singh et al., 2024). The larvae feed, tunnel away from the gallery, and develop into new adults, thereby increasing the population to 15-fold from the parent generation. This way, the beetle increases in number and with an enormous population, they can attack healthy trees, leading to the epidemic stage or outbreak phase.

3.3. Host-Pest Interaction

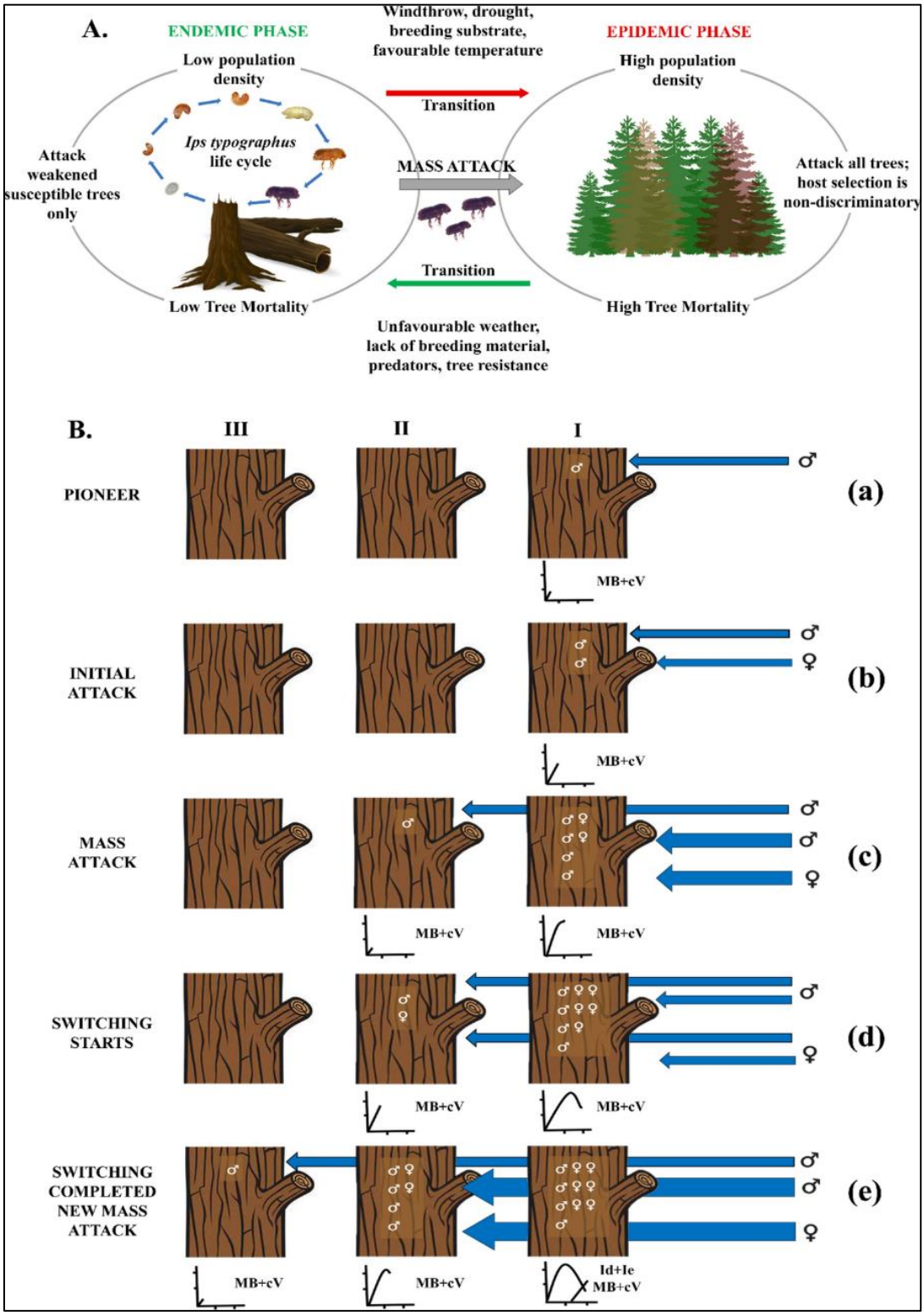

Figure 4. Scheme of *Ips typographus* population dynamics. (A.) In the endemic phase, beetles feed and reproduce in windthrown trees, trunks, and stumps and later move to the epidemic phase under favourable conditions. There are several factors, such as favourable weather, availability of breeding substrate, droughts, and windthrows, that influence the transition of the beetle populations to the epidemic phase. In the epidemic phase, the beetle population is high enough to mass attack healthy trees and cause widespread mortality. Later, certain factors such as unfavourable weather, exhaustion of the breeding substrate, abundance of predators, and increased tree resistance of surviving trees can cause high beetle mortality and limit the population to the endemic phase (Adapted from (Kautz et al., 2024). (B.) a. The initial host colonisation stage is usually initiated by pioneer beetles, males in *Ips* and females in *Dendroctonus*. In *Ips typographus*, aggregation pheromones, 2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (*c*V) are released to attract additional male and female beetles. b. If the pioneering beetle survives host tree defences, with the arrival of additional beetles, they produce larger amounts of aggregation pheromones which usually ends the mass colonisation of host trees. The goal of mass colonisation is to exhaust tree defence to secure reproduction. c. During the mass colonisation stage, while the initial tree (I) is mass colonised, a small portion of male beetle land on a neighbouring uncolonized tree (II) and starts a new colonisation sequence. d. As the production of attractive pheromone components diminishes, the attraction towards the initial tree declines, while simultaneously, attraction towards the neighbouring tree increases, starting a switch in attraction. e. When the initial "switching" process is completed, only small amounts of aggregation pheromones are produced, while larger amounts of inhibitory compounds (ipsdienol & ipsenol) are continuously produced. The attraction is now shifted to the neighbouring tree (II), where a new mass attack starts, with a small proportion of males once more being deflected and ending up on the new uncolonized neighbouring tree (III). The small graphs represent log pheromone release from Birgersson et al. (1984). The marks on the x-axis represent the attack phases 1, 3 and 6, where (1) males are bored in bark, (3) have completed nuptial chamber formation, and (6) are joined with females. (Adapted from (Schlyter et al., 1987). Figure source- Singh et al. (2024).

Interestingly, the chemical-ecological co-evolution between the Norway spruce trees and the ESBB has enabled the beetles to formulate new strategies for overcoming plant defences upon encountering new hosts (Singh et al., 2024). The ability to infest new host thought comes with multiple challenges but offers several advantages, such as lesser competition, utilizing plant metabolites as recognition or aggregation signals, and formulating chemical defences against potential predators and parasitic or pathogenic attacks**.** Two terms are quite frequently used in this regard: resistance and tolerance. While the former means the ability to survive the effects of a toxicant due to genetic selection, which goes on increasing from one generation to the other until a maximal point is reached, the latter means a relative comparison between the population/ species/ strains of their ability to tolerate a particular toxicant generally expressed in terms of LD50 or LC50 (Heckel, 2014).

3.3.1. Insect Detoxification

To counteract plant defence compounds, insects employ several strategies, which can be grouped into three categories: a) sequestration, b) excessive excretion, and c) biochemical alteration, also known as detoxification. Sequestration is among the many ways of defence adaptation, mimicry, plant-insect coevolution, and chemical ecology, where plant secondary metabolites are stored in specialized organs/ glands or tissues of the attacking insect without its alteration. A classic example is the sequestration of *α*-pinene to produce *cis*-verbenol, an aggregation pheromone produced by ESBB (Ramakrishnan et al., 2022a; Ramakrishnan et al., 2022b; Ramakrishnan et al., 2024). However, some detoxification is involved before the actual sequestration occurs (Duffey, 1980). During excessive excretion, the insect tends to remove the unaltered toxins from its body (Dowd et al., 1983; Heckel, 2014; Kshatriya and Gershenzon, 2024).

When an insect comes across a uniform and predictable compound, it may deploy its countermeasures and/or use these compounds as oviposition and feeding stimulants (Heckel, 2014). The generalist herbivores come across a wide variety of defence chemicals, and hence, they use diverse detoxification strategies, while the specialist herbivores feed on a narrow range of host plants and thus have specialized mechanisms of detoxification and sequestration strategies (van Veen, 2015; Erb and Robert, 2016; Bras et al., 2022). Studies on an aposematic butterfly, diurnal moth, and beetle suggest these species use sequestration strategies against toxic host compounds rather than manufacturing chemical defences of their own (Nishida, 2002). Reports on generalist insect *Spodoptera exigua* also suggest that the secondary metabolites (e.g., gossypol) of the plants, if not toxic in itself to the insect, could help in enhancing its defence against other toxic compounds (deltamethrin) (Hafeez et al., 2019).

Detoxification refers to the bio-chemical alteration of the endogenous or exogenous lipophilic compound to more hydrophilic substances that can be excreted out of the insect body via body fluids (Jakoby and Ziegler, 1990; Heckel, 2014; Heidel-Fischer and Vogel, 2015; Kshatriya and Gershenzon, 2024).

3.3.2. Enzyme Systems Involved

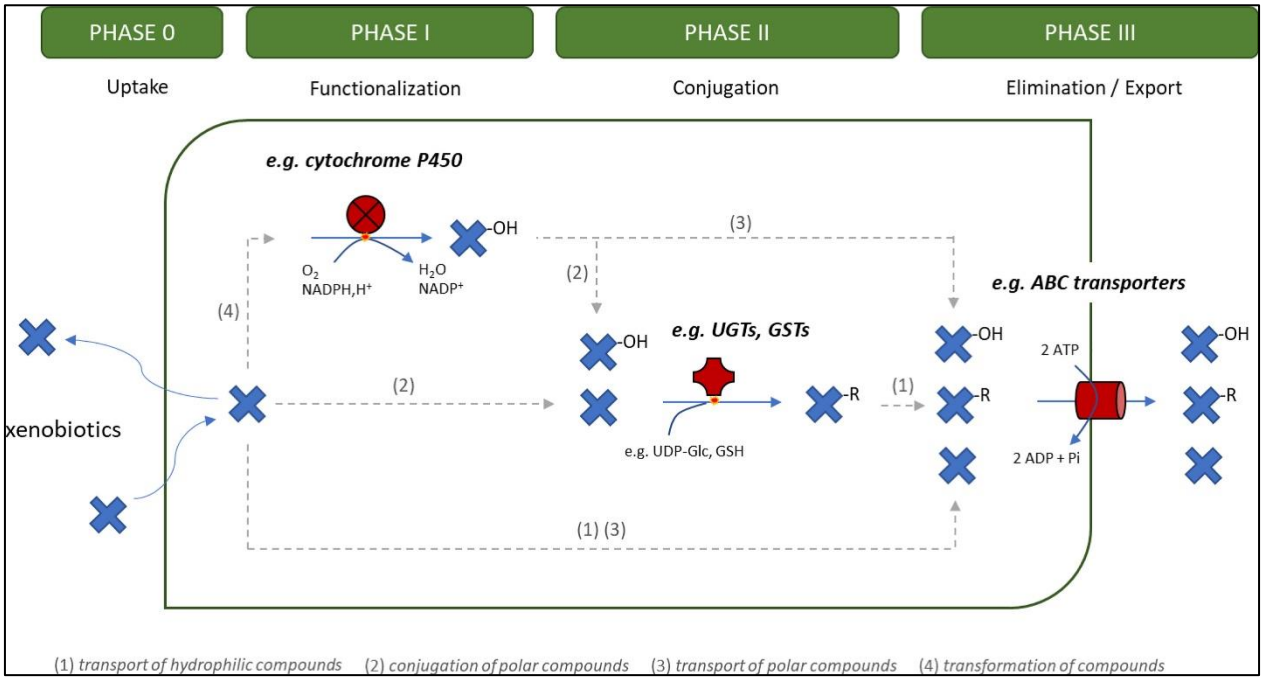

Figure 5. General scheme highlighting different pathways of xenobiotic elimination. Detoxification mediated by Phase I and II enzymes are characterised by reactions catalysed by cytochrome P450 monooxygenases (P450s) and UDP-glycosyl transferases (UGTs), respectively. The elimination pathways shown can work simultaneously, but depending on the xenobiotic, individual pathways can dominate. Figure source- Amezian et al. (2021).

The detoxification of host allelochemicals involves a repertoire of genes and their enzyme products. Such enzyme systems can be divided into Phase I (the lipophilic group attacking enzymes), Phase II (attacking the epoxides intermediates of the phase I) metabolic processes, and Phase III enzymes that transport the detoxified products out of the cells and insect body (Figure 5). The major groups of Phase I enzymes include Cytochrome P-450-dependent monooxygenases (P450s), reductases, esterases, epoxide hydrolases, peroxidases, and dehydrogenases. Phase II enzyme systems involve Glutathione-S-transferases (GSTs), UDP-glucuronosyltransferase (UGTs), sulfotransferases, N-acetyltransferases or acyltransferases. Phase III enzymes include multidrug resistance proteins, which are members of ATB-binding cassette transporters (ABC transporters) that cause the ATP-dependent transport of the hydrophobic products of phase I and II to

extracellular medium such that they can be excreted out of the insect body through body fluids (Ahmad, 1986; Jakoby and Ziegler, 1990; Váradi and Sarkadi, 2003; Wu et al., 2019; Hilliou et al., 2021; Kshatriya and Gershenzon, 2024). Recently, five signalling pathways that carry out detoxification in insects were described, *viz*. GPCR signalling pathway, MAPKs-CREB pathway, AhR/ARNT pathway, HR96 pathway, and CncC/Keap1 pathway (Amezian et al., 2021). These pathways contain the xenobiotic sensors, which activate the cascading mechanism leading to the activation of respective transcription factors and, therefore, the upregulation of the detoxification genes. Previous reports have identified insect gut and fat body as important sites for the detoxification reaction as most of the important genes are reported to be over-expressed in these tissues during toxin treatments (Tittiger et al., 2005; Fernanda López et al., 2011; Cano-Ramírez et al., 2013; Noriega et al., 2020; Ashraf et al., 2023).

3.3.3. Examples of Detoxification in Agricultural Pests

3.3.3.1. Cytochrome P450 Mixed Function Oxidases

P450s are one of the most important groups of enzymes involved in insecticide detoxification by strategically converting the lipophilic toxins to polar compounds that can be excreted (Feyereisen, 2012) and are abundantly present in the midgut and fat bodies of the insects that facilitate not only the metabolism of endogenous substrates but also the biotransformation of xenobiotics. The midgut is assumed to be an essential site for its activity (Hodgson, 1983; Berenbaum and Calla, 2021). Cytochrome P450s are the most critical groups in both generalist and specialist herbivores for the detoxification of xenobiotics via oxidation of its iron atom (Feyereisen, 2012; Heckel, 2014; Alyokhin and Chen, 2017). The complete cycle of the reaction depends upon the type of reaction- hydroxylation, epoxidation, dealkylation, oxidative deamination, oxidative de-sulphuration, *N*-hydroxylation, and sulphoxidation (Guengerich et al., 2009; Heckel, 2014). The most crucial detoxification families are CYP4 and CYP6.

The gene CYP6B1 was first described in *P. polyxenes*. This gene, when expressed in the baculovirus Sf9 cells along with the housefly P450 reductase enzyme, could efficiently detoxify the linear furanocoumarin (xanthotoxins) more than its angular form angelicin (Wen et al., 2003; Heckel, 2014). The detoxification behaviour depends upon the specialists and generalist behaviour of the insect. In the case of *Helicoverpa zea*, a generalist lepidopteran insect, the CYP6B8 can metabolize a wide variety of plant chemicals like quercetin, flavone, chlorogenic acid, indole-3-carbinol, and rutin. Whereas CYP6B1 from *P. polyxenes* could detoxify xanthotoxins better than CYP6B8 (Wen et al., 2003).

Various other pests of the Apiaceae have evolved detoxification strategies against the plant toxins. One such example is illustrated by the parsnip webworm, *Depressaria pastinacella*, a Pastinaca and Heracleum fruit pest that produces 10-fold higher furanocoumarin in fruits than its leaves (Berenbaum, 1990). It was reported that the overall expression of the P450s was ten times higher in this insect. The most characterized enzyme from *D. pastinacella* is CYP6AB3v1, which specifically detoxifies imperatorin (Mao et al., 2006).

Helicoverpa is a notorious agricultural pest known to detoxify gossypol and capsaicin. Capsaicin is a well-established defence compound found in *Capsicum annum* and *C. frutescens* for protection against herbivores and pathogens. It was reported that four P450 genes CYP6B6, CYP9A12, CYP9A14, and CYP9A17, cause the depletion of capsaicin in *Helicoverpa*; out of these genes, CYP6B6 has the highest depletion rate (Tian et al., 2019).

Gossypol is a secondary metabolite compound produced by the cotton plant and other related members to defend against pests (Celorio-Mancera et al., 2011; Celorio-Mancera et al., 2012; Jin et al., 2019). The multi-step oxidation of gossypol to gossic acid requires P450s as prominent entities. The study reported the up regulation of 13 P450 in the gut when *H. armigera* was fed with gossypol at the highest concentrations. Among these genes, CYP6AE14, worth mentioning, was highly regulated in the midgut of the insect (Celorio-Mancera et al., 2011). This

gene is expressed in midgut, and its silencing via RNAi causes retarded and reduced larval growth (Mao et al., 2007). Upregulation of two more P450s, CYP6AB9, and CYP9A17, was reported by Celorio-Mancera et al. (2012), which may be involved in detoxification. Functional validation of two P450s and one CCE, CYP4L11, CYP6AB9, and CCE001b, led to reduced larval growth in *H. armigera* larvae when fed on a gossypol diet (Jin et al., 2019). Furthermore, Hafeez et al. (2019) reported that P450 is involved also in providing resistance to other insecticides. They confirmed that the gossypol-treated larvae of the *Spodoptera exigua* showed overexpression of CYP6AB14 and CYP9A98 that provides tolerance to the insect towards insecticide deltamethrin and their knockdown induced sensitivity in the larvae to the insecticide.

Spodoptera is a well-studied agricultural pest for its detoxification genes. The role of P450 as a detoxification gene has also been studied in *Spodoptera exigua* (Hafeez et al., 2019; Hafeez et al., 2020). They used RNAi to functionally validate the role of three P450s, CYP6AB14, CYP9A98, and CYP9A10, in insecticide resistance. The abamectin exposure on *S. exigua* showed tissue specificity expression of eight P450 and four UGT genes when exposed to λ-cyhalothrin, Chlorantraniliprole, metaflumizone, and indoxacarb, showing similar up-regulated response (Hu et al., 2019). In *S. frugiperda*, CYP6B39, CYP321A, CYP9A31, and CYP9A27 were induced on xanthotoxin feeding within 24 hours (Giraudo et al., 2015).

In an endoparasitoid wasp *Meteorus pulchricornis*, which is a natural enemy of lepidopteran pests *Helicoverpa* and *Spodoptera* species, 28 P450s were identified to be overexpressed when exposed to chemical insecticides (Xing et al., 2021). These CYPs belong to the CYP2, CYP3, CYP4, and mitochondrial clans. They further characterised CYP369B3 using RNAi, which increased insect mortality after gene silencing.

Transcriptomics analysis of a coleopteran agricultural weevil of camphor by *Pagiophloeus tsushimanus* revealed that there exists a co-expression between the P450s expression and that of cuticular proteins during the developmental stages when exposed to camphor (Li et al., 2023).

Such an adaptation leads to the thickening of the cuticle, thus preventing the entry of the toxin into the body via fumigation.

3.3.3.2. UDP-glycosyltransferases and Glutathione S-transferases

Uridine diphosphate (UDP)-glycosyltransferases/ glucuronosyltransferase (UGTs) are major phase II enzymes specialized in the detoxification of glycosylated chemical defences of the plant by attaching sugar to it, hence diminishing its activity and elevating its solubility. After the activation of the monosaccharide xenobiotic via attachment to uridine diphosphate (UDP), UGTs catalyse the conjugation of the sugar, thus increasing its solubility in water and excretion henceforth (Ahn et al., 2012; Heckel, 2014; Bretschneider, 2016). Apart from their occurrence in humans and plants, over 310 insect UGT have been identified in *H. armigera* (42), *Bombyx mori* (45), *Tribolium casteneum* (43), and *Apis* species (10). These were classified into 68 families, UGT50 being the only family expressed universally in insects, and their occurrence varies from fat body, midgut, and Malpighian tubules, suggesting their role in detoxification of host compounds and antennae suggesting a role in pheromone deactivation (Ahn et al., 2012; Bock, 2016; Bretschneider, 2016). Although the role of cytochromes is well established in gossypol detoxification, Krempl et al. (2016) demonstrated the role of two UGTs, UGT41B3 and UGT40D1, in partially metabolising gossypol via glycosylation in *Helicoverpa armigera*. Similarly, 23 UGTS were significantly up-regulated in *Aphis gossypii* on thiamethoxam-resistant strains (Pan et al., 2018). Gene silencing of UGT2 in Colorado potato beetles increases the susceptibility of these insects towards imidacloprid (Hu et al., 2019). Functional characterisation using RNAi knockdown of more UGT2 family genes has proven strong association with detoxification like UGT2B17 involved with exogenous toxin Chlorantraniliprole in diamond moth (*Plutella xylostella*) (Li et al., 2017) and UGT2B20 being involved in malondialdehyde resistance in *Apis cerana cerana* (Cui et al., 2020). Chlorantraniliprole resistance was also reduced in rice stem borer (*Chilo suppressalis*) after CsUGT40AL1 and CsUGT33AG3 knockdown (Zhao et al., 2019). A single UGT of *S. frugiperda*, SfUGT33F28 provides defence against major maize-defensive benzoxazinoids (Israni et al., 2020). Apart from detoxification, certain UGTs are involved in developmental functions. For example,

ecdysteroid UDP glucosyltransferase (EGT) can hinder moulting by interrupting cocoon formation in larvae of silkworm *Bombyx mori* (Shen et al., 2018).

Glutathione S-transferases (GSTs) are another phase II gene family involved in the detoxification of the host allelochemical by conjugation of electrophilic molecules with reduced glutathione (Francis et al., 2005; Koirala et al., 2022). In other words, they remove toxic oxygen free radicals by either dehydrochlorination or conjugating with reduced glutathione (Enayati et al., 2005). In 1995, Hung et al. showed that GST and P450s also facilitate furanocoumarin detoxification. It was shown in *Manduca sexta* that there was GST gene upregulation in response to plant feeding, yet the response was not specific (Francis et al., 2005). Additionally, when *H. armigera* larvae fed on different hosts, various gene products involved in xenobiotic metabolism were up-regulated, such as a GST (GST23), and the UGTs (UGT41B2, UGT41D1, and UGT33T1) (Celorio-Mancera et al., 2012). Another study identified thirty differentially expressed UGTs and GSTs that participate in detoxification in *Heliconius melpomene* (Yu et al., 2016). Previous reports have also identified three genes in brown plant hopper involved in insecticide resistance *viz.* NlGST1-1, NlGSTd2, and NlGSTE1 (Yang et al., 2021). Similarly, Zhang et al. (2016) demonstrated that chlorpyrifos leads to the induction of GSTs in *Spodoptera litura* larvae. Another study by Meng et al. (2020) demonstrated the putative involvement of the GSTD9 gene in *Bactrocera dorsalis* responsible for malathion resistance. GSTS6 induced susceptibility towards insecticides and reduced embryogenesis in *Tribolium casteneum* (Song et al., 2022). However, not many functional studies have been performed in bark beetles for UGTs and GSTs. One of the few reports, used phloem-feeding and monoterpene fumigation on the Chinese white pine beetle, *Dendroctonus armandi*, to study GST genes across various tissues and life stages and reported sixteen full-length GSTs belonging to the category delta, epsilon, sigma, and theta (Gao et al., 2020).

3.3.3.3. ABC Transporters

Adenosine triphosphate (ATP)-binding cassettes (ABC) are conserved transmembrane proteins crucial for the ATP-driven export of a wide range of substrates across cellular membranes. In insects, ABC transporters play significant roles in xenobiotic detoxification, resistance to pesticides, and the action of *Bacillus thuringiensis* (Bt) toxins (Bretschneider, 2016; Wu et al., 2019). Structurally, these proteins consist of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs), where ATP hydrolysis induces conformational changes that drive substrate transport. ABC transporters in insects are divided into eight subfamilies (ABCA-ABCH), each with unique functional properties. The study of ABC transporter genes in *Tribolium casteneum* revealed ABCA and ABCC subfamily genes to be involved in detoxification. The expression of several ABC genes was lowered after gene knockdown, and gene knockdown of ACCG4C also led to lesser egg hatching, thus preventing development (Broehan et al., 2013). In the leaf beetle *Chrysomela populi*, Strauss et al. (2014) studied how ABC transporters help detoxify and sequestrate plant-derived chemicals. They identified sixty-five ABC transporter genes from the ABCB, ABCC, and ABCG subfamilies inked to the absorption of plant metabolites in the gut and their excretion via Malpighian tubules. In Asian corn borer O*strinia furnacalis*, Gao et al. (2022) reported the significant role of the ABCG4 family gene in Bt-toxin resistance using CRISPR/Cas9 gene knockout. Researchers utilized CRISPR/Cas9 in *Helicoverpa zea* to create an ABCA2 knockout strain, revealing that the absence of this gene increased resistance to Bt toxins (Fabrick et al., 2022). These findings indicate that ABCA2 may be a receptor for Bt Cry1 toxins. Other reports suggested that ABC transporter genes are linked to deltamethrin resistance in *Culex pipiens pallens* (Xu et al., 2023). They identified sixty-three ABC transporter genes, and those belonging to ABCB, ABCC, and ABCG subfamilies were highly expressed in deltamethrin-resistant mosquitoes. Further, the RNAi gene knockdown of ABCG6032427 increased the mosquitoes' susceptibility to deltamethrin, suggesting its role in enhancing resistance by altering cuticle thickness and structure as the cuticle became thinner and looser, as revealed by SEM and TEM.

3.4. Monoterpene Detoxification in Bark Beetles

Bark beetles detoxify the monoterpenes of their host by their hydroxylation to alcohol. More than a hundred P450s have been identified in the insects. Several studies on *Ips pini* reported that P450s play an essential role in the hydroxylation of myrcene to ipsdienol, which acts as a semiochemical to attract the conspecifics (Blomquist et al., 2010; Nadeau et al., 2017; Tittiger and Blomquist, 2017; Blomquist et al., 2021). For the detoxification study, P450s belonging to families 4 and 6 are considered the most important (Powell et al., 2021). Previously, the detoxifying role of P450s has been elaborately studied in different species of *Dendroctonus*, a pine beetle which is a close relative of ESBB (Cano-Ramírez et al., 2013; López et al., 2013; Liu et al., 2022). Whole-genome transcript analysis of *D. ponderosae* revealed the presence of several P450s, GSTs, and esterases putatively involved in the detoxification of the host compound. These genes were present in the midgut, fat body, and other tissues of the adult, and some of those P450s were overexpressed in a sex-specific manner, suggesting the role involvement early colonisation of the host and of females being the pioneer sex (Robert et al., 2013). More recently, the functional characterisation of three P450s, CYP6DJ1, CYP6BW1, and CYP6BW3, involved in detoxifying pine monoterpenes and diterpenes was reported (Chiu et al., 2019). Similar whole-genome analysis in *D. armandi* reports the overexpression of various P450s in the whole body and gut of the insect upon monoterpene vapor exposure and feeding, respectively (Dai et al., 2015). Two P450 genes, CYP6CR2 and CYP6DE5 were characterized in *D. armandi* via gene knockdown (Liu et al., 2022). Silencing these genes significantly increased the mortality of the beetles exposed to monoterpenes like *α*-pinene, 3-carene, limonene, and turpentine, thus, demonstrating their involvement in detoxification. Up-regulation of five P450s was also reported in the midgut of *D. rhizophagus* during the early stages of feeding on the host (Sarabia et al., 2019). Several ultrastructural changes were also reported in monoterpene-induced cells on the midgut of *D. valens* (Fernanda López et al., 2011). These studies suggest that the detoxification of host monoterpenes mainly occurs in the midgut, and P450s play a crucial role.

3.5. Current Knowledge of *Ips typographus* at the Molecular Level

Based on the literature research, we acknowledge that although agricultural pests and some forest pests are well studied for their detoxification genes, the bark beetle detoxification machinery, in *Ips* species remains unexplored. The availability of the *I. typographus* genome has highly facilitated our knowledge and research in *Ips* for their genetic regulation (Powell et al., 2021). Insect olfaction is one important phenomenon that helps the beetles locate their suitable host during attack and has been the most studied field in *I. typographus* at the gene level. Various studies have reported and characterised olfactory receptors and chemosensory genes in *I. typographus* for their evolution and sensation to individual plant volatiles (Andersson et al., 2013; Johny et al., 2024). For instance, characterisation of ItypOR46 for *(S)*-(-)-ipsenol, ItypOR49 for *(R)*-(-)-ipsdienol, ItypOR25 for (+)-3- carene, ItypOR6 for 2-phenylethanol, ItypOR41 for (4S)-*cis*-verbenol, and ItypOR33a for amitinol (Hou et al., 2021; Yuvaraj et al., 2021; Roberts et al., 2022; Biswas et al., 2024; Johny et al., 2024). Advances in -*omics* studies have identified several gene families related to pheromone biosynthesis in *Ips* beetles (Ramakrishnan et al., 2022a). A follow-up study on hormone regulation, using juvenile hormone III (JH III) on *I. typographus*, further advanced the identification of specific gene families involved in pheromone production. Interestingly, research on JH III has also highlighted the role of genes which are not only involved in pheromone biosynthesis but also play a key role in detoxification mechanisms, such as the breakdown of monoterpenes. This suggests that JH III influences both pheromone production and related processes, including detoxification, in *I. typographus* (Ramakrishnan et al., 2024). Various reports have tried to explain the role of ESBB-associated microbiome and how it facilitates ESBB's resistance towards complex host chemicals (Zhao et al., 2019; Chakraborty et al., 2020a; Chakraborty et al., 2020b). To facilitate the RNA-seq., Sellamuthu et al. (2022) have provided a comprehensive catalogue of optimal reference genes for validation studies. The occurrence of RNAi core machinery genes and the future possibilities of targeted pest management strategies for ESBB using molecular gene targets was recently reported (Joga et al., 2021). The current study focuses on identifying potential targets in detoxification mechanisms using the RNA-seq

approach. The compiled report in the presented thesis enhances our current understanding of *Ips typographus* detox-mechanisms and its allelochemical resistance that have been unknown so far. Notably, these are the very first published reports on *I. typographus* detoxification mechanisms and could pave the way for gene silencing mediated management strategies in the future.

4. Material and Methods

4.1. Sample Collection

4.1.1. Insect Collection

Study I

For the study in Chapter 5.2 *Ips typographus* (F0) were sourced from Rouchovany in Czechia (49°04’08.0” N 16°06’15.4” E), 360 m above sea level, a warm and drought-prone area with regular forest management by State Forest Enterprise during the infestation season in May 2019. The average temperature was around 15°C during collection. Different life stages (larval stages 1, 2, 3, pupae, callow beetles, and adult beetles) were collected from the infested logs and snap-frozen in liquid nitrogen. Callow and adult (sclerotised) beetles were dissected to isolate desired tissues (midgut, fat body, and head) in sterile conditions and stored in RNAlater^{TM} (Thermo Fisher Scientific (Waltham, MA, USA).

Study II

For the study in Chapter 5.3, newly infested Norway spruce logs were collected from research plots near Kostelec nad Černými lesy in the Central Bohemian region of Prague, Czech Republic. The area experiences warm, dry summers, ideal for beetle development, with a growing season of 150-160 days, annual temperatures of 7-7.5°C, and 600 mm of rainfall (Tolasz et al., 2007; Singh et al., 2023). After felling, the logs were stored at 4°C for up to two weeks at the Czech University of Life Sciences. *I. typographus* were collected, reared on fresh spruce logs, and kept in controlled lab conditions (25°C, 65% humidity) (Sellamuthu et al., 2022; Naseer et al., 2023). F1 beetles were sexed, weighed, and used immediately, excluding compromised individuals.

Study III

Ips sexdentatus, ISx (F0) used in the study of Chapter 5.4 were collected from fresh pine logs sourced from Kostelec nad Černými lesy and were reared on fresh pine and spruce logs to produce F1 under lab conditions: temperature 27 ± 1°C under 70 ± 5% humidity and a 16:8 h light/dark (L:D) photoperiod (Sellamuthu et al., 2021).

4.1.2. Plant Material Collection

Uninfested and non-pesticide treated logs of *Pinus sylvestris* L. (Scots pine) and *Picea abies* L. (Norway spruce) trees (80-100 years in age, diameter 30–45 cm, length 38–42 cm) were sourced from Kostelec nad Černými lesy and Rouchovany for host-switch and metabolomic studies in Chapter 5.4.

4.2. Bioassay

4.2.1. Fumigation (Study II)

For the study in Chapter 5.3, five monoterpenes (Table 1): *α*-pinene, *(R)*-(+)-limonene, *(S)*-(-)-limonene, myrcene, and sabiene were tested using fumigation (details in Chapter 5.3) using the protocol of Chiu et al. (2017) with slight modification as detailed in Naseer et al. (2024). Monoterpenes were tested at five doses (volume monoterpene applied/volume airspace of the assay vial) of 50 µL/L, 100 µL/L, 200 µL/L, 400 µL/L, and 800 µL/L which are represented at 1 µL, 2 µL, 4 µL, 8 µL, and 16 µL per 20 mL of vial used for the study. A piece of Whatman filter paper (1.5 cm × 1.5 cm) was placed in the vial, a definite dose of a single monoterpene was applied, a moist filter paper for maintaining humidity was placed, a beetle was inserted, and the vial was sealed. For control, no monoterpene was applied to the Whatman filter paper. The set-up was placed inside the climate chamber (Memmert HPP2200ECO, , Schwabach Germany) at 25°C temperature and 20 h/4 h light/dark photoperiod (Faccoli and Schlyter, 2007) and was observed for 72 h, and mortality was recorded at 12 h interval. 60 insects (30 ♀ and 30 ♂) were tested for

each dose of monoterpene and control for bioassay. A total of 360 insects (180 ♀ and 180 ♂) were used for each monoterpene. Dose-response analyses were conducted after 48 h to calculate the LC_{70} using 48 h mortality data. Adult emerging F0 and F1 ESBB were again treated with the resulting LC_{70} dose for four chemicals (*α*-pinene, *(R)*-(+)-limonene, myrcene, and sabiene). The whole body of the live beetles was collected after 48 h and snap-frozen in liquid nitrogen for RNA isolation. F0 beetles were also treated with a double dose of the LC_{70} of R-limonene and sabiene to assess the effect of generational vigour of ESBB. The whole body was crushed in liquid nitrogen using a pre-chilled mortar pestle to make powder, which was used for RNA and protein isolation. Details of sample pooling and biological replicates used can be found in Chapter 5.3.

Table 1: List of chemicals used in the bioassay.

Sr. No.	Name	Purity	Manufacturer	CAS
1	*α*-Pinene	97%	Thermo Scientific Chemicals	80-56-8
2	(*S*)-(-)-Limonene	97%	Thermo Scientific Chemicals	5989-54-8
3	(*R*)-(+)-Limonene	~90%	Sigma-Aldrich	5989-27-5
4	(1*S*)-(+)-3-Carene	99%	Sigma-Aldrich	498-15-7
5	Myrcene	≥ 90.0 %	Sigma-Aldrich	123-35-3
6	Sabiene	75%	Sigma-Aldrich	3387-41-5

4.2.2. Offspring Performance and Fecundity (Study III)

In Chapter 5.4, to assess beetle performance, we compared the elytra length, weight, and fecundity of ISx beetles after being transferred between species (pine to spruce, F1-spruce) and within the same species (pine to pine, F1-pine). Adult ISx (F0), equally divided between males and females reared on *Pinus sylvestris* (pine), were transferred to both *Pinus sylvestris* and *Picea abies*

(spruce) logs for the next generation (F1) in a climate-controlled chamber (Sellamuthu et al., 2021). Ten pairs from the F0 generation were placed on each type of log, and fecundity was measured by counting larval galleries or hatched larvae per square centimetre of phloem. F1 adults' body weight and elytra length (n = 60) were recorded to evaluate their performance.

4.3. Total RNA Isolation, cDNA Synthesis, and RT-qPCR Analysis

4.3.1. Samples Used for RNA Isolation

Study I

For the study in Chapter 5.2, the whole body, and tissues of ESBB were used as: eight L1 larvae, eight L2 larvae, five L3 larvae, five pupae, and two adults were pooled; ten guts, ten fat bodies, and five heads were dissected and pooled as one biological replicate for RNA and protein isolation (Naseer et al., 2023).

Study II

For the RNA-sequencing in Chapter 5.3, three emerging F1 ESBB were pooled for R-limonene and sabiene treatment. For RT-qPCR and enzyme assays performed for four chemicals (*α*-pinene, *R*-limonene, sabiene, and myrcene), four ESBB whole body for F1 and F0 were pooled for treatment at LC_{70}, and two beetle whole body of F0 were pooled for treatment at double LC_{70} as one biological replicate (Naseer et al., 2024).

Study III

For the study in Chapter 5.4, ten gut tissues of ISx F0-Pine, F1-pine, and F1-spruce were pooled as one biological replicate after feeding on *P. sylvestris* and *P. abies*, respectively, to be used as the source of RNA and protein (Sellamuthu et al., 2024).

4.3.2. Protocols Used

RNA was isolated using the RNA extraction kit PureLink™ RNA Kit from Ambion (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Isolated RNA was immediately treated with DNase I (TURBO DNase Kit, Ambion, Austin, TX, USA). Integrity and concentration of RNA were checked on a 1.2% agarose gel and using a NanoDrop2000 spectrophotometer (Thermo Scientific, MA, USA) in Chapters 5.2 and 5.3. For Chapter 5.4, the concentration of RNA was measured with a Qubit 2.0 Fluorometer (Life Technologies) and integrity with 2100 Bioanalyzer (Agilent, CA, USA). cDNA was synthesised with one µg of RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems Life Technologies, Waltham, MA, USA). cDNA was diluted 10-fold (for study II, Chapter 5.2) and 5-fold (study III and IV, Chapters 5.3 and 5.4). cDNAs were stored at -20°C until use.

The RT-qPCR primers were designed using the IDT PrimerQuest software (IDT, Leuven, Belgium). The 10 µL RT-qPCR reactions contained 5.0 µL of SYBR Green PCR Master Mix (Applied Biosystems Life Technologies, Waltham, MA, USA), 1.0 µL of cDNA, 1.0 µL of 10 µM forward and reverse primers, and 3.0 µL RNase-free water (Invitrogen, Waltham, MA, USA). The reactions were performed in an Applied Biosystems^{TM} StepOne^{TM} Real-Time PCR System (Applied Biosystems Life Technologies, Waltham, MA, USA) with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and dissociation curve analysis during which temperature was increased from 60 to 95°C. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Ribosomal proteins L7 and S7 (RPL7 and RPS7) were used as reference genes for expression normalization.

4.4. Transcriptome Sequencing, Library Preparation, and Analysis

Study I & II

Transcriptome libraries of different life stages and tissues prepared in the study following Powell et al. (2021) were used in Chapter 5.2. The RNA sequencing data were submitted to NCBI

(PRJNA679450) and reused for the present gene expression study. The raw reads were mapped back to the *I. typographus* reference genome (Powell et al., 2021). The differential gene expression studies in Chapters 5.2 and 5.3, were performed using the CLC workbench (CLC version 21.0.5, Qiagen, Denmark) as detailed in section 5.2 and 5.3. The data for Chapter 5.3 was submitted to NCBI under Bioproject PRJNA1149972 (Naseer et al., 2024).

Study III

For the study in Chapter 5.4, RNA-seq data analysis was performed using the OmicsBox transcriptomics module (version 1.4.11), and *de novo* assembly of the cleaned reads were performed as explained in detail in Chapter 5.4. The data was submitted to NCBI under Bioproject PRJNA846690 (Sellamuthu et al., 2024).

4.5. Enzyme Assay

Enzyme activity assays were formed for three enzymes *viz.* NADPH–Cytochrome P450 Reductase (CPR), glutathione S-transferase (GST), and esterases (EST) following the detailed protocol of Sellamuthu et al. (2024) (Chapter 5.4, supplement protocol file). Samples (whole body and/or tissues) were homogenized in 100 µL of sodium phosphate buffer (50 mM, pH 7.0). The microplate reader (Agilent BioTek Cytation 5, CA, USA) used definite wavelength and time interval to measure the enzyme activities as described in Chapters 5.2 and 5.4.

4.5. Gas Chromatography-mass Spectrometry (GC-MS) Analysis for Hostplant

For the study in Chapter 5.4, phloem samples from pine and spruce logs were freeze-dried, homogenised into powder, and 200 mg was extracted in hexane using sonication for 10 minutes. The compounds were separated and identified using GC-MS (Agilent 7890B, CA, USA) with a Pegasus 4D mass analyser (LECO, MI, USA), following modified methods (Ramakrishnan et al., 2022a). A 1 µL extract was injected into a cold PTV injector, heated to 275°C, and separated on an

HP-5MS UI column. The total run time was 33 minutes, and ions were collected in the 35–400 Da range. Data were processed, normalized, and analysed using PCA and OPLS-DA in SIMCA 17 software (Sartorius Stedim Data Analytics AB, Malmö, Sweden). Compound identity was confirmed using standards and National Institute of Standards and Technology (NIST) indexes (2017).

4.6. Statistical Analysis

Study I

In Chapter 5.2, we used Student's *t*-test for pairwise comparisons to analyse differences in RT-qPCR gene transcript levels for 19 detoxification-related genes. These comparisons included the callow beetle gut versus the sclerotised beetle male gut and the callow beetle female fat body versus the callow beetle male fat body. Tukey's HSD was applied for multiple comparisons of relative gene expression across developmental stages and gut tissues of ESBB. Finally, one-way ANOVA tested the significance of differences in enzymatic activities between ESBB stages and tissues. All analyses were conducted in RStudio (version 4.2.2).

Study II

In Chapter 5.3, the percentage mortality of the beetles was calculated using Henderson-Tilton's formula (Henderson and TILTON, 1955). The dose-response analysis was performed using XLSTAT 2020 (v 3.1.1011) to calculate the LC_{70}. F-test and Student's *t*-test were performed to check the sex-based mortality among the populations. To compare the relative expression of detoxification genes between the control and treated beetles in RT-qPCR, and enzyme activity assay. First, the normality of each group was checked using the Shapiro-Wilk test, and then variance homogeneity was calculated between the control and treatment groups using Levene's test. Then independent *t*-test was performed with equal variance-Student's *t*-test (if Levene's $p>0.05$) or unequal variance- Welch's t-test (if Levene's $p<0.05$) accordingly, and *p*-values were generated based on the significant differences between control and treatment groups at 95 C.I. using RStudio (version 4.2.3).

Study III

In Chapter 5.4, ANOVA was used to analyse differences in ISx body size and weight after feeding on different hosts in the no-choice bioassay. A paired *t*-test was conducted to assess significant variations in fecundity. For DGE data, a negative binomial Generalized Linear Model (GLM) with a Likelihood Ratio Test was applied. One-way ANOVA with Šídák's multiple comparisons test was used to evaluate differences in RT-qPCR results and enzyme activity levels.

5. Results

The dissertation thesis consists of four published articles distributed in four sub-chapters. The first sub-chapter (5.1) is a review article that details the bark beetle population dynamics regarding forest tree health, attack mechanism, and present and prospects of future management strategies. Sub-chapters second to fourth contain original data in the form of published articles. The second sub-chapter (Chapter 5.2, Study I) explores the detoxification machinery across different life stages and tissues of ESBB and their abundance (Objective I). The third sub-chapter (Chapter 5.3, Study II) examines the effect of fumigation of two monoterpene *(R)*-(+)-limonene and sabiene, on ESBB at the gene level and details the conserved mechanism of overexpression of detoxification genes and suppression of developmental genes on different monoterpene exposure (Objective II). The fourth sub-chapter (Chapter 5.4, Study III) reveals the changes in the gene expression on host switch in another important *Ips* species, *I. sexdentatus*.

5.1. Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management.

Published as: Singh, V.V., **Naseer, A.,** Mogilicherla, K., Trubin, A., Zabihi, K., Roy, A., Jakuš, R. and Erbilgin, N., 2024. Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management. Reviews in Environmental Science and Bio/Technology, pp.1-34. https://doi.org/10.1007/s11157-024-09692-5

Contributed to: visualisation, writing- section of the first draft, reviewing and editing.

This study provides a comprehensive overview of the bark beetle ecology, their population dynamics at the stand and landscape levels, and major drivers of the outbreak in the context of climate change. It preliminary examines how changes in forest structure influence the initial colonisation and spread of bark beetles, how drought and water stress impact the physiology and vitality of host trees, and how temperature regimes affect bark beetle activity. The article covers the overall objective 'to understand spruce-beetle interaction' and evaluates current forest management strategies and their effectiveness in safeguarding forests and emphasised the importance of exploring innovative, molecular-based techniques for managing bark beetles.

The article highlights the inadequacy of traditional management approaches in dealing with escalating bark beetle outbreaks, especially in the context of climate change. It stresses the importance of integrating novel molecular tools alongside conventional methods. Emerging technologies like RNA interference (RNAi) and CRISPR/Cas9 offer a species-specific, targeted approach and have shown potential in controlling agricultural pests. However, their applicability in managing bark beetles, particularly *Ips typographus*, has not been thoroughly explored. While these techniques have not yet been tested in this species, the article discusses their potential future use and efficiency in bark beetle management. Nonetheless, further research is required to validate their effectiveness in forest ecosystems. We propose incorporating these advanced molecular techniques to address new challenges, which could enhance future forest management practices in the Anthropocene.

Rev Environ Sci Biotechnol
https://doi.org/10.1007/s11157-024-09692-5

REVIEW PAPER

Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management

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Received: 31 December 2023 / Accepted: 7 May 2024

Abstract Climate change has increased the susceptibility of forest ecosystems, resulting in escalated forest decline globally. As one of the largest forest biomasses in the Northern Hemisphere, the Eurasian boreal forests are subjected to frequent drought, windthrow, and high-temperature disturbances. Over the last century, bark beetle outbreaks have emerged as a major biotic threat to these forests, resulting in extensive tree mortality. Despite implementing various management strategies to mitigate the bark beetle populations and reduce tree mortality, none have been effective. Moreover, altered disturbance regimes due to changing climate have facilitated the success of bark beetle attacks with shorter and multivoltine life cycles, consequently inciting more frequent bark beetle-caused tree mortality. This review explores bark beetle population dynamics in the context of climate change, forest stand dynamics, and various forest management strategies. Additionally, it examines recent advancements like remote sensing and canine detection of infested trees and focuses on cutting-edge molecular approaches including RNAi-nanoparticle complexes, RNAi-symbiotic microbes, sterile insect technique, and CRISPR/Cas9-based methods. These diverse novel strategies have the potential to effectively address the challenges associated with managing bark beetles and improving forest health in response to the changing climate.

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Published online: 23 May 2024

Keywords *Ips typographus* · Norway spruce (*Picea abies*) · Outbreak dynamics · Tree-bark beetle interaction · Environmental stress · Forest management strategies · Traditional and molecular approaches · Biosafety issues

1 Introduction

Over the last several decades, a shift in climatic conditions has increased the frequency of severe windstorms and drought events, favouring the emergence and proliferation of insect outbreaks. Bark beetles (Coleoptera: Curculionidae: Scolytinae) are widespread globally, and several species are associated with extensive mortality of major tree species in the Northern Hemisphere (Vega and Hofstetter 2015; Hlásny et al. 2021). For instance, the Eurasian spruce bark beetle, *Ips typographus* (L.) plays a vital role in forest succession and nutrient recycling by decomposing dead and dying trees (Edmonds and Eglitis 1989; Hofstetter et al. 2015; Raffa et al. 2015). However, once their population density reaches the epidemic threshold, they have the potential to cause widespread forest mortality (Berryman et al. 1989; Hlásny et al. 2021). These outbreaks have gone beyond economic implications by disrupting forest succession and nutrient cycling and turning forests from carbon sink to source (Seidl et al. 2014; Aldea et al. 2023).

As the primary host for *I. typographus* and one of the major tree species in the Eurasian boreal forests, Norway spruce (*Picea abies*) is highly vulnerable to rising temperatures. For instance, unprecedented Norway spruce mortality occurred from 1970 to 2010 due to frequent drought events and heatwaves, which resulted in an average annual loss of up to 14 million m^3 per annum, especially 118 million m^3 in 2019 alone (Ebner 2020). Furthermore, its primary pest, *I. typographus* has benefited from warming temperatures, which resulted in accelerated and extended brood development and early swarming (Hlásny et al. 2011; Netherer et al. 2014; Hinze and John 2020).

Management of bark beetles is driven by two primary objectives: prevention and containment (Wermelinger 2004). Prevention strategies aim to keep the beetle populations at low densities by implementing measures such as removing infested trees, employing traps, and clearing fallen trees. Containment involves salvage and sanitation logging in outbreak areas or nearby areas to halt or slow the spread of outbreaks. However, the current management strategies are still inadequate, and their effectiveness has been frequently questioned (Dobor et al. 2020). Consequently, exploring innovative strategies for bark beetle management is imperative, leveraging techniques emerging from functional genomics. Methods such as RNA interference (RNAi) and CRISPR/Cas9 are rapidly advancing as pest control tools, with their effectiveness proven in controlling agricultural pests, such as *Spodoptera* and the Colorado potato beetle (Gui et al. 2020; Mezzetti et al. 2020; Vatanparast and Park 2022). Recent reports demonstrate the presence of core machinery genes that can be utilized for gene silencing for *Ips* management using molecular tools (Powell et al. 2021; Joga et al. 2021). However, previous reports have not highlighted or addressed the applicability of these techniques. This review emphasizes the future usability and efficiency of such techniques in *Ips* bark beetle management. Unlike many conventional pest management approaches, these molecular methods are tailored to target specific species. Nevertheless, they require further exploration and functional validation for *I. typographus* management.

Despite a century of extensive research on bark beetles, there are several gaps in our understanding of the main drivers of bark beetle outbreak dynamics and the strategies for their management. Moreover, the recent advancements in the bark beetle management techniques are poorly elaborated. Here, we aim to provide a brief overview of the ecology and population dynamics of *I. typographus* and discuss the major drivers of outbreaks in the context of climate change. This overview focuses on *I. typographus*, but we also discuss other eruptive bark beetle species. We preliminarily examine how changes in a forest structure affect the initial host colonization and spread, how droughts and water stress affect host tree physiology and vigour, and how temperature regimes affect bark beetle activity. We analysed the present management approaches and their efficacy in protecting the forests and suggested cutting-edge molecular-based approaches to face new challenges that could be used to guide future forest management practices in the Anthropocene.

2 Eurasian spruce bark beetle ecology

Ips typographus is a relatively small insect (4.5–5 mm) and exhibits sexual dimorphism. They are univoltine (*producing one generation per year*) but may shift to polyvoltine (*more than two generations per year*) due to the influence of warm temperatures, resulting in accelerated development (Hlásny et al. 2011). During the endemic phase, the beetles infest mature trees that are stressed and weakened due to various biotic or abiotic factors (e.g., high temperature, drought, disease, herbivory). The beetles are associated with a variety of microbial symbionts, including bacteria and ophiostomoid fungi, that play a significant role in exhausting tree defenses (Lieutier et al. 2009; Zhao et al. 2019b; Chakraborty et al. 2023). After overwintering, a new generation of beetles emerge from the litter or Norway spruce trunks and disperses between 100 m to tens of kilometers while searching for a suitable host tree (Biedermann et al. 2019; Fig. 1A). Host tree-bark beetle interaction is generally considered multifaceted, and various factors across different spatial and temporal scales shape their interactions (Jakuš et al. 2011; Kautz et al. 2011; Netherer et al. 2024). Furthermore, *Ips typographus* shows density-dependent host colonization behaviour. Beetle populations in the endemic phase rely on the availability of windthrown, dead, or weakened trees; in contrast, in the epidemic phase, they colonize healthy trees (Økland and Berryman 2004; Økland and Bjørnstad 2006). We will review factors in the context of the population dynamics of *I. typographus*.

2.1 Stand-level dynamics

At the stand level, once the pioneer male beetles overcome the host defenses, they produce aggregation pheromones, 2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (cV) (Keeling et al. 2021; Ramakrishnan et al. 2022; Fig. 1B.**a**). Male beetles produce high amounts of MB when initiating the nuptial chamber, while the production of cV changes depending on the amount and enantiomeric composition of α-pinene in Norway spruce phloem (Birgersson et al. 1984; Fig. 1B.b). Emission of these aggregation pheromones from trees under attack brings additional male and female beetles to the foci trees. Arriving male and female conspecifics release more of the same pheromones to coordinate mass attacks on the host (Blomquist et al. 2010). Upon entering the host, adult beetles create mating chambers, mate, and excavate oviposition galleries where eggs are deposited. Eggs hatch into larvae, feed, complete their development under bark, and exit from the parental trees. Depending on the host characteristics, such as phloem thickness, *I. typographus* can increase its population size by up to 15-fold from the preceding generation (Hlásny et al. 2019).

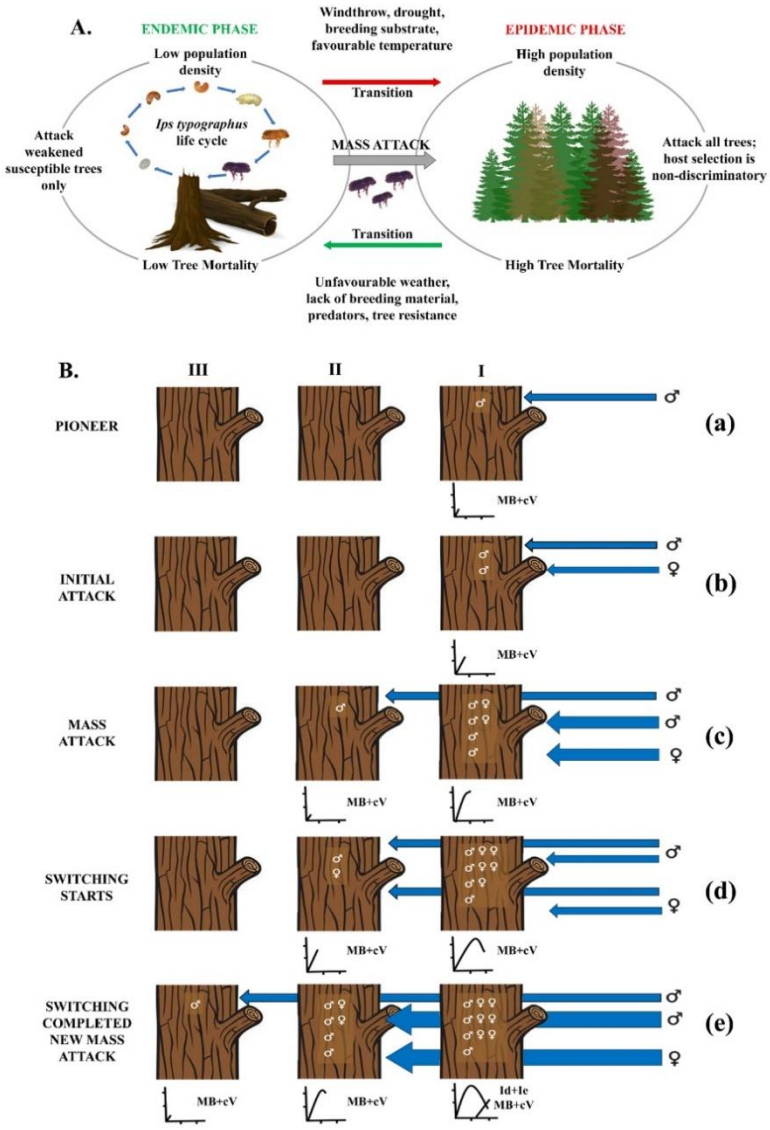
Once the foci host trees are fully occupied, the production of anti-attractant compounds such as verbenone starts to avoid overcrowding (Xu et al. 2015) while the production of aggregation pheromones declines. This increases attraction towards nearby uninfested trees, initiating a new wave of mass attacks (Fig. 1B.c, 1B.d). This event marks the completion of "switching" of bark beetle attacks from heavily colonized trees to neighbouring uncolonized trees (Schlyter et al. 1987; Fig. 1B.e). This process may result in several mass-attacked trees at the landscape level.

2.2 Landscape-level dynamics

The spatial distribution of mass-attacked trees, the host availability, and environmental factors can influence the initiation and spread of bark beetle infestations. However, the effects of these factors vary between endemic and epidemic phases of *I. typographus* (Mezei et al. 2014a; Potterf et al. 2019).

2.2.1 Endemic phase

When bark beetle population densities are low, they are primarily associated with stressed or defensively compromised trees (Raffa et al. 2008; Boone et al. 2011; Biedermann et al. 2019). Factors such as tree resistance, stand structure, abundance of natural enemies and competitors, and weather can potentially limit the beetle's population size at the endemic phase, whereas natural disturbances and climatic events can disrupt this equilibrium by reducing tree resistance and hence increasing the beetle population (Raffa et al. 2008; Ruel et al. 2023). The capacity of bark beetle populations to cross the epidemic threshold and move from the endemic to the outbreak phase depends critically on the availability of suitable breeding materials, such



as susceptible host trees, stumps, and windblown trees (Hroššo et al. 2020). Weather events like extensive wind damage or intense droughts can provide ample breeding grounds for population build-up. As the population has built up and depleted the available substrate, they begin to attack healthier trees (Biedermann et al. 2019).

2.2.2 Epidemic (outbreak) phase

Once the beetle population reaches the epidemic phase, the discriminatory behaviour in host selection becomes less adaptive because there is a greater possibility of recruiting sufficient numbers of conspecifics to overcome the defenses of large healthy trees, which tend to have greater resources

◂**Fig. 1** Scheme of *Ips typographus* population dynamics. (**A.**) In the endemic phase, beetles feed and reproduce in windthrown trees, trunks, and stumps and later move to the epidemic phase under favourable conditions. There are several factors, such as favourable weather, availability of breeding substrate, droughts, and windthrows, that influence the transition of the beetle populations to the epidemic phase. In the epidemic phase, the beetle population is high enough to mass attack healthy trees and cause widespread mortality. Later, certain factors such as unfavourable weather, exhaustion of the breeding substrate, abundance of predators, and increased tree resistance of surviving trees can cause high beetle mortality and limit the population to the endemic phase (Adapted from Kautz et al. 2014). (**B.**) **a.** The initial host colonization stage is usually initiated by pioneer beetles, males in *Ips* and females in *Dendroctonus*. In *Ips typographus*, aggregation pheromones, 2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (cV) are released to attract additional male and female beetles. **b.** If the pioneering beetle survives host tree defenses, with the arrival of additional beetles, they produce larger amounts of aggregation pheromones which usually ends the mass colonization of host trees. The goal of mass colonization is to exhaust tree defense to secure reproduction. **c.** During the mass colonization stage, while the initial tree (I) is mass colonized, a small portion of male beetles land on a neighbouring uncolonized tree (II) and starts a new colonization sequence. **d.** As the production of attractive pheromone components diminishes, the attraction towards the initial tree declines, while simultaneously, attraction towards the neighbouring tree increases, starting a switch in attraction. **e.** When the initial *"switching"* process is completed, only small amounts of aggregation pheromones are produced, while larger amounts of inhibitory compounds (ipsdienol & ipsenol) are continuously produced. The attraction is now shifted to the neighbouring tree (II), where a new mass attack starts, with a small proportion of males once more being deflected and ending up on the new uncolonized neighbouring tree (III). The small graphs represent log pheromone release from Birgersson et al. (1984). The marks on the x-axis represent the attack phases 1, 3 and 6, where (1) males are bored in bark, (3) have completed nuptial chamber formation, and (6) are joined with females. (Adapted from Schlyter et al. 1987)

(i.e., thicker phloem) to support substantial beetle populations, resulting in higher number of broods (Raffa et al. 2008). The factors governing the bark beetle outbreaks also change as the infestation progresses (Walter and Platt 2013).

In the early stages of the outbreak, beetles still infest susceptible trees with weakened defenses (Raffa et al. 2008), with most beetles targeting windthrown trees. As a result, the beetle infestation is still relatively small at this stage, and tree mortality is primarily attributed to windthrow events (Potterf et al. 2019). However, the infestation of standing trees intensifies up to three years after a major windthrow event and enters a patch-driven outbreak phase, signified by the establishment of many infestation spots, benefiting from a large pool of available healthy host trees (Økland et al. 2016; Potterf et al. 2019). Still, the rate of infestation spread is minimal, and the distance between new and old infestation sites is maximum (Jakuš et al. 2002). These spots typically emerge close to windthrow areas within previously unaffected stands, appearing as small, rounded patches (Potterf et al. 2019).

As the epidemic spreads across the landscape, beetle pressure becomes the most crucial determinant of tree mortality because the population size of bark beetles is large enough to mass attack healthy trees and overcome their defenses (Walter and Platt 2013; Mezei et al. 2014b). The outbreak emerges within one to three years following a major disturbance event (Økland and Berryman 2004). Although healthy trees can withstand a certain number of attacks, once a certain threshold is surpassed, such a tree cannot repel the attackers (Krokene 2015). As a result of the coalescence of nearby infestation spots, the distance between freshly and previously infested locations is smaller during the epidemic phase than the endemic phase (Kautz et al. 2011; Potterf et al. 2019). As each successive generation of bark beetles exhausts resources needed by subsequent generations (Raffa et al. 2008), higher population pressure further facilitates the initiation and spread of bark beetle infestations at higher elevations (Jakuš et al. 2002) and other neighbouring less affected areas.

2.2.3 Epidemic to the endemic phase transition

In the later phase of the outbreak, as the significant portion of available host trees has already been depleted, bark beetles target marginal host trees in less favourable habitats. This phase is characterized by relatively lower densities of beetle populations than the earlier phases of the outbreak, and the beetle numbers are too low to kill healthy trees. Surviving trees are likely to fend off attacking beetles due to their anatomical and chemical defenses (Nelson et al. 2007; Raffa et al. 2008; Erbilgin et al. 2017; Zhao and Erbilgin 2019; Zhao et al. 2019a). In the post-epidemic phase, while the number of new bark beetle infestation sites decreases, the expansion of existing infestation spots reaches its maximum, with the beetles more likely to target less suitable resources

neighbouring old spots due to limited available resources.

The mechanisms behind the decline in bark beetle populations in the later stage of an outbreak are not well understood. However, when the host tree supply is depleted, the beetle population decreases significantly (Walter and Platt 2013). Additionally, adverse weather conditions, pathogens, and natural enemies can significantly increase beetle mortality, ultimately limiting their population to the endemic phase (Boone et al. 2011; Wegensteiner et al. 2015).

3 Effects of forest stand structure on population dynamics of *I. typographus*

The structure of the forest stand (e.g., stand density and age classes) strongly influences bark beetle outbreak (Fettig et al. 2007; Sproull et al. 2015). Windthrow alters stand structure as a primary form of disturbance by creating openings in the forest canopy (Marini et al. 2017). Promoting forest diversity (discussed in Sect. 6.2) and thinning are the highly recommended practices to reduce overall vulnerability and have been effective against *Dendroctonus ponderosae, D. frontalis,* and *D. brevicomis* (Egan et al. 2010; Fettig and McKelvey 2010; Zhang et al. 2013; Hood et al. 2016).

Within outbreak stands, foresters employ intensive measures like sanitation felling and salvage logging to reduce damage, including cutting and removing infested or dead trees (Stadelmann et al. 2013). However, intensive management activities such as sanitation can further amplify the destabilization of the stand. For example, intense sanitary interventions in outbreak-impacted stands could cause the fragmentation of forest stands by creating canopy openings and numerous margins or edges (Hanson and Lorimer 2007; Zabihi et al. 2021; Özçelik et al. 2022; Singh et al. 2023). Notably, fragmented forests exhibit increased susceptibility to wind damage and render freshly formed forest edges to bark beetle infestation (Kautz et al. 2013; Marešová et al. 2020). This susceptibility is likely due to sudden changes in microclimatic conditions, such as increased solar radiation and temperature, reduced humidity, increased vapour pressure demands, and greater wind exposure (Hanson and Lorimer 2007; Herbst et al. 2007; Marešová et al. 2020; Özçelik et al. 2022). The resulting changes may further facilitate increased bark beetle generations and temporal scale of infestation and increase the damage risk.

Likewise, highly dense plantations, often driven by commercial goals, exhibit poor growth and resilience to stress. Increased tree density can increase intraspecific competition among individual trees and constrain resource availability (Zabihi et al. 2023; Thomas et al. 2024). Research indicates that increased competition within a forest stand can exacerbate the impacts of drought and increase the likelihood of mortality, particularly if water is the limiting resource (Zhang et al. 2015; Young et al. 2017; Korolyova et al. 2022). Consequently, constraints on resource availability can influence many plant functions, such as resin exudation, photosynthesis, and subsequent biosynthesis of carbon-dependent defense metabolites (Bazzaz et al. 1987; Netherer et al. 2014; Erbilgin et al. 2021), and predispose them to bark beetle attacks (Netherer et al. 2019).

Practices like thinning can enhance forest resilience by providing growing space to individual trees (Fettig et al. 2007; Hood et al. 2016; Knapp et al. 2021) but its impact on the dynamics of *I. typographus* remains unknown. Forest structures vary across elevation, slope, and aspect gradients, with elevation playing a crucial role in tree mortality during all phases of *I. typographus* infestation (Mezei et al. 2014a; Sproull et al. 2015). For example, trees tend to be more densely packed at lower elevations within a limited altitude range (Mazón et al. 2020; Zabihi et al. 2023). This difference in tree density due to elevation also affects tree characteristics like bark temperature and diameter (Fig. 2; Zabihi et al. 2023) which are important predictors of host susceptibility to bark beetles. Since topography and inter-tree spacing significantly modulate ecosystem functions, forest management strategies should be tailored considering these variables to foster improved overall health and resilience of secondary spruce forests (Zabihi et al. 2023; Thomas et al. 2024).

4 Impact of drought stress on population dynamics of *I. typographus*

The intense and recurring drought events compromise the host tree defense mechanisms, rendering them more susceptible to infestation by bark beetles

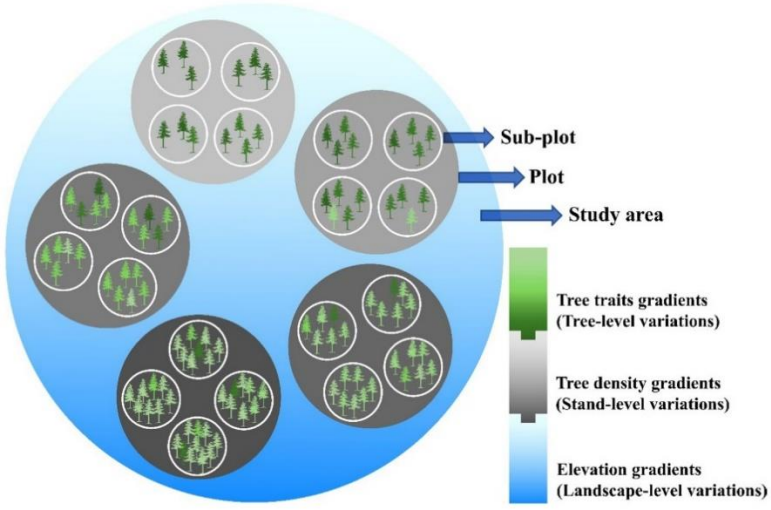


Fig. 2 A representation showing how variations in landscape, stand, and tree characteristics interact at different scales of elevation, tree density, and tree traits. The five circles in the diagram represent tree density gradients, with darker grey indicating higher density and lighter grey indicating lower density. The blue circle represents changes in elevation, with darker blue indicating higher altitude and lighter blue indicating lower altitude. The individual tree traits, such as bark temperature and diameter at breast height, shown in green color gradients, were found to be positively and negatively related to tree density, respectively. (Adapted from Zabihi et al. 2023)

(Berryman 1972; Christiansen et al. 1987; Erbilgin et al. 2021). Research indicates that severe drought events limit the production of defense metabolites, unlike moderate drought, which is hypothesized to limit tree growth but enhance carbon allocation to defenses (Desprez-Loustau et al. 2006; Ferrenberg et al. 2015). Drought stress occurs when the available water in the soil falls below a specific threshold, leading to decreased soil water content and increased hydraulic resistance at the root-soil interface. Water transfer through xylem tissues may become irreversibly disrupted due to water cohesion breakdown and vessel embolism, increasing the risk of premature mortality of roots and twigs (Cruiziat et al. 2002; Cochard et al. 2009).

Isohydric conifer species avoid or delay the decline in xylem water potentials by restricting transpiration through stomatal closure (Rothe et al. 2002; Schume et al. 2004). Although stomatal regulation helps in maintaining water potential in the xylem, reduction in sap flow and leaf-atmosphere gas exchange declines the photosynthetic product assimilation regardless of continued respiratory consumption of carbon (Bréda et al. 2006; Rennenberg et al. 2006). Eventually, stored carbon reserves (i.e., non-structural carbohydrates) are depleted by repeated bark beetle attacks, and due to no or low replenishment as trees cannot sustain the production of defense metabolites (Erbilgin et al. 2021). At the same time, while tighter stomatal regulation can help avoid a decline in lethal xylem water potentials, the tension in the water conduits may cause shrinkage in tracheid diameters. This, together with a decrease in turgor pressure inside the epithelial cells lining the resin ducts, reduces the physical pressure on the oleoresin and subsequently reduces the exudation rate upon wounding of the bark, which may compromise the tree's defense against frost, another drought episode and pest attacks, ultimately leading to tree mortality (Cruiziat et al. 2002; Bréda et al. 2006; Cochard et al. 2009; Rissanen et al. 2016).

Despite the well-known negative impacts of drought events on tree physiology and inherent defensive mechanisms, there is no conclusive evidence connecting drought-induced physiological stress and the tree's attractiveness to bark beetles

(Netherer et al. 2014). The Rosalia Roof experiment on drought manipulation addresses the issue of resource distribution in Norway spruce, focusing mainly on secondary metabolism (Netherer et al. 2014, 2019; Matthews et al. 2018). The project aimed to determine the impact of drought stress on both inherent and triggered defense mechanisms of trees against bark beetle attacks, including factors like resin flow and hypersensitive reactions to blue stain fungus inoculation. Furthermore, a recent field study has shown that when bark beetles infest drought-stressed trees, it not only decreases the local availability of carbohydrates crucial for essential tree functions but also hinders the tree's capacity to replenish carbohydrate reserves (Erbilgin et al. 2021). Nevertheless, further research is needed to determine whether there is a difference in the attractiveness of stressed and control trees and validate the hypothesis that susceptible host trees are more attractive to pioneer beetles (Wermelinger 2004; Netherer et al. 2014).

5 Changing temperature regimes and their impact on population dynamics of *I. typographus*

Bark beetle outbreaks have been correlated with shifts in temperature and precipitation regimes as host tree vigor is affected by warmer spring and summer temperatures combined with increased water stress (Powell and Logan 2005; Berg et al. 2006). Bark beetle population growth and survival depend on thermal conditions as it can shorten development time and increase the number of generations per year (Bentz et al. 2010; Marini et al. 2012). Moreover, winter mortality is another critical component in bark beetle population dynamics (Hinze and John 2020). While the cold temperature adaptations and cold hardening mechanisms of many bark beetle species remain relatively unexplored, some *Ips* and *Dendroctonus* beetles stand out for their ability to accumulate cryoprotectant compounds (e.g., glycerol) during the colder periods of autumn, resulting in the decreased mortality (Lombardero et al. 2000; Bentz et al. 2010; Koštál et al. 2011). Most bark beetle species have symbiotic relationships with microorganisms like blue-stain fungi and bacteria that increase their tolerance to cold temperatures and provide nutrients to the larvae (Bleiker and Six 2007; Ayres et al. 2000; Guevara-Rozo et al. 2020).

The ambient temperature also influences the flight activities of bark beetles. The optimal flying activity occurs from 22 to 26 °C, while *I. typographus* do not swarm below 16.5 °C (Hinze and John 2020). Swarming of beetles in search of suitable breeding material is affected by two factors: the emergence from overwintering, which can be anticipated by thermal sum, and the mass flight of beetles, which occurs at a temperature above 20 °C (Annila 1969; Wermelinger 2004). A recent study showed that most *I. typographus* were caught on the hottest day (maximum temperature of 33.4 °C) of the observation period (mean air temperature 19.2 °C), suggesting that its ability to find hosts and mass flight is not compromised by increased thermal conditions (Hinze and John 2020). Furthermore, the average flight distance of *I. typographus* increases significantly on days with moderate temperatures than cold temperatures (Wermelinger 2004; Hinze and John 2020). Due to the rapid genetic adaptation of insects to seasonal changes in temperature regimes, range expansion of bark beetles beyond their habitat has also been observed where species move into new niches facilitated by increasing temperature (Balanyá et al. 2006; Battisti et al. 2006; Bradshaw and Holzapfel 2006; Nealis and Peter 2008; Erbilgin et al. 2014; Erbilgin 2019). Bark beetle dispersal-related ecological and environmental factors are reviewed in detail by Jones et al. (2019).

Regional-scale flight activity periods of *I. typographus* have been established by analysing climate data, focusing on temperature parameters. For instance, in southern Sweden, the onset of spring flight among *I. typographus* occurred when the accumulated thermal sum, averaging above 5 °C, persisted for roughly 47 ± 24 days (Öhrn et al. 2014). This period aligns with the findings from Denmark (Harding and Ravn 1983) and southern Finland (Annila 1969; Öhrn et al. 2014), where a similar flight duration of 45 days was observed. The flight period in Denmark lasted from early May to mid-August, whereas, in central Europe, it occurred between April and September (Faccoli and Stergulc 2004; Baier et al. 2007; Öhrn et al. 2014). In the region of southern Sweden, the flight activity period of *I. typographus* was observed to take place from mid-April to mid-August, which can be attributed

to the effects of climate change resulting in warmer spring and summer temperatures compared to three decades ago (Öhrn 2012; Öhrn et al. 2014). In central European forests, sister broods of *I. typographus* with a minimum of two subsequent generations have been documented during the summer (June to August). Consequently, these temperature conditions have led to an increased frequency and extension of the flight activity periods of bark beetles (Netherer et al. 2019).

6 The current and potential future management practices

Bark beetle management strategies involve a range of approaches and typically start with removing windthrown and trees recently infested by *Ips typographus*. Anti-attractants or trap trees are commonly used to control beetle populations, whereas methods like remote sensing and canine detection are employed for early detection and mitigating attack damage. Furthermore, modern molecular tools are being investigated for controlling forest pests. In this section, we will discuss the traditionally adopted measures (salvage and sanitation harvesting, pheromone or trap-tree, short rotation forestry), progressive-practical methods (remote sensing, detection dogs) and explore promising cutting-edge molecular methods (Sterile insect technique (SIT), clustered regulatory interspaced short palindromic repeats/cas9 (CRISPR/Cas9) and RNA interference (RNAi)).

6.1 Reduction of rotation periods

Norway spruce forests often have rotation periods exceeding 100 years in many regions of Europe, resulting in extensive forest areas that are particularly prone to wind and bark beetle disturbances. For example, in Slovakia, between 1998 and 2009, less than a quarter of Norway spruce could reach 100 years of age (Hlásny et al. 2017). Because of these vulnerabilities, reducing the duration of rotation can be an effective strategy for forests to adapt to the growing bark beetle pressures (Zimová et al. 2020). Given that the optimal rotation period varies depending on the forest management system, site productivity, and species mixture (Hlásny et al. 2017), forest managers should consider the length of the current rotation period based on regional conditions. However, reducing the rotation period may also result in a decline in biodiversity and the amount of carbon stored in forests.

6.2 Optimizing forest composition to enhance resilience against bark beetles

Due to rapid population and economic growth, forests are under increasing pressure to meet demands for wood and associated products (Liu et al. 2018). Consequently, extensive areas across the globe are being cleared and transformed into plantation forests to cater to this demand (West 2014). Plantation forests vary in their intended purposes, and different species are chosen accordingly. Monocultures emerged in Europe during the eighteenth and nineteenth centuries as a response to timber scarcity, aiming to achieve high quality products (Griess and Knoke 2011). For instance, Norway spruce trees were extensively harvested in most parts of Europe due to their rapid growth rate and favourable timber characteristics (Spiecker 2000; Caudullo et al. 2016). However, frequent drought events are threatening the harvesting plantations. For instance, the significant increase in spruce mortality in Europe from 1970 to 2019 was partly attributed to the frequent drought events and heatwaves, with alterations in the structure and composition of forests accounting for approximately half of the documented increase in bark beetle outbreaks (Seidl et al. 2011). Furthermore, ecosystem services such as carbon sequestration, water regulation, and habitat provision are compromised in monocultures (Barrette et al. 2023) because a higher number of species with diverse functional traits can collectively contribute to a wide range of ecological functions (Lefcheck et al. 2015). Apart from the deliberate cultivation of Norway spruce beyond its native range, the augmentation of growing stocks and alterations in age-class distributions have significantly enhanced the susceptibility of spruce forests to *Ips typographus* attacks (Seidl et al. 2011; Hlásny et al. 2021).

In response to diebacks of monoculture forests, increasing tree diversity and age classes has been tested and recommended to increase forest resilience against biotic and abiotic disturbances (Lohbeck et al. 2016; Singh et al. 2023). In fact, there is a large number of empirical evidence suggesting

that planting multiple species can provide several environmental, economic, and social benefits, and careful selection of species in mixed plantations can facilitate the promotion of complementary structural and functional traits (Hartley 2002; Forrester et al. 2005; Pawson et al. 2013; Carnol et al. 2014; Alem et al. 2015; Drössler et al. 2015). Thus, adopting mixed plantations as a strategy is more favourable than monocultures (Zhang et al. 2022). For instance, Dedrick et al. (2007) evaluated risks for several forest types and found that monoculture Norway spruce forests were more susceptible to biotic and abiotic disturbances than mixed-species forests. Including trees with varying age classes in mixed forests offers the potential for improved canopy coverage, leading to reduced solar radiation reaching the forest floor, which may minimize ground-level evaporation (Singh et al. 2023).

The argument that fostering tree species diversity lowers the risk of bark beetle infestation (Klapwijk et al. 2016) can be attributed to two main factors: Firstly, as discussed, mixed forests provide greater resilience against windthrow and storm damage; both of which are crucial drivers of the initiation of bark beetle outbreaks. In addition, an abundance of diverse tree species fosters various natural enemies of bark beetles including predators, pathogens, and parasitoids (Jactel and Brockerhoff 2007; Klapwijk and Björkman 2018; Stemmelen et al. 2022) suppressing the bark beetle population. Secondly, bark beetles rely on several cues for host selection, including olfactory, gustatory, and visual (Campbell and Borden 2006), and these cues help beetles to discriminate, for instance, defensively compromised trees (Rodriguez and Redman 2008; Schiebe et al. 2019). However, such cues may be masked by diverse volatile emissions from non-host trees in mixed forests during the host selection (Schiebe et al. 2011). Furthermore, tree diversity benefits the host tree species preferred by bark beetles; as such trees benefit from being hidden among non-host trees in the stand (Berthelot et al. 2021). However, tree species richness only works to suppress bark beetle outbreaks and not to avoid them, and even when the proportion of host plants is below 40% in the stand (de Groot et al. 2023). These facts demonstrate the importance of species richness in the context of bark beetle infestation. Yet, more evidence from field studies involving choice bioassays is needed to determine the effects of non-host volatiles on beetle infestation dynamics.

6.3 Early detection of infested trees, monitoring, and mass trapping

6.3.1 Detection dogs

Successfully implementing a management strategy that relies on rapid detection of bark beetle infestations and removing recently infested trees is necessary for forest protection. However, human detection generally requires close inspection (≤ 1 m) of trees and is therefore time-consuming, costly, and not always possible (Svensson 2007). Thus, aerial detection of infested trees generally occurs 2–3 months after an infestation, when tree crown colour fades, and bark falls off. At this point, the majority of bark beetles have already left infested trees and might target other uninfested trees. Using trained detection dogs has proven an effective alternative to locating infested trees (Johansson et al. 2019). The main advantages of using trained detection dogs in finding infested trees are their incredible sense of smell and capacity to explore large areas quickly (Hepper and Wells 2015; Mosconi et al. 2017). Vošvrdová et al. (2023) reported that trained sniffer dogs can locate infested trees up to 150 m away. Thus, such dogs can extend the time window for finding and removing infested trees, potentially avoiding the growth of larger infestations. As a limitation, detection dogs cannot be used for larger and remote areas.

6.3.2 Pheromone traps

Various trapping techniques have been widely used in bark beetle management in addition to sanitation felling and removal of windthrown trees. The rationale for using pheromone traps is to monitor beetle populations or reduce beetle numbers below the outbreak density by mass-trapping. The approach uses pheromone trap barriers, which aim to reduce the bark beetle population to a level where trees can successfully defend themselves against attacks (Jakuš 1998, 2001). Trapping techniques include using trap trees or log traps baited with species-specific pheromones or host compounds that may attract beetles. Once attracted, beetles can be killed

with pesticides or by removing the infested trees or logs. Limited scientific evidence supports the efficacy of using trap trees to effectively reduce beetle populations or the number of attacked trees (Klutsch et al. 2017). However, there is a shortage of research on the efficacy of trapping techniques in reducing the risks of outbreaks and rates of damage, particularly for large-scale applications. While pheromone traps typically capture only 3–10% of beetle populations at a relatively high cost (Wermelinger 2004), using pheromone trap barriers as part of the integrated forest protection system makes it possible to reduce tree mortality significantly (Jakuš 1998, 2001). Due to relatively high labour costs, using pheromone traps has been discontinued in Scandinavia and most parts of Germany and France. The use of pheromone traps for mass trapping of bark beetles is further constrained by the "spillover effect," where commercial pheromones attract more beetles than the traps could handle, causing attacks in trees adjacent to the traps (Niemeyer 1997; Jakuš et al. 2022). Nevertheless, the primary purpose of such traps should continue to monitor the bark beetle population rather than population reduction and outbreak suppression. Further improvement in using pheromone trap barriers is possible with the new mixtures of attractants (Blaženec et al. 2021; Jirošová et al. 2022).

6.3.3 Anti-attractants

Anti-attractants are semiochemicals used to disrupt the host-finding behavior of pests. By emitting repelling signals to pests or masking the attractive signals emitted by potential hosts, anti-attractants can protect trees from being located and colonized by these pests. Among the range of active anti-attractant compounds identified for *I. typographus* (Schiebe et al. 2011), the first notable one is verbenone, generated from the host compound α-pinene or converted from *I. typographus*'s main pheromone component *cis*-verbenol (Birgersson and Leufvén 1988). The second group comprises non-host volatiles (NHVs): *trans*-conophthorin, an important synergistic compound found in the bark of broad-leaf trees (Zhang and Schlyter 2003); green leaf volatiles (GLV; 1-hexanol; (Z)-3-hexen-1- ol; I-2-hexen-1-ol), detected in non-host birch (*Betula* spp.) and aspen (*Populus tremula*) (Zhang et al. 1999); and C8 alcohols (3-octanol; 1-octen-3-ol), emitted from the bark of the mentioned species. Attempts to protect logs or fallen trees with anti-attractants have not been successful in stopping bark beetle attacks, including *I. typographus* (Jakuš and Blaženec 2003).

Currently, the most promising methods for defending standing spruce trees against *I. typographus* attacks involve dispensers with a blend of verbenone and NHV compounds (Schiebe et al. 2019). A recent study reported a strong "switching effect," of using anti-attractants, which involves pushing away beetles from areas with anti-attractants into areas without non-attractants (Jakuš et al. 2022). Furthermore, installing anti-attractant dispensers at two different heights on trees showed no difference, suggesting that using anti-attractants is ineffective in areas affected by severe drought and extremely high bark beetle populations (Jakuš et al. 2022). Unusually, (+)-*trans*-4-thujanol repelled female *I. typographus*, demonstrating its efficacy to be on par with known anti-attractants such as 1,8-cineole and verbenone, making it an innovative anti-attractant for forest protection (Jirošová et al. 2022). A recent meta-analysis by Afzal et al. (2024) on the push–pull strategy, which involves using attractive and repellent semiochemicals, reports that this strategy can reduce *Ips* and *Dendroctonus* populations by 66% and 54%, respectively, compared to the control.

6.3.4 Remote sensing (RS) of forests

Gathering precise and current spatial information on the presence and dynamics of the bark beetle infestation is still challenging in large areas with limited access (Stereńczak et al. 2019). Recently, several research teams in Europe and North America have focused on studying the spatio-temporal analyses of bark beetle population dynamics (Simard et al. 2012; Kärvemo et al. 2014; Meddens and Hicke 2014; Senf et al. 2015; Havašová et al. 2017; Mezei et al. 2017; Marvasti-Zadeh et al. 2024). Forest managers will likely need to face widespread and frequent infestations of bark beetles in the near future, necessitating the need to develop efficient tools that can assess the current spread and dynamics of insect outbreaks in a given area rapidly and precisely. Precise and up-to-date spatial information on bark beetle outbreaks (e.g., locations of dead trees) is essential when planning protective and sanitary

actions (e.g., pheromone or tree trapping) to control or limit the intensity of an outbreak in a given area (Fassnacht et al. 2014; Fettig and Hilszczański 2015). The ability to identify and map tree mortality caused by bark beetle outbreaks depends mainly on the forest structure. For example, detecting and monitoring forest stands that consist solely of coniferous host species is relatively straightforward. New dead trees in such stands mostly appear in large, easily detectable groups as bark beetles primarily attack trees adjacent to those already killed (Lausch et al. 2011; Seidel et al. 2016). Detection of individual-infested trees can change in complex forest structures consisting of mosaics of various stands with mixed tree species where the typical host tree species occur in smaller scattered groups or individual trees. Identifying individual infested trees in such a situation is a challenging task, and as financial resources typically limit fieldwork, RS methods are an effective alternative and supplement to field surveys. Multispectral aerial and satellite imagery, in combination with artificial intelligence and machine learning, has been successfully used for mapping insect outbreaks and other forest disturbances (Roberts et al. 2003; White et al. 2007; Long and Lawrence 2016; Senf et al. 2017; Marvasti-Zadeh et al. 2024). For instance, the significant spectral differences between the healthy and stressed trees (i.e., drought) were found on imageries of the Enhanced Vegetation Index (EVI) and Visible Atmospherically Resistant Index (VARI) at the beginning of the growing season before the new attacks. The results highlight the potential of using SVIs derived from high-resolution multispectral imagery to detect pest infestations early and manage forest ecosystems (Trubin et al. 2023, 2024).

We could rely on various RS data and sources to estimate tree health characteristics. Remote sensing generally uses satellite- or aircraft-based (e.g., Unmanned Aerial Vehicle) sensor technologies. In forestry, especially in the case of examination of the spectral properties of trees, we can use active (such as LIDAR) and passive sensors (such as Muti- and Hyperspectral sensors) (Niemann et al. 2015). The most recent methods in using, processing, and analysing RS in the context of tree predisposition determination combine sensor data and represent multi-temporal GIS analysis based on hyperspectral and airborne laser scanning data (Abdullah et al. 2019; Stereńczak et al. 2019). Hyperspectral and airborne laser scanning data are widely used to identify prominent forest disturbances in the bark beetle outbreak sites and dead trees in the red and grey attack stages. The change in foliage colour in trees affected by infestation becomes noticeable after approximately 6 to 8 months from the onset of the infestation. This transformation in the crown of infested trees from green to yellow to red is due to the gradual loss of moisture and the deterioration of pigments within the foliage. These distinct stages in the colour change are referred to as the "red-attack" and "grey-attack" phases, respectively (Safranyik and Carroll 2007). Analysis of red and grey attack data allows us to understand the dynamics of the bark beetle outbreak, and the accumulation of additional data, such as climatic (temperature, wind speed, precipitation), will allow us to understand the most critical drivers of the outbreak.

The possibility of identifying all attacked trees is limited by the output data available for analysis from visible dominant trees in the first forest layer. Another associated challenge is incorrectly identifying dead trees as living due to reflection from the lower layer (Stereńczak et al. 2019). Similarly, early detection of trees attacked by bark beetles depends on the availability of ground truth data (Nardi et al. 2023; Trubin 2024; Kautz et al. 2024). However, acquiring ground truth data is often labour-intensive (Zabihi et al. 2021) and only limited to areas accessible by the field crew. Additionally, early attacked trees are challenging to identify using remote sensing when there are stressed trees around and due to changes in the foliar colour of spruce trees at the beginning of the season. Another limitation relates to the limited computational power for analysing large-scale data.

6.4 Biological control agents

6.4.1 Natural enemies

Biological control involves suppressing a pest by introducing a naturally occurring antagonist to achieve sustainable and eco-friendly control over insect pests (Kenis et al. 2017). Natural enemies such as predators, parasites and parasitoids impact bark beetles' population dynamics and ecology. The variety of antagonists that target bark beetles is extensive and includes the order Hymenoptera,

beetles (Coleoptera), flies (Diptera), true bugs (Heteroptera), snake flies (Raphidioptera), and mites (Acari) (Table 1).

Bark beetle predators are highly diverse, and can consume all life stages of bark beetles, from eggs to adults, and significantly influence their population dynamics. Although numerous predator species are associated with bark beetle galleries, only a few actively prey on eggs, larvae, pupae, and/or adults. Most predators targeting bark beetles belong to Coleoptera (beetles), including Cleridae, Trogossitidae, and Rhizophagidae (Hopping 1947; Mills 1985; Wermelinger 2002). The most notable coleopteran predators of bark beetles are the checkered beetles (Cleridae). This small family includes several early-season predatory species, such as *Thanasimus dubius* (F.) from North America and *Thanasimus formicarius* (L.) from Europe (Stephen and Dahlsten 1976; Herard and Mercadier 1996; Lawson et al. 1997). Clerid beetles arrive at the infested trees shortly after bark beetles and consume the beetles directly on the bark surface. *Thanasimus formicarius* is among the most studied predators of *I. typographus* (Kenis et al. 2007). Its females lay approximately 100 eggs, and adults can consume up to three beetles per day, while larvae can consume approximately 50 bark beetle larvae throughout their larval development stages (Mills 1985; Dippel et al. 1997). While clerid beetles are typically considered generalist predators, trogossitids are believed to have a more specialized approach (Kohnle and Vite 1984; Lawson and Morgan 1992). For instance, *Temnochila virescens* primarily responds to attractants produced by *Ips* species, and their larvae feed on bark beetle larvae and pupae within the tree's phloem, whereas adult *Temnochila* species consume the adults of various other bark beetles (Billings and Cameron 1984).

Furthermore, parasitoids can be an important part of the population dynamics of bark beetles. The parasitoids prefer a specific developmental stage of the host. Most parasitoids associated with Scolytids belong to the Hymenoptera order, including *Braconidae* and *Pteromalidae*. They can attack various developmental stages of bark beetles. Each parasitoid larva typically consumes one beetle larva or pupa. The most effective parasitoid of *I. typographus* is *Coeliodes bostrichorum,* and it appears to be exclusively associated with bark beetle species breeding in Norway spruce (Feicht 2006; Kenis et al. 2007).

Various organisms, including wasps, ants, birds, shrews, mites, and pathogens like viruses and microsporidia are potential antagonists of *I. typographus*, but their impact on population dynamics requires further understanding (Kenis et al. 2007). Muratoğlu et al. (2011) identified five pathogenic bacterial genera associated with *I. typographus* mortality, with *Serratia liquefaciens* showing notable effectiveness, causing 53.3% mortality. Commercially produced entomopathogenic microbes, including fungal species like *Beauveria bassiana, Metarhizium anisopliae*, *Hirsutella guignardii*, *Isaria farinosa,* and *Lecanicillium lecanii* have been utilized as microbial control agents against *Ips* and *Dendroctonus* (Kreutz et al. 2004; Popa et al. 2012; Lacey et al. 2015; Mann and Davis 2021; Rosana et al. 2021; Fernandez et al. 2023). Entomopathogens serve as effective pest controllers, efficiently managing insect populations while also being environmentally friendly towards non-target organisms (Hajek and Bauer 2009). However, despite their potential, challenges such as susceptibility to ultraviolet light, low moisture, temperature fluctuations, plant secondary metabolites, and competition with other microorganisms limit the widespread field application (Mann and Davis 2021).

While biological control strategies offer innovative and eco-friendly methods for bark beetle management, only a few biological control strategies against bark beetles have been implemented so far. The bark beetles are often attacked by polyphagous enemies, which typically have minimal impact on regulating the beetle populations (Kenis et al. 2007). In most cases, the lack of specificity of many natural enemies of bark beetles would make them unsuitable as biological controls, except the successful use of the specific predatory beetle *Rhizophagus grandis* to effectively control the great spruce bark beetle in areas like the Caucasus and Western Europe (Grégoire et al. 1992; Averbeke and Grégoire 1995). Apart from that, biological control programs also struggle to prove their financial benefits. Politically, this impedes government investment in biological control research and development and reduces academic interest in further exploration. At the management level, lack of engagement leads to skepticism about the effectiveness and financial advantages compared to pesticides (Barratt et al. 2018). Understanding

Table 1 Overview of Bark Beetle Management Approaches: A summary outlining key strategies employed for bark beetle management, highlighting traditionally used, progressive-practical, and cutting-edge molecular methods to mitigate infestations and promote forest health

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Resilience	Rotation period	Coniferous sp.	Shortening of the rotation period	De Groot et al. (2019), Zimová et al. (2020)
	Mixed plantations		Mixing diverse species	Fares et al. (2015), De Groot et al. (2019), De Groot et al. (2023)
Sanitation	Salvage		Harvesting of windthrown timber	Göthlin et al. (2000), Stadelmann et al. (2013)
	Sanitation felling		Removal of infested trees	Stadelmann et al. (2013), Seidel et al. (2016)
Monitoring/population control	Pheromone traps	*Ips* and *Dendroctonus* sp.	Use of pheromones to lure beetles	Jakuš (1998), Jakuš and Blaženec (2003), Faccoli and Stergulc (2004), Blaženec et al. (2021), Duduman et al. (2022), Jakuš et al. (2022)
	Tree traps		Use of standard tree traps with large diameters	Raty et al. (1995), Faccoli and Stergulc (2004), Lubojacký and Holuša (2014), Holuša et al. (2017)
	Anti attractants		Spruce forest protection with chemicals to achieve the anti-attractive effect	Zhang and Schlyter (2003), Schiebe et al. (2019), Jakuš et al. (2022), Jirošová et al. (2022), Jakuš et al. (2024)
Early Detection	Remote sensing	Coniferous sp.	Satellite-mediated detection of susceptible host tree/attack detection	Candotti et al. (2022), Dalponte et al. (2022), Safonova et al. (2022), Gao et al. (2023), Huo et al. (2023), Jamali et al. (2023), Trubin et al. (2023, 2024)
	Detection dogs	*Ips typographus*	Faster detection of bark beetle-infested trees	Johansson et al. (2019), Vošvrdová et al. (2023)

Table 1 (continued)

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Biological Control Agents	Coleopteran predators A. Cleridae 1. *Thanasimus* sp. 2. *Enoclerus* sp. B. Histeridae 3. *Plegaderus* sp. 4. *Platysoma* sp. C. Monotimidae 5. *Rhizophagus* sp. D. Staphylinidae 6. *Phloeonomus* sp. 7. *Phacophallus* sp. 8. *Quedius* sp. E. Tenebrionidae 9. *Corticeus* sp. F. Trogossitidae 10. *Tenebroides* sp. 11. *Temnochila* sp. G. Zopheridae 12. *Lasconotus* sp. 13. *Aulonium* sp.	*Ips* and *Dendroctonus* sp.	Natural enemies of *Ips* and *Dendroctonus* sp.	Otvos (1965), Billings and Cameron (1984), Kohnle and Vite (1984), Grégoire et al. (1985, 1992), Mills (1985), Lawson and Morgan (1992), Lawson et al. (1996, 1997), Dippel et al. (1997), Reeve (1997), Aukema et al. (2000a, 2000b), Wermelinger (2002), Erbilgin and Raffa (2001), Dahlsten et al. (2003), Aukema and Raffa (2005), Boone et al. (2008), Wermelinger et al. (2012, 2013), Martín et al. (2013)
	Non-Coleopteran Predators A. Diptera B. Heteroptera C. Acari			Dahlsten (1970), Moser and Roton (1971), Nicolai (1995), Ulrich (2004), Feicht (2004), Hedgren and Schroeder (2004), Hulcr et al. (2005), Moser (1975), Moser et al. (1978, 1989, 2005), Hofstetter et al. (2009), Sarikaya and Avci (2009), Wermelinger et al. (2012), Sousa et al. (2023)
	Parasitoids A. Hymenoptera *Braconidae* sp. *Pteromalidae* sp. *Coeliodes* sp. B. Microbial parasitoids *Bacillus* sp. *Acinetobacter* sp. *Proteus* sp. *Serratia* sp. *Beauveria* sp. *Metarhizium* sp. *Hirsutella* sp. *Isaria* sp.			Kreutz et al. (2004), Feicht (2006), Kenis et al. (2007), Wegensteiner (2007), Muratoğlu et al. (2011), Popa et al. (2012)

Table 1 (continued)

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Molecular techniques	Sterile insect technique (SIT)	*I. typographus*	Induce sterility in insects via irradiation	Turčáni and Vakula (2007), Čičková et al. (2018)
	RNA interference (RNAi)	Coleoptera (*Dendroctonus ponderosae, D. frontalis, Agrilus planipennis, Anoplophora glabripennis*)	mRNA degradation-mediated gene silencing	Rodrigues et al. (2017a, 2017b, 2018), Kyre and Rieske (2022), Kyre et al. (2019, 2020), Dhandapani et al. (2020a, 2020b)
	Clustered regulatory interspaced short palindromic repeats (CRISPR/cas9)	*Coleoptera	Gene editing tool	*Successfully tested and proven effective on agricultural beetles like Colorado potato beetles but not yet tested on forest beetles such as *Ips* and *Dendroctonus* species

ecological and environmental factors (i.e., bark beetle attack dynamics, predator population and their co-existences, the impact of changing climates on prey and predators, and the effect of other pesticide-based control measures on natural enemies) determining the efficiency of biological control methods against bark beetles need to be thoroughly investigated for successful incorporation of natural enemies in forest pest management programs.

6.4.2 Sterile insect technique (SIT)

The sterile insect technique involves dispersing a large number of sterile male insects into the population in a given area, with the expectation that these males mate with normal females, resulting in non-viable offsprings and lower the population densities of pests in the generation that follows (Lance and McInnis 2005; Diallo et al. 2019; Tam et al. 2023). Both sterile sexes can be released when it is impossible to distinguish between the sexes, making the procedure more successful while helping to keep the pest population below the epidemic threshold (Hendrichs et al. 2021; Ikegawa et al. 2021). The SIT relies on the irradiation of insects by gamma radiation from isotopic elements (cobalt-60 or cesium-137) as well as high-energy electrons and X-rays. To effectively sterilize the insect's reproductive cells while still keeping them alive enough to compete for mating, the radiation dose must be tightly managed because radiation can harm the sex cells of insects by causing chromosome fragmentation (dominant lethal mutation, translocations, and/or chromosomal aberrations), which results in the production of unbalanced gametes. Hence, mitosis is inhibited, and fertilized egg/embryo development is impaired (Bakri et al. 2021; Klassen and Vreysen 2021; Robinson 2021). The lethality of the dose–effect on two lepidopteran pests (*Ostrinia nubilalis* and *Phyllocnistis citrella*) indicates that higher doses of gamma radiation reduce the life span of these insects in both sexes. However, the larval emergence of the F1 was skewed towards males. The effectiveness of this technique is studied and confirmed in agricultural insect pests like sweet potato weevil (*Cylas formicarius*), pepper weevil (*Anthonomus eugenii*), and *I. typographus* (Turčáni and Vakula 2007; Čičková et al. 2018; Ikegawa et al. 2022; Basso et al. 2023). While SIT proves to be an

economical, sustainable, and efficacious approach, its potential for extensive implementation in large-scale field scenarios has not been examined and continues to pose a constraint.

6.5 Genetic approaches

6.5.1 Utilizing genetic information of host-tree

The degree and frequency of drought events have recently increased for forests worldwide due to rapid climate change, and adaptation of trees to the new disturbance regimes can be locally scaled at the gene level and includes substantial selection pressure on tolerant phenotypes (Zacharias et al. 2022). Norway spruce exhibits higher lignin synthesis and increased expression of defense-related genes in response to shade, potentially enhancing its resilience against pest attacks (Ranade et al. 2019, 2022). Tree responses to heat stress are influenced by signaling factors (protein kinases and transcription factors), heat shock proteins, heat stress factors, and catalase enzymes. In contrast, ascorbate peroxidase and histidine kinases remove reactive oxygen species during heat stress, while dehydration-responsive element-binding proteins shield trees from osmotic stress.

According to spatial and differential gene expression examination of conifers, stomatal closure and cuticular wax on the surface of needles can reduce water loss. A study on needles of maritime pine (*Pinus pinaster*) and Norway spruce reported that in non-irrigated saplings, the cuticular wax and the genes involved in its synthesis were overexpressed as compared to the irrigated ones (Blödner et al. 2007; Le Provost et al. 2013). With the advancement of molecular techniques and high throughput sequencing methods, the use of molecular tools to target genes for pest management is highly popularized (Singh et al. 2024). Various genes have been employed in agricultural pest management since the *cry* gene was identified in the 1980s, which was among the initial unique genes utilized for GM crops to combat insect pests. These advancements, however, are still in their early stages and are constrained by factors like tree size, density, and application at the forest stand level. Hence, additional technologies were diverted to insect-level genetic modifications, which led to the development of various new techniques such as sterile insect technique, gene silencing via RNAi and CRISPR, and other pre- and post-translation level modifications. Identifying genetic markers of tree resistance can be applied to forest protection, tree health diagnosis, and breeding bark beetle-resistant trees (Korecký et al. 2023).

6.5.2 RNA interference (RNAi)

In insects, RNAi is a conserved cellular process that turns off gene function by interfering with mRNA breakdown and protein production (Fire et al. 1998; Zhu and Palli 2020; Mogilicherla and Roy 2023a). This process involves silencing specific genes by using short RNA molecules to target and degrade messenger RNA (mRNA), preventing the production of proteins encoded by those genes. The silencing or "turning off" of vital genes for the survival of insects ultimately results in mortality (Zhu and Palli 2020).

Three distinct RNAi mechanisms have been identified: small interfering RNA (siRNA), microRNA (miRNA), and piwiRNA (piRNA). The siRNA pathway has received the most attention in insect pest management (Zhu and Palli 2020). In a nutshell, the siRNA machinery is activated by the successful delivery of double-stranded RNA (dsRNA) into the cell, then Dicer-2 (ribonuclease type III) enzyme converts the dsRNA into siRNAs (~21–24 bp), which are subsequently integrated into the RNA-induced silencing complex. Eventually, Argonaute2 cleaves and removes the sense strand of the siRNA. The remaining antisense strand then directs the RNA-induced silencing complex to sequence-specific targeting of complementary mRNA strand, which leads to degradation of the mRNA strand and post-transcriptional gene silencing (Zhu and Palli 2020). This sequence-specific mechanism of RNAi can be harnessed to effectively target vital genes in insects, including bark beetles, offering a means of pest management (Zhu and Palli 2020; Joga et al. 2016, 2021; Mogilicherla and Roy 2023b).

Researchers have developed RNAi-biopesticides that effectively silenced the target genes and caused decent mortality in beetles such as the Colorado potato beetle (*Leptinotarsa decemlineata*), emerald ash borer (*Agrilus planipennis*), Asian long-horned beetle (*Anoplophora glabripennis*), mountain pine beetle (*Dendroctonus ponderosae* Hopkins) and southern pine beetle (*Dendroctonus frontalis*) (Table 1) (Yoon et al. 2016, 2018; Rodrigues et al.

2017a, 2017b; Máximo et al. 2020; Dhandapani et al. 2020a, 2020b; Kyre et al. 2019, 2020; Kyre and Rieske 2022). The putative variability of RNAi among genetically variable beetles in geographically distinct populations was also recently documented (Kyre et al. 2024). Recent research has led to the development of the RNAi-based biopesticide Ledprona against *L. decemlineata*, which prevents the expression of enzymes, promotes protein breakdown, and ultimately results in mortality (Pallis et al. 2023). RNAi provides excellent promise for minimal environmental impact, attributed to its precise targeting and the transient nature of its active molecules. However, additional research is needed before widespread use of such commercial products in forestry. To ensure its safety, a combination of bioinformatics and ecologically sound bioassays with selected focal insect species will help a thorough understanding of potential off-target effects and impacts on non-target organisms (Christiaens et al. 2022; Mezzetti et al. 2022). These products must be used only to maintain the bark beetle populations under endemic conditions and preserve the beneficial role of bark beetles as decomposers in forest ecosystems.

Bark beetles are more susceptible to RNAi, but the effectiveness depends on the target gene selection, dsRNA stability, and expression of the RNAi core machinery genes (Mogilicherla et al. *unpublished data*). Effectiveness, off-target and non-target effects, and a lack of reliable dsRNA delivery mechanisms are the main obstacles to the widespread use of RNAi for bark beetle pest management. In contrast, feeding techniques combined with advanced development technologies (symbiont-mediated and nanoparticle-enabled) are critical for improved dsRNA transport, stability, endosomal escape, and dsRNA processing (Joga et al. 2021; Mogilicherla and Roy 2023b). Recently, Mogilicherla and Roy (2023b) comprehensively reviewed chitosan-dsRNA nanopesticides and their applications in managing bark beetles. The bacteria and fungi that live symbiotically with bark beetles have been successfully isolated and identified and can be considered putative candidates for symbiont-mediated RNAi (SMT) for tropical application to control bark beetles (Chakraborty et al. 2020a, 2020b, 2023; Gupta et al. 2023; Mogilicherla and Roy 2023b). These studies may pave the way for developing and using RNAi-biopesticides as a secure, efficient, and innovative method to safeguard forest trees. Fortunately, current research conducted globally and recent work on the genome, transcriptome, and proteome of bark beetles and their symbiotic microbes will significantly enhance the information on these insects and facilitate the development of species-specific RNAi-based biopesticides in the future (Powell et al. 2021; Ashraf et al. 2023; Naseer et al. 2023; Sellamuthu et al. 2023). Nonetheless, only a limited number of RNAi-based insecticides have obtained licensing and are on the verge of becoming accessible in the market (Li et al. 2023; Pallis et al. 2023).

6.5.3 CRISPR/Cas9

Numerous studies have made considerable use of the ground-breaking genome editing tool known as clustered regulatory interspaced short palindromic repeats/cas9 (CRISPR/Cas9) (Sun et al. 2017; Singh et al. 2022; Yan et al. 2023). CRISPR/Cas9 is a gene editing technology used to alter the DNA of organisms. In the CRISPR/Cas system, CRISPR comprises DNA sequences found in prokaryotic organisms like bacteria and archaea, while Cas9 is an enzyme that uses CRISPR sequences as a guide to locate and open specific DNA strands. The Cas9 enzyme attaches to the target DNA and cleaves it, deactivating the targeted gene. This process, known as gene "knock-out," is a reliable method for identifying genes of interest or gaining deeper insights into genome complexities (Doudna and Charpentier 2014; Jiang and Doudna 2017).

CRISPR is a more intuitive and user-friendly technology because it needs only one guide RNA (gRNA) for target identification and Cas9 nuclease for implementation (Richter et al. 2013; Upadhyay 2021). The relevant derivative has a single chimeric guide RNA (sgRNA) that recognizes and binds to the intended target sequence, as well as a CRISPR-associated (Cas) nuclease that cleaves DNA at specific locations by producing site-specific double-strand breaks (Horvath and Barrangou 2010; Wiedenheft et al. 2012). The plasmid DNA, RNA, or ribonucleoprotein complex (RNPC) are some of the different delivery mechanisms that can be used to introduce the CRISPR/Cas components (sgRNA and Cas9 protein) into the target organism

(Ogaugwu et al. 2013). CRISPR/Cas9 has an advantage over RNAi due to its ability to bring about enduring and inheritable genomic alterations. In contrast, RNAi only produces immediate effects unless a steady supply of dsRNA is kept available. Nevertheless, CRISPR technology has the potential to revolutionize pest control, but both the advantages and disadvantages must be carefully considered (Perkin et al. 2016). The CRISPR/Cas9 technology has been used against a wide variety of insect species; however, in the case of coleopteran insects, it has only been applied to the red flour beetle (*Tribolium castaneum*) and Colorado potato beetle (*Leptinotarsa decemlineata*) (Gilles et al. 2015; Gui et al. 2020; Singh et al. 2022).

6.5.4 *Ethical and legal implications of RNAi and gene editing technologies in forest pest management*

Development of regulatory protocols for modern biotechniques such as RNAi and CRISPR is essential before intended deployment. However, the regulatory protocols can vary across jurisdictions, predominantly emphasizing the process or the end product due to associated risks and the need to effectively manage toxicity levels to non-target organisms (Ahmad et al. 2021; Távora et al. 2022). The regulatory framework for genetically modified (GMO) products in the European Union and New Zealand is process-based and necessitates significant time and financial expenditure. In contrast, the end-product-based regulatory structure used by the US, Canada, China, and selected European countries is comparatively efficient in terms of time and cost. Before commercialization, the RNAi and gene-edited products must be checked for potential adverse effects on non-target organisms, including microorganisms in the habitat (i.e., forest soil). Therefore, discussing several regulatory regimes regarding RNAi and CRISPR systems in numerous jurisdictions worldwide is necessary.

Only a limited number of RNAi and CRISPR-based products have been approved for global commercial release, likely due to persistent regulatory obstacles and unsettling consumer perception and acceptance (Mat Jalaluddin et al. 2019). Besides, several important issues must be addressed before society accepts biotechnological products (Taning et al. 2021). Regular communication among researchers, foresters, and other pertinent participants (i.e., state forest enterprises and forest owners) is essential for reporting biotechnological breakthroughs. Furthermore, the contemporary acceptance of CRISPR and RNAi-based bioproducts, such as biotic stress-resistant plant varieties and biopesticides, depends on transparent communication referring to technical intricacies like gene editing and silencing mechanisms alongside a thorough exploration of potential risks and benefits. Scientists from relevant sectors are pivotal in facilitating open dialogue with forest organizations and sustaining educational efforts to foster informed decision-making (Rank and Koch 2021; Taning et al. 2021). Nevertheless, it is crucial to address ethical and moral considerations at the outset of developing CRISPR/Cas and RNAi-based technological solutions (Frewer et al. 2013; Gupta et al. 2015; Beghin and Gustafson 2021).

Interestingly, RNAi technique does not always require a transgenic expression of target dsRNA; the use of topically applied RNAi-based biopesticides or spray-induced gene silencing (SIGs) for the management of tree diseases may see a rise in public acceptance (Shew et al. 2017). Regulatory frameworks for topical RNAi-based products in agriculture or forestry are still nascent worldwide. Despite this, extensive research has been conducted into plant protection using topically applied RNAi-based bioproducts, driven by their promising benefits. Topical RNAi-based products offer clear advantages over most used crop protection methods using chemical pesticides. However, the environmental fate and stability of the naked dsRNA or dsRNA conjugates used in SIGs and their impact on beneficial microorganisms in the habitat must be assessed from case to case.

Although there is still much debate about the regulations surrounding CRISPR-edited organisms, a few nations have established regulatory frameworks designed to assess these products (reviewed by Devos et al. 2022). Some genetic engineering methods do not entail the introduction of exogenous DNA sequences. Such methodologies may not produce a final product classified as genetically modified (Podevin et al. 2013; Entine et al. 2021). In many countries, legislation governing GMOs or traditional chemical and biological pesticides does not cover these genetic engineering methods. Nevertheless, an

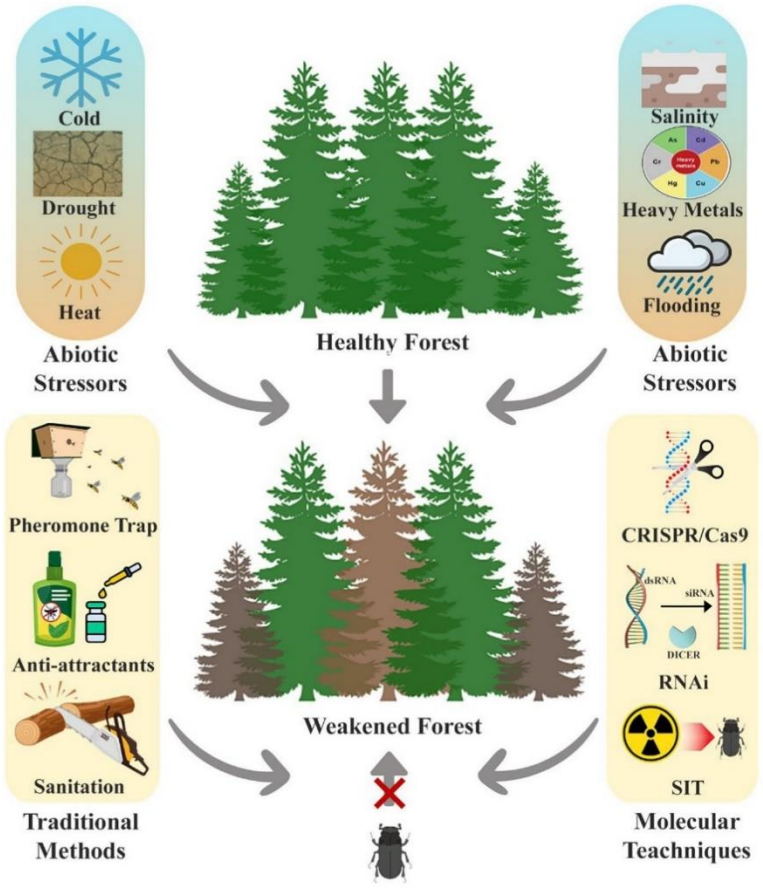


Fig. 3 The Scheme illustrates the currently used traditional methods and prospective molecular techniques that can help manage bark beetles in weakened forest ecosystems. The upper panel shows the major abiotic factors that weaken the forest ecosystem and increase its susceptibility to bark beetle infestations. The traditional methods are helpful in monitoring and controlling bark beetle populations to some extent, but novel cutting-edge technologies can play a crucial role in eliminating pest outbreaks

approach based on scientific parameters to develop and validate biotechnological products is fundamental to formulating the most appropriate risk assessment protocols (Mezzetti et al. 2020).

7 Conclusions and future prospectives

A considerable proportion of global plantation forests consisting of monocultures, preferred for timber production owing to their uniformity and ease of management, are under threat. However, as extreme weather conditions like droughts and rising temperatures favour forest pest outbreaks, there is a pressing need for eco-friendly strategies to reduce the vulnerability of forests to climatic change. To enhance overall forest resilience, it is proposed that cultivating plantations with diverse species and promoting complementary traits could be a viable solution. However, despite being a longstanding priority on the political agenda in central Europe, realizing such goals has been slow. A recent report from the European Commission aims to promote a transition toward a sustainable and resilient silviculture practice by 2030 (European Commission 2021).

Understanding the mechanism of bark beetle infestation and outbreak dynamics at different spatial scales has serious implications for protecting and managing the Norway spruce ecosystem. Despite the ecological and economic impacts of bark beetle outbreaks, the current control measures are ineffective in stopping the spread of bark beetle outbreaks.

Existing methods like pheromone and tree traps, anti-attractants, remote sensing, and canine detection of infested trees against bark beetles will continue to aid in managing bark beetles amidst climate change. However, additional approaches such as nanoparticle-coated RNAi complexes and symbiont-mediated RNAi, SIT, and CRISPR/Cas9-based approaches are needed and may potentially replace some of the current management approaches in combating bark beetles in forest management (Fig. 3). These new approaches have been very successful in controlling agricultural pests. They are relatively less explored for forest pest management, especially for bark beetles, for various reasons, including the availability of sequenced genomes, difficulties in rearing on an artificial diet, and obtaining viable eggs from beetles for injection. Regardless, extensive product development and testing their effectiveness in field studies are required before commercial manufacturing and widespread field application. In addition, the success of the potential application of the recent molecular techniques is contingent upon understanding their mechanisms, assessing potential risks, and navigating the global regulations governing similar genetic materials. It can be anticipated that molecular approaches will continue evolving in the research domain aimed at combating forest pests, and thus, the environmental safety of such approaches should be carefully monitored for safe deployment.

Acknowledgments We thank Dr. Jiří Synek for providing beetle pictures. We also thank the editor and anonymous reviewers for their constructive comments and suggestions.

Author contributions VVS and NE conceptualized the article. VVS searched the literature and wrote the first draft of the manuscript. AN, KM, AT, and KZ wrote sections of the manuscript. VVS, AN, and KZ prepared figures. KM, AR, RJ, and NE supervised the work. All authors contributed to reviewing and editing the article.

Funding Open access publishing supported by the National Technical Library in Prague. This work was funded by Grant No. CZ.02.1.01/0.0/0.0/15_003/0000433, "EXTEMIT—K Project," Operational Program Research, Development and Education (OP RDE), and Internal Grant Agency (Grant No. 43950/1312/3123) at the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic. KM and AR were supported by "Excellent Team Grant" at the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic. KM acknowledges the Ramalingaswami re-entry grant (BT/RLF/Re-entry/11/2023 from the Department of Biotechnology, Govt. of India.

Availability of data and material (data transparency) Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

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

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5.2. Age matters: Life-stage, tissue, and sex-specific gene expression dynamics in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae)

Published as: **Naseer, A.**, Mogilicherla, K., Sellamuthu, G. and Roy, A., 2023. Age matters: Life-stage, tissue, and sex-specific gene expression dynamics in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae). Frontiers in Forests and Global Change, 6, p.1124754. https://doi.org/10.3389/ffgc.2023.1124754

Contributed to: methodology, formal analysis, data curation, data analysis, visualisation, writing-original draft, review, and editing.

The study was conducted 'to identify the detoxification machinery genes in *I. typographus* and elucidate their roles in overcoming host plant toxins during feeding (Objective I).' The study utilised mRNA sequencing from 13 samples across different life stages [larvae (L1, L2, and L3), pupa, callow, and sclerotised adult], and male/female tissues (gut, fat body, and head) from callow beetles and sclerotised adult beetles to generate 11 transcriptomes used in the study. The study focussed on the detoxification gene regulated across different life stages, and the feeding (larval and adults) and the non-feeding stage (pupa) revealed that detoxification gene expression is high during feeding on host tissues, especially in the second instar larva and the gut of callow male beetles. A few gene families of pheromone biosynthesis were also reported to be upregulated in the callow beetle fat bodies. In addition to detoxification, the study also identified genes and their dynamics related to digestion, resistance, signalling, and transport across different comparisons.

The results denote that ESBB's ability to detoxify spruce monoterpenes is a testament to its evolutionary adaptation to its host environment. By upregulating key detoxification genes and enzymes, including P450s, GSTs, UGTs, and ABC transporters, the beetle can neutralise the toxic effects of host chemical defence and continue feeding on spruce trees. This detoxification system is highly dynamic, with gene expression varying across different life stages and tissues, allowing the beetle to cope with varying levels of toxic exposure during its development under the bark. The study signifies that the larva and newly emerged callow beetles (being a rapidly feeding stage), and the insect gut serves as a specialised site for detoxification activity.

frontiers | Frontiers in Forests and Global Change

TYPE Original Research
PUBLISHED 14 March 2023
DOI 10.3389/ffgc.2023.1124754

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pests, Pathogens and Invasions, a section of the journal Frontiers in Forests and Global Change

RECEIVED 15 December 2022
ACCEPTED 13 February 2023
PUBLISHED 14 March 2023

CITATION
Naseer A, Mogilicherla K, Sellamuthu G and Roy A (2023) Age matters: Life-stage, tissue, and sex-specific gene expression dynamics in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae). *Front. For. Glob. Change* 6:1124754. doi: 10.3389/ffgc.2023.1124754

Age matters: Life-stage, tissue, and sex-specific gene expression dynamics in *Ips typographus* (Coleoptera: Curculionidae: Scolytinae)

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The Eurasian spruce bark beetle (ESBB), *Ips typographus*, has recently caused catastrophic damage to Norway spruce (*Picea abies*) forests in Europe, resulting in the loss of more than 100 million cubic meters of wood. Traditional forest management strategies have failed to constrain the growing infestation rate; hence, novel measures must be deployed. A better understanding of ESBB physiology and adaptation to host allelochemicals may provide a platform for future management strategies using molecular tools such as RNA interference. To understand ESBB physiology and adaptation, the current study unraveled the gene expression dynamics of ESBB in different life stages and tissues. We obtained ESBB transcriptomes for different life stages [larvae (L1, L2, and L3), pupa, callow, and sclerotized adult] and male/female tissues (gut, fat body, and head) from callow and sclerotized adult beetles. Differential gene expression analysis (DGE) identified multiple gene families related to detoxification, digestion, resistance, and transport in different life stages and tissues of the beetle. Gene Ontology (GO) enrichment revealed 61 critical metabolic pathways enriched across all DGE comparisons. DGE analysis further pinpointed the differential expression of essential genes involved in detoxification, digestion, transport, and defense in various tissues and life stages. RT-qPCR experiments and enzymatic assays corroborated the findings further. The catalogue of differentially expressed genes identified in ESBB could aid better understanding of ESBB physiology and adaptation to hosts and serve as targets for future RNAi-based ESBB management.

KEYWORDS

Norway spruce, Eurasian spruce bark beetle, DGE analysis, allelochemicals, detoxification, RT-qPCR, enzyme assays, forest pest management

Introduction

Ips typographus, the Eurasian spruce bark beetle, is one of the most destructive forest pests of Norway spruce (*Picea abies*), causing considerable damage throughout Europe (Huang et al., 2020; Fang et al., 2021; Hlásny et al., 2021). They attack trees weakened due to drought or windthrow when the bark beetle population is in an endemic phase

(Jakuš et al., 2011; Biedermann et al., 2019; Hlásny et al., 2019; Netherer and Hammerbacher, 2022). The host selection by ESBB is based on the attraction *via* pheromones and kairomones, viz. 2-methyl-3-buten-2-ol and 4S-(−)-cis-verbenol, and repellence due to non-host volatiles/anti-attractants such as verbenone (Faccoli et al., 2005; Zhang et al., 2012; Unelius et al., 2014; Netherer et al., 2021). Males are the pioneering sex, and after colonizing the host, they release aggregation pheromones to attract conspecifics, resulting in mass attacks. ESBBs infest healthy trees during an epidemic stage and subsequently kill them (Biedermann et al., 2019). The defense mechanism of the conifer trees can be overwhelmed by the detoxification machinery of the beetle and their resident symbionts, such as bacteria and fungi that can metabolize plant defense chemicals to semiochemicals or sequester them to less toxic and more usable carbon resource forms (Krokene and Solheim, 1998; Faldt et al., 2006; Hammerbacher et al., 2013; Davis, 2015; Cale et al., 2019; Chakraborty et al., 2020a,b). Upon encountering a suitable host, the aggressive beetles bore into the intercortical regions where they feed on the phloem tissues, lay eggs, and the emerging larvae develop by feeding on the surrounding tissues, forming characteristic galleries. After completing the development, ESBB adults leave these galleries, swarm around the forest, and attack new trees to start the cycle again (Hlásny et al., 2019).

Conifers have evolved complex constitutive and inducible defenses to protect themselves against herbivores and their associates, including anatomical barriers and secondary metabolites, such as phenolics and terpenes (mono-, sesqui-, and diterpenes) produced *via* phenylpropanoid and terpene synthase pathways (Franceschi et al., 2005; Biedermann et al., 2019; Celedon and Bohlmann, 2019). Herbivore insects counteract these responses by employing various coping strategies, such as avoidance, sequestration, target site mutation, and enhanced metabolism/detoxification (Heckel, 2014; Meng et al., 2015; Gaddelapati et al., 2018; Hilliou et al., 2021; Lu et al., 2021; Bras et al., 2022). During detoxification, insects produce enzymes that convert the toxic allelochemicals to less toxic, easily excretable forms by oxidation, hydroxylation, or glycosylation. Such complex biochemical reactions are carried out by enzymes categorized as phase I, phase II, and, more recently, phase III enzymes (Hilliou et al., 2021). The primary or phase I enzymes target the lipophilic groups *via* oxidation, dihydroxylation, or dealkylation, rendering them water-soluble forms. In contrast, the phase II or secondary enzymes attack the epoxide intermediates of the phase I molecules, which can then be excreted through body fluid. The enzymes involved in phase I detoxification include cytochrome P450s (P450), carboxyl/choline esterases (CCE), and other esterases (i.e., acetylcholinesterase-AChE, JH esterase) that carry out the oxidation, hydrolysis, and reduction of primary toxic molecules. These molecules are then conjugated with glutathione molecules by glutathione S-transferases (GSTs) or glycosylated by UDP-glucuronosyltransferases/UDP-glycosyltransferases (UGTs), which are considered phase II enzymes (Heidel-Fischer and Vogel, 2015; Krempl et al., 2016; Jin et al., 2019). Secondary enzymes also include sulfotransferase and ATP-binding cassette (ABC) transporters. However, ABC transporters have recently been reported as phase III enzymes that obtain energy from the hydrolysis of ATP to transport the substrate across the lipid membrane and facilitate the excretion of entomotoxic compounds (Jin et al., 2019; Hilliou et al., 2021).

Dendroctonus species, close relatives of ESBB, have been well studied regarding their gene expression toward host toxins. In *D. armandii*, sixty-four P450s were identified in different developmental stages (larvae, pupa, and adult) and validated with RT-qPCR; nineteen CYP genes were upregulated upon host feeding (Dai et al., 2015). The role of the CYP4 and CYP9 families in detoxification was also confirmed in *D. rhizophagus* (Cano-Ramírez et al., 2013; Sarabia et al., 2019). In *D. ponderosae*, three CYPs were identified as involved in monoterpene oxidation, and one CYP was implicated in aggregation. Furthermore, transcript levels of several GSTs, ABC transporters, esterases, and dehydrogenases have been reported to increase significantly in mountain pine (*D. ponderosae*) beetles upon host feeding (Robert et al., 2013; Chiu et al., 2019a,b). Various *Ips* species, like *I. paraconfusus* and *I. pini*, are reported to deploy sex-specific expression of CYPs to convert host allelochemicals to *de novo* produced aggregation pheromones (Huber et al., 2007; Song et al., 2013; Tittiger and Blomquist, 2017; Blomquist et al., 2021). For ESBB, the roles of most of the detoxifying enzymes and the related genes upon mass attack have yet to be identified. ESBBs are exposed to various levels of host bark chemicals throughout the life cycle; as a result, the expression or catalytic activity of beetle defense enzymes may fluctuate according to the life stage or phase of the detoxification process. Frequent ESBB outbreaks in the recent past and the projected increased frequency of such outbreaks have created a high demand for target-specific control of ESBBs, as conventional methods have proved inefficient (Hlasny et al., 2021; Joga et al., 2021). Hence, detailed knowledge of the detoxification enzyme expression dynamics in the different life-stages of the ESBB is necessary to obtain the target genes for future species-specific management. Furthermore, the recent release of the ESBB genome has opened such captivating research avenues by giving insights into the ubiquitous presence of phase I, II, and III enzymes (84 P450s, 58 GSTs, 86 UGTs, 75 ABC transporters, and 59 CCE families) in the species (Powell et al., 2021). In the present study, we performed screening of the various developmental stages from larval, callow adults, and fully emerged sclerotized beetles, and different tissues of ESBB to catalogue genes differentially expressed and putatively involved in the processes of detoxification, digestion, and transport. We captured the expression dynamics of detoxification machinery and other physiologically essential genes in ESBB. Our findings have led to a better understanding of ESBB physiology and offer valuable targets for RNAi-based control measures (Joga et al., 2021).

Materials and methods

Sample collection and dissection

All ESBB samples (**Figure 1** and **Supplementary material 1**) were taken directly from beetle-infested logs from Rouchovany in Czechia (49°04′08.0″N 16°06′15.4″ E, 360 m above sea level, a warm and drought-prone area with regular forest management by State Forest Enterprise) during the infestation season in May 2019. The average temperature was around 15°C during collection. The larvae (L1, L2, and L3), pupae, callow, and sclerotized adult beetles were snap-frozen in liquid nitrogen, and the target tissues (gut, head, and fat body) were dissected in sterile conditions

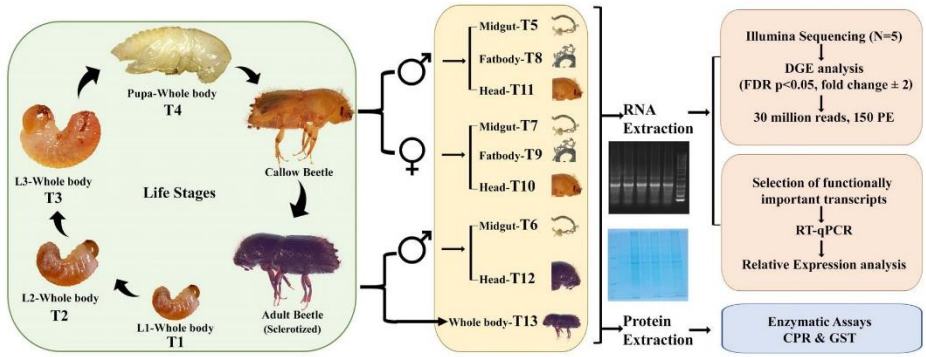

FIGURE 1
Study design. Larvae 1 (L1/T1), Larvae 2 (L2/T2), Larvae 3 (L3/T3), Pupa (T4), Callow male gut (T5), Sclerotized adult gut (T6), Callow female gut (T7), Callow male fat body (T8), Callow female fat body (T9), Callow female head (T10), Callow male head (T11), Sclerotized adult male head (T12), Sclerotized adult whole body (T13) (CPR, Cytochrome P450; GST, Glutathione S-transferase).

and collected in RNAlater^{TM}. The sex of the collected beetles was determined by pronotum hair density, as has been described previously (Chakraborty et al., 2020a; Powell et al., 2021). The collected developmental stages and tissues were stored at −80°C until total RNA extraction, as we previously described (Powell et al., 2021). ESBBs were also reared in an insect-rearing chamber with fresh Norway spruce logs at 27 ± 1°C, 70 ± 5% humidity, and a 16:8-h light/dark (L: D) photoperiod. A new set of samples (developmental stages and tissues) was obtained from the F1 generation, and the extracted RNA and protein were used in RT-qPCR and enzymatic experiments, respectively.

Transcriptome sequencing, library preparation, and analysis

Various ESBB life stages and tissues were used to create transcriptome libraries [larval stage 1 (T1), larval stage 2 (T2), larval stage 3 (T3), pupa (T4), callow male gut (T5), sclerotized adult male gut (T6), callow female gut (T7), callow male fat body (T8), callow female fat body (T9), callow female head (T10), callow male head (T11), sclerotized adult male head (T12), and sclerotized adult whole-body (T13)] (**Figure 1** and **Supplementary material 1**) during the *Ips typographus* genome study (Powell et al., 2021). Precisely, mRNA in samples was enriched using oligo (dT) beads, and cDNA libraries were prepared using NEB Next® Ultra^{TM} RNA Library Prep Kit followed by Illumina sequencing (Illumina Novaseq6000) to generate 30 million reads (150 paired-end) for each sample. Five biological replicates were sequenced per sample (Powell et al., 2021). The raw reads were processed using CLC workbench (CLC version 21.0.5, Qiagen, Denmark). The sequence data were submitted to NCBI (PRJNA679450) and reused for the present gene expression study. For differential gene expression analysis (DGE), raw reads were mapped back to the *I. typographus* reference genome (Powell et al., 2021), and the read counts were obtained using CLC workbench (version 21.0.5). Gene expression quantification was obtained by employing standard pre-optimized settings and parameters, such as mapping to exon regions. The TMM-adjusted log CPM counts (similar to the EdgeR approach) were also used to correct biases in the sequence datasets and abundance, resulting in accurate estimations of relative expression levels as described by Robinson et al. (2010). A multi-factorial statistical analysis based on a negative binomial Generalized Linear Model (GLM) was used to identify differentially abundant transcripts between developmental stages and tissues in varied comparisons deploying an FDR corrected p-value < 0.05; fold change ± 2 was used as a threshold for differentially expressed transcripts (DET). Using the GLM model permitted fitting the curves to expression values without presuming that the error on the values was normally distributed. DEGs were functionally annotated using the "cloud blast" feature within the "Blast2GO Plugin" in the CLC Genomic Workbench 21.0.5 (Qiagen, Denmark). A nucleotide blast was done against the NCBI Nr database with an E-value cut-off of 1.0E-5. Both annex and GO slim were used to improve the GO term identification further by crossing the three GO categories (biological process, molecular function, and cellular component) to search for name similarities, GO term, and enzyme relationships within KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Roy et al., 2017; Powell et al., 2021).

Total RNA extraction, cDNA synthesis, and RT-qPCR analysis

Ribonucleic acid was isolated from each life stage and tissue using the PureLink^{TM} RNA Kit from Ambion (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For the ESBB tissue samples, 10 guts, 10 fat bodies and 5 heads were dissected and pooled. Eight L1 larvae, eight L2 larvae, five L3 larvae, five pupae, and two adults were used to obtain enough RNA from each developmental phase for downstream processing. The total extracted RNA was then treated with DNase I (TURBO DNase Kit, Ambion, Austin, TX, USA). The integrity of the purified total RNA was checked on 1.2% agarose gel. One μg

of total RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems-Life Technologies, Waltham, MA, USA) and stored at −20°C. The cDNA samples were diluted 10-fold before being used as a template in RT-qPCR experiments. Four biological replicates from each sample were used in each RT-qPCR assay. The IDT PrimerQuest software (IDT, Belgium) was used to design the primers (**Supplementary material 2**). RT-qPCR was carried out for all the samples (T1-T9 and T13). The 10 μL RT-qPCR reactions contained 5.0 μL of SYBR® Green PCR Master Mix (Applied Biosystems), 1.0 μL of cDNA, 1.0 μL of 10 μM forward and reverse primers, and 3.0 μL RNase-free water (Invitrogen). The reactions were performed in an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems) with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and dissociation curve analysis during which temperature was increased from 60 to 95°C. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). RPL7 and RPS7 were used as reference genes for expression normalization (Sellamuthu et al., 2022).

Enzyme assays

To consider differences in the enzyme activity levels between the ESBB developmental stages, biochemical assays for Glutathione S-transferase (GST) and Cytochrome P450 Reductase (CYP450) were performed as described previously (Habig and Jakoby, 1981; Ortego et al., 1999; Bosch-Serra et al., 2021). The developmental stages and gut samples of ESBB were collected and homogenized in sodium phosphate buffer (50 mM) solution at pH 7.0. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. Precisely, eight L1 larvae (T1), six L2 larvae (T2), four L3 larvae (T3), three pupae (T4), two sclerotized adults, and ten guts were pooled to extract enough protein from each sample category. The extract was collected to serve as a source of the enzyme, and enzyme activities were corrected using the protein concentration as a standard correction factor. The total protein content of the enzyme solution was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

The activity of glutathione S-transferase (GST) was measured using a spectrophotometric method with 5 mM reduced glutathione (GSH) and 1.5 mM 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates (Habig and Jakoby, 1981; Bosch-Serra et al., 2021). One unit of glutathione S-transferase activity is the amount of enzyme-catalyzing 1 μmol of CDNB -GSH conjugate per minute at 30°C. For the GST activity, the reaction solution contained 20 μl of protein extract (**Supplementary Table 2**), 170 μl of 5 mM GSH in sodium phosphate buffer (pH 7.0), and 10 μL1.5 mM CDNB to make a 200 μl total reaction volume in a transparent micro-plate (96-well Thermo Scientific™ Sterilin™ Clear Microtiter™ Plates). For blanks, 20 μl of sodium phosphate buffer (50 mM pH 7.0) was used instead of protein extracts. After a 1 min incubation period at 30°C, the absorbance of the reaction product [CDNB-glutathione (GSH) conjugate] was measured at 340 nm at intervals of 1 min for up to 10 min using the microplate reader (Agilent BioTek Cytation 5). GST activity was expressed as nmol substrate conjugated·min-1 ·mg-1 protein using a CDNB molar extinction coefficient (9.6 $mM^{-1} \times cm^{-1}$). The final specific activity was expressed in μmol of CDNB-glutathione min^{-1} mg^{-1} of total protein extracted (Habig and Jakoby, 1981).

Cytochrome P450 Reductase (CYP450) activity was measured according to Ortego et al. (1999) and Bosch-Serra et al. (2021). The 200 μl reaction was prepared in a clear micro-plate (96-well Thermo Scientific™ Sterilin™ Clear Microtiter™ Plates). It comprised 20 μl of protein extract or sodium phosphate buffer (blank) (**Supplementary Table 2**) and 160 μl of NADPH-generating system [0.6 mM β-nicotinamide adenine dinucleotide phosphate (NADP), 2.8 mM D-glucose-6-phosphate (G6P), and 0.28 units of glucose-6-phosphate dehydrogenase (G6PD)]. The reaction was initiated by adding 20 μl of 1 mM cytochrome C in sodium phosphate buffer (pH 7.0), and the absorbance was measured at 550 nm at intervals of 1 min for up to 25 min using the microplate reader (Agilent BioTek Cytation 5). CYP450 activity was expressed as nmol substrate conjugated·min^{-1} ·mg^{-1} protein using a cytochrome C molar extinction coefficient (27.6 $mM^{-1} \times cm^{-1}$) (Margoliash and Frohwirt, 1959). The enzymatic activity was measured in cytochrome P450 equivalent unit min^{-1} mg^{-1} of the total protein extracted.

Statistical analysis

First, we conducted pairwise comparisons with Student's *t*-test to consider the differences in RT-qPCR determined gene transcript levels for 19 selected genes related to detoxification. The pairwise comparisons included callow gut vs. sclerotized male gut, and callow female fat body vs. callow male fat body. For multiple comparisons in the relative gene expression of 19 genes between different developmental stages and gut tissues of ESBB, Tukey's HSD was used. Lastly, one-way ANOVA was used to test the significance of differences in enzymatic activities between ESBB developmental stages and tissues. All these analyses were performed in RStudio (version 4.2.2). The statistical analysis deployed for DGE data analysis is described above in the "Transcriptome sequencing, library preparation, and analysis" section.

Results

DGE analysis

From 65 libraries (13 samples × 5 biological replicates), 5,199,150,714 reads were retrieved, and 3,728,549,638 reads were mapped back to the reference genome of *I. typographus* (Powell et al., 2021) to generate the count table (**Supplementary Table 1**). Using CLC workbench 21.0.5 (QIAGEN Aarhus, Denmark), DGE comparisons were performed among the 13 samples (T1 to T13) (**Figure 1**). The number of DEGs, i.e., up- or downregulated genes, were reported based on the cut-off of FDR $p < 0.05$ and fold change ± 2 considered as the threshold for differential expression. We performed DGE for the following perspectives: (A) life-stage comparison: T1, T2, T3, T4, and T13 (from larvae to adult); (B) tissue-specific comparison: (i) T7, T8, and T9, and (ii) T5,

T9, and T11; (C) sex-specific comparison: (i) T7 vs. T5, and (ii) T9 vs. T8; and (D) comparison between gut tissues in different adults stages: T6 vs. T5 (sclerotized vs. callow male guts) (Table 1, Supplementary Figure 1, and Supplementary materials 1, 3–13).

A. DGE in ESBB life stages

A.1. Group-wise comparison between T1-T4 and T13

A group-wise comparison was made between the five samples (three larval stages, pupa, and adult) to explore the differential gene expression across the developmental stages (Supplementary material 3). The principal component analysis (PCA plot) resulted in the segregation of samples from different life stages, indicating life-stage-specific gene expression in ESBB (Figure 2A). The gene expression dynamics in different life stages of ESBB were represented as a heatmap (Figure 2B). We found that detoxifying enzyme families (i.e., cytochrome P450s/CYPs, UDP-glucuronosyltransferases/UGTs, acetylcholinesterase/AchE, hydrolases, dehydrogenases, peroxidases, carboxylesterases, esterases, ABC transporters, and glutathione S-transferases/GSTs) showed differential gene expression (up- and downregulation) in larvae and adult stages (Figures 2C, D). A graph was plotted using mean CPM values (FDR corrected $p < 0.05$) for essential detoxification genes/enzymes (P450s, GSTs, UGTs, carboxylesterases, esterases, and ABC transporters). The expression of detoxification-related genes increased from L1 to L2, decreased in L3 and pupa, and increased in adults (Figure 2D). Some other detoxifying enzymes, such as dehydrogenases, hydrolases, and aminoacylases, also revealed a similar pattern (Supplementary Figure 2).

Most of the cytochrome and esterase family genes showed no variation in gene expression across the life stages of ESBB, indicating their importance for beetle survival under the bark (Figure 3A). The expression of cytochrome P450 genes was high in the larval and adult stages. The present study identified differentially expressed cytochrome P450 genes primarily for family 4, with a few belonging to families 6 and 9 (Figure 3). In the upregulated category, CYP4BH1, CYP6CR2, chorion peroxidase, sphingomyelin phosphodiesterase-like and esterase FE4-like gene were only over-expressed in adults, whereas CYP306A1, CYP6A1-like, cAMP and cAMP-inhibited cGMP 3′,5′-cyclic phosphodiesterase 10A-like, and palmitoleoyl-protein carboxylesterase NOTUM-like were overexpressed only in pupae (Figure 3A). In the downregulated category, most genes were downregulated in pupae compared to other ESBB life stages, with CYP410A1 showing the lowest gene expression level. In adults, the most highly upregulated gene was CYP4G27 (Figure 3A), and the lowest gene expression was recorded for GST 1 isoform D (Figure 3B). The 19 transcripts involved in pheromone biosyntheses, like ipsdienol dehydrogenase, juvenile hormone (JH) epoxide hydrolase, JH esterase, and JH acid O-methyltransferase, were also reported to be primarily overexpressed in adults (T13) and larval stage L2 (T2) and L3 (T3) and were least expressed in pupae (T4) (Supplementary material 3).

A.2. Feeding vs. non-feeding stage comparison (T1, T2, T3, T13 vs. T4)

The effect of bark feeding and encountering the host at different developmental stages was studied by comparing the feeding stages (larva and adult; T1, T2, T3, and T13) against the non-feeding stage (pupae; T4) (Supplementary materials 4–7). This revealed the complete segregation of gene expression between the feeding and non-feeding stages (Figures 4A–D). Compared to T4, the transcript levels of most of the genes of interest were significantly elevated in T1, T2, T3, and T13. The T2 vs. T4 comparison revealed the most differentially expressed gene transcripts, i.e., 8,319 (4,896 downregulated, 3,423 upregulated). We discovered that 1,798 gene transcripts were commonly upregulated, and 1,060 transcripts were commonly downregulated in all feeding stages, including larvae and adults, with the dehydrogenases and cytochromes being the most differentially expressed (Figures 5, 6 and Supplementary materials 36, 37). Two GST transcripts, namely, GST-like isoform X2 and microsomal GST 1-like, had higher expression in the feeding stages (adults and larvae) than the non-feeding pupal stage, indicating their putative involvement in digestion and the detoxification of host allelochemicals.

B. Tissue-specific DGE

We compared tissues from both females and males separately to capture similarities and sex-specific differences in gene expression in different tissues, which are crucial to understand ESBB physiology (Figures 7A, B). In both males and females, the expression of genes related to detoxification, digestion, defense, and metabolism was highest in the gut, followed by in the fat body, and lowest in head tissues.

B.1. Female tissue comparison (T7, T8, and T10)

The callow female head (T10), fat body (T8), and gut (T7) were compared and clustered on a group-by-group basis (Figure 7A, i, ii and Supplementary material 8). There were 6,024 genes upregulated (FDR corrected $p < 0.05$, fold change ≥ 2), including 74 dehydrogenases, 37 CYP/P450s, 20 hydrolases, 9 ABC transporters, 8 sulfotransferases, 5 GSTs, 3 peroxidases, 2 each of the AchE, aminoacylase, and esterase sub-families, and 1 UGT and carboxylesterase (Figure 7A, iii). Among the 4,112 downregulated transcripts (FDR corrected $p < 0.05$, fold change ≤ -2) were 40 dehydrogenases, 15 CYP/P450s, 11 UGTs, 15 hydrolases, 9 ABC transporters, 6 GSTs, 3 of each sulfotransferase and esterase subfamilies, and 1 peroxidase. The upregulated transcripts have molecular functions that relate to defense (21 transcripts), signaling (176 transcripts), growth (22 transcripts), transport (116 transcripts, excluding ABC transporter families), digestion (120 transcripts, excluding esterase sub-families), and metabolism (40 transcripts).

B.2. Male tissue comparison (T5, T9, and T11)

The callow male head (T11), fat body (T9), and gut (T5) were compared and clustered on a group-by-group basis (Figure 7B, i, ii and Supplementary material 9). There were 10,464 transcripts substantially expressed (FDR corrected $p < 0.05$), with 6,178 upregulated (fold change ≥ 2) and 4,287 downregulated (fold change ≤ -2). The upregulated transcripts included 40 CYP/P450s, 1 UGT, 2 AchEs, 7 GSTs, 22 hydrolases, 60 dehydrogenases, 9 sulfotransferases, 3 peroxidases, 1 carboxylesterase, and 2 esterase subfamily genes. The downregulated transcripts included 16 CYP/P450s, 11 UGT, 8 GSTs, 2 aminoacylases, 16 hydrolases, 50 dehydrogenases, 5 sulfotransferases, 1 peroxidase, and 3 esterase subfamily genes (Figure 7B, iii). A total of 17 transcripts had molecular functions associated with defense, 130 with digestion, 30

TABLE 1 The number of differentially expressed genes in various comparisons.

	Comparison		Sample	Life stage or tissues	Sample ID	Downregulated (FDR $p < 0.05$, Fold change ≤ -2)	Upregulated (FDR $p < 0.05$, Fold change ≥ 2)
A	Developmental stages		ITL1, ITL2, ITL3, ITP, ITB	Larval stage 1, 2,3; pupa; sclerotized adult beetle	T1, T2, T3, T14, T13	4,975	7,153
	Feeding vs. non-feeding	(i)	ITL1 vs. ITP	Larval stage 1, Pupa	T1 vs. T4	3,610	3,429
		(ii)	ITL2 vs. ITP	Larval stage 2, Pupa	T2 vs. T4	4,896	3,423
		(iii)	ITL3 vs. ITP	Larval stage 3, Pupa	T3 vs. T4	4,027	3,199
		(iv)	ITB vs. ITP	Sclerotized adult whole body, Pupa	T13 vs. T4	2,473	3,386
B	Tissue-specific	(i)	ITGFG, ITGFF, ITGFH	Callow female gut, callow female fat body, callow female head	T7, T8, T10	4,112	6,024
		(ii)	ITGMG, ITGMF, ITGMH	Callow male gut, callow male fat body, callow male head	T5, T9, T11	4,287	6,177
C	Sex-specific	(ii)	ITGFG vs. ITGMG	Callow female gut, callow male gut	T7 vs. T5	541	99
		(i)	ITGFF vs. ITGMF	Callow female fat body, callow male fat body	T9 vs. T8	3,604	2,847
D	Sclerotized male gut vs. Callow male gut		ITFMG vs. ITGMG	Sclerotized male gut, callow male gut gut	T6 vs. T5	2,767	4,501
E	All gut tissues*		ITGFG, ITGMG, ITFMG	Callow female gut, callow male gut, sclerotized male gut	T5, T6, T7	2,504	5,326

*Supplementary information (Supplementary material 13, 34, 35).

with growth, 170 with signaling, 122 with transport (excluding ABC transporters), and digestion (excluding esterase subfamilies).

C. Sex-specific DGE

We compared the callow male and female ESBB guts (T7 vs. T5) and fat bodies (T9 vs. T8) for gene expression dynamics during maturation feeding (Supplementary Figures 3A, B).

C.1. ESBB gut comparison (T7 vs. T5)

The callow male gut had higher expression levels than the callow female gut for most of the transcripts involved in vital physiological functions, such as detoxification, defense, digestion, transport, metabolism, signaling, and growth (Supplementary Figure 3A, i, ii, iii and Supplementary material 10). Most of the transcripts were upregulated in the male gut (T5), including four cytochrome P450s (301A1, 4G15-like, 4C21-like, CYP410A1), 14 dehydrogenases, 1 ubiquinone oxidoreductase (defense), 6 serine/threonine-protein kinases and 1 phosphatase (signaling), 1 transporter protein and 1 trehalose transporter (transport), 1 serine protease, and 1 phosphodiesterase.

C.2. ESBB fat body comparison (T9 vs. T8)

The callow female and male fat body (T9 vs. T8) comparison showed 7,268 differentially expressed transcripts (FDR-corrected $p < 0.05$); 4,501 were upregulated (fold change ≥ 2), and 2,767 were downregulated (fold change ≤ -2) (Supplementary Figure 3B,i, ii and Supplementary material 11). Most transcripts with molecular functions related to detoxification, defense, digestion, and metabolism had higher expression in the callow male fat body, which is plausibly linked to future host colonization as pioneers (Supplementary Figure 3B iii). Thirteen transcripts related to pheromone biosynthesis were differentially expressed, such as juvenile hormone esterases, epoxide hydrolase, ipsdienol dehydrogenases, juvenile hormone acid O-methyltransferase, and pheromone binding proteins between males and female ESBB fat body. Of these, nine were upregulated in male (T9) and four in female ESBB fat bodies (T8). None of these genes were found to be expressed in the gut comparison.

D. DGE in callow and sclerotized male gut (T6 vs. T5)

The current comparison dealt with the gene expression dynamics in ESBB adults during maturation feeding (callow beetles) and post-maturation feeding (sclerotized). As demonstrated by the PCA plot and heatmap (Figures 8A, B and Supplementary material 12), there was a significant difference in gene expression between sclerotized (black) and callow male gut (T6 vs. T5). A total of 7,268 gene transcripts were significantly expressed (FDR-corrected $p < 0.05$), with 4,501 being upregulated (fold change ≥ 2) and 2,767 being downregulated (fold change ≤ -2). The upregulated gene transcripts include 17 P450s/CYPs, 1 UGT and AchE, 19 hydrolases, 46 dehydrogenases, 6 sulfotransferases, 2 peroxidases, 2 carboxylesterases, and 7 ABC transporters. The downregulated gene transcripts include 26 P450s, 8 UGTs, 12 GSTs, 2 aminoacylases, 14 hydrolases,

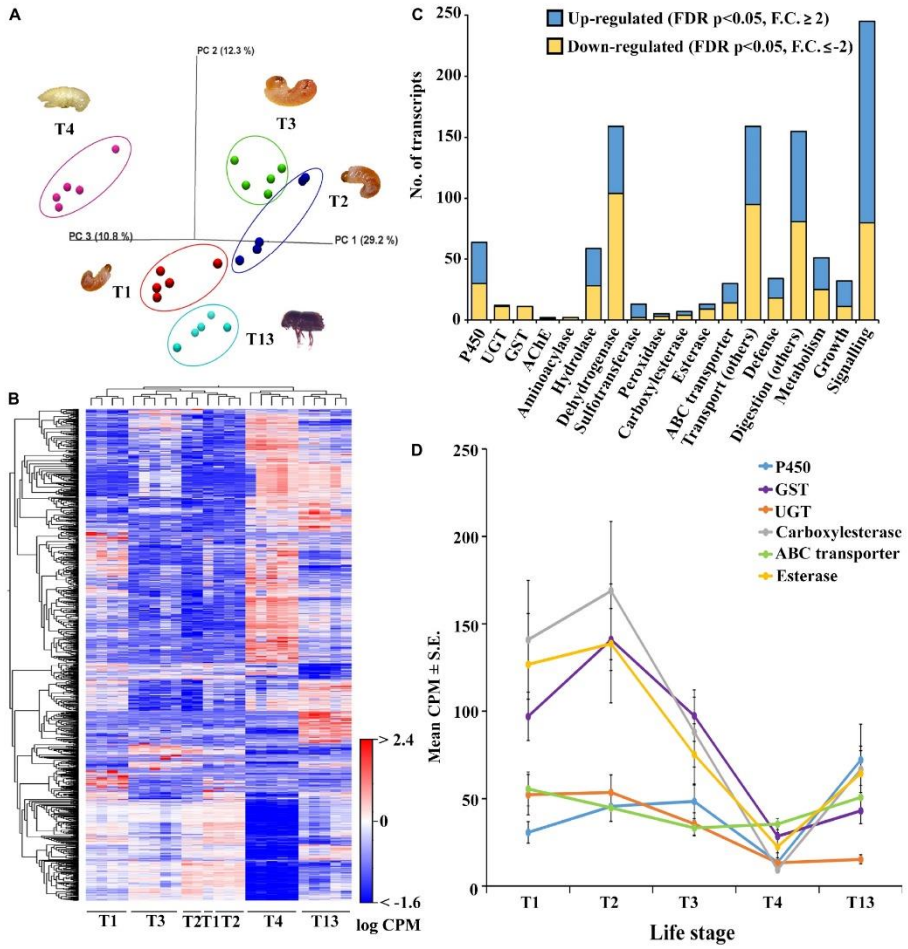

FIGURE 2
Group-wise comparison between different developmental life stages; larvae 1-3 (T1-T3), pupae (T4), and sclerotized adult whole body (T13) (N = 5). **(A)** PCA plot showing sample clustering. **(B)** Heat maps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. **(C)** Bar graphs showing the number of transcripts (CPM) for differentially expressed contigs of specific detoxification-related enzyme families and other essential enzymes related to defense, digestion, transport, metabolism, signaling, and growth after group-wise comparison between different developmental life stages. **(D)** Detoxification enzyme dynamics during different life stages. Average CPM ± standard error values of significantly expressed genes are plotted against each enzyme on the x-axis corresponding to the life stages on the y-axis. The total CPM was calculated by taking the average of all the differentially expressed contigs of each gene family. Analysis was done using CLC workbench (21.0.5, Qiagen, Denmark) with FDR $p < 0.05$ and a fold change ± 2 cut-off.

62 dehydrogenases, 3 sulfotransferases, 2 peroxidases, 1 carboxylesterase, 9 esterase subfamilies, and 43 ABC transporters (Figure 8C). A total of 15 gene transcripts had molecular functions predicted to be associated with defense, 145 with signaling, 18 with growth, 103 with transport (excluding ABC transporter families), 107 with digesting (excluding esterase sub-families), and 27 with metabolism. The detoxification-related gene transcripts were plotted based on average CPM values (mean ± STD error) (Figure 8D), and the majority of these were considerably overexpressed in the callow male gut (T6), indicating that callow beetles need those enzymes in high amounts during maturation feeding under the bark before they sclerotize. When comparing the sclerotized male gut, callow male gut, and callow female gut (T6, T5, and T7) (Supplementary material 13, 34, 35), a similar propensity was observed, with the sclerotized male gut (T6) segregating from the other two (T5 and T7), thus indicating the fine-tuning of gene expression for new colony establishment in the pioneer sex (Supplementary Figure 4). The

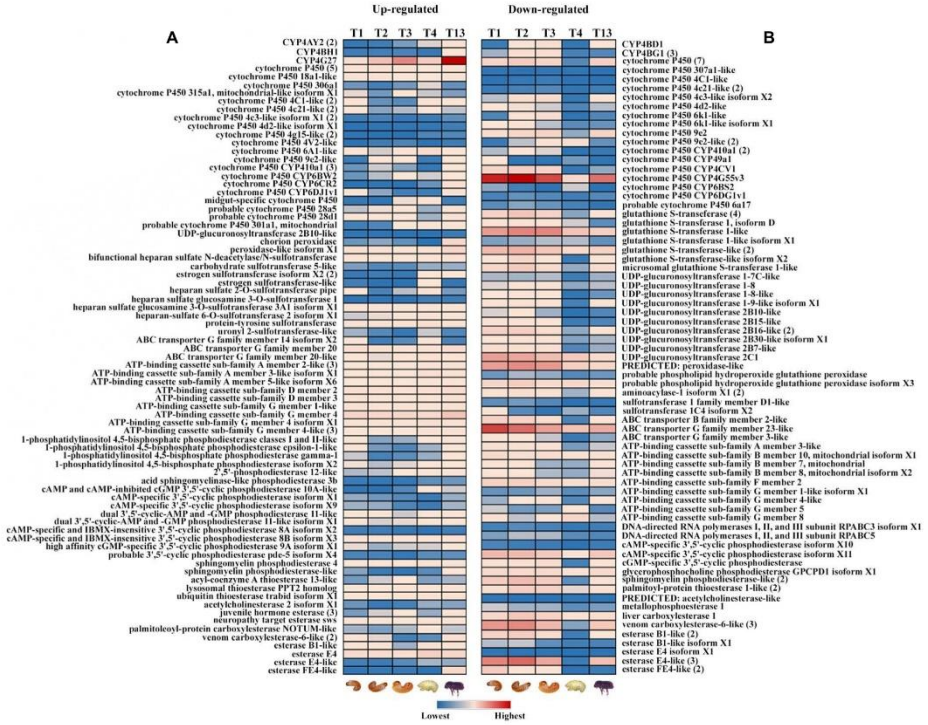

FIGURE 3
Expression profile of detoxification-related genes in different life stages of ESBB. **(A)** Upregulated genes; **(B)** downregulated genes. The details of the genes are given in **Supplementary Excel 3**. The expression level is represented in different colors indicating the TMM-adjusted log CPM expression mean values. Analysis was done with FDR $p < 0.05$ and fold change $\pm$ 2 cut-off in CLC workbench (21.0.5, Qiagen, Denmark).

cytochrome P450s expressed substantially in the sclerotized gut (T6) belong to the known detoxification families 4 (5), family 6 (3), and family 9 (1) (**Figure 9A**). However, the downregulated detoxification-related genes had lower mean CPM values or transcript levels in the sclerotized male gut than in the callow one (**Figure 9B**).

Gene ontology enrichment analysis

Gene ontology enrichment analyses for upregulated and downregulated transcripts were performed in all ten comparisons using the FDR-corrected $p < 0.05$ and a fold change threshold of $\pm$ 2 (**Supplementary materials 14–33**). According to the GO enrichment, the 62 crucial metabolic pathways enriched across seven comparisons could be involved in detoxification, digestion, and defense. Most of these were related to molecular functions (28) and biological processes (34) in the GO classification system (**Figure 10**). In the comparison of developmental stages (T1, T2, T3, T4, vs. T13), 37 critical pathways were enriched, including acyltransferase activity, hydrolase activity, transferase activity, immune response, stress response, and gene expression regulation. The comparison of callow and sclerotized male gut (T5 vs. T6) showed enrichment of 21 pathways in callow beetles, including catalytic activity, enzyme binding, cellular response to stress, and detection of biotic stress. Concerning specific tissues, 19, 11, 16, and 11 metabolic pathways were enriched in callow male and female fatbody (T8 vs. T9), callow male and female guts (T5 vs. T7), callow male gut, fat body, and head (T5, T9, vs. T11) and callow female gut, fat body, and head (T7, T8, vs. T10), respectively. The pheromone-related activity was only enriched in the callow male fat body vs. callow female fat body (T8 vs. T9), implying that pheromone production occurs in the ESBB fat body (**Figure 10**).

RT-qPCR analysis and enzymatic assay

The RT-qPCR was performed for 19 differentially regulated genes in different life stages, sex-specific guts, and fat body comparisons. Most of these genes revealed strong similarities in transcript levels with respect to expression patterns observed in their respective transcriptome expression (**Figures 11–13**).

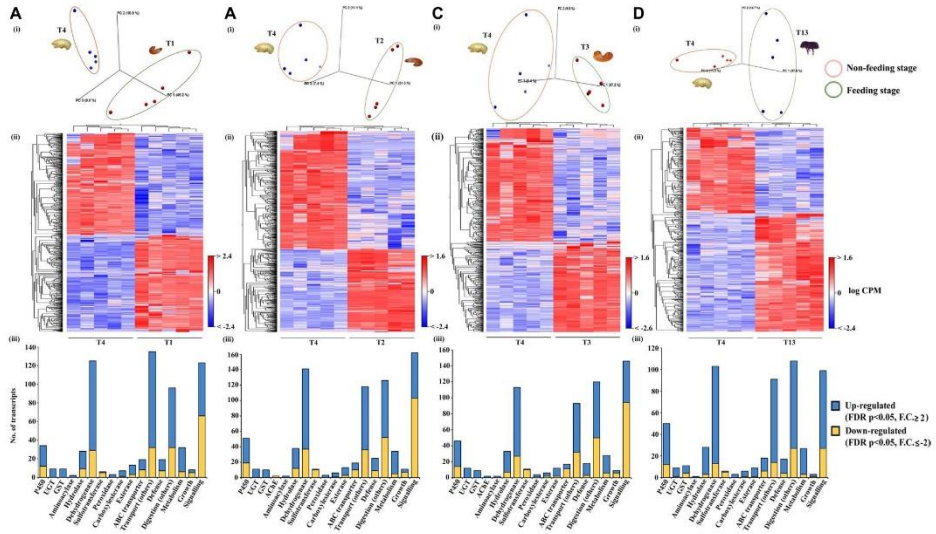

FIGURE 4
Feeding vs. non-feeding stage comparison. Pupa (T4, non-feeding stage) was compared with different feeding stages (T1, T2, T3, and T13) (N = 5). **(A)** PCA plot showing sample clustering. **(B)** Heat maps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. **(C,D)** Bar graphs showing differentially expressed contigs of detoxification-related enzyme families and other essential enzymes related to defense, digestion, transport, metabolism, signaling, and growth between the feeding vs. the non-feeding stage. Analysis was done using the CLC workbench using FDR $p < 0.05$ and fold change ± 2 as the threshold for significance.

The enzyme activity assay of cytochrome P450 reductase (CYP450) and glutathione S-transferase (GST) showed stage-specific expression (Figure 14). When compared against the larval stage 1 (T1), the pupa (T4) and sclerotized adult (T13) stages had significantly higher enzyme activities of GST, while the larval stages showed constitutive expression of GST. The activity of CYP450 decreased from T1 stage to T4 and was lowest in the sclerotized adult stage (T13). Similarly, the callow male ESBB gut (T5) had a relatively low expression of CYP450 but a high expression of GST.

Discussion

The family Curculionidae of the order Coleoptera comprises a massive number of bark beetles that colonize tissues of a wide range of tree species. The damage caused by bark beetles has been extensive in recent years, leading to the destruction of more than 100 million m^3 of spruce forest during a single year in Europe and Asia (Hlásny et al., 2019). This included 14.5 (67% forest cover) million m^3 of spruce forest within Czechia in 2021 (Lubojacký et al., 2022). The revenue loss in the timber industry was so huge that the Czechia state had to support it with ca 260 million euros in 2018–2019 (Hlásny et al., 2021). The management of ESBB necessitates a thorough knowledge of its physiology and adaptation to a nutritionally limiting host. ESBB has four main phases in their life cycle: egg, larva, pupa, and adult (callow and sclerotized). The ESBB larvae (L1, L2, and L3), pupa, and adults are developed under the bark upon exposure to the host allelochemicals. When their development is complete, the sclerotized males, being the pioneer sex, attack new host trees displaying constitutive and induced defenses. The shift from callow (early) to sclerotized (black) adult is a critical phase, during which the beetles undergo various physiological and gene-transcript-level changes that prepare them to establish themselves in a new host. The mechanisms that control the growth and development of ESBBs and their resistance toward spruce allelochemicals are still unknown. RNA-seq has always been a standard method for studying complex gene expression and molecular mechanisms in non-model insects. Hence, we used RNA-seq data (PRJNA679450) from developmental stages (larvae to fully emerged sclerotized beetles) and diverse tissues of ESBB produced during the in-house ESBB genome study to evaluate the gene expression dynamics further. RT-qPCR and enzymatic assays further corroborated the transcriptome data.

A. Gene expression dynamics across life stages of ESBB

A.1. Gene family wise comparison

All detoxifying enzyme families were downregulated in the pupae, non-feeding stage, suggesting that the expression of genes related to detoxification was costly and should be optimally expressed as per requirements, for instance, during host feeding. This phase-specific variation (Figure 2D) showed that the

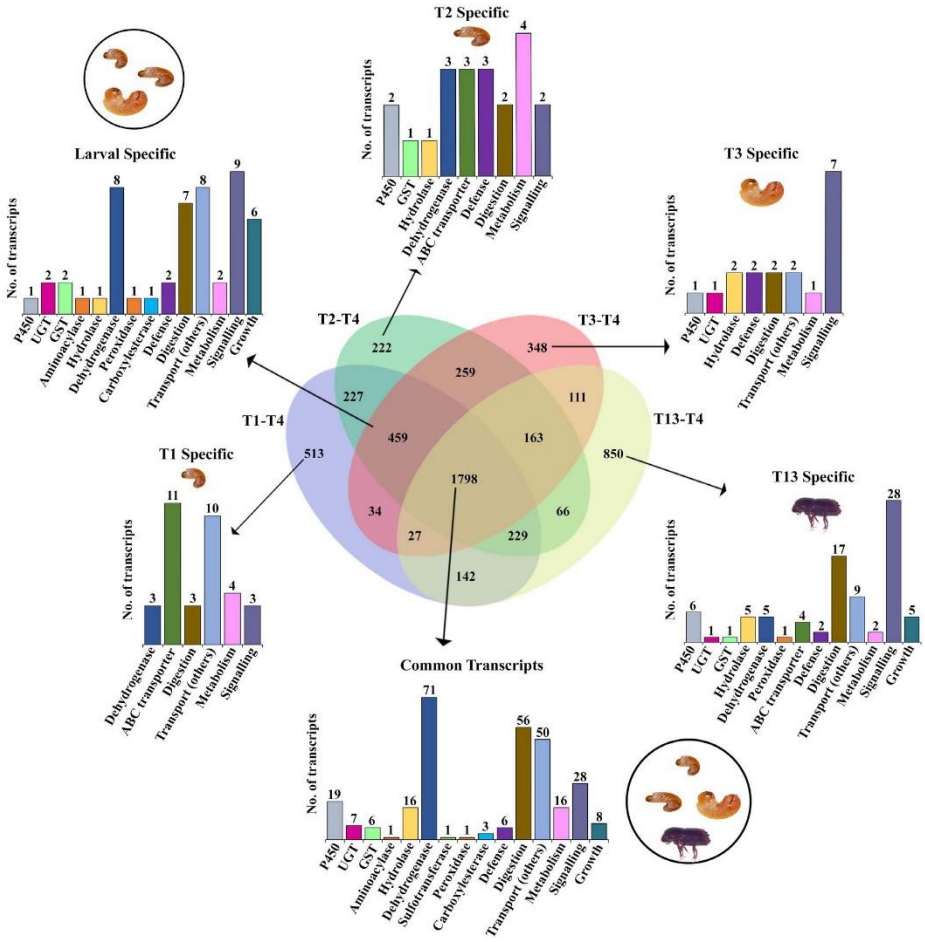

FIGURE 5
Venn diagram representation of feeding vs. non-feeding stage comparison for upregulated enzymes using FDR $p < 0.05$ and fold change ≥ 2 as the threshold for significance. Numbers within the Venn diagram represent the number of contigs found for each group. The bar graph corresponds to the number of contigs of each gene family identified from the list (**Supplementary material 36**).

expression of detoxifying enzymes considerably relies on host encounters and active feeding in ESBB.

A.1.1. Differentially expressed Cytochromes

A.1.1.(a). Cytochromes involved in detoxification

In this study, many detoxification-related cytochrome families (CYP 4, 6, and 9) were overexpressed during the adult life stage. The cytochrome gene expression of the *Dendroctonus* species, close relatives of *I. typographus*, has been extensively researched concerning the detoxification of host allelochemicals. For example, 64 P450s were identified in *D. armandii* at various life stages (larvae, pupae, and adults), and RT-qPCR showed upregulation of 41 of these genes in later life stages as compared to larvae. After host feeding, 19 CYP genes of these 41 genes showed overexpression as compared to the unfed beetles (Dai et al., 2015). *Dendroctonus* bark beetles showed a significant, non-sex-specific change in gene expression of CYP6DG1, CYP6BW5, CYP6DJ2, CYP9Z18, and CYP9Z20 during the early hours of host feeding, implying that cytochrome families 6 and 9 were also involved in the xenobiotic response in beetles as well (Sarabia et al., 2019). In *D. rhizophagus*, the role of CYPs from the CYP4, CYP6, and CYP9 families in detoxification was also confirmed. Recently, three CYPs involved in monoterpene oxidation and one CYP involved in aggregation were identified and characterized in *D. ponderosae* (Chiu et al., 2019a,b). CYP6CR2 is an epoxidase involved in exo-brevicomin

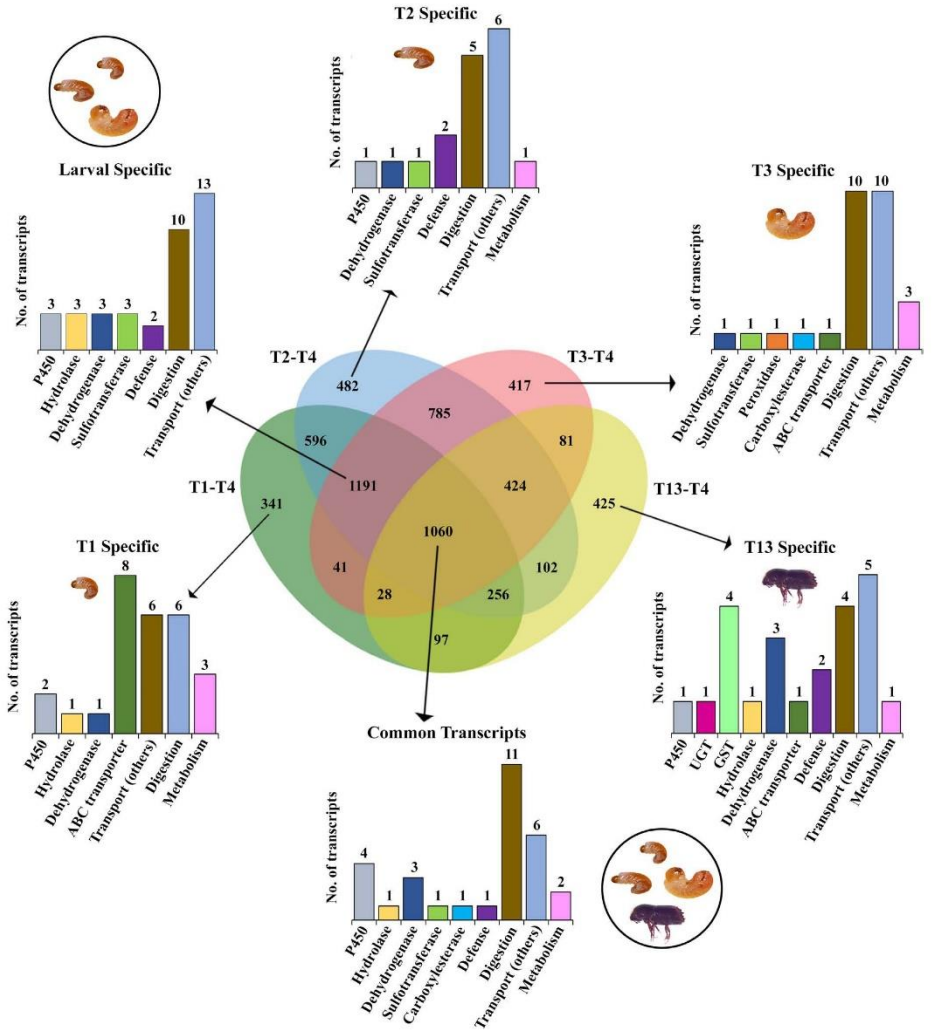

FIGURE 6
Venn diagram representation of feeding vs. non-feeding stage comparison for downregulated enzymes deploying FDR $p < 0.05$ and fold change ≤ -2 the threshold for significance. Numbers within the Venn diagram represent the number of contigs found for each group. The bar graph corresponds to the number of contigs for each gene family identified from the list (**Supplementary material 37**).

biosynthesis and pheromone generation in male mountain pine beetles after they leave the brood tree, but CYP6CR2 decreases during host tree selection and mating (Song et al., 2014). In *Sitophilus zeamais*, terpinen-4-ol fumigation induced differential regulation of cytochrome genes related to detoxification, such as CYP4BH1 (up), CYP6BW2 (up), CYP6DJ1 (up), CYP6DG1 (up), and P450 9E2 (down) (Huang et al., 2018, 2019). Hence, cytochromes are vital components in insect adaptations, including bark beetles.

CYPs, particularly those belonging to the CYP6 gene family, are reported to play a role in detoxification, toxin resistance, and other functions such as pheromone synthesis (Nadeau et al., 2017; Ramakrishnan et al., 2022). In previous investigations, the CYP4 and CYP9 genes have also been reported to have a sex-specific expression when bark beetles are exposed to host tree allelochemicals. For instance, the sex-specific expression of four CYP4 (AY2, G27, BD1, and BG1) and cytochrome P450 9E2 genes were identified in *I. paraconfusus*. The CYP4AY1, CYP4BG1,

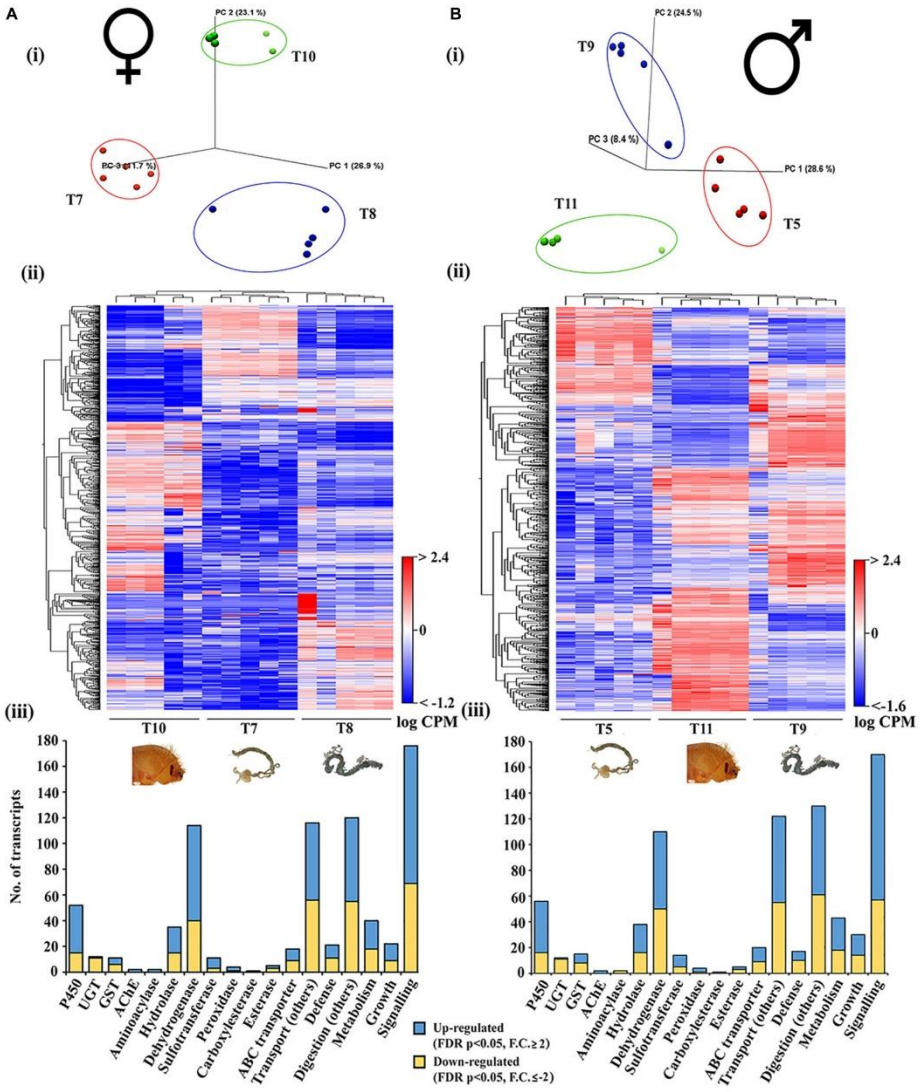

FIGURE 7
Tissue-specific comparison in callow male and female beetles. **(A)** Callow female head (T10), Callow female gut (T7), and Callow female fat body (T8) (N = 5). **(B)** Callow male head (T11), Callow male gut (T5), and Callow male fat body (T9) (N = 5). (i) PCA plots showing sample clustering. (ii) Heat maps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. (iii) Bar graphs showing differentially expressed contigs of detoxification-related enzyme families and other essential enzymes related to defense, digestion, transport, metabolism, signaling, and growth after group-wise comparison between different developmental life stages. Analysis was done using the CLC workbench using FDR $p < 0.05$ and fold change ± 2 as cut-offs for significance.

and CYP9T1 genes are expressed in male *I. paraconfusus*, suggesting their putative involvement in synthesizing male-specific aggregation pheromones (Huber et al., 2007). Similarly, after exposing *D. rhizophagus* to host-tree monoterpenes, differential expression of the CYP4G27, CYP4AY1, and CYP4AY2 genes was observed in the female and male antenna and gut (Cano-Ramirez et al., 2013). In our data, the CYP4BH1 gene was highly expressed in ESBB adults, whereas the CYP4BG1 and CYP4BD1 gene expression were low. The differential expression of these genes suggested that feeding regulates their expression. While we

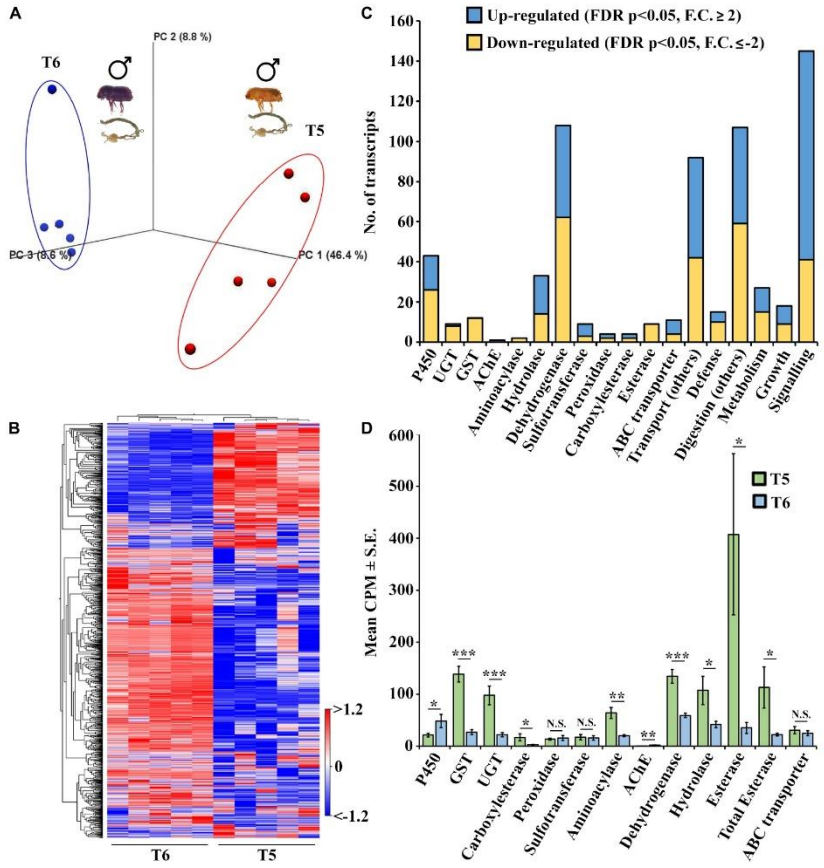

FIGURE 8
Comparison between sclerotized male gut (T6) vs. callow male gut (T5) $N = 5$. **(A)** PCA plots showing sample clustering. **(B)** Heat maps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. **(C)** Bar graphs showing the differentially expressed contigs of specific detoxification-related enzyme families and other important enzymes related to defense, digestion, transport, metabolism, signaling, and growth between sclerotized male gut (T6) vs. callow male gut (T5). **(D)** Detoxification genes expressed as the average of total differentially expressed genes in each family between the sclerotized male vs. the callow guts. Gene families on the x-axis plotted against their corresponding mean CPM values ± S.E. One-tailed *T*-test was performed to obtain the significant differences between the two stages. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, and NS represents non-significant difference. DGE analysis was done using CLC workbench with FDR $p < 0.05$ and a fold change ± 2 cut-off.

did not determine the sex of these ESBB adults, the regulation may be sex-specific and related to pheromone biosynthesis pathways, as reported earlier for *Dendroctonus ponderosae* (Nadeau et al., 2017). However, this needs to be functionally validated in ESBB.

Previous research has verified that some of the identified CYP6 family cytochromes (CYP6DJ1, CYP6BW1, and CYP6BW3) in *Dendroctonus* spp. are involved in pheromone biosynthesis pathways during the oxidation of host terpenes (Chiu et al., 2019b). Symbiotic blue-stain fungi vectored by bark beetles synthesize bicyclic ketals, i.e., pheromones and other semiochemicals (exo-brevicomin, endo-brevicomin, and trans-conophthorin) and help the beetles to colonize healthy trees (Lee et al., 2006; Zhao et al., 2019). A recent study showed that the CYP6CR2 and CYP6DE5 genes were highly expressed under the treatment of terpenoids, whereas silencing of these genes significantly reduced the activity of P450 and increased the mortality of adults after exposure to terpenoids. CYP6CR2 expression was considerably higher in larvae and sclerotized adults, with lower expression in starving adult mountain pine beetles (Robert et al., 2013, 2016; Liu et al., 2022). A transcriptome study of the imidacloprid resistance (IR) and isogenic susceptible (IGS) strains of *Aphis gossypii* revealed that the resistant strain had high gene expression of cytochrome P450 4G15-like (Kim et al., 2015). We found that the CYP6CR2 gene was expressed 400-fold higher in sclerotized adult beetles than in the other stages. Cytochrome P450 4G15-like gene expression

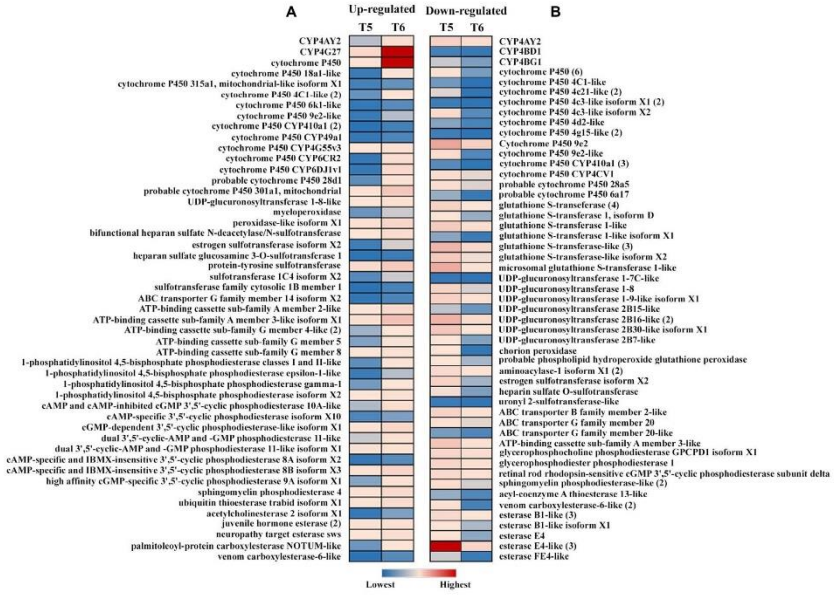

FIGURE 9
Expression profiles of detoxification-related genes in the sclerotized male gut (T6) vs. callow male gut (T5) (N = 5). **(A)** Upregulated genes FDR corrected $p < 0.05$ and equal or above the threshold of fold change of 2; **(B)** downregulated genes FDR corrected $p < 0.05$ and equal or below the threshold of fold change of −2. The expression level is represented in different colors indicating the TMM-adjusted log CPM expression mean values.

was also higher in adult ESBB, suggesting that both genes may play a role in detoxifying plant-allelochemicals.

A.1.1.(b). Cytochromes involved in other physiological functions

In general, cytochrome genes are involved in many critical physiological functions in addition to detoxification. For instance, ecdysteroid 26-hydroxylase encoded by CYP18A1 is a key hormone inactivation enzyme. CYP18A1 is expressed in many of the target tissues of ecdysteroid (20E) in *Drosophila* larvae, and CYP18A1 inactivation slows the larval development and causes pupal mortality, while CYP18A1 overexpression causes late embryonic lethality. According to these findings, the inactivation of 20E is required for proper growth, and CYP18A1 is a crucial enzyme in this process (Guittard et al., 2011). In the current study, the CYP18A1-like gene was constitutively expressed from the initial larvae to the adult, which showed that uniform expression of this gene may aid larval development and pupal formation and adult development (**Figure 3B**). The enzyme Cytochrome P450 306A1 is involved in the metabolism of insect hormones, such as ecdysteroid C25-hydroxylase activity, and the breakdown of synthetic pesticides. The effective conversion of keto-diol to keto-triol in CYP306A1 transfected S2 cells *via* carbon 25 hydroxylation suggests that CYP306A1 works as a carbon 25 hydroxylase and plays an essential role in ecdysteroid production during insect development (Niwa et al., 2004). Cytochrome P450 306A1 gene was highly expressed in the pupal stage, suggesting that it may aid in the pupal transition into an adult. The enzyme CYP4C1 is involved in abiotic stress tolerance. For instance, silencing of CYP4C1 causes *B. tabaci* to have much lower heat resistance and higher cold tolerance, implying that CYP4C1 is a significant regulator in temperature adaptation and geographical distribution (Shen et al., 2021). The CYP4C1-like gene was highly expressed in the sclerotized adult ESBB, suggesting its putative involvement in winter survival, range expansion, and adaptability to different environments. Chalkbrood is the most common fungal disease in honeybees, and transcriptome investigation of the immunological defenses of *A. cerana* larvae to *A. apis* infection revealed that the fungal-infected strain had a high expression of the cytochrome P450 6A1-like gene (Guo et al., 2019). The cytochrome P450 6A1-like gene was significantly expressed in the ESBB pupal stage, suggesting that it may aid pupal resistance to fungal infection (**Figure 3B**). Interestingly, CYP4BD1 was expressed in all larval stages and sclerotized adults but not in the pupa (**Figure 3B**). CYP4BD1 expression was higher in males and did not differ significantly in females compared to unfed individuals; it might be required for male-specific physiological activities such as pheromone production and/or juvenile hormone biosynthesis (Tillman et al., 1998, 2004; Helvig et al., 2004; Huber et al., 2007).

A.1.2. Differentially expressed UGTs and GSTs

We found UGTs and GSTs expressed in various life stages and gut tissues of ESBB (**Figure 3**). UGTs are endoplasmic reticulum-associated enzymes that perform glycosylation using

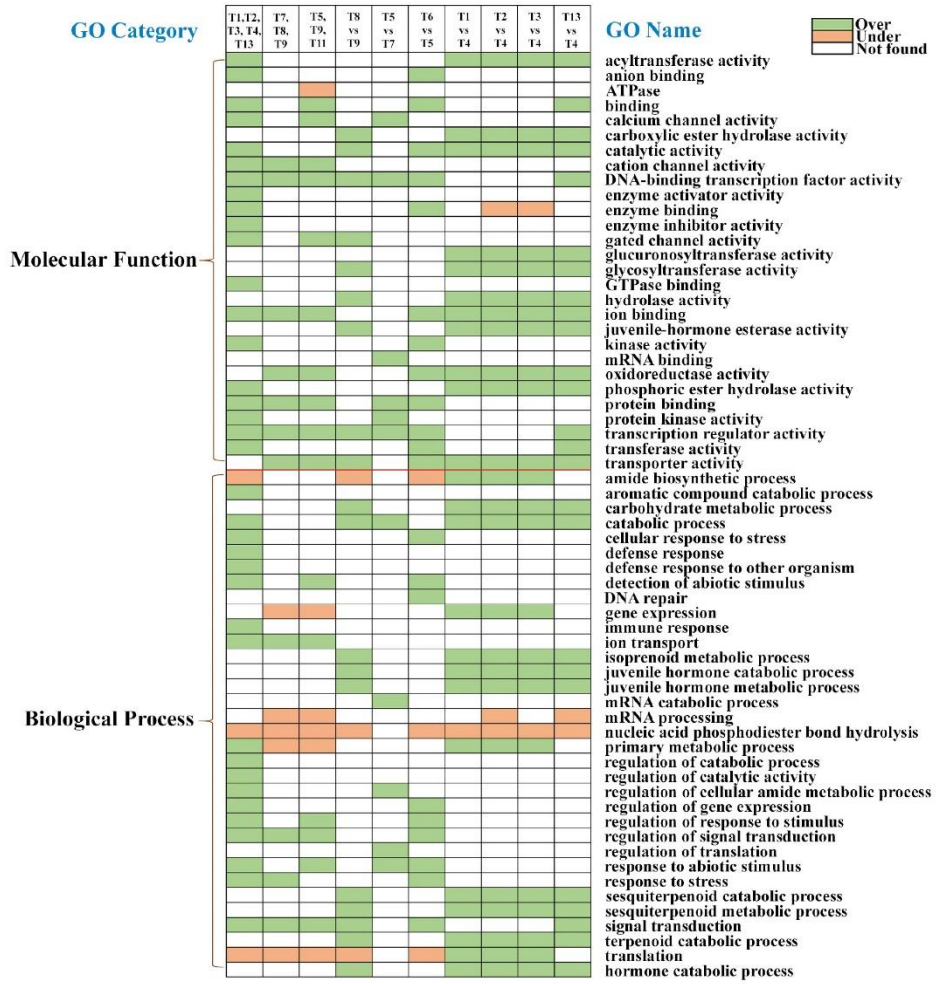

FIGURE 10
Critical physiological processes in different ESBB sample comparisons after GO enrichment analysis (FDR $p < 0.05$, fold change ≥ 2). Green boxes are over-enriched, orange boxes are under-enriched, and white boxes represent processes not occurring in a particular comparison.

UDP-glucose as an active sugar source. The protein structures are composed of N-terminal aglycone substrate-binding and C-terminal UDP-glycoside binding domains. About 40 UGTs in *H. armigera* and 44 UGTs in *Bombyx mori* were identified, and two UGT genes, UGT41B3 and UGT40D1, have been linked to metabolizing gossypol *via* glycosylation in *H. armigera* (Ahn et al., 2012; Krempl et al., 2016). Ecdysteroid UDP-glycosyltransferase (EGT), a baculovirus-encoded protein, activates and regulates insect molting by ecdysteroid hormones (Shen et al., 2018). UGTs were reported to aid host plant allelochemical detoxification in *Spodoptera* (Roy et al., 2016). The UDP-glucuronosyltransferase 2B10 (UGT2B10) is a detoxifying enzyme specializing in the N-linked glucuronidation of numerous drugs and xenobiotics. In our investigation, the UDP-glucuronosyltransferase 2B10-like gene was significantly expressed in sclerotized adults, suggesting that it may play a role in detoxification (**Figure 3B**). The UDP-glucuronosyltransferase 1-8 (UGT1A8) is an enzyme that participates in the glucuronidation pathway, which converts small lipophilic compounds into water-soluble, excretable metabolites (Wang et al., 2013). UDP-glucuronosyltransferase 1-8 and UDP-glucuronosyltransferase 1-8-like genes were expressed in ESBB larvae, probably facilitating food digestion and excretion.

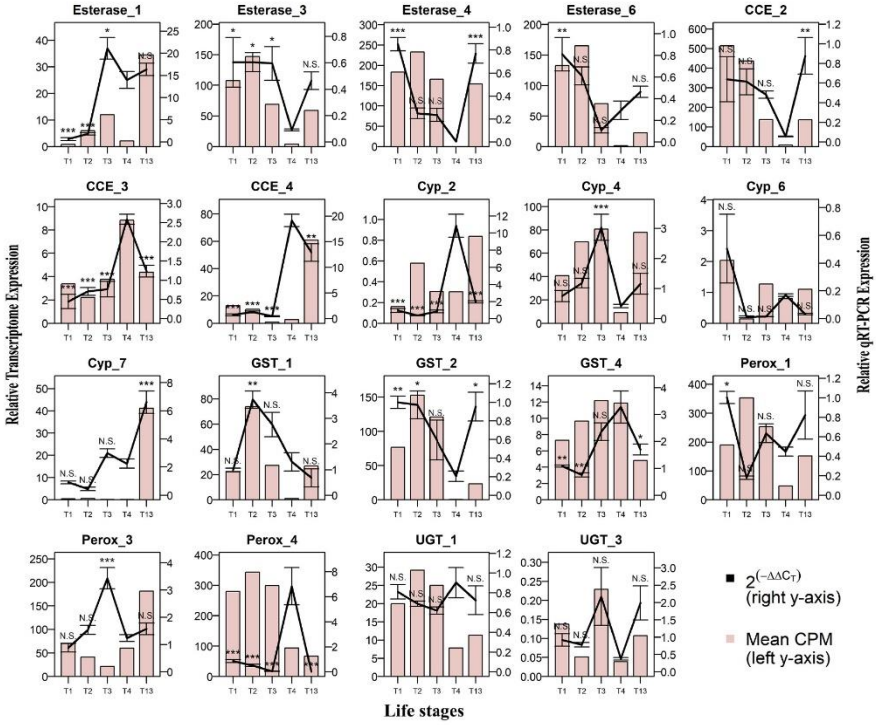

FIGURE 11
Comparison of RNA-seq and RT-qPCR data for the relative expression of 19 genes in developmental life stages. Life stages: 1st instar larvae (T1), 2nd instar larvae (T2), 3rd instar larvae (T3), pupae (T4), and sclerotized adult beetles (T13) on the *x*-axis plotted against relative expression in RNA seq ($N = 5$) represented as mean CPM on the left *y*-axis, and relative expression in RT-qPCR ($N = 4$) represented as mean 2^{-}-($\Delta\Delta$Ct) ± S.E. on the right y-axis. The bar graph represents CPM-based expression from RNA-seq, and the solid line represents RT-qPCR expression. Tukey's HSD was performed with a 95% confidence level with T4 as the control group, and *p*-values were generated as per the fit model. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, and *N.S.* represents non-significant difference.

Glutathione S-transferases are well-known for mediating insecticide resistance by reductive dehydrochlorination or conjugation reactions (Kostaropoulos et al., 2001; Enayati et al., 2005; Song et al., 2022). In *S. litura*, RNA-seq-based DGE studies were used to identify 8 GSTs and verify the involvement of GSTS1 in tomatine metabolism (Li et al., 2019). Previous studies have reported 16 GSTs from four different groups (delta, epsilon, sigma, and theta) in the Chinese white pine beetle, and these GSTs were expressed in all developmental stages and tissues (antennae, gut, and reproductive tissues) (Gao et al., 2020). Similarly, the present study observed GSTs expression during the different life stages and tissues of ESBB, implying its physiological importance in bark beetles (**Figure 3**).

A.1.3. Differentially expressed esterases

The overexpression of esterases (ESTs), particularly those belonging to class I (clade A-C), aids detoxification, while class III (clade I-M), containing CCE and AChEs (clade I-M), works primarily through target site mutation-based resistance to pyrethroids, organophosphates, and carbamates, but their role in detoxification has also been documented concerning malathion detoxification (Wei et al., 2020). The NOTUM gene encodes a palmitoleoyl-protein carboxylesterase that works as a negative regulator of the Wnt signaling pathway. NOTUM knockdown lentivirus inhibits colon adenocarcinoma development *in vitro* and *in vivo* by reducing tumor proliferation, shrinking tumor size, and enhancing apoptosis (Gong et al., 2021). The palmitoleoyl-protein carboxylesterase NOTUM-like gene was significantly expressed in ESBB pupae, implying that it regulates apoptosis in the non-feeding stage. Carboxylesterase 6 has the highest expression level in the social wasp *P. varia*, while solitary wasps have little or no expression, suggesting that this protein may play a defensive role in social wasps (Yoon et al., 2020). The venom carboxylesterase-6-like gene was highly expressed in ESBB sclerotized adults, suggesting its putative involvement in the ESBB defense mechanism (**Figure 3A**). In insects, chorion peroxidase (pxt) has a role in the formation of a rigid and insoluble egg chorion by catalyzing chorion protein crosslinking through dityrosine formation and phenol oxidase-catalyzed chorion melanization (Li et al., 2004). Chorion peroxidase and peroxidase-like isoform X1 were highly expressed in sclerotized

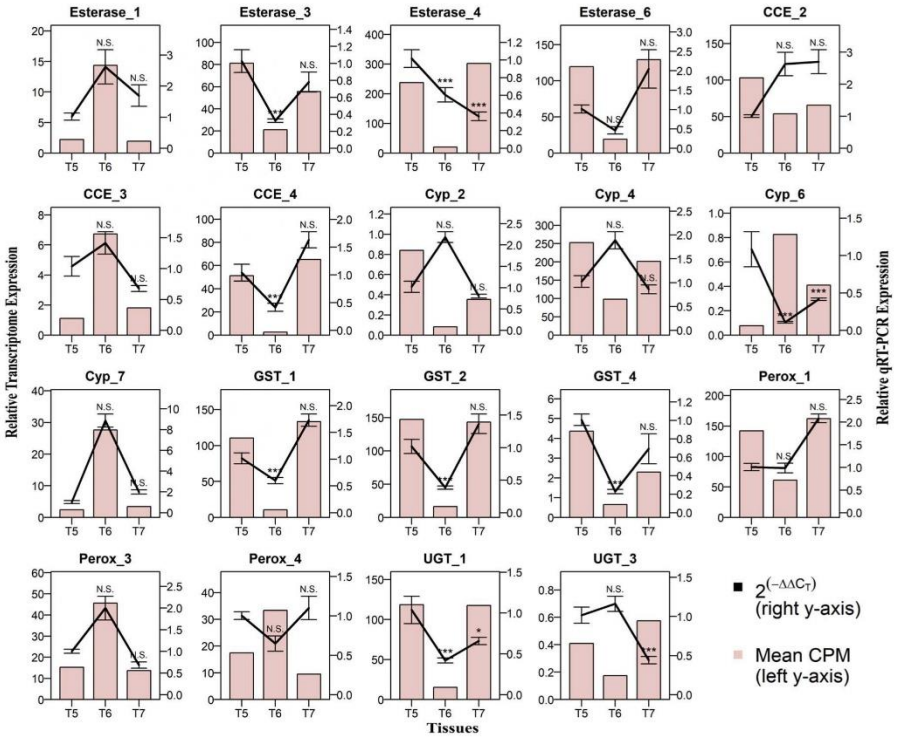

FIGURE 12
Comparison of RNA-seq and RT-qPCR data for the relative expression of 19 genes in gut tissues. Callow male gut (T5), sclerotized adult male gut (T6), and callow female gut (T7) on the *x*-axis plotted against relative expression in RNA seq ($N = 5$) represented as mean CPM on the left *y*-axis and relative expression in RT-qPCR ($N = 4$) represented as mean 2^{-}-($\Delta\Delta$Ct) ± S.E. on the right *y*-axis. The bar graph represents the mean CPM-based expression from RNA-seq transcriptome data, and the solid line represents RT-qPCR expression. Relative expressions in T6 and T7 were compared against those in T5 using Tukey's HSD with a 95% confidence level, and *p*-values were generated as per the fit model. * represents $p < 0.05$, *** represents $p < 0.001$, and *N.S.* represents non-significant difference.

adults, suggesting that they may function in egg production and development (**Figure 3A**). In *Myzus persicae*, the overexpression of E4 and FE4 genes have a role in insecticide resistance (Field and Devonshire, 1998). In our study, the esterase FE4-like gene was expressed in all larval and sclerotized adult stages but not in the pupae, suggesting that it may influence the detoxification of host allelochemicals during feeding (**Figures 3A, B**). However, esterase B1-like and esterase E4 genes were expressed in pupae, suggesting that they may be involved in fat consumption for energy production during pupa-to-adult development. However, our observations need further experimental validation.

A.1.4. Differentially expressed hydrolases

The phospholipase activity of the mitochondrial cardiolipin hydrolase gene is essential for mitochondrial fusion and fission, allowing cells to cope with the increased nucleotide demand during DNA synthesis (Huang et al., 2011). In the current study, the mitochondrial cardiolipin hydrolase-like gene was highly expressed in late larval and adult stages, suggesting its involvement in larvae-to-pupae conversion and adult development. The protein deubiquitination carried by the ubiquitin C-terminal hydrolase (UCH) and ubiquitin-specific processing protease (UBP) protein families is involved in various biological activities in animals, fungi, and plants (Wang et al., 2018). Ubiquitin carboxyl-terminal hydrolase 1-like gene was significantly expressed in pupae and adult ESBB stages compared to the larvae, suggesting its putative role in later stages of ESBB development.

A.1.5. Differentially expressed transporter enzymes

ATP-binding cassette transporter proteins transport a variety of molecules across cell membranes. ATP binding cassette subfamily A member 12 (ABCA12) protein is essential for transporting fats (lipids) and enzymes in the cells and maintaining the layers of lipids within the epidermis to prevent water loss (dehydration) and to allow normal skin development (Fukuda et al., 2012; Akiyama, 2014). The expression of ABC transporters remained primarily constant throughout development, suggesting their importance

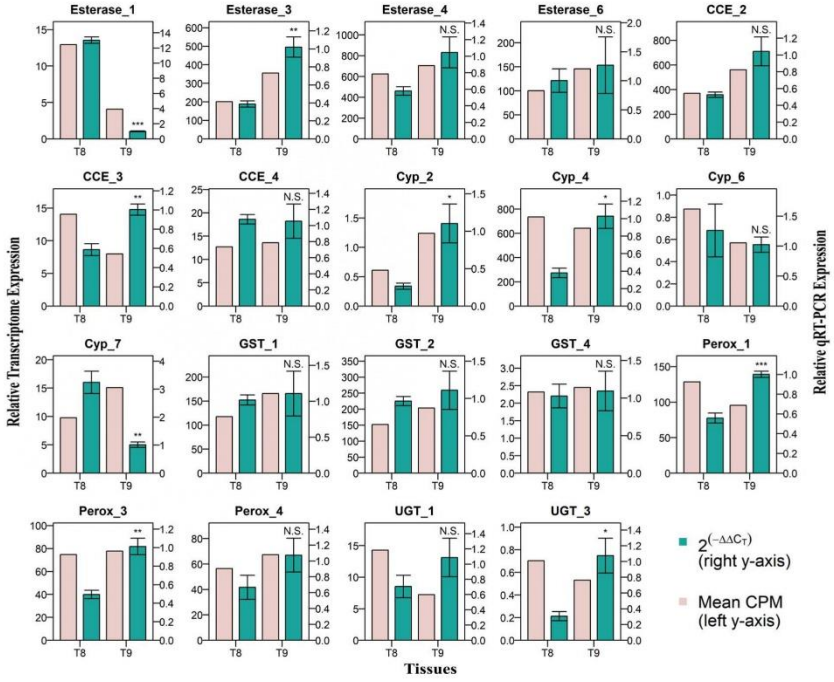

FIGURE 13
Comparison of RNA-seq and RT-qPCR data for the relative expression of 19 genes during RT-qPCR in the fat body tissues. Callow female fat body (T8) and callow male fat body (T9) on the *x*-axis plotted against relative expression in RNA seq ($N = 5$) represented as mean CPM on the left *y*-axis, and relative expression in RT-qPCR ($N = 4$) represented as mean 2^{-}-(ΔΔCt) ± S.E. on the right *y*-axis. Light brown bars represent mean transcriptome expression, and dark green bars represent RT-qPCR expression. Student's *T*-test was performed with a 95% confidence level, and *p*-values were generated after comparing RT-qPCR expression between T9 vs. T8. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, and *N.S.* represents the non-significant difference.

throughout the life cycle of ESBB. In the larval and adult stages, the ABC transporter subfamily genes (D member 3, G member 1-like, and G member 4) exhibited higher expression than in the pupal stage (**Figure 3**). Interestingly, ABC transporter expression dropped dramatically during the early ESBB larval stage, but increased again in the pupal and adult stages, demonstrating its role in later developmental stages in ESBB. Interestingly, all ABC transporter subfamily A genes had higher expression in pupae, suggesting that these genes might play an essential role during the pupal stage. The ABC transporter subfamilies D and G genes expressed in other ESBB life stages may be involved in detoxification during feeding. Furthermore, ATP-binding cassette sub-family A member 2-like and ATP-binding cassette sub-family A member 3-like isoform X1 were highly expressed in ESBB pupae, suggesting that they may facilitate proper epidermis formation in ESBB. The ATP-binding cassette sub-family G member 4 (ABCG4) subunit of heterodimeric precursor transporters for eye-pigment synthesis was identified in *Drosophila's* brain and eye (Oldfield et al., 2002). In the current study, the ATP-binding cassette sub-family G member 4, ATP-binding cassette sub-family G member 4-like, and ATP-binding cassette sub-family G member 4 isoform X1 genes were significantly expressed in ESBB sclerotized adults as compared to larvae and pupae, indicating that they may play a similar role in eye pigment synthesis. The ATP binding cassette subfamily G member 8 (ABCG8) and member 5 (ABCG5) genes produced sterolin-1 and sterolin-2, respectively, and combinedly formed sterolin protein (Calandra et al., 2011). Sterolin is a plant sterol transporter protein that moves substances across cell membranes and is found mainly in intestines and liver cells. Sterolin also helps cholesterol regulation; typically, about 50 percent of the cholesterol in the diet is absorbed by the body (Calandra et al., 2011). The ATP-binding cassette sub-family G member 8 and ATP-binding cassette sub-family G member 5 genes were significantly expressed in ESBB pupae, implying that they may be involved in fat absorption and accumulation to provide energy for adult conversion.

A.1.6. Differentially expressed dehydrogenases

The mitochondrial glutamate dehydrogenase catalyzes the conversion of L-glutamate into alpha-ketoglutarate, an essential intermediate in the tricarboxylic acid cycle. It plays a role in insulin homeostasis and is also involved in learning and memory reactions by increasing the turnover of the excitatory neurotransmitter glutamate (Plaitakis et al., 2000, 2017). In this

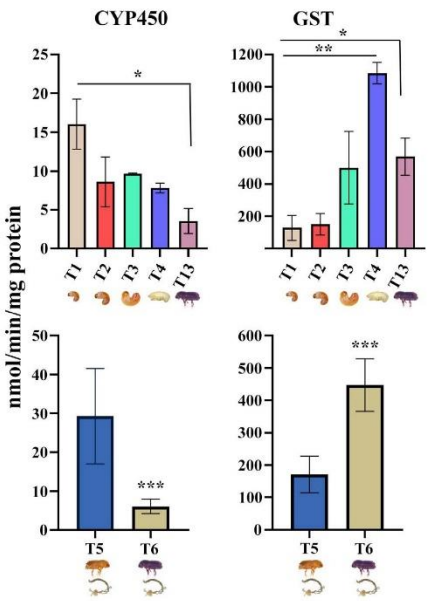

FIGURE 14
The activities of selected enzymes, glutathione S-transferase (GST) and cytochrome P450 (CYP450), measured in the developmental and tissue-specific stages. The bars represent mean values and whiskers standard deviations in four biological replicates. One-way ANOVA was performed between the life stages (T1, T2, T3, T4, and T13) and Student's *t*-test between guts of callow and sclerotized males (T6 vs. T5). * indicates that values are significantly different from each other at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. GST, and CYP450 activity are expressed as nmol/min/mg protein.

study, glutamate dehydrogenase mitochondrial-like protein and glutamate dehydrogenase mitochondrial-like isoform X2 genes were highly expressed from late larvae to adults, suggesting that they may be involved in energy production and ESBB development (**Supplementary material 3**). In the glycolytic pathway, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a highly conserved enzyme that catalyzes the transformation of glyceraldehyde-3-phosphate to glycerate-1, 3-biphosphate, and produces NADH. In the *Mortierella alpina* fungus, the overexpression or knockdown of GAPDH1 and GAPDH2 genes significantly accumulated or reduced lipid content, respectively (Wang et al., 2020). In the current study, the glyceraldehyde-3-phosphate dehydrogenase-like and glyceraldehyde-3-phosphate dehydrogenase 2-like genes were highly expressed from late larvae to adults. Fungi and insects share common metabolic pathways, and our data implied that these genes may have a role in lipid accumulation to satisfy the energy requirement for ESBB while searching for a suitable host. The glucose dehydrogenase genes are expressed at all stages of *Drosophila* development and play an essential role in the eclosion process and cuticular remodeling (Krasney et al., 1990). Similarly, the glucose dehydrogenase (FAD, quinone)-like gene was expressed in all ESBB developmental stages, suggesting its involvement in the eclosion process and cuticular remodeling in ESBB. In *Ips pini*, the short-chain oxidoreductase ipsdienol dehydrogenase (IDOLDH) converts (-)-ipsdienol to ipsdienone and plays a role in determining pheromone composition (Figueroa-Teran et al., 2016). In the current study, ipsdienol dehydrogenase transcripts were expressed from initial larvae to adults, suggesting that they may have a similar role in pheromone biosynthesis and composition in ESBB (Ramakrishnan et al., 2022). The glutaryl-CoA dehydrogenase (GCDH) is an enzyme found in mitochondria and involved in the breakdown of amino acids like lysine, hydroxylysine, and tryptophan, which are building blocks of proteins (Basinger et al., 2006). The glutaryl-CoA dehydrogenase mitochondrial gene was expressed in all larval and adult stages in our study but not in the pupae, implying a function in protein formation during ESBB feeding stages. However, such observations need to be further empirically validated.

B. Tissue-specific comparison

We observed tissue-specific regulation of many genes; for instance, CYP6K1-like and CYP410A1 had higher expression in the head and lower expression in the gut and fat body, respectively, implying a role in olfaction as odorant degrading enzymes (ODEs) (Baldwin et al., 2021). Similarly, juvenile hormone epoxide hydrolase 1-like (JHEH 1-like) gene transcripts were more abundant in the gut and fat body than in the head, as these two tissues are the site of production and metabolism of aggregation pheromone in ESBB (Ramakrishnan et al., 2022). Our findings (**Figure 7**) were consistent with earlier research showing the upregulation of detoxification-related genes and pheromone genes in the gut and fat body (Keeling et al., 2016; Li et al., 2019; Ramakrishnan et al., 2022). Cytochrome P450 4C1 (CYP4C1) transcripts are mainly engaged in the metabolism of insect hormones and the breakdown of synthetic insecticides. Whiteflies, after silencing CYP4C1, showed significantly lower heat resistance and higher cold tolerance, which indicates that CYP4C1 is a critical regulator in temperature adaptation and influences the geographical distribution and dispersal of whiteflies (Shen et al., 2021). In the present study, the cytochrome P450 4C1-like gene transcripts showed high levels in male and female fat bodies and heads of callow ESBB, suggesting a putative role in thermal adaptation during new host finding. In bark beetles, the CYP9T2 (gut-specific cytochrome P450) gene hydroxylates myrcene to ipsdienol and functions toward the end of the pheromone biosynthesis pathway (Sandstrom et al., 2006). Gut-specific cytochrome P450 transcript was expressed in both male and female callow ESBB fat body, suggesting a putative role in pheromone biosynthesis and odor degradation.

The glutathione S-transferases (GSTs) play an essential role in detoxifying xenobiotic toxins in insects, including insecticides (Balakrishnan et al., 2018). Most GSTs, such as glutathione S-transferase-like, glutathione S-transferase-like isoform X2, and microsomal glutathione S-transferase 1-like transcripts, are expressed in the callow beetle's gut, suggesting roles in food digestion and detoxification of plant xenobiotics. UDP-glucuronosyltransferases (UGTs) are significant phase II drug metabolism and multifunctional detoxification enzymes that play an essential role in insect resistance to various plant allelochemicals and pesticides (Neumann et al., 2016; Roy et al., 2016; Cui

et al., 2020). Most UGTs, such as UDP-glucuronosyltransferase 1-8, UDP-glucuronosyltransferase 1-9-like isoform X1, UDP-glucuronosyltransferase 2B10-like, UDP-glucuronosyltransferase 2B16-like, UDP-glucuronosyltransferase 2B30-like isoform X1, and UDP-glucuronosyltransferase 2C1, are expressed in the male and female callow ESBB gut, implying a role in multifunctional detoxification during maturation feeding of ESBB. Aminoacylase 1 (ACY1) was found in many tissues and organs, where it may be involved in breaking down proteins that are no longer needed. A novel aminoacylase isolated from *Burkholderia* sp. strain LP5_18B effectively catalyzes N-lauroyl-L-amino acids synthesis (Takakura and Asano, 2019). In the current study, aminoacylase-1 isoform X1 was expressed in both callow ESBB male and female guts, suggesting a role in dietary protein breakdown. Epoxide hydrolases (EH) are essential regulators of lipid epoxides and play a role in detoxification processes, and their inhibition can have a physiological and pathological impact (Morisseau, 2013). Epoxide hydrolase 4-like gene was expressed highly in the fat bodies of both male and female callow ESBB, suggesting that it may be required for lipid regulation and detoxification and also help in the physiological process upon conversion of immature callow to mature sclerotized (black) beetles. Glycosyl hydrolases hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The enzymatic activity and ancestral origin suggest that glycoside hydrolase families 45 (GH45s) were likely essential for the adaptation of phytophagous beetles to feed on plants (Busch et al., 2019). GH45 transcript was highly expressed in both the gut and fat bodies of males and females of callow ESBB, suggesting roles in food digestion, detoxification, and host adaptation. The juvenile hormone epoxide hydrolase (JHEH) is an essential enzyme in the breakdown pathways of juvenile hormone (JH) in insects; it converts JH to JH-diol and hydrolyzes JH acid to JH acid-diol, and JHEH titers regulate the entire process of insect development. In the gypsy moth *Lymantria dispar*, the knockdown of JHEH1 slightly delayed larval development (Wen et al., 2018). The juvenile hormone epoxide hydrolase 1-like transcripts were highly expressed in the male and female guts and fat bodies of callow ESBB, suggesting that they may be involved in JH pathway regulation. The genome-wide microarray study in gut tissue of Indian silkworm *Bombyx mori* Sarupat race resistant against BmNPV infection of gut showed upregulation of the lactase-phlorizin hydrolase-like gene, indicating that the gene plays a role in the antiviral immune response (Lekha et al., 2015). The lactase-phlorizin hydrolase-like transcript was highly expressed in the gut of callow ESBB, probably performing a similar role by protecting the beetles from getting infected with pathogenic viruses during maturation feeding.

Sulfotransferase catalyzes the sulfate conjugation of catecholamines such as dopamine, prostaglandins, leukotriene E4, drugs, and xenobiotic substances by using 3'-phospho-5'-adenylyl sulfate (PAPS) as a sulfonate donor. Sulfonation enhances the water solubility of most chemicals and facilitates their excretion, but it can also lead to bioactivation and the formation of active metabolites (Shimada et al., 2004). In the present study, the sulfotransferase 1 family member D1-like transcript was highly expressed in the gut of callow ESBB, pointing toward similar xenobiotic catabolism. The chorion peroxidase is expressed in mature eggs of *Aedes aegypti* mosquitoes and is involved in forming a rigid and insoluble chorion (egg shell) by catalyzing chorion protein cross-linking (Han et al., 2000). Esterases are often present in antennae and help in odor desensitization (Guo and Smith, 2022). Esterase E4 transcripts were expressed in the head of callow ESBB, possibly performing a similar function. The ABCB subfamily contains both full-transporters and half-transporters; ABCB2 and ABCB10 are half-transporters with evolutionarily conserved roles and protect arthropods from oxidative stress (Wang et al., 2019). ABC transporter B family member 2-like and ATP-binding cassette sub-family B member 10 mitochondrial isoform X1 transcripts were expressed in both the gut and fat body of callow ESBB, suggesting that they may be involved in the transport and subsequent excretion of entomotoxic substances during maturation feeding.

C. Sex-specific comparison

C.1. Sex-specific gene expression dynamics in callow ESBB gut

Most of the upregulated genes were linked to cytochromes and peroxidases in the male gut, indicating roles in establishing an attack on the new host tree as the pioneer sex and capacity to aid digestion and detoxification of dietary material. Other key genes expressed in the gut, such as UGTs, GSTs, esterase sub-families, hydrolases, and peroxidases, were not differentially regulated, indicating conserved expression requirements. Cytochrome P450s are essential for insecticide tolerance in the endoparasitoid wasp *Meteorus pulchricornis*; the cytochrome P450 301A1 (mitochondrial) gene was expressed highly after insecticide exposure compared to control (Xing et al., 2021). In the current study, a probable cytochrome P450 301A1 (mitochondrial) transcript was highly expressed in a callow male gut and fat body of ESBB, suggesting a role in supporting male beetles to deal with host allelochemicals during host colonization (**Supplementary Figure 3**).

C.2. Sex-specific gene expression dynamics in callow ESBB fat bodies

Apart from the detoxification-, digestion-, and defense-related genes, we observed that the genes related to pheromone biosynthesis were expressed in the fat body tissues, which are the site of pheromone production in ESBB. Our finding was coherent with the copulation and infestation mechanism where ESBB male, the pioneer sex, releases sex aggregation pheromones to attract the conspecifics during new host colonization (Ramakrishnan et al., 2022). Various *Ips* species, including *I. paraconfusus* and *I. pini*, have been observed to use the sex-specific expression of CYPs to convert host allelochemicals into aggregation pheromones (Huber et al., 2007; Song et al., 2013; Tittiger and Blomquist, 2017; Blomquist et al., 2021). However, the function of these genes is yet to be confirmed in *I. typographus*. Furthermore, additional validation of the pheromone biosynthesis pathway genes of ESBB may reveal sex-specific roles. All stages of ESBB are exposed to the chemical defenses of the trees to varying degrees. These enzymes are produced at different times in different tissues throughout life, and it is believed that variations in their expression or catalytic activity are related to the need for compound detoxification. The transcriptome of the sex pheromone gland of the sandfly, *Lutzomyia longipalpis,* revealed the expression of NADP + dependent farnesol dehydrogenase, which is implicated in the isoprenoid pathway (Gonzalez-Caballero et al., 2013).

Furthermore, a farnesol dehydrogenase-like transcript was highly expressed in callow male ESBB fat body, implying its involvement in pheromone synthesis.

D. Gene expression differences between callow and sclerotized male gut (T6 vs. T5)

As the gene expression in the gut reflects the feeding behavior, differences in gene expression between callow and sclerotized adult guts have additional adaptive significance. Many physiologically important genes are differentially regulated in these tissues. For instance, the cytochrome P450 9E2-like gene showed higher gene expression in the sclerotized male gut of *Ips typographus* than in the callow male gut (Ramakrishnan et al., 2022). In the current study, the cytochrome P450 9E2-like was extensively expressed in the larval and adult stages, suggesting a role in ESBB feeding and aggregation pheromone production (**Figure 9**). In *Aedes aegypti*, transcriptome comparison between an insecticide-susceptible strain (Bora7) and insecticide-resistant strain (KhanhHoa7) showed upregulation of cytochrome P450 4C1, 4C3, 4C21, 4D1, 4D1 isoform X2, 4D2, 4D2 isoform X2, 4G15, 6A2, 6A8, 6D3, and 9E2 in the resistant strain (Lien et al., 2019). The comparative genome-wide analysis of single-base nucleotide polymorphisms between insecticide-resistant MED whitefly lines and insecticide-susceptible MED whitefly lines showed potential resistance markers (SNP) in cytochrome P450 4C21-like transcripts (Wang et al., 2022). In the current study, the cytochrome P450 4C1-like, cytochrome P450 4C21-like, cytochrome P450 4C3-like isoform X1, cytochrome P450 4C3-like isoform X2, cytochrome P450 4D2-like, cytochrome P450 4G15-like, cytochrome P450 6K1-like, Cytochrome P450 9E2, and cytochrome P450 9E2-like transcripts were expressed in the callow male gut, suggesting that they may have a role in detoxification mechanism during maturation feeding.

Furthermore, glutathione S-transferase, glutathione S-transferase 1, isoform D, glutathione S-transferase 1-like, glutathione S-transferase 1-like isoform X1, glutathione S-transferase-like, glutathione S-transferase-like isoform X2, and microsomal glutathione S-transferase 1-like transcripts were highly expressed in the callow male gut, suggesting that they may help freshly emerged callow beetles to detoxify plant allelochemicals. UDP-glucuronosyltransferase 1-8, UDP-glucuronosyltransferase 1-9-like isoform X1, UDP-glucuronosyltransferase 2B15-like, UDP-glucuronosyltransferase 2B16-like, UDP-glucuronosyltransferase 2B16-like, UDP-glucuronosyltransferase 2B30-like isoform X1, and UDP-glucuronosyltransferase 2B7-like transcripts were highly expressed in the callow male gut, suggesting roles in detoxification.

In the mosquito, *Culex pipiens*, the evolution of overproduced esterases is implicated in organophosphate pesticide resistance (Raymond et al., 1998). Esterase B1-like, esterase B1-like isoform X1, esterase E4, and esterase E4-like transcripts were highly expressed in the callow male gut, implying a role in ESBB digestion and detoxification. The mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 1 (MT-ND1) gene encodes a protein called NADH dehydrogenase 1. This protein is part of a large enzyme complex known as complex I, active in mitochondria that convert the energy from food to power the biochemical reactions in the cell (Valentino et al., 2004). NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 (mitochondrial), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11 (mitochondrial), subunit 5 (mitochondrial), subunit 8 (mitochondrial), NADH dehydrogenase [ubiquinone] 1 subunit C2 and NADH dehydrogenase [ubiquinone] flavoprotein 1 (mitochondrial), and NADH dehydrogenase [ubiquinone] iron-sulfur protein 6 (mitochondrial) transcripts were highly expressed in the callow male gut, possibly to aid food digestion and energy metabolism.

In summary, many physiologically essential genes, such as cytochromes, GSTs, UGTs, esterases, and dehydrogenases, were differentially expressed in the callow or sclerotized ESBB gut, probably supporting different behavioral traits (i.e., such as maturation feeding, host finding, and new colony establishments). The ecological relevance of such fine-tuned gene expression demands further functional corroboration.

Enzymatic assay endorsing the RNA-seq findings

The enzymatic assay results drew attention toward fine-tuned protein expressions that reflect the feeding and non-feeding behavior of ESBB. The larvae are in an intense feeding and growth stage, which involves detoxification and digestion of host materials. During the pupal stage, the beetle becomes sedentary and non-feeding, and most of its energy reserves are deployed in ecdysis and metamorphosis. The pupae develop into the callow stage and again start feeding and become sclerotized, as they require energy to fly out, start a new attack, and aggregate the conspecifics (by male ESBB). Our assay results pinpointed that most GST proteins are abundant in pupae and have putative functions in body development and overwintering (for pupae). In contrast, cytochromes were expressed more in both larvae and adult ESBB and participate primarily in detoxification and digestion during host feeding. These results were in accordance with the previous studies suggesting life stage-specific expression of various genes in different insect groups (Perkin and Oppert, 2019; Oppert et al., 2020; Ernst and Westerman, 2021).

Study limitations

The science of insect physiology has dramatically benefited from RNA-seq-based studies. This trend will continue as omics technology becomes more accessible and less expensive. However, there are some limitations in transcriptomics studies. Regulatory proteins are more likely to influence physiological response than mRNAs with short half-lives, i.e., an increase or decrease in mRNA may not always equate to a corresponding change in protein abundance or activity. Such limitations also exist in the current study. To alleviate some of these limitations, we performed RT-qPCR to validate the most crucial DEGs with independently collected fresh samples. Furthermore, we used an enzymatic assay to cross-check the protein expression and activity of two key proteins in ESBB, namely, cytochromes and GSTs. The results of the enzymatic and RT-qPCR assays correlated positively with the transcriptome findings, with minor exceptions. The physiological

functions of several crucial genes discussed in the present study need additional validation.

Conclusion

Overall, the current transcriptome study combined with RT-qPCR validation and enzymatic assays reveals gene expression dynamics specific to ESBB life stages, sex, and tissues. Our findings indicated that ESBB has allelochemical resistance mechanisms that are an essential precondition for the hormetic response in larval and adult ESBB growth, ultimately contributing to the widespread successful colonization of coniferous trees. The physiologically essential genes identified in this work should be functionally validated to better understand ESBB physiology and adaptation to a nutritionally challenging diet containing host defense allelochemicals. Furthermore, the RNAi-based approach can target key genes for survival and adaptation, identified in the present study, for ESBB management as described in detail by Joga et al. (2021).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/- PRJNA679450.

Author contributions

AR conceptualized the study and performed the RNA-seq analysis. AN and GS did the lab work. AR, AN, and KM interpreted the data. AN and AR prepared the figures. AN wrote the first draft. AR, KM, AN, and GS prepared the final draft. All authors read and approved the final draft.

Funding

This research was supported by grant no. CZ.02.1.01/0.0/0.0/15_003/0000433, "EXTEMIT–K project," financed by the Operational Program Research, Development and Education (OP RDE), and Internal Grant Agency (A_06_22, AISHA NASEER) at the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czechia. AR and KM are supported by grant no. CZ.02. 1.01/0.0/0.0/16_019/0000803 financed by OP RDE.

Acknowledgments

We acknowledge the support from Faculty for Forestry and Wood Sciences (FLD), CZU, for conducting the present study. We also acknowledge Dr. Roman Modlinger and Dr. Jiri Synek, FLD, CZU, for assistance in beetle collection and maintenance. We acknowledge the *Ips typographus* genome manuscript team's contribution in generating the valuable RNA-seq data. We appreciate the helpful feedback provided by the reviewers and especially the handling editor. Special thanks to Prof. Fredrik Schlyter for his constructive comments and encouragement.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1124754/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Volcano plots of different comparisons were made for the study with the number of differentially expressed contigs (FDR $p < 0.05$, fold change ± 2). **(A)** Group-wise comparison between L1-L3 (T1-T3), pupa (T4), and sclerotized adult whole-body (T13); **(B)** T1 vs. T4; **(C)** T2 vs. T4; **(D)** T3 vs. T4; **(E)** T13 vs. T4; **(F)** tissue-specific group-wise comparison between the callow female head (T10), gut (T7) and fat body (T8); **(G)** tissue-specific group-wise comparison between the callow male head (T11), gut (T5) and fat body (T9); **(H)** sex-specific comparison between callow male gut (T5) vs. callow female gut (T7); **(I)** sex-specific comparison between callow female fat body (T8) vs. callow male fat body (T9); **(J)** comparison between sclerotized male gut (T6) vs. callow male gut (T5); and **(K)** group-wise comparison between all gut tissues sclerotized male gut (T6), callow male gut (T5), and callow female gut (T7). $N = 5$. Up-head arrows represent upregulation (fold change ≥ 2), and down-head arrows represent downregulation (fold change ≤ -2). Red dots represent significantly expressed contigs (FDR $p < 0.05$), and blue dots represent non-significantly expressed contigs (FDR $p > 0.05$). The X-axis represents the $\log_2$ fold-change plotted against $-\log_{10}$ (p-values).

SUPPLEMENTARY FIGURE 2
Dynamics of other detoxification-related genes. Different life stages of ESBB on the x-axis are plotted against the average CPM values of all the contigs of a gene family on the y-axis. Different line colors represent individual gene families. The total CPM was calculated by taking the average of all the differentially expressed contigs (FDR $p < 0.05$, fold change ± 2) of each gene family. T1, T2, and T3 represent the first, second, and third larval stage, respectively, T4 represent pupal stage, and T13 represent the sclerotized adult stage of *I. typographus*.

SUPPLEMENTARY FIGURE 3
Sex-specific comparison. **(A)** Callow male gut (T5) vs. Callow female gut (T7) ($N = 5$). **(B)** Callow female fat body (T8) vs. Callow male fat body

(T9) (N = 5). (i) PCA plot showing sample clustering. (ii) Heatmaps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. (iii) Bar graphs showing differentially expressed contigs of specific detoxification-related enzyme families and other essential enzymes related to defense, digestion, transport, metabolism, signaling, and growth. Analysis was done using the CLC workbench (FDR $p < 0.05$ and fold change $\pm$ 2).

SUPPLEMENTARY FIGURE 4
Group-wise comparison between sclerotized male gut (T6), callow male gut (T5), and callow female gut (T7) (N = 5). (i) PCA plot showing sample clustering. (ii) Heatmaps representing gene clustering. The color spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. (iii) Bar graphs showing differentially expressed contigs of specific detoxification-related enzyme families and other essential enzymes related to defense, digestion, transport, metabolism, signaling, and growth after group-wise comparison between the three tissues. Analysis was done using CLC workbench with FDR $p < 0.05$ and fold change $\pm$ 2 cut off.

SUPPLEMENTARY TABLE 1
RNA-seq statistics for ESBB life stage and tissue transcriptomes (T1-T13).

SUPPLEMENTARY TABLE 2
Protein concentrations for four replicates used in enzymatic assays (T1-T6 and T13).

SUPPLEMENTARY MATERIAL 1
Metadata file for all the life stages and tissues used for the study.

SUPPLEMENTARY MATERIAL 2
Functional information about the genes used for designing primer for RT-qPCR and their functional information in NCBI BLASTn.

SUPPLEMENTARY MATERIAL 3
Expression browser for group-wise comparison between all life stages (T1, T2, T3, T4, and T13) containing all the differentially expressed genes (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 4
Expression browser for all the differentially expressed genes in T1 compared against T4 as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 5
Expression browser for all the differentially expressed genes in T2 compared against T4 as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 6
Expression browser for all the differentially expressed genes in T3 compared against T4 as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 7
Expression browser for all the differentially expressed genes in T13 compared against T4 as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 8
Expression browser for group-wise comparison for tissue-specific female comparison between callow female gut (T7), callow female fat body (T8), and callow female head (T10) containing all the differentially expressed genes (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 9
Expression browser for group-wise comparison for tissue-specific male comparison between callow male gut (T5), callow male fat body (T9), and callow male head (T11) containing all the differentially expressed genes (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 10
Expression browser for all the differentially expressed genes in the callow female gut (T7) compared against callow male gut (T5) as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 11
Expression browser for all the differentially expressed genes in callow female fat body (T8) compared against callow male fat body (T9) as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 12
Expression browser for all the differentially expressed genes in the sclerotized male gut (T6) compared against callow male gut (T5) as control (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 13
Expression browser for group-wise comparison for tissue-specific male comparison between callow male gut (T5), sclerotized male gut (T6), and callow female gut (T7) containing all the differentially expressed genes (FDR $p < 0.05$, fold change $\pm$ 2) and their mean CPM values for each stage (N = 5).

SUPPLEMENTARY MATERIAL 14
Fisher extract test result for all life stages (T1, T2, T3, T4, and T13) comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 15
Fisher extract result for all life stage (T1, T2, T3, T4, and T13) comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 16
Fisher extract test result for T1 vs. T4 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 17
Fisher extract test result for T1 vs. T4 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 18
Fisher extract test result for T2 vs. T4 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 19
Fisher extract test result for T2 vs. T4 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 20
Fisher extract test result for T3 vs. T4 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 21
Fisher extract test result for T3 vs. T4 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 22
Fisher extract test result for T13 vs. T4 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 23
Fisher extract test result for T13 vs. T4 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 24
Fisher extract test result for T7, T8, and T10 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 25
Fisher extract test result T7, T8, and T10 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 26
Fisher extract test result for T5, T9, and T11 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 27
Fisher extract test result T5, T9, and T11 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 28
Fisher extract test result for T7 vs. T5 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change $\geq$ 2).

SUPPLEMENTARY MATERIAL 29
Fisher extract test result for T7 vs. T5 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 30
Fisher extract test result for T8 vs. T9 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change ≥ 2).

SUPPLEMENTARY MATERIAL 31
Fisher extract test result for T8 vs. T9 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 32
Fisher extract test result for T6 vs. T5 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change ≥ 2).

SUPPLEMENTARY MATERIAL 33
Fisher extract test result for T6 vs. T5 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 34
Fisher extract test result for T5, T6, and T7 comparison for differentially upregulated transcript IDs (FDR $p < 0.05$, fold change ≥ 2).

SUPPLEMENTARY MATERIAL 35
Fisher extract test result T5, T6, and T7 comparison for differentially downregulated transcript IDs (FDR $p < 0.05$, fold change ≤ -2).

SUPPLEMENTARY MATERIAL 36
List of common and unique differentially upregulated (FDR $p < 0.05$, fold change ≥ 2) transcript IDs between life stages (T1, T2, T3, T4, and T5) described in **Figure 5**.

SUPPLEMENTARY MATERIAL 37
List of common and unique differentially downregulated (FDR $p < 0.05$, fold change ≤ -2) transcript IDs between life stages (T1, T2, T3, T4, and T5) described in **Figure 6**.

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Supplementary Table 1 | RNA-seq statistics for ESBB life stage and tissue transcriptomes (T1-T13).

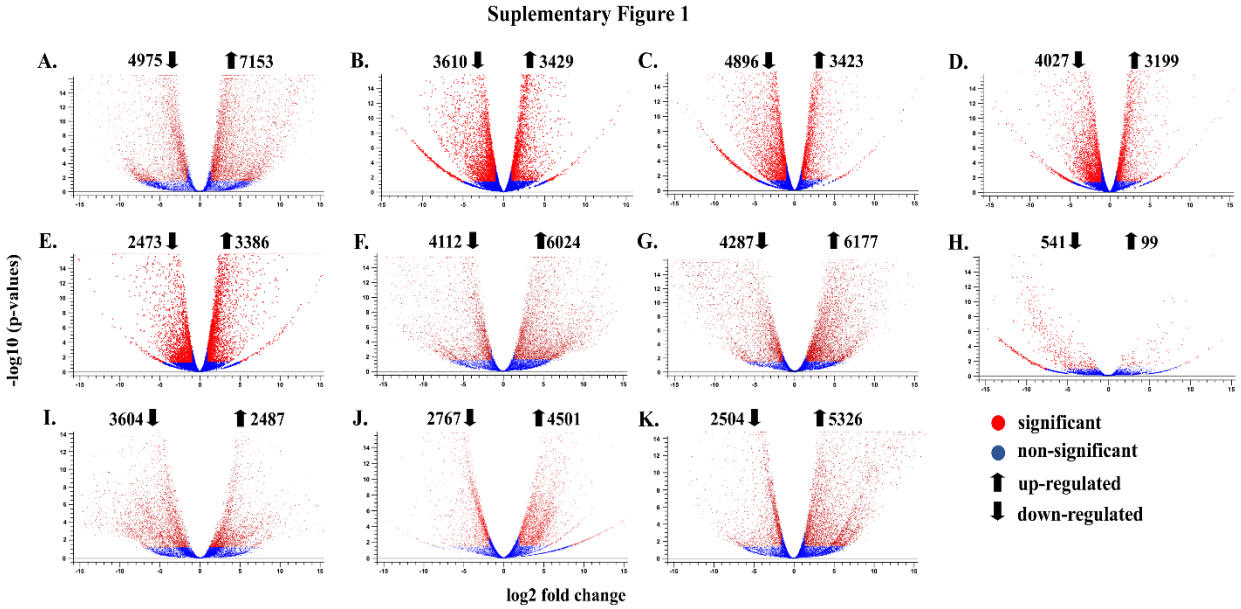
Table S1. RNA-seq. statistics

Sample (N=5)	Treatment	Samples	Mean total number of reads	Mean no. of reads mapped in pairs	Percentage reads mapped (%)	Mean no. of Unique Fragments
LARVAL STAGE 1	T1	ITL1	75936269.6	51943861.2	64 -74	25971930.6
LARVAL STAGE 2	T2	ITL2	70945550	55041472.4	76-79	27520736.2
LARVAL STAGE 3	T3	ITL3	71505554	52769624	71-76	26384812
PUPAE	T4	ITP	71115348.8	49325786.4	69-71	24662893.2
CALLOW MALE GUT	T5	ITGMG	77119550.8	55041556.8	70-73	27520778.4
SCLEROTIZED ADULT MALE GUT	T6	ITFMG	83262577.2	61155543.6	71-75	30577771.8
CALLOW FEMALE GUT	T7	ITGFG	84040242	61648464.8	72-76	30824232.4
CALLOW FEMALE FAT BODY	T8	ITGFF	111302670.4	84415630	74-78	42207815
CALLOW MALE FAT BODY	T9	ITGMF	84587229.2	64721005.2	76-78	32360502.6
CALLOW FEMALE HEAD	T10	ITGFH	69250893.6	43442953.2	50-68	21721476.6
CALLOW MALE HEAD	T11	ITGMH	82077204.4	53581683.6	58-68	26790841.8
SCLEROTIZED ADULT MALE HEAD	T12	ITFMH	78049898.4	52849436.8	63-71	26424718.4
WHOLE BODY	T13	ITB	80637154.4	59772909.6	73-75	29886454.8

Supplementary Table 2 | Protein concentrations for four replicates used in enzymatic assays (T1-T6 and T13).

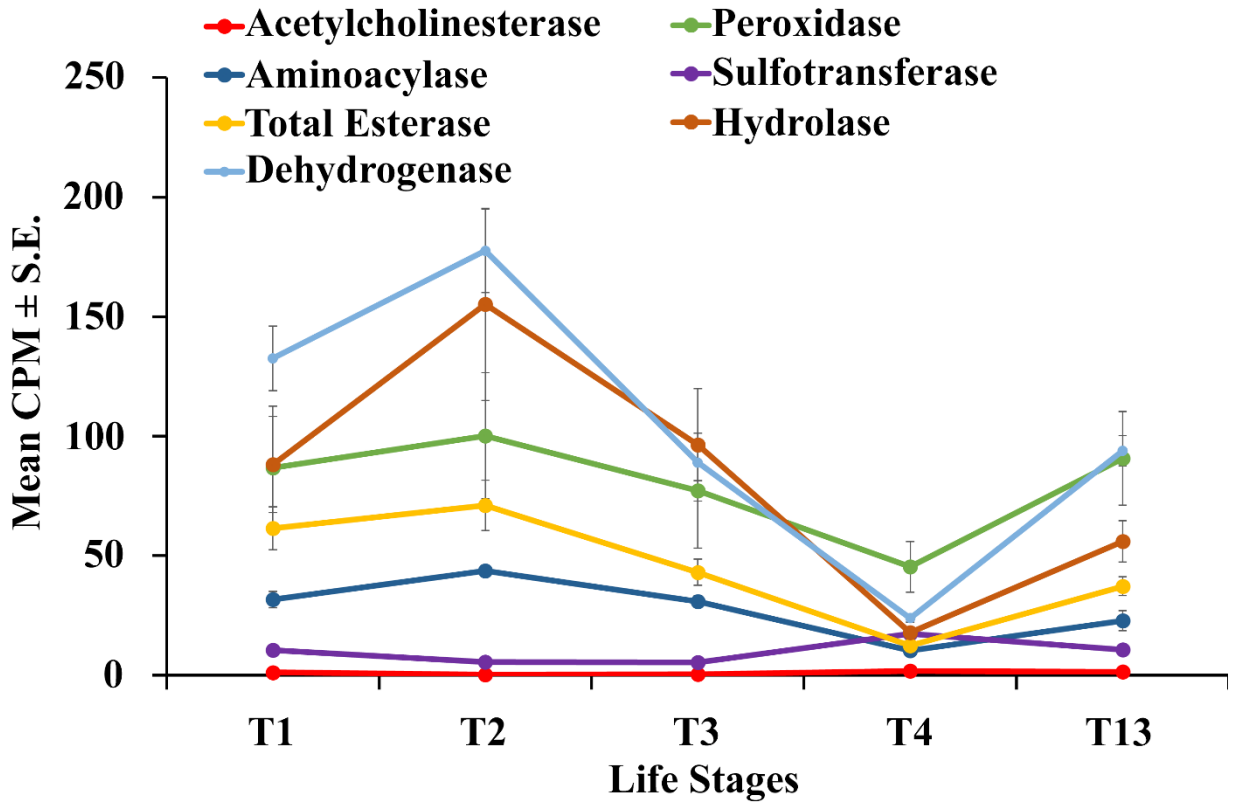
Table S2. Protein Concentrations

Treatment	N	R1 (mg/ml)	R2 (mg/ml)	R3 (mg/ml)	R4 (mg/ml)
T1	8	1.049614	1.124408	1.249065	1.623037
T2	6	7.127649	5.636998	1.722762	3.268512
T3	4	6.235353	8.105211	7.531788	9.077537
T4	3	7.40713	5.562204	5.936176	7.456993
T13	2	7.107953	5.886313	6.434804	6.684119
T5	10	0.130607	0.199511	0.101711	0.223961
T6	10	0.297311	0.219515	0.270638	0.343988



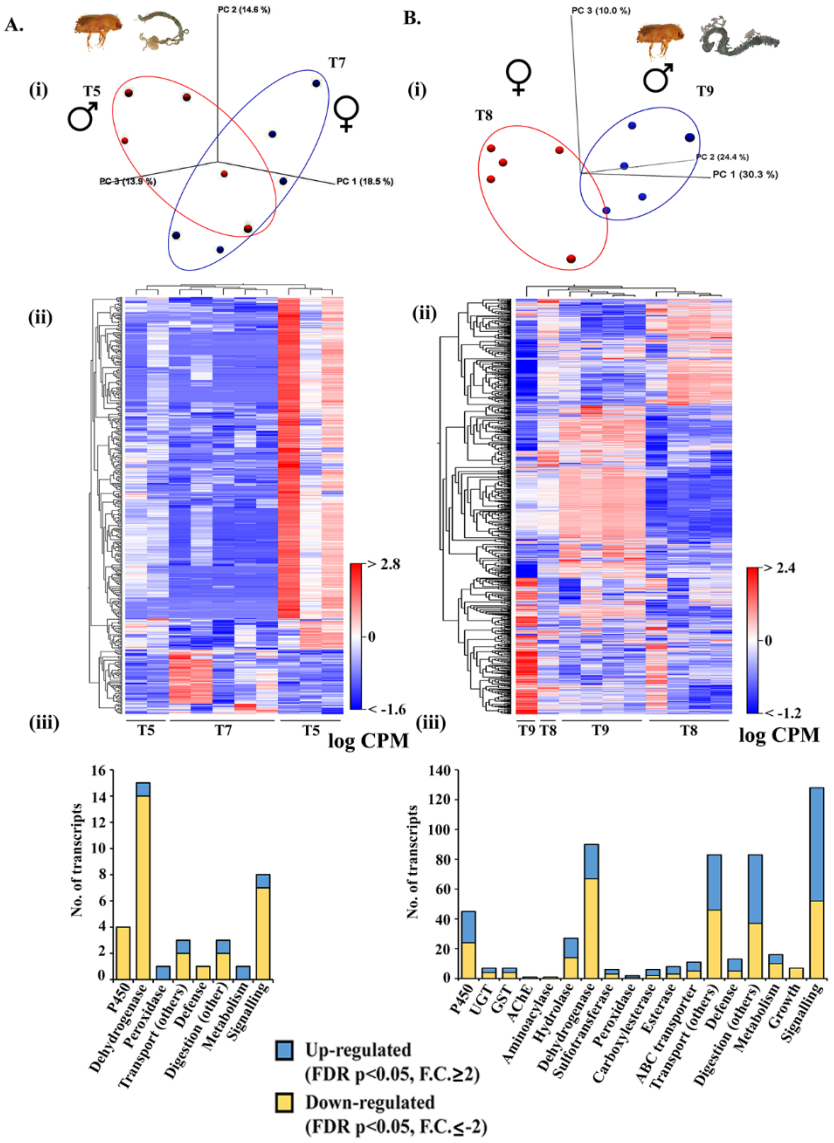
Supplementary Figure 1: Volcano plots of different comparisons were made for the study with the number of differentially expressed contigs (FDR $p < 0.05$, fold change ± 2). **(A)** Group-wise comparison between L1-L3 (T1-T3), pupa (T4), and sclerotised adult whole-body (T13); **(B)** T1 vs. T4; **(C)** T2 vs. T4; **(D)** T3 vs. T4; **(E)** T13 vs. T4; **(F)** tissue-specific group-wise comparison between the callow female head (T10), gut (T7) and fat body (T8); **(G)** tissue-specific group-wise comparison between the callow male head (T11), gut (T5) and fat body (T9); **(H)** sex-specific comparison between callow male gut (T5) vs. callow female gut (T7); **(I)** sex-specific comparison between callow female fat body (T8) vs. callow male fat body (T9); **(J)** comparison between sclerotised male gut (T6) vs. callow male gut (T5); and **(K)** group-wise comparison between all gut tissues sclerotised male gut (T6), callow male gut (T5), and callow female gut (T7). N = 5. Up-head arrows represent upregulation (fold change ≥ 2), and down-head arrows represent downregulation (fold change ≤ −2). Red dots represent significantly expressed contigs (FDR $p < 0.05$), and blue dots represent non-significantly expressed contigs (FDR $p > 0.05$). The X-axis represents the $\log_2$ fold-change plotted against $-\log_{10}$ (p-values).

Supplementary figure 2



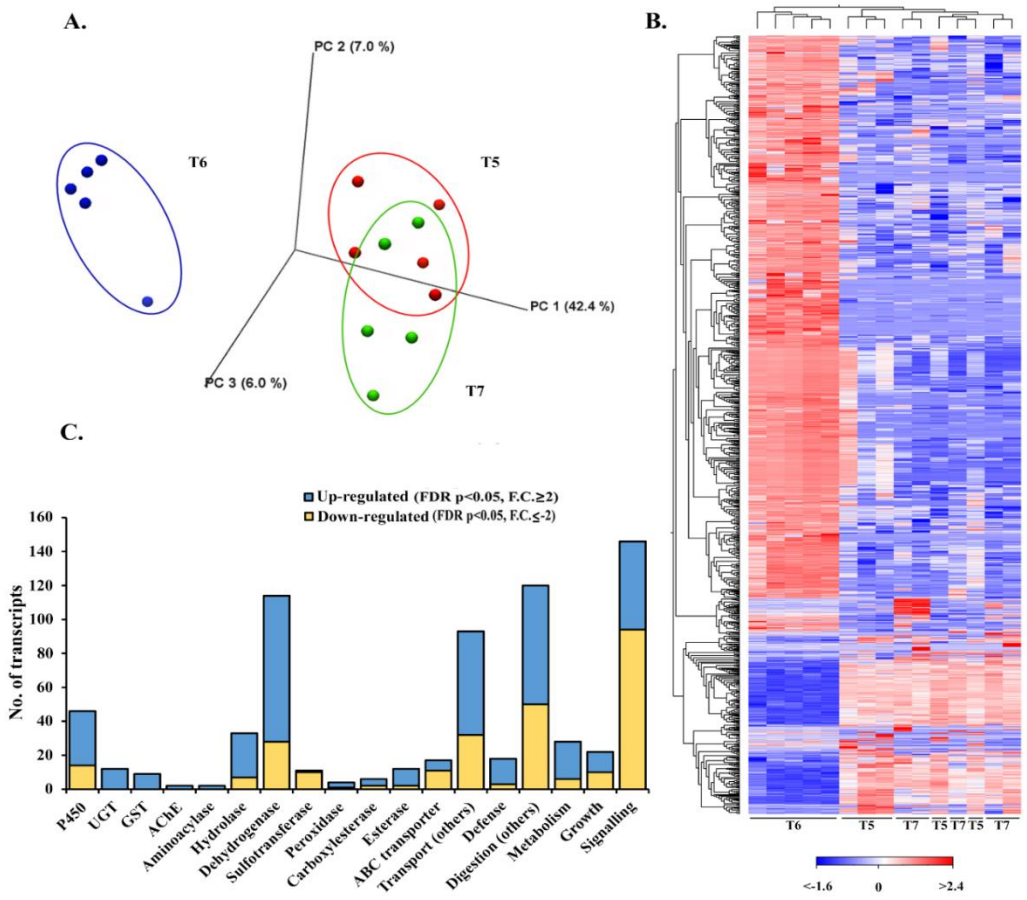
Supplementary Figure 2: Dynamics of other detoxification-related genes. Different life stages of ESBB on the x-axis are plotted against the average CPM values of all the contigs of a gene family on the y-axis. Different line colours represent individual gene families. The total CPM was calculated by taking the average of all the differentially expressed contigs (FDR $p < 0.05$, fold change ± 2) of each gene family. T1, T2, and T3 represent the first, second, and third larval stage, respectively, T4 represent pupal stage, and T13 represent the sclerotised adult stage of *I. typographus*.

Supplementary Figure 3



Supplementary Figure 3: Sex-specific comparison. **(A)** Callow male gut (T5) vs. Callow female gut (T7) (N = 5). **(B)** Callow female fat body (T8) vs. Callow male fat body (T9) (N = 5). **(i)** PCA plot showing sample clustering. **(ii)** Heatmaps representing gene clustering. The colour spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. **(iii)** Bar graphs showing differentially expressed contigs of specific detoxification-related enzyme families and other essential enzymes related to defence, digestion, transport, metabolism, signalling, and growth. Analysis was done using the CLC workbench (FDR $p < 0.05$ and fold change ± 2).

Supplementary figure 4



Supplementary Figure 4: Group-wise comparison between sclerotised male gut (T6), callow male gut (T5), and callow female gut (T7) (N = 5). **(A)** PCA plot showing sample clustering. **(B)** Heatmaps representing gene clustering. The colour spectrum, stretching from blue to red, represents TMM-adjusted log CPM expression values obtained after DGE analysis. **(C)** Bar graphs showing differentially expressed contigs of specific detoxification-related enzyme families and other essential enzymes related to defence, digestion, transport, metabolism, signalling, and growth after group-wise comparison between the three tissues. Analysis was done using CLC workbench with FDR $p < 0.05$ and fold change ± 2 cut off.

Remaining Supplementary Material (excel datasets) for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1124754/full#supplementary-material.

5.3. Insights into the Detoxification of Spruce Monoterpenes by Eurasian Spruce Bark Beetle

Published as: **Naseer, A.**, Singh, V.V., Sellamuthu, G., Synek, J., Mogilicherla, K., Kokoska, L. and Roy, A., 2024. Insights into the Detoxification of Spruce Monoterpenes by the Eurasian Spruce Bark Beetle. International Journal of Molecular Sciences, 25(18), p.10209. https://doi.org/10.3390/ijms251810209

Contributed to: conceptualisation, methodology, formal analysis, data curation, data analysis, visualisation, writing- original draft, review, and editing, project administration.

This study was conducted 'to identify the key genes in *I. typographus* that are responsible for the detoxification of specific allelochemicals of the host (Objective II).' For this study, we performed a fumigation bioassay using five key monoterpenes—*α*-pinene, sabinene, myrcene, *(R)*-(+)-limonene, and *(S)*-(-)-limonene—to assess their toxicity on *I. typographus*. Following exposure, the beetles were analysed to determine how their gene expression changed in response to these compounds using RNA-seq, RT-qPCR, and enzyme assays to identify differentially expressed genes related to detoxification processes using the whole body.

The bioassay results show that sabinene and *(R)*-(+)-limonene were particularly toxic to the beetles, significantly reducing their survival rates in a dose-dependent manner. The RNA-seq data showed that exposure to these monoterpenes led to the upregulation of several detoxification genes, particularly those from the CYP (family 4 and 6), GST-theta class, and UGT family 2. For example, CYP4 and CYP6, known for their roles in metabolizing toxic substances, were among the most upregulated genes. GSTs and UGTs were also highly expressed, which help conjugate and excrete toxins. On the contrary, there was a concurrent downregulation of genes related to development, such as moulting and pupation (CYP314A1, CYP306A1, and CYP315A1), eggshell formation (chorion peroxidase), and diapause initiation (peroxiredoxin-6). Further, validation of key detoxification genes by RT-qPCR and enzyme activity assay conducted on ESBB fumigated on LC_{70} and double LC_{70} with four monoterpenes showed a conserved mechanism underlying the upregulation of these genes on chemical over-exposure. This suggests that the beetles may be diverting resources from growth and reproduction towards detoxification when exposed to high levels of toxic compounds.

International Journal of
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Insights into the Detoxification of Spruce Monoterpenes by the Eurasian Spruce Bark Beetle

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Abstract: Plant defence mechanisms, including physical barriers like toughened bark and chemical defences like allelochemicals, are essential for protecting them against pests. Trees allocate non-structural carbohydrates (NSCs) to produce secondary metabolites like monoterpenes, which increase during biotic stress to fend off pests like the Eurasian spruce bark beetle, ESBB (*Ips typographus*). Despite these defences, the ESBB infests Norway spruce, causing significant ecological damage by exploiting weakened trees and using pheromones for aggregation. However, the mechanism of sensing and resistance towards host allelochemicals in ESBB is poorly understood. We hypothesised that the exposure of ESBB to spruce allelochemicals, especially monoterpenes, leads to an upsurge in the important detoxification genes like P450s, GSTs, UGTs, and transporters, and at the same time, genes responsible for development must be compromised. The current study demonstrates that exposure to monoterpenes like R-limonene and sabiene effectively elevated detoxification enzyme activities. The differential gene expression (DGE) analysis revealed 294 differentially expressed (DE) detoxification genes in response to R-limonene and 426 DE detoxification genes in response to sabiene treatments, with 209 common genes between the treatments. Amongst these, genes from the cytochrome P450 family 4 and 6 genes (CP4 and CP6), esterases, glutathione S-transferases family 1 (GSTT1), UDP-glucuronosyltransferase 2B genes (UDB), and glucose synthesis-related dehydrogenases were highly upregulated. We further validated 19 genes using RT-qPCR. Additionally, we observed similar high expression levels of detoxification genes across different monoterpene treatments, including myrcene and α-pinene, suggesting a conserved detoxification mechanism in ESBB, which demands further investigation. These findings highlight the potential for molecular target-based beetle management strategies targeting these key detoxification genes.

Keywords: Norway spruce; monoterpenes; bark beetles; detoxification; RNA-seq; enzyme assay; RT-qPCR; bioassay

Citation: Naseer, A.; Singh, V.V.; Sellamuthu, G.; Synek, J.; Mogilicherla, K.; Kokoska, L.; Roy, A. Insights into the Detoxification of Spruce Monoterpenes by the Eurasian Spruce Bark Beetle. *Int. J. Mol. Sci.* **2024**, *25*, 10209. https://doi.org/10.3390/ijms251810209

Academic Editor: Francesca Barbero

Received: 23 August 2024
Revised: 16 September 2024
Accepted: 18 September 2024
Published: 23 September 2024

1. Introduction

Plants have evolved a diverse array of defence mechanisms to protect themselves against biotic and abiotic stressors. These defences include physical barriers such as toughened bark and resin ducts, as well as chemical deterrents like allelochemicals. Among the carbon reserves of the tree, non-structural carbohydrates (NSCs) play a critical role by providing the nutrient pool required for various physiological processes, including the production of constitutive and induced secondary metabolites. These secondary metabolites, including terpenes and phenolics, are crucial for plant defence. Monoterpenes, C10-compounds composed of two isoprene units, are particularly prominent qualitatively and

quantitatively compared to other terpenoids as defence compounds, due to their significant role in deterring herbivores and pathogens [1]. Previous studies have elaborated on a significant increase in the proportion of monoterpenes in response to stressors such as fungal inoculation, drought, and methyl jasmonate (MeJA) infusion, indicating their role in both constitutive and induced defence mechanisms [2–5].

Among many herbivores that challenge these defences, the Eurasian spruce bark beetle (ESBB, *Ips typographus*) represents one of the most destructive pests in coniferous forests, particularly targeting Norway spruce (*Picea abies* L. Karst.). Despite its sophisticated chemical defence, including producing toxic allelochemicals, *I. typographus* has exploited Norway spruce trees, leading to severe economic and ecological damage to the central European conifer forest [6–8]. The host colonisation starts by targeting mature, weakened trees by pioneer males using aggregation pheromones to attract additional beetles. They feed on the phloem, construct mating chambers, and lay eggs, leading to extensive damage as the larvae create radial galleries in the bark. The success of bark beetles in infesting and damaging trees despite their chemical defences is primarily due to their evolved mechanisms for overcoming these defences through the sequestration and detoxification of host allelochemicals [9,10]. Such attacks are also overwhelmed by the microbiome of the bark beetle (together referred to as bark beetle holobiont) that also uses the carbon sources of the host trees (mostly phenols) and metabolises them to semiochemicals or detoxifies them to less toxic forms [11–17].

I. typographus employs a sophisticated detoxification strategy involving several enzymatic systems, which allows it to use plant chemicals for pheromone biosynthesis and detoxification. It utilises the mevalonate and geranyl pyrophosphate (GPP) pathways to convert α-pinene into various pheromones, including 4S-(−)-*cis*-verbenol and 2-methyl-3-buten-2-ol [10,18–20]. The detoxification pathways are complex and comprise three main phases: phase I includes lipophilic attacking enzymes such as cytochrome P450 mono-oxygenase (P450), dehydrogenase, peroxidase, hydrolysis enzymes like esterases: carboxycholine (CCE)/acetylcholine esterases (AChE), and esterase families (EST), epoxide hydrolase, and reductase like NADPH-cytochrome P450 reductase (CPR); phase II involves the conjugation of the activated intermediates by enzymes like glutathione S-transferases (GST), UDP-glucuronosyltransferase (UGT), sulfotransferases, N-acetyltransferases, or acyltransferases, rendering them hydrophilic; and lastly, phase III entails the transport of these less toxic forms out of the cells via multidrug resistance proteins [21–24]. The MRPs are members of ATB-binding cassette transporters (ABC transporters) that cause the ATP-dependent transport of the hydrophobic products of phase I and II to an extracellular medium such that they can be excreted out of the insect body through body fluids [25–29].

Recent reports detail the detoxification, digestion, and defence mechanisms in *I. typographus* and the eight-toothed bark beetle (*Ips sexdentatus*) across different life stages and feeding behaviours [30–32]. The key detoxification genes upregulated during feeding or exposure to toxins include cytochrome P450s from families 4, 6, and 9, also found in related beetles like *Dendroctonus* [33–35]. Our previous work catalogued detoxification enzymes such as GSTs, UGTs, ABC transporters, and esterases in *I. typographus* [31]. Our lab studies (*unpublished data*) further revealed that beetles feeding on Norway spruce treated with MeJA, which increases toxin levels, exhibit detoxification gene expression compared to those feeding on long-term stored Norway spruce logs. Based on these findings, we hypothesised that the level and type of host chemical exposure directly affect gene expression in *I. typographus*, influencing their survival and successful establishment on the host. Since the allelochemicals in the host bark are present in mixtures, the individual roles and toxicity of monoterpenes in the interaction between Norway spruce and *I. typographus* have not yet been evaluated. In this study, we selectively evaluated the toxicity of five important Norway spruce monoterpenes, *viz*, α-pinene, sabiene, myrcene, R-limonene, and S-limonene against *I. typographus* using the fumigation bioassay [36]. Our results showed that the survival of the beetles decreased inversely with time of exposure in a dose-dependent manner. We examined the effects of the most effective monoterpenes,

sabiene, and R-limonene, on gene expression in *I. typographus* using RNA-seq. Notably, exposure to monoterpenes induced the upregulation of a common set of detoxification genes throughout the different chemicals tested and suppressed developmental genes, affirming that specific gene sets are consistently expressed in response to host chemicals and could be potential targets for future pest management strategies for *I. typographus*.

2. Results

2.1. Toxicity Assay

The toxicity assay was performed for five monoterpenes by fumigating a single beetle for 72 h for each chemical (Table 1). From the sex-specific bioassay, we found no sex-specific effect on the mortality of the male and female beetles after 48 h ($p > 0.05$ in all chemicals). The bioassay revealed that the survival of the beetles is inversely proportional to the time of fumigation in a dose-dependent manner. The mortality increased and was highest at 72 h, with the control mortality surpassing the optimal limit of 15–20% (Figure 1). Hence, the lethal concentration for 70% mortality (LC_{70}) was calculated at 48 h. The LC_{70} for the 48 h incubation of *I. typographus* was recorded as v/v per 20 mL per individual (Table 1, Supplementary File S1). The LC_{70} values at 48 h ranged from ~6 to 10 µL/20 mL. Sabiene was recorded as the most toxic chemical at the lowest LC_{70} of 6.01 µL/20 mL, followed by α-pinene with an LC_{70} of 6.57 µL/20 mL and myrcene with 8.05 µL/20 mL, (R)-(+)-limonene with 8.42 µL/20 mL and the highest LC_{70} of (S)-(−)-limonene at 10.64 µL/20 mL. To see the gene expression difference, we selected the two monoterpenes, R-limonene (moderate LC_{70}) and sabiene (lowest LC_{70}).

Table 1. Toxicity assay. Monoterpenes and their respective LC_{50} and LC_{70} values against *Ips typographus*.

Chemical	48 H			%Corrected Beetle Mortality for Different Doses *				
	LC_{50} (µL/20 mL)	LC_{70} (µL/20 mL)	% Control Mortality	1 (µL/20 mL)	2 (µL/20 mL)	4 (µL/20 mL)	8 (µL/20 mL)	16 (µL/20 mL)
Sabiene	4.4	6.0	10.0	−4.0	8.0	20.0	84.0	100.0
α-Pinene	3.6	6.6	6.7	16.1	28.6	37.5	71.4	96.4
Myrcene	5.3	8.1	16.7	6.0	12.0	12.0	72.0	90.0
(R)-(+)-Limonene	6.2	8.4	5.0	8.8	3.5	21.1	57.9	98.3
(S)-(−)-Limonene	7.3	10.6	23.0	8.7	6.5	17.4	26.1	89.1

** The percentage corrected mortality was calculated for each dose using Henderson–Tilton's formula* [37].

2.2. Reference Gene Selection

Among the twelve housekeeping genes proposed by Sellamuthu et al. (2022) [38] for the expression normalisation in monoterpene-treated *I. typographus*, the best combination of stable genes was RPS3-a (Ityp04549) and RPL7 (Ityp01351) (Figure 2, Table 2). These two genes have previously been reported by Sellamuthu et al. (2022) [38] as the best reference genes for *I. typographus* study for various tissues and development stages. RPS3a was consistently ranked the best across all five analyses: the ΔCt method, BestKeeper, RefFinder, NormFinder, and geNorm.

2.3. Differential Gene Expression Analysis

From three samples × one biological replicate, a raw library size of approximately 91 million reads was retrieved, which resulted in a 91.8 million normalised library size (Supplementary Files S2 and S3). All the features (reads) generated (Table 3) were mapped back to the reference genome of *I. typographus* [30] to generate the count table. After applying the cut-off for probability >0.9, a total of 5363 and 6815 reads were generated in the R-limonene and sabiene comparison, respectively. These reads/transcripts were further filtered based on M values ± 1 (Figure 3A,B).

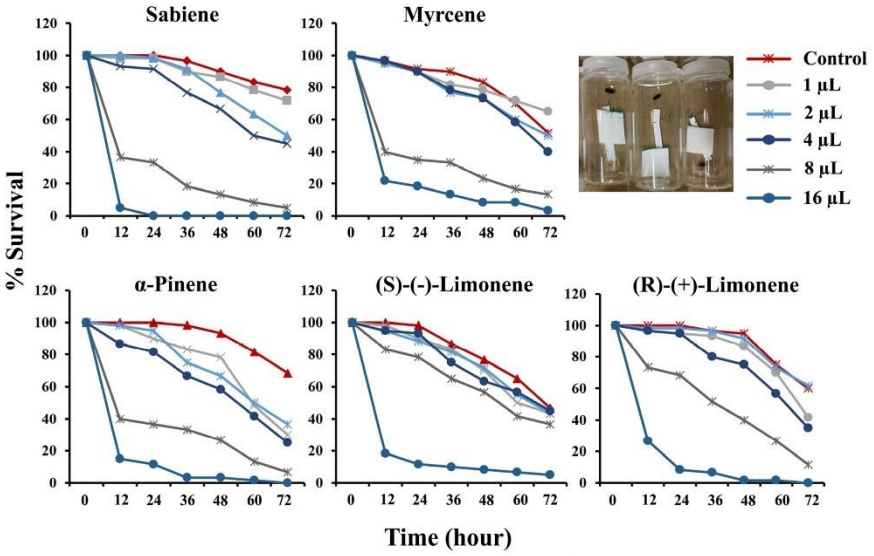

Figure 1. Monoterpene bioassay (via fumigation method). The bark beetle survival distribution curve against the five tested monoterpenes was plotted against a 12 h interval for 72 h against the tested dose (n = 60 per dose per chemical). Different colours represent the dose applied in µL per 20 mL of air in the vials.

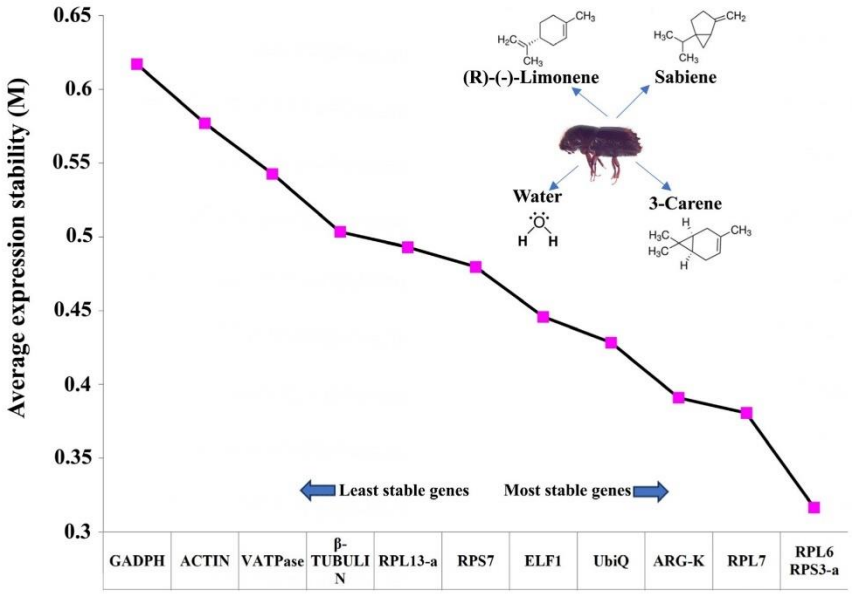

Figure 2. The geNorm comprehensive ranking of the least stable and most stable reference gene combination after monoterpene treatment based on their stability value plotted on the y-axis for each of the 12 genes on the x-axis. Three individual monoterpene treatments, R-limonene, sabiene,

and 3-carene, were tested to select the suitable reference gene. Five different algorithms were used based on the Ct-values generated for each of the 12 housekeeping genes after RT-qPCR ($n = 4$), *viz.*, the ΔCt method, BestKeeper, RefFinder, and NormFinder. The comprehensive ranking was generated using geNorm.

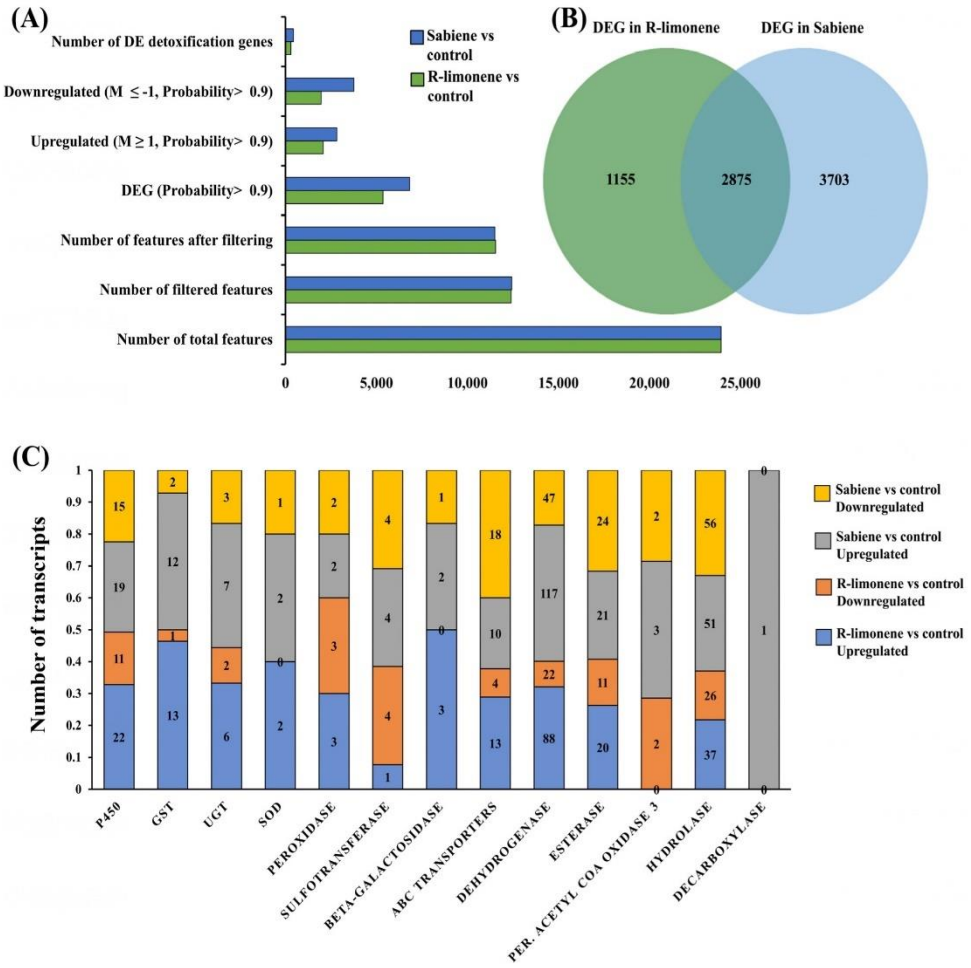

Figure 3. Differential gene expression analysis in fumigated *I. typographus* for the two selected monoterpenes. (**A**) Bar graph representing the number of total genes and DEGs in R-limonene vs. control in green colour and those in sabiene vs. control in blue colour after a cut-off of probability value > 0.9 and log fold change, M ± 1. (**B**) Venn diagram representing the number of common DEGs between the two comparisons. (**C**) Stacked bar graphs comparatively represent the number of expressed detoxification genes in the two treatments. Different colours represent the number of upregulated and downregulated genes in the two chemical treatments, with the individual number of transcripts of each gene family plotted on the *y*-axis. Number inside each bar represents number of transcripts for the corresponding comparison.

Table 2. Reference gene identification. Ranking of the 12 candidate reference genes based on their stability values performed by ΔCt, BestKeeper, RefFinder, and NormFinder after fumigation with three monoterpenes.

Sr. No.	Genes	ΔCt Method		BestKeeper		RefFinder		NormFinder		Recommended Genes
		Stability	Rank	Stability	Rank	Stability	Rank	Stability	Rank	
1	RPS3-a	0.52	1	0.31	2	1.19	1	0.144	1	**RPS3-a** and **RPL7**
2	Arg-K	0.54	2	0.38	6	3.25	4	0.152	2	
3	RPL7	0.55	3	0.32	3	3	3	0.161	4	
4	RPS7	0.56	4	0.27	1	2.99	2	0.223	8	
5	UbiQ	0.56	5	0.33	4	4.95	6	0.196	6	
6	RPL13-a	0.58	6	0.37	5	5.96	7	0.221	7	
7	RPL6	0.6	7	0.37	5	4.14	5	0.181	5	
8	V-ATPase	0.65	8	0.51	8	8.49	9	0.240	10	
9	ELF1	0.69	9	0.44	7	8.49	8	0.160	3	
10	Actin	0.74	10	0.7	11	10.72	10	0.269	11	
11	GADPH	0.74	11	0.6	10	10.74	11	0.295	12	
12	β-Tubulin	0.77	12	0.56	9	11.47	12	0.238	9	

Table 3. RNA-seq analysis results.

Description	R-Limonene vs. Control	Sabiene vs. Control
Number of total features	23,937	23,937
Number of filtered features	12,393	12,435
Number of features after filtering	11,544	11,502
DE (probability > 0.9)	5363	6815
Upregulated (M $\geq$ 1, probability > 0.9)	2069	2817
Downregulated (M ≤ -1, probability > 0.9)	1961	3761
Number of DE detoxification genes	294	426

2.3.1. R-Limonene Treatment vs. Control

To screen the effects of the R-limonene fumigation treatment on detoxification-related genes in *I. typographus* using DGE, we applied a cut-off probability >0.9 and M value $\geq +1$ for upregulation and M ≤ -1 for the downregulation of genes. We reported a total of 2069 upregulated and 1961 downregulated genes, out of which 208 detoxification genes were upregulated and 86 were downregulated. We reported a total of 33 cytochrome P450s, 14 GSTs, 8 UGTs, 2 SODs (superoxide dismutase), 6 peroxidases, 5 sulfotransferases, 3 beta-galactosidase, 17 ABC transporters, 110 dehydrogenases, 31 esterases, 2 peroxisomal acyl-coenzyme A oxidase 3, and 63 hydrolases (Figure 3C, Supplementary Files S3 and S4).

2.3.2. Sabiene Treatment vs. Control

Out of the 2817 upregulated genes (probability > 0.9, and M value $\geq$ 1) and 3761 downregulated genes (probability > 0.9, and M value $\leq$ 1), 208 detoxifications were upregulated, and 86 downregulated genes were reported. We reported a total of 24 cytochrome P450s, 11 GSTs, 5 UGTs, 2 SODs, 3 peroxidases, 2 sulfotransferases, 1 beta-galactosidase, 11 ABC transporters, 98 dehydrogenases, 20 esterases, 3 peroxisomal acyl-coenzyme A oxidase 3, and 46 hydrolases. In addition, a single decarboxylase was upregulated (Figure 3C, Supplementary Files S3 and S4).

We recorded more DEGs in the sabiene treatment than in the R-limonene treatment, as also suggested by the lower LC70 value of sabiene compared with R-limonene. In both comparisons, many overexpressed cytochromes belonging to CYP6 and CYP4, the GST sigma class, GSTT1, and UGT B families were found, which are already reported to be mainly associated with detoxification. A large number of dehydrogenases associated with glucose, alcohol metabolism, and aldo-keto conversion were found to be highly upregulated. All the EST family genes were also upregulated. An interesting hydrolase family gene, myrosinase, was reported, which specialises in catalysing the hydrolysis of

glucosinolates via the cleavage of thio-linked glucose. Glucosinolates are an important ovipositioning and feeding stimulant in Coleoptera [39].

In the comparison between the DE genes of R-limonene and sabiene, we found 2875 gene transcripts in common. However, 1155 transcripts were explicitly differentially expressed in the R-limonene comparison, and 3703 were expressed only in the sabiene comparison (Figure 3B, Supplementary File S4). A total of 209 common detoxification genes were found between the two chemical treatments. On comparing these 209 genes with other existing in-house RNA-seq data, we found 77 in common with the genes induced after carene monoterpene treatment (*unpublished data*), 92 in common with genes induced in L2 (the actively feeding stage) compared to ESBB pupa, 33 in common with MeJA-induced spruce log-fed ESBB (*unpublished data*), and 24 in common with the proteome data of the callow vs. sclerotised gut of male ESBBs (Figure 4, Supplementary File S4). Most of them showed the same expression patterns across the two transcriptome comparisons. Over 90% of the genes that occurred in comparison (I) R-limonene and (II) sabiene also have the same expression pattern in carene treatment (III). Similar expressions are also revealed with comparisons like MeJA (IV). However, the expression pattern differs with no external treatment comparisons like the larval–pupal stage (V) and adults (VI) and male guts (VII) (Figure 5). Such expression patterns reveal that a conserved mechanism might be activated when the beetles are exposed to the external overexposure of host allelochemicals or the induced chemical defences of the tree. Interestingly, the expression of genes related to development, such as ecdysone 20-monooxygenase, cytochrome P450 315a1, and chorion peroxidase, was suppressed.

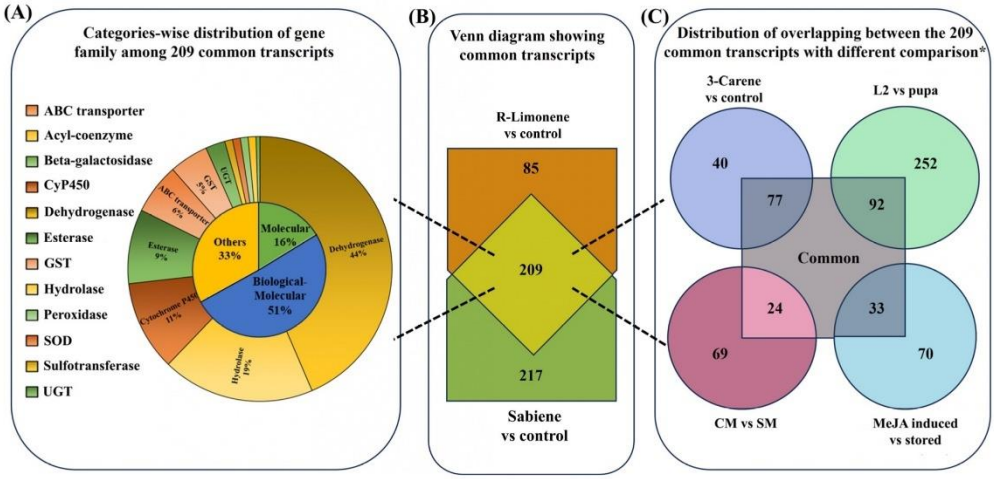

Figure 4. Comprehensive figure representing the distribution of 209 common detoxification genes in *I. typographus* between the two tested monoterpene fumigations. (**B**) Venn diagram showing the common 209 detoxification genes. (**A**) Percentage representation of the detoxification family genes among the 209 common transcripts and the GEO function distribution. (**C**) Comparison of the 209 common genes with four different available transcriptome datasets of *I. typographus* with different treatments: 3-carene vs. control (*unpublished data*); larval stage 2 vs. pupal stage [31]; callow male (CM) vs. sclerotised male (SM) [40]; and *I. typographus* fed on MeJA-treated bark vs. stored bark (Sellamuthu et al., *unpublished data*). * Non-intersecting circles (**C**) do not mean that the comparisons do not have common transcript sequences between them.

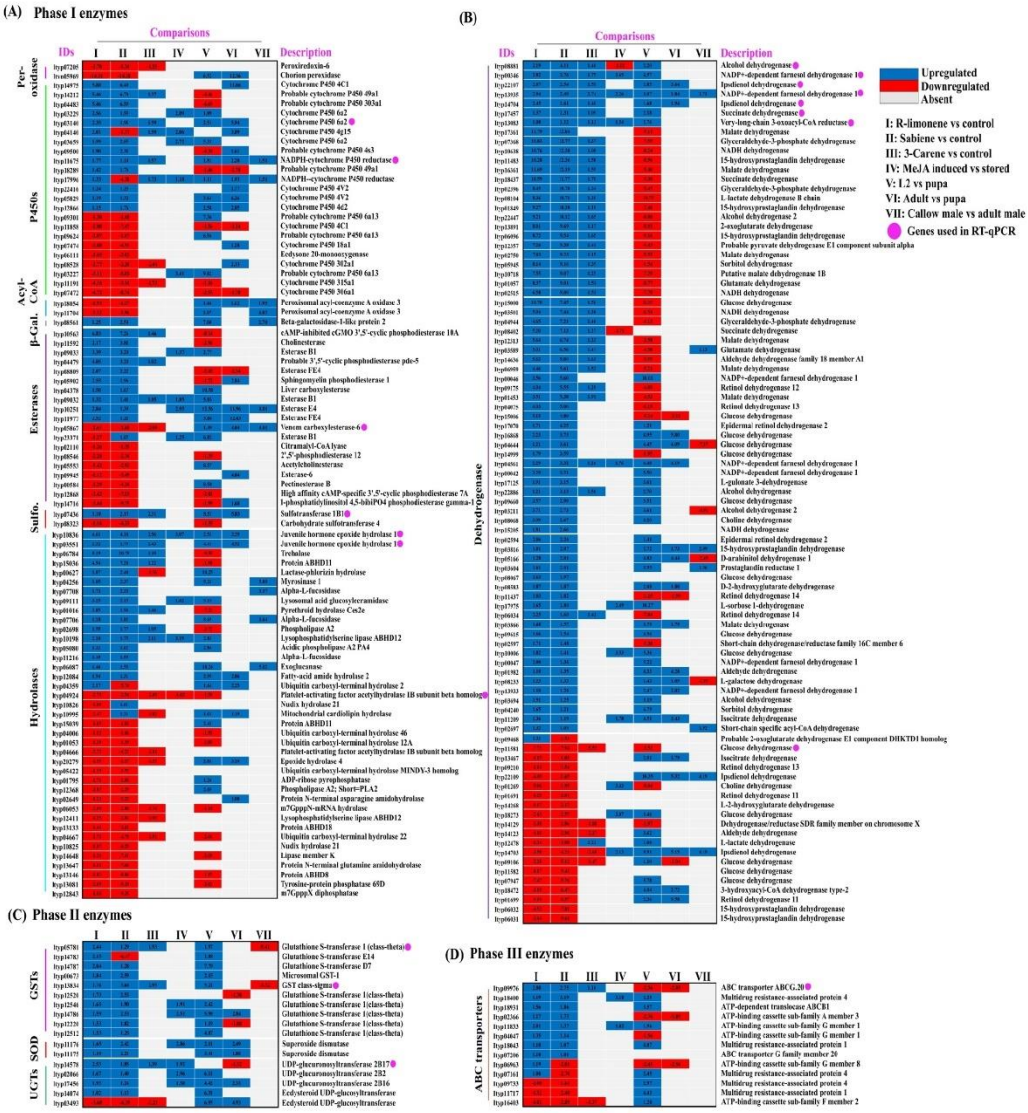

Figure 5. Heat map showing the categorical fold change expression of the 209 common detoxification gene families across the various comparisons used in the study. (**A**,**B**) phase I detoxification enzymes, (**C**) phase II detoxification enzymes, and (**D**) phase III detoxification enzymes. Different comparisons were formulated: (I) R-limonene vs. control, (II) sabiene vs. control, (III) 3-carene vs. control, (IV) MeJA-induced (high) vs. stored (low), (V) L2 vs. pupa, (VI) adult vs. pupa, (VII) callow male vs. sclerotised male. Blue colour represents upregulation, red colour represents downregulation, and white represents that the transcript was either absent or not differentially expressed in the respective comparison (more details in Supplementary File S4). Pink dots mark genes selected for RT-qPCR (refer to Table 5). Expression levels (numbers) for comparisons (I) and (II) were represented based on M value ≥ 1 with probability ≥ 0.9; the rest of the comparisons (III–VII) were based on logFC $\pm$ 1.

2.4. RT-qPCR Analysis and Enzymatic Assay Analysis

To validate the RNA-seq-based gene expression data, we performed an RT-qPCR analysis on F1 beetles treated with R-limonene and sabiene for 19 selected detoxification genes. These included cytochrome P450, GST, esterase, ABC transporter, juvenile hormone epoxide hydrolase, and dehydrogenases. The RT-qPCR expression showed a similar expression pattern of these genes as the RNA-seq data (Figure 6, Supplementary File S5). Most of the genes had a significantly high expression fold change. Further, to assess the effect of generation and conserved upregulated detoxification genes on monoterpene overexposure, we tested some of these genes with an RT-qPCR on the F0 beetle population treated with R-limonene, sabiene, myrcene, and α-pinene (Figure 7). The expression in α-pinene was the highest throughout all the nine tested genes, followed by myrcene, which can be attributed to the involvement of these chemicals in sequestration and pheromone biosynthesis pathways. The mortality assay of F0 beetles with an LC70 dose showed a lower mortality (~30% only) than the expected percentage observed for F1 beetles. Such a lower mortality can be attributed to the higher vigour of the F0 population due to their already-induced detoxification genes. However, with a double LC70 dose, the mortality increased to up to 80% (Supplementary File S1). When the fumigation dose was doubled, the gene expression was higher in the double LC70 dose-treated beetles than the beetles treated at just LC70 in the F0 and F1 populations. These findings assert that the F0 beetles possess a higher vigour, likely due to a higher resistance, associated with a higher expression of detoxification genes.

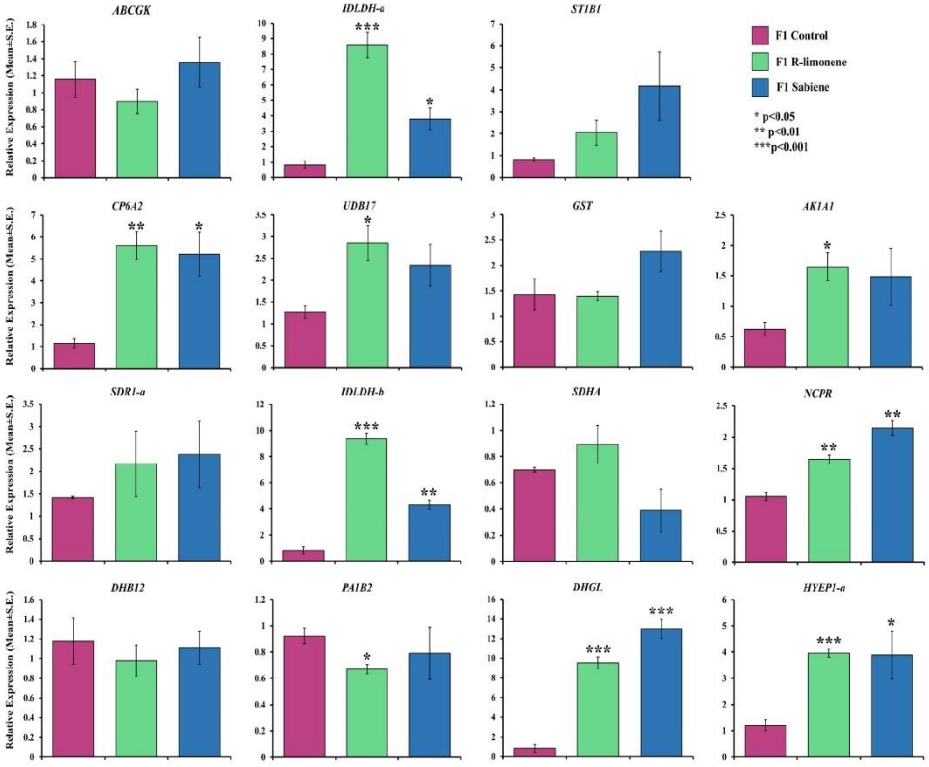

Figure 6. *Cont.*

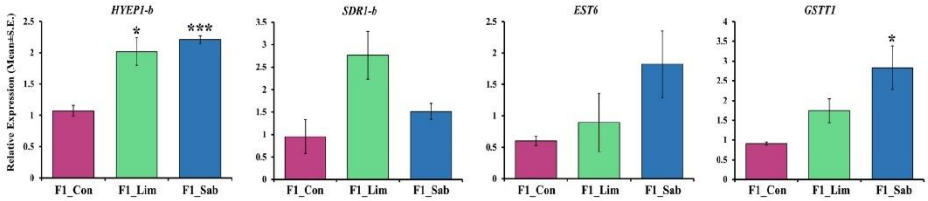

Figure 6. Relative fold change expression ($2^{(-\Delta\Delta Ct)} \pm$ S.E.) of the 19 important detoxification genes in F1 beetles fumigated with R-limonene and sabiene (n = 3). F1 control (pink), F1 R-limonene (green), and F1 sabiene (blue) on the x-axis plotted against their fold change on the y-axis. An independent t-test was performed to check the statistical difference between the control and the treatment, and accordingly, p-values were generated. * represent $p < 0.05$, ** represent $p < 0.01$, and *** represent $p < 0.001$. *ABCGK*–ABC transporter G family member 20, *IDLDH-a*–Ipsdienol dehydrogenase, *ST1B1*–Sulfotransferase 1B1, *CP6A2*–Cytochrome P450 6a2, *UDB17*–UDP-glucuronosyltransferase 2B17, *GST*–Glutathione S-transferase, *AK1A1*–Alcohol dehydrogenase, *SDR1-a*–Farnesol dehydrogenase, *IDLDH-b*–Ipsdienol dehydrogenase, *SDHA*–Succinate dehydrogenase, *NCPR*–NADPH-cytochrome P450 reductase, *DHB12*–17-beta-hydroxysteroid dehydrogenase 12, *PA1B2*–Platelet-activating factor acetylhydrolase IB subunit beta homolog, *DHGL*–Glucose dehydrogenase, *HYEP1-a*–Juvenile hormone epoxide hydrolase 1, *HYEP1-b*–Juvenile hormone epoxide hydrolase 1, *SDR1-b*–Farnesol dehydrogenase, *EST6*–Venom carboxylesterase-6, and *GSTT1*–Glutathione S-transferase 1.

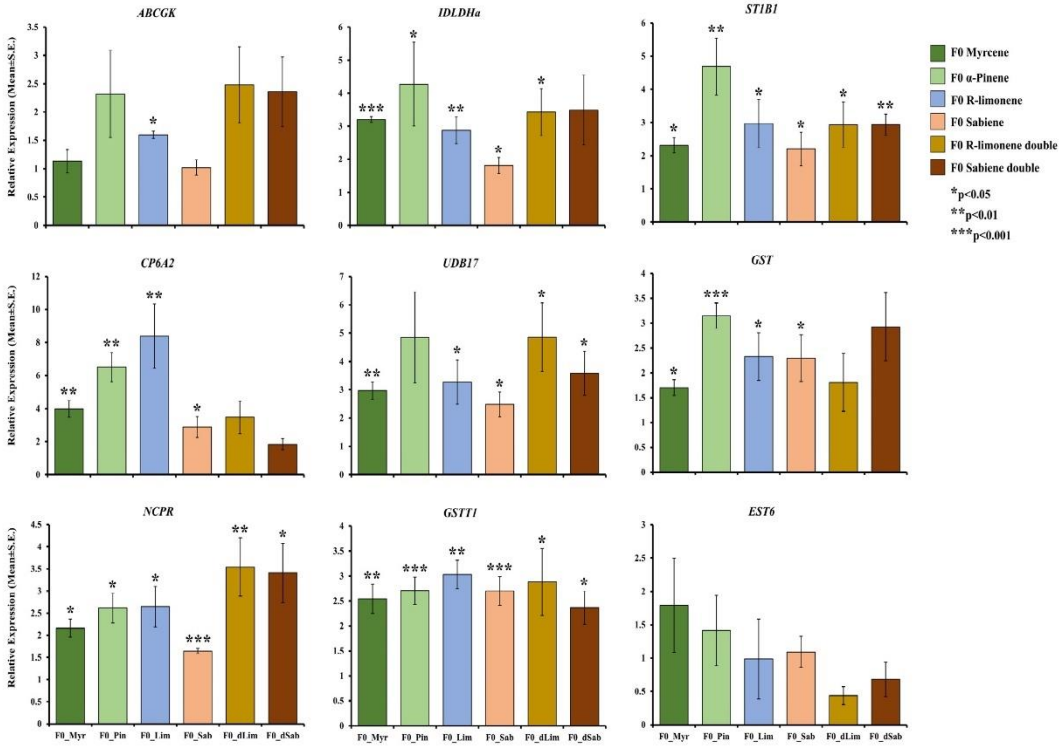

Figure 7. Relative fold change expression ($2^{(-\Delta\Delta Ct)} \pm$ S.E.) of the selected 9 important detoxification genes in F0 beetles fumigated with myrcene (dark green), α-pinene (green), R-limonene (blue), and

sabiene (orange) at LC_{70}-48 h (n = 4); and F0 beetles treated with a double dose of LC_{70} of R-limonene (yellow) and sabiene (brown) each (n = 5) for 48 h on the x-axis plotted against their fold change on the y-axis. An independent t-test was performed to check the statistical difference between the control and the treatment, and accordingly, p-values were generated. * represent $p < 0.05$, ** represent $p < 0.01$, and *** represent $p < 0.001$. *ABCGK*–ABC transporter G family member 20, *IDLDH-a*–Ipsdienol dehydrogenase, *ST1B1*–Sulfotransferase 1B1, *CP6A2*–Cytochrome P450 6a2, *UDB17*–UDP-glucuronosyltransferase 2B17, *GST*–Glutathione S-transferase, *AK1A1*–Alcohol dehydrogenase, *SDR1-a*–Farnesol dehydrogenase, *IDLDH-b*–Ipsdienol dehydrogenase, *SDHA*–Succinate dehydrogenase, *NCPR*–NADPH-cytochrome P450 reductase, *DHB12*–17-beta-hydroxysteroid dehydrogenase 12, *PA1B2*–Platelet-activating factor acetylhydrolase IB subunit beta homolog, *DHGL*–Glucose dehydrogenase, *HYEP1-a*–Juvenile hormone epoxide hydrolase 1, *HYEP1-b*–Juvenile hormone epoxide hydrolase 1, *SDR1-b*–Farnesol dehydrogenase, *EST6*–Venom carboxylesterase-6, and *GSTT1*–Glutathione S-transferase 1.

Enzymatic activity assays on CPR, GST, and EST reflected the same expression pattern as the RNA-seq and RT-qPCR, showing the downregulation of esterases and the upregulation of GST and CPR (Figure 8).

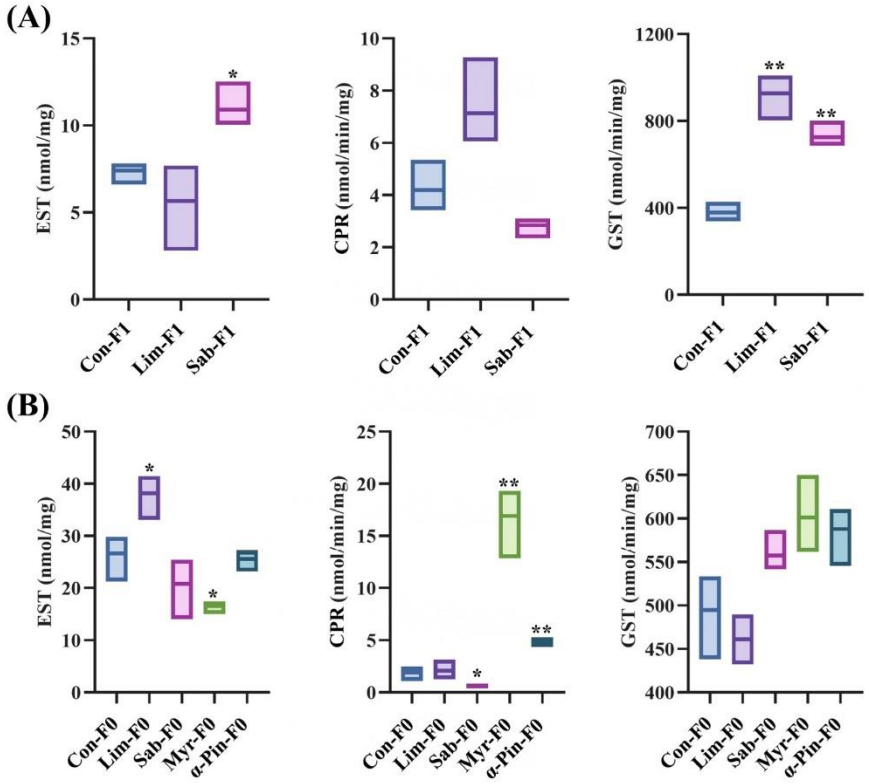

Figure 8. The activities of esterase (EST), cytochrome P450 reductase (CPR), and glutathione S-transferase (GST) measured on (**A**) F1 population of *I. typographus* fumigated with R-limonene and sabiene and (**B**) wild beetle (F0) population fumigated with R-limonene, sabiene, and additionally with myrcene and α-pinene (n = 3). An independent t-test was performed to check the statistical difference between the control and the treatment, and accordingly, p-values were generated. Esterase

enzyme activity is expressed as nmol/mg, and that of CPR and GST is expressed as nmol/min/mg. * represent $p < 0.05$, and ** represent $p < 0.01$. F0–wild beetles, F1–first lab-reared generation, Con–control, Lim-(R)-limonene, Sab–sabiene, Myr–myrcene, and α-Pin–α-pinene.

3. Discussion

The detoxification of spruce monoterpenes by *Ips typographus* plays a pivotal role in the beetle's ability to colonise and damage spruce forests, leading to significant ecological and economic impacts in Central Europe [6,8]. The beetle's success in thriving despite exposure to these toxic allelochemicals highlights its highly specialised adaptation involving enzymatic systems that degrade or transform monoterpenes into less harmful forms [41,42]. Often, the divergent response of beetle holobionts against host monoterpenes may determine their niche partitioning strategies. Interestingly, the expression of the gene in specific monoterpenes has not been explored. This study provides new insights into the specific molecular mechanisms that enable *I. typographus* to metabolise toxic monoterpenes such as α-pinene, sabiene, myrcene, and R-limonene, potent defensive allelochemicals produced by spruce trees. The fumigation bioassay conducted in this study revealed that these monoterpenes exhibit significant toxicity to *I. typographus*, with sabiene and R-limonene emerging as particularly effective inhibitors.

Previous research has shown the induction of defence priming and the elicitation of the induced defense system of the tree during biotic and abiotic stresses [43–46]. Although some monoterpenes, such as α-pinene and myrcene, have been found to have attractant properties in other beetle species [47,48], they can exhibit inhibitory effects at higher concentrations [49]. Additionally, R-limonene has been reported to cause a higher mortality in other beetle species than α-pinene [33], while its effectiveness varies in different contexts [36]. The direct toxicity of sabiene to bark beetles remains less explored, although its increased production in response to MeJA and fungal inoculation is noted [50]. In this study, the fumigation assays revealed significant differences in the effectiveness of the five monoterpenes tested, with R-limonene and sabiene showing the highest potency. The lower LC_{70} value for sabiene indicates its high effectiveness as a fumigant, whereas R-limonene, although moderately toxic, may require higher concentrations or alternative formulation strategies for optimal pest control (Figure 1, Table 1). Similarly, Chiu et al. (2017) [36] reported that R-limonene exhibits the highest toxicity when used via fumigation against the mountain pine beetle. However, *I. typographus* has developed sophisticated detoxification strategies to overcome these chemical defences, as demonstrated by the changes in gene expression and enzyme activity in response to monoterpene exposure. Previous studies have identified detoxification-related gene families in *I. typographus* at both the RNA and protein levels, showing tissue-specific and feeding-specific behaviour induced by feeding on host tissues [30,31,40].

This study, encompassing a gene expression analysis using RNA-seq, RT-qPCR, and enzymatic assays, indicates that the evolution of the detoxification pathways in *I. typographus* is driven by intense selective pressures from the spruce host defence chemistry. The upregulation of detoxification enzymes in response to monoterpene exposure reflects a dynamic, inducible defence mechanism that allows the beetle to rapidly adapt to fluctuating levels of these toxic compounds based on the physiology of the host [51,52]. The involvement of key detoxification enzymes highlights the beetle's sophisticated metabolic activity tailored to overcoming the host's chemical defences [32,52,53]. The redundancy and robustness observed in the beetle's detoxification pathways suggest a high level of metabolic flexibility, allowing *I. typographus* to survive across diverse environmental conditions and variations in host tree chemotypes [32,54,55].

3.1. Expression of Phase I Detoxification Enzymes after Monoterpene Exposure

CYP4, CYP6, and CYP9 are the most important detoxification groups reported in other insect species such as *Dendroctonus* [34,35,56,57], *Sitophilus zeamais* [58,59], and *Aphis gossypii* [60]. Dai et al. (2015, 2021) [33,52] also reported several CYPs being expressed due

to monoterpene exposure and feeding, and the molecular characterisation of one of the CYP6 genes denotes that their expression is regulated by juvenile hormone (JH) levels. Our study revealed that the most common cytochromes belong to families 4 and 6, the most abundant being CYP6A2. We also report higher levels of JH-related hormones like juvenile hormone epoxide hydrolase 1, a pheromone biosynthesis pathway gene [10,61,62] across chemical treatment comparisons I to III (Figure 5). The most highly expressed cytochrome reported is CYP4C1, which is supposedly involved in breaking down synthetic insecticides and providing cold tolerance and heat resistance in *Bemisia tabaci* [63]. CYP49A1 was overexpressed in three chemical exposures in our study (Figure 5, comparisons I, II, and III). This gene was previously reported in the *D. melanogaster* hindgut of the larval stage and was only overexpressed during the feeding larval stage and not the wandering larval stage [64]. However, Naseer et al. (2023) [31] reported that in *I. typographus*, CYP49A1 was downregulated in the L2 stage, which is a high feeding stage. In our data, the most abundantly upregulated gene across most comparisons was CYP6A2, which is associated with insecticide metabolism, mainly DDT in *Drosophila* and imidacloprid in *Aphidius gifuensis* [65–67]. Recently, Tsuji et al. (2024) [68] reported a higher accumulation of CYP6A2 in the gut and salivary glands of *Drosophila* larvae after sesamin feeding. Apart from R-limonene and sabiene, CYP6A2 was also upregulated in the L2 and adult feeding stages, as previously reported (Figure 5A) [31]. Interestingly, in our report, the two most downregulated CYPs were the Halloween genes CYP306A1 and CYP315A1. The expressions of CYP306A1 and CYP315A1 were two times lesser in the R-limonene treatment and four times lesser in sabiene treatment when compared with the pupal stage of ESBB (Figure 5A). CYP306A1 has been previously reported to take part in insect moulting and ecdysone biosynthesis in flies and a lepidopteran insect, *Chilo suppressalis* [69,70]. Another gene, ecdysone 20-monooxygenase, also known as CYP314A1, was also downregulated in our data (Figure 5A, Supplementary File S3), revealing that at a higher toxin exposure, the expression of these developmental genes is lower, resulting in hindered moulting or insect development (Figure 9). We also report that peroxiredoxin-6, reported earlier to be associated with diapause induction in locusts [71], is downregulated in all the chemical treatments. A chorion peroxidase was downregulated in R-limonene and sabiene treatment. Chorion peroxidase is linked with eggshell formation and has been reported to be highly expressed in adult (sclerotised) *I. typographus*, which can start oviposition [31,71].

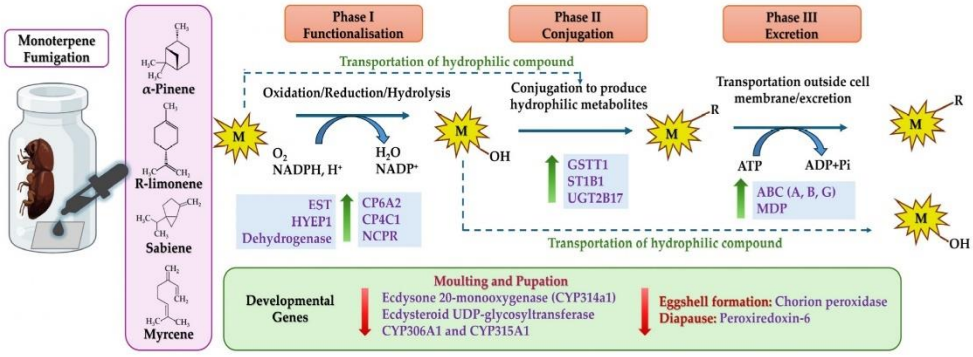

Figure 9. Summary of the key findings in the RNA-seq analysis and RT-qPCR validation of important genes. Monoterpenes (M) are applied to filter paper, and beetles are fumigated for 48 h. M passes through phase I and produces a reactive intermediate (M-OH) after oxidation, reduction, or hydroxylation of the lipophilic compound (M), or it passes directly to phase II. M-OH get conjugated with reduced glutathione or UDP-glucose in phase II to produce a hydrophilic compound (M-R). This hydrophilic compound is transported through the cell membrane into body fluid and then excreted

out of the insect body in phase III. Key detoxification genes reported to be upregulated in this study in each phase are denoted by green up-head arrows. Simultaneously, some development-related genes were downregulated, denoted by red down-head arrows. *EST*–esterases, *HYEP1*–juvenile hormone epoxide 1, *CP6A2*–Cytochrome P450 6a2, *CP4C1*–Cytochrome P450 4c1, *NCPR*–NADPH-cytochrome P450 reductase, *GSTT1*–Glutathione S-transferase 1, *ST1B1*–Sulfotransferase 1B1, *UDB17*–UDP-glucuronosyltransferase 2B17, *ABC*–ABC transporter family members, *MDP*–Multidrug resistance-associated protein.

3.2. Expression of Phase II Detoxification Enzymes after Monoterpene Exposure

Glutathione S-transferases (GSTs) facilitate the metabolism of the endo- or exogenous oxidative stress molecules (e.g., insecticides) by reducing them via dehydrochlorination or by conjugating them with reduced glutathione to produce hydrophilic metabolites that can be excreted [72,73]. In our study, we reported 10 GSTs, which are upregulated in both the chemical treatments, denoting their involvement in endogenously catalysing the intermediates produced during the detoxification mechanism and facilitating their solubility and excretion out of the body. Most of them belong to the theta class of GST (Figure 5C). Gao et al. (2020) [74] reported 16 full-length GSTs belonging to class delta, epsilon, theta, and sigma in Chinese pine beetles. GSTT1 possesses peroxidase activities and is also involved in protein binding for organophosphates [75]. The expression of GSTs and esterases are putatively dependent on the host tree chemical composition and have been reported to significantly change with the alteration of the host via diet switch in *I. sexdentatus* [32]. The consistently high significant expression of GSTT1 throughout all the tested monoterpenes in both the F1 and F0 populations in the RT-qPCR reflects the importance of this gene in monoterpene detoxification (Figures 6 and 7), which can be functionally validated in the future. Another important gene consistently upregulated in the RNA-seq and RT-qPCR was sulfotransferase 1B1 (ST1B1), which is reported to have specialised xenobiotic substrate response sites and an involvement in resistance against xenobiotic stress [76].

Insects have evolved to utilise UGTs to glycosylate endogenous or exogenous lipophilic compounds by conjugating them to UDP-glucose as an activated sugar donor, facilitating detoxification [77,78]. In the recent past, UGTs have been established as one of the important genes for metabolising dietary toxins and insecticides in many crop pests like silkworms (*Bombyx mori*), the fall armyworm (*Spodoptera frugiperda*), cotton aphid (*Aphis gossypii*), brown planthopper (*Nilaparvata lugens*), cotton bollworm (*Helicoverpa armigera*), and peach-potato aphid (*Myzus persicae*), to name some [79–85]. Also, in sucking insects like the Asiatic honeybee, *Apis cerana cerana*, the UGT2B20-like gene plays an important role in pesticide resistance [86]. UGT 2B17 was the most upregulated gene of treatment comparisons I–II (Figure 5C). Li et al. (2017) [87] functionally characterised UGT 2B17 in *Plutella xylostella* using RNA interference (RNAi). Following the knockdown of UGT 2B17, the sensitivity of third-instar larvae to chlorantraniliprole increased by 27.4% in the susceptible population and by 29.8% in the resistant populations. In this study, the RT-qPCR expression of UGT 2B17 was also reported to be significantly high through the different chemical treatments and with the respective generations (Figures 6 and 7). Alternatively, we reported the constant downregulation of ecdysteroid UDP-glycosyltransferase (EGT) in the three chemical treatments (Figure 5C). EGT is a baculovirus-encoded protein, transferred to initially lepidopteran insects via horizontal gene transfer, which inactivates the ecdysone hormone formation in the host insects, thus preventing moulting and pupation [88,89]. Naseer et al. (2023) [31] showed that this gene was highly upregulated during the pupal stage compared to larvae or adults. The downregulation of EGT after the overexposure of allelochemicals in adult beetles shows the conserved nature of insects to save their energy during oxidative stress rather than reproduce. Hence, we can re-infer that exogenous overexposure to plant defence chemicals hinders developmental and reproductive genes and promotes the overexpression of conserved detoxification genes (Figure 9). Two superoxide dismutase (SOD) genes were upregulated in the R-limonene and sa-

biene comparison (Figure 5C). These genes are involved in tolerance against oxidative stresses [90].

3.3. Expression of Phase III Detoxification Enzyme after Monoterpene Exposure

Phase III detoxification enzymes play a crucial role in detoxification by facilitating the removal of xenobiotics and endogenous toxins. ABC transporter genes help the hydrophilic, less toxic metabolised product to be excreted from the body of the insects with body fluid. Previously, Naseer et al. (2023) [31] reported that the expression of these transported genes drastically increases at the second-instar larva stage compared to the pupal stage of *I. typographus*. The pupae are in the non-feeding sedentary stage, where the exposure of the host chemicals is close to none, and only moulting and development occur. The beetle development requires energy derived from already metabolised host tissues ingested during feeding. Hence, the detoxification genes are downregulated during the pupal stages. However, during the callow stage, beetles start to feed again, and the transportation and excretion via body fluid continue. Instead, they must increase the supply of nutrition to keep it circulating for moulting into sclerotised adults. In our study, we reported the ABC transporter being highly upregulated during the vigorous feeding stage of the beetle (Figure 5D). Sun et al. (2017) [91] identified 40 ABC transporters from the ABCA–ABCH subfamilies in *Laodelphax striatellus*, with over 20% of these genes significantly upregulated in resistant strains. Eight genes from the ABCB, ABCC, ABCD, and ABCG subfamilies were consistently upregulated across all resistant strains compared to the susceptible strain. Knocking down genes encoding ABC transporters, either individually or simultaneously, confirmed their role in resistance [92–95]. We assume that overexposure to the monoterpene leads to a hastened detoxification process, and hence, the need to eliminate the toxins from the beetle body increases, which can be achieved by the overexpression of ABC transporter genes.

4. Materials and Methods

4.1. Insect Collection and Rearing

Freshly infested Norway spruce logs were collected from research plots managed by the School Forest Enterprise (ŠLP) near Kostelec nad Černými lesy (49.9940° N, 14.8592° E) in the eastern district of the Central Bohemian region of Prague, Czech Republic. During the summer, the area's climate is drier and warmer, with a growing season of 150 to 160 days, an average annual temperature of 7 to 7.5 °C, and a mean annual precipitation of 600 mm [96,97]. During the summer season, the climatic conditions are suitable for the progeny development and swarming of beetles. The infested trees were cut down, and their logs were then transported to a rearing facility at the Faculty of Forestry and Wood Sciences of the Czech University of Life Sciences, where they were stored at 4 °C until used. Logs were stored for a maximum of two weeks from the date of felling. Beetles were collected from the wild logs and reared on fresh, uninfested spruce logs to produce the F1 generation population. The logs were placed in mesh cages within a laboratory environment where conditions were carefully controlled: a temperature of 25 °C, a relative humidity maintained at 65%, and ample air supplied [31,38]. Emerging F1 beetles were collected, sexed based on pronotum hair and knob dimorphism, weighed, and used on the same day, and those in compromised physiological conditions were excluded from the bioassay.

4.2. Fumigation Bioassay and Toxicity Calculation

For the toxicity assays, five monoterpenes (Table 4) were used individually for fumigation assays based on previous reports [3,44,45,98,99] following the protocol of Chiu et al. (2017) [36]. A 1.5 cm × 1.5 cm piece of Whatman filter paper was placed in a 20 mL scintillation vial, onto which defined volumes of undiluted monoterpenes were applied using a pipette (Eppendorf) immediately before adding a single beetle to the vial. A moist filter paper was placed in each vial to maintain humidity, and the vial was closed with a cap and sealed with parafilm. Only moist filter paper was placed in the scintillation

vial for the control, a beetle was inserted, and the vial was sealed without any monoterpene application. Monoterpenes were tested at five defined doses (volume monoterpene applied/volume airspace of the assay vial) of 50 µL/L, 100 µL/L, 200 µL/L, 400 µL/L, and 800 µL/L. To achieve the doses of monoterpenes, undiluted monoterpenes were applied at volumes of 1 µL, 2 µL, 4 µL, 8 µL, and 16 µL per 20 mL of the vial, respectively. The set-up was placed inside the climate chamber (Memmert HPP2200ECO, Schwabach, Germany), which maintained a temperature of 25 °C and a 20 h/4 h light/dark photoperiod [100]. The beetles were exposed to volatiles for the pre-optimised time duration of 72 h, and mortality was assessed at every 12 h interval. Beetles were considered dead if they did not show any movement while the vial was being agitated. In total, 360 beetles were tested for each of the monoterpenes. At each chemical dose and control for each monoterpene, 60 insects with 30 females and 30 males were used (n = 60/dose/monoterpene). The beetles were distributed so that their average body weight was almost the same for all concentrations. Trials were conducted on each monoterpene in multiple technical replicates due to uneven numbers of beetles collected each day; however, the beetles collected on each day were randomly sorted into control and treatments (for all doses) in equal numbers to minimise the sample distribution biases. There was no significant difference between the body weight of the males and females used for the individual chemical treatment (Supplementary File S1).

Table 4. List of chemicals used in the bioassay with their purity.

Sr. No.	Name	Purity	Manufacturer	CAS
1	α-Pinene	97%	Thermo Scientific Chemicals, Waltham, MA, USA	80-56-8
2	(S)-(−)-Limonene	97%	Thermo Scientific Chemicals	5989-54-8
3	(R)-(+)-Limonene	~90%	Sigma-Aldrich, St. Louis, MO, USA	5989-27-5
4	(1S)-(+)-3-Carene	99%	Sigma-Aldrich	498-15-7
5	Myrcene	≥90%	Sigma-Aldrich	123-35-3
6	Sabiene	75%	Sigma-Aldrich	3387-41-5

To calculate the LC_{70}, 48 h mortality data was used, and dose–response analyses were conducted. Out of all the monoterpenes used in the bioassay, the top two most suitable chemicals (one with the lowest LC_{70} and another with a moderate LC_{70}) were used for the RNA-seq. For the RNA-seq, freshly emerging F1 *I. typographus* were treated individually again at LC_{70} with R-limonene and sabiene for 48 h; then, the collected beetles were snap-frozen in liquid nitrogen and stored at −80 °C. A total of three treated beetles were pooled together to make one biological replicate, and their whole body was crushed using a pre-chilled mortar and pestle. The crushed beetle samples were stored at −80 °C for further RNA extraction. For the RT-qPCR, the bioassay was repeated with new F1 and F0 ESBB populations and four chemicals (R-limonene, sabiene, α-pinene, and myrcene) at their respective LC_{70} for 48 h.

To check the effect on beetle generation, a double dose of the LC_{70} was applied to F0 beetles for R-limonene and sabiene. For the RT-qPCR and enzyme assays, RNA was extracted from the raised samples after 4 beetles were pooled together to make one biological sample for the LC70, and 2 beetles were pooled for the double LC_{70}.

4.3. Total RNA Extraction, cDNA Synthesis, and RT-qPCR Analysis

Total RNA was isolated using the PureLink™ RNA Kit from Ambion (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The isolated RNA was treated with DNase I (TURBO DNase Kit, Ambion, Austin, TX, USA). One µg of RNA was used to synthesise cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Waltham, MA, USA) and stored at −20 °C until further

use. The cDNA was diluted 5-fold to be used as a template for RT-qPCR. Three biological replicates were used for F1 beetles, four biological replicates per treatment were used for F0 beetles at LC_{70}, and five biological replicates were used for the double LC_{70} for F0 beetles for RT-qPCR. The primers were designed using IDT PrimerQuest software (IDT, Leuven, Belgium) (Table 5). The RT-qPCR was performed as reported by Naseer et al. (2023) [31]. Briefly, a 10 µL reaction mixture was prepared using 5 µL of 2x SYBR® Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA), 3 µL RNase-free water (Invitrogen, Waltham, MA, USA), 1.0 µL of cDNA, and 0.5 µL each of 10 µM forward and reverse primers. The Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems) was set up with the following reaction conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 1 min, and a dissociation curve analysis during which temperature was increased from 60 to 95 °C. The $2^{(-\Delta\Delta Ct)}$ method [101] was used to calculate the relative expression levels of the target genes. Ribosomal protein L7 (RPL7) served as a reference gene for expression normalisation [31,38].

Table 5. Primer designed for the RT-qPCR study.

Sr. No.	Primer Name	Transcript ID	Transcript Name		Primer Sequence	Amplicon Length	Tm (°C)
1	EST6	Ityp05867	Venom carboxylesterase-6	*F* *R*	CAACCGAAATGGTGAACTG ATTCTCAACCACCGTAGAC	119	60
2	HYEP1-a	Ityp10836	Juvenile hormone epoxide hydrolase 1	*F* *R*	CGGCCTGACTAAACACTTT AGCCAAACCCTTCAGAATAC	110	60
3	AK1A1	Ityp08881	Alcohol dehydrogenase	*F* *R*	TCCGAACAACTGCAAAGG TAGCACCAGGACTTCCTAAA	160	60
4	GST	Ityp13834	Glutathione S-transferase	*F* *R*	CTACTCTGGAAGTGGATGG AATCCGTGACTGTGTCG	138	58, 59
5	SDR1-a	Ityp00346	Farnesol dehydrogenase	*F* *R*	GGCAATAACCACAAGAGAGG CGCAAATTTACTGGCTGGA	139	60, 61
6	ABCGK	Ityp09976	ABC transporter G family member 20	*F* *R*	CCACTGACGCTATTAAGCAC CAGGTACGCCTTGATTTCTC	143	61, 60
7	IDLDH-a	Ityp22107	Ipsdienol dehydrogenase	*F* *R*	GGACAATAATCGGGACGAAG GGTTGGTCATGGAGATGATG	131	60
8	SDR1-b	Ityp13935	Farnesol dehydrogenase	*F* *R*	GCGTTAACGGAAACTGTTAG AGGTCTGTCATTTGCTAGAG	153	59
9	ST1B1	Ityp07436	Sulfotransferase 1B1	*F* *R*	AACCACGTTCTGCCATTC GCTTCAGTGAGGTCTTTCTC	134	60
10	CP6A2	Ityp03140	Cytochrome P450 6a2	*F* *R*	TTGAGACATCGGCTACCA GGCGAAGATAGGTCATTTCC	156	60
11	UDB17	Ityp14578	UDP-glucuronosyltransferase 2B17	*F* *R*	GATTCCAACGCCGCTAAA GACGATGCTTCACTTGACTT	139	60
12	HYEP1-b	Ityp03551	Juvenile hormone epoxide hydrolase 1	*F* *R*	GAGAGATAGTCCGGT GTCCAGTAATTGGTC	127	60
13	IDLDH-b	Ityp14704	Ipsdienol dehydrogenase	*F* *R*	CAGACAGTTGGGACCTTTAG CACGTGGTTTGATCATTCTG	146	60
14	SDHA	Ityp17457	Succinate dehydrogenase	*F* *R*	CGTTCTGGATCTGTTGATGG GGCTGTGCAGGAGAAATATG	145	60
15	GSTT1	Ityp05781	Glutathione S-transferase 1	*F* *R*	GTAGATCAGCGCCTCCATTT GGGACTGATAAGCTTGCACCACT	136	60
16	NCPR	Ityp11675	NADPH-cytochrome P450 reductase	*F* *R*	GCAAACACTGCGAGAAGA AAACGTGAGTTCTGGGATTC	153	60
17	DHB12	Ityp13083	17-beta-hydroxysteroid dehydrogenase 12	*F* *R*	ATCAACAACGTCGGGATG CACCATTCCAGGAAGTACAA	141	60
18	PA1B2	Ityp04924	Platelet-activating factor acetylhydrolase IB subunit beta homolog	*F* *R*	ACTACCTCGAGGACAGAATC ACCATCAGGTTGGATAAAGC	136	60
19	DHGL	Ityp11581	Glucose dehydrogenase	*F* *R*	GGCTTTCAGAAGTGGAGAAT GTTCTGGACGGTGGTATTG	140	60

4.4. Reference Gene Selection

Twelve candidate reference genes for the fumigation of ESBB with (R)-(+)-limonene, sabiene, and 3-carene were selected based on a previous study (Table 2) [38]. Four different programs (NormFinder, ΔCt, BestKeeper, and RefFinder) were used to check the expression stability of the candidate genes. The genes were ranked according to their overall performance in three chemical treatments and a control using geNorm. Four biological replicates were used for each treatment and control.

4.5. RNAseq Analyses

A pairwise differential expression analysis (without replicates) was performed using OmicsBox (version 3.2.2) [102], using the software package NOISeq [103,104], in a nonparametric approach. The contrasting log fold change difference (M) and absolute expression difference (D) between the test (chemical treatment-R-limonene and sabiene) and reference (control) were ascertained. A single replicate was fed into the NOISeq pipeline, and five technical replicates were simulated for each experiment condition, assuming that the read counts followed the multinomial distribution. Preprocessing of the raw read was performed using the following criteria: CPM—1.0, normalisation method—TMM (trimmed mean of M values), number of simulated replicates—5, size of simulated replicates—0.2, and variability—0.02. For the differential expression analysis, the probability value > 0.9 was chosen. A gene set enrichment analysis (GSEA) was performed using the ranking generated using the formula: $-\text{sign}(M)*\text{sqrt}(M^2 + D^2)$ (Supplementary File S2).

4.6. Comparison between Multiple In-House Data

To compare the differential gene expression data of the two chemicals and identify the vital detoxification gene across the two comparisons (I: R-limonene vs. control and II: sabiene vs. control), and in-house carene-fumigated beetles vs. control (III) (*unpublished data*), ESBB feeding on MeJA-treated bark vs. those feeding on stored bark (IV) (*unpublished data*), active feeding stage vs. non-feeding stage (L2-larval stage two vs. T4-pupa (VI) and adult vs. pupa stage (VI)) data of ESBB from Naseer et al. (2023) [31], and callow male vs. sclerotised male (VII) (CM vs. SM) data from Ashraf et al. (2023) [40] were used (Supplementary File S4). The cut-off used for each of the comparisons (III–VII) was FDR $p < 0.05$ and log fold change $\pm$ 1 (M value in case of I and II). The fold change value of the common gene transcripts between I and II, among all other comparisons after applying the cut-off, was retrieved and plotted in the heat map (Figure 5).

4.7. Enzyme Activity Assay

An enzyme activity assay was performed for three genes, glutathione S-transferase (GST), cytochrome P450 reductase (CPR) and esterase (EST), to comparatively assess the enzyme activity in *I. typographus* treated with four different chemicals following the preoptimised protocols of Naseer et al. (2023) and Sellamuthu et al. (2024) [31,32]. The enzyme activities were assessed following the published protocol with minor modifications [105]. Briefly, the beetle whole body powder was homogenised in 50 mM of Na_3PO_4 (pH 7) containing 1 mM EDTA and 0.1 mM DTT. The solution was centrifuged, and the supernatant containing total protein was collected as an enzyme source. The protein concentration was measured using the Bradford method [106] with BSA and used to correct the enzyme activities as standard.

4.8. Statistical Analysis

The percentage mortality of the beetles was calculated using Henderson–Tilton's formula [37]. The dose–response analysis was performed using XLSTAT 2020 (v 3.1.1011) to calculate the LC_{70}. An F-test and Student's *t*-test were performed to check the sex-based mortality among the populations (Supplementary File S1). To compare the relative expression of detoxification genes between the control and treated beetles in the RT-qPCR and enzyme activity assay, first, the normality of each group was checked using the

Shapiro–Wilk test, and then the variance homogeneity was calculated between the control and treatment groups using Levene's test. Then, an independent *t*-test was performed with an equal variance Student's *t*-test (if Levene's $p > 0.05$) or unequal variance Welch's *t*-test (if Levene's $p < 0.05$) accordingly, and *p*-values were generated based on the significant differences between control and treatment groups at 95 C.I. using RStudio (version 4.2.3) (Supplementary File S5). The statistical and numerical analysis used in the DGE data is described in Section 4.5 under 'RNA-seq analyses'.

5. Study Limitations

The RNA study is conducted based on a single replicate (n − 1) and may have some biases. We used an RT-qPCR and other in-house transcriptome data to gain a higher reliability for and insight into our current findings. Though the RT-qPCR data show considerable correspondence to the transcriptome data, we recommend including more biological replicates to enhance the robustness of the RNA-seq analysis.

6. Conclusions

This study extends our understanding of the detoxification mechanisms of allelochemicals in *I. typographus* by investigating the toxicity of five key monoterpenes (α-pinene, sabiene, myrcene, R-limonene, and S-limonene) found in Norway spruce bark. With the integration of mortality assays and chemical treatments, our findings demonstrate a dose-dependent decrease in beetle survival upon exposure to these monoterpenes, with sabiene and R-limonene proving particularly effective. The RNA-seq analysis revealed a significant upregulation of detoxification genes and suppression of developmental genes in response to these potent monoterpenes. Furthermore, the RT-qPCR and enzymatic assays corroborated the RNA-seq results, suggesting the conserved regulation of detoxification pathways triggered by exogenous host allelochemicals that demands further experimental corroboration. In conclusion, this study deepens our understanding of monoterpene toxicity in forest beetles, and the integration of bioassay and molecular data provides a solid foundation for future bark beetle adaptation research, illuminating the complex interactions between monoterpenes and beetle physiology. These insights pave the way for developing innovative target gene-based management strategies (i.e., RNA interference) for a more effective and sustainable control of bark beetles, including *I. typographus*, addressing critical needs in forest pest management and promoting the advancement of targeted pest control solutions [107,108].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms251810209/s1. Reference [109] is cited in Supplementary File S2.

Author Contributions: A.N.—conceptualisation, methodology, formal analysis, data curation, data analysis, visualisation, writing—original draft, review and editing, project administration. V.V.S.—methodology, data curation, writing—review and editing. G.S.—methodology, data curation, writing—review and editing. J.S.—methodology, writing—review and editing. K.M.—writing—review and editing. L.K.—infrastructure, writing—review and editing. A.R.—conceptualisation, RNA-seq data analysis, supervision, project administration, funding administration, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grant no. CZ.02.1.01/ 0.0/0.0/15_003/0000433, "EXTEMIT–K project", financed by the Operational Program Research, Development, and Education (OP RDE). A.N. was supported by the Internal Grant Agency (IGA; 43950/1312/3120_A_06_22) of the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czechia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding author. The RNA-seq raw reads were submitted under NCBI bioproject PRJNA1149972.

Acknowledgments: V.V.S. was supported by project FORSOMICS (grant no. 09I03-03-V03-00103) financed by NextGenerationEU through the Recovery and Resilience Plan for Slovakia. K.M. acknowledges the Ramalingaswami re-entry grant (BT/RLF/Re-entry/11/2023 from the Department of Biotechnology, Govt. of India. A.N., A.R., and G.S. are supported by excellent team grants from the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czechia. The summary figure was prepared using biorender.com.

Conflicts of Interest: The authors declare no conflicts of interest.

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Remaining Supplementary Material (excel datasets) for this article can be found online at: **https://www.mdpi.com/article/10.3390/ijms251810209/s1**. Reference (109) is cited in Supplementary File S2.

5.4. Gene expression plasticity facilitates different host feeding in *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae)

Published as: Sellamuthu, G., **Naseer, A.**, Hradecký, J., Chakraborty, A., Synek, J., Modlinger, R. and Roy, A., 2024. Gene expression plasticity facilitates different host feeding in *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae). Insect Biochemistry and Molecular Biology, 165, p.104061. https://doi.org/10.1016/j.ibmb.2023.104061

Contributed to methodology, data curation, writing- reviewing and editing.

Study III was designed 'to understand the host selection and host chemical defences adaptation in *Ips* beetles and understand host shift.' This chapter helps us elaborate more on the overall objective 'understanding spruce-beetle interaction'. We investigated the gene expression plasticity of the six-toothed bark beetle, *Ips sexdentatus* (ISx), as it adapted to feeding on different conifer hosts, specifically Scots pine and Norway spruce. Remarkably, when ISx was made to shift (no-choice assay) from pine to spruce, it not only survived but thrived, as evidenced by its high fecundity on spruce logs. Such notable adaptations were also reflected in gene expression, particularly in gut tissues. The critical physiological changes involved in digestion, detoxification, and stress response were studied using RNA sequencing, enzymatic assays, and metabolomic analyses to uncover the molecular mechanisms underlying this host shift.

The findings reveal that ISx exhibits significant transcriptional plasticity when shifting from its native pine host, allowing the beetle to modulate the expression of genes involved in digestion, detoxification, and molecular transport, enabling it to process and neutralize the allelochemicals present in spruce. For instance, genes associated with detoxification enzymes such as CYP4C1, UDP-glucose: glycoprotein glucosyltransferase, ABC transporter (ABCC and ABCB6), and glucose dehydrogenase were differentially upregulated when ISx was fed on spruce (F1 ISx-Spruce) compared to pine (F1 ISx-pine). Additionally, transporter genes associated with sugars and nutrient transport were abundant, and trehalose essential for stress tolerance and homeostasis were upregulated in F1 ISx-spruce, suggesting their importance in maintaining energy balance and stress tolerance under the new dietary conditions.

Insect Biochemistry and Molecular Biology 165 (2024) 104061

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Gene expression plasticity facilitates different host feeding in *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae)

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ARTICLE INFO

Keywords:
Bark beetles
Ips sexdentatus
Host switch
Differential gene expression (DGE)
Detoxification
Enzyme assay
Chitin metabolism
Host adaptation
Metabolomics
RT-qPCR

ABSTRACT

Host shift is ecologically advantageous and a crucial driver for herbivore insect speciation. Insects on the non-native host obtain enemy-free space and confront reduced competition, but they must adapt to survive. Such signatures of adaptations can often be detected at the gene expression level. It is astonishing how bark beetles cope with distinct chemical environments while feeding on various conifers. Hence, we aim to disentangle the six-toothed bark beetle (*Ips sexdentatus*) response against two different conifer defences upon host shift (Scots pine to Norway spruce). We conducted bioassay and metabolomic analysis followed by RNA-seq experiments to comprehend the beetle's ability to surpass two different terpene-based conifer defence systems. Beetle growth rate and fecundity were increased when reared exclusively on spruce logs (alternative host) compared to pine logs (native host). Comparative gene expression analysis identified differentially expressed genes (DEGs) related to digestion, detoxification, transporter activity, growth, signalling, and stress response in the spruce-feeding beetle gut. Transporter genes were highly abundant during spruce feeding, suggesting they could play a role in pumping a wide variety of endogenous and xenobiotic compounds or allelochemicals out. Trehalose transporter (TRET) is also up-regulated in the spruce-fed beetle gut to maintain homeostasis and stress tolerance. RT-qPCR and enzymatic assays further corroborated some of our findings. Taken together, the transcriptional plasticity of key physiological genes plays a crucial role after the host shift and provides vital clues for the adaptive potential of bark beetles on different conifer hosts.

1. Introduction

Host shift-driven exposure to new host chemistry paves the way for speciation and evolution in generalist insect pests (Groman, 2000). Insect host selection depends on finding a suitable host that facilitates the development and survival of the next generation (Carrasco et al., 2015; Tadmor et al., 2022). Many phytophagous insects are specialists by restricting themselves to a single host species or closely related host plant families (Lin et al., 2010; Novotny and Basset, 2005; Novotny et al., 2002; Strong et al., 1984). Acceptance of sympatric novel hosts by phytophagous insects is commonly influenced by the ancestral or native host and less rivalry and escape from natural enemies (Dres and Mallet, 2002). Host selection is influenced by chemical and physical defences, nutritional resources, mating, and oviposition site of the new host (Carrasco et al., 2015; Jaenike, 1978; Salgado and Saastamoinen, 2019; Wang et al., 2020). Host plants produce secondary metabolite-based defence chemicals, providing direct and indirect defences against phytophagous insects (Fürstenberg-Hägg et al., 2013; Gatehouse, 2002; Li et al., 2022a; War et al., 2012). The adaptive mechanisms against the new host apply strong selection pressure on phytophagous insects (de la Paz Celorio-Mancera et al., 2013; Dermauw et al., 2018; Kant et al., 2015). The genetic variation and allelochemical environment of the new host are essential drivers of phenotypic and genetic changes in phytophagous insects (De Castro et al., 2021; Yu et al., 2018).

Phytophagous insects, including bark beetles, can adapt and utilize the novel host toxin compounds as nutrients through detoxifying host allelochemicals by themselves or symbiotic microbes in the gut (Ashraf et al., 2023; Chakraborty et al., 2020a, 2020b; Müller et al., 2017; Singh

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https://doi.org/10.1016/j.ibmb.2023.104061
Received 28 September 2023; Received in revised form 30 November 2023; Accepted 15 December 2023
Available online 25 December 2023

et al., 2020). Phytophagous insects have also evolved *in-vivo* enzymatic solutions to overcome the effects of host allelochemicals. Three different phases of xenobiotic detoxification enzymes, including cytochrome P450 monooxygenase (P450s), UDP-glycosyltransferase (UGTs), carboxylesterase (CEs), glutathione S-transferase (GST), ATP-binding cassette (ABC) transporter, convert host toxic compounds to non-toxic ones and excrete out or sequester them (Beran and Petschenka, 2022; Rand et al., 2015; Roy et al., 2016; Torres-Banda et al., 2022; Wu et al., 2019; Xu et al., 2005). Prolonged exposure to novel hosts can lead to xenobiotic resistance in insects (Müller et al., 2017; Tadmor et al., 2022). Understanding such xenobiotic resistance mechanisms upon host switch in bark beetles is still limited, although there is ample evidence of host shifts and multiple conifer species feeding by bark beetles.

Ips sexdentatus (Börner) (Coleoptera: Curculionidae: Scolytinae) is a major pine-feeding beetle [scots pine (*Pinus sylvestris*)] in Eurasia, which attacks weakened pine trees by climatic disturbances such as drought, windstorms, wildfires (Fernández, 2006; Hlávková and Doležal, 2022; Knížek et al., 2022; Pineau et al., 2017; Rossi et al., 2009). Beetle larvae and adults (primarily, callow stage) feed on the phloem tissues under the bark of conifers. Climate change has a positive effect on herbivore performance, particularly on bark beetle species, and increases their host range (Akkuzu et al., 2017; Colombari et al., 2012; Cooke and Carroll, 2017; Cudmore et al., 2010; Jönsson et al., 2007; Venäläinen et al., 2020; Wermelinger et al., 2021). The mass attack during epidemic conditions and also as the vector of phytopathogenic fungi belonging to the group of *Ophiostomatales* results in massive conifer tree mortality worldwide (Bueno et al., 2010; Lieutier et al., 1991, 2009; Ramanenka et al., 2021; Wingfield et al., 2017). The oligophagous beetle *I. sexdentatus* (hereby called ISX) thrives on a wide range of pine trees such as *P. sylvestris, P. pinaster, P. heldreichii, P. nigra, P. leucodermis, P. sibirica,* and *P. koraiensis* as primary hosts in Europe (Health et al., 2017; Knížek et al., 2022; Pfeffer, 1995). Interestingly, it occurs in Turkey, Georgia, and Southern Russia on *Picea orientalis* (Norway Spruce) as their primary host (Cebeci and Baydemir, 2019; Health et al., 2017; Schimitschek, 1939; Schönherr et al., 1983). Often, *I. sexdentatus* (ISX) is also captured from infested spruce logs in Czechia. It is captivating how ISX deals with spruce and pine defences. No studies delineate the molecular readjustments these bark beetles have made to thrive in different toxin-laden environments after host switch. Shifting from one host to another demands readjustments in insect enzymatic machinery and defence arsenal against an unexposed combination of xenobiotics (Hou and Wei, 2019; Mackay-Smith et al., 2021; Müller et al., 2017; Noriega et al., 2020). We hypothesize that bark beetles (here, ISX) also readjust defence and detoxification machinery through gene expression plasticity upon conifer host switch. To evaluate this hypothesis, we established short-term generation of ISX on two different hosts (pine and spruce) in the laboratory conditions to explore the phenotypic performance and transcriptional expression changes. Our results provided the first insights on transcriptomic plasticity in ISX gut gene expression upon spruce feeding (alternative host). RT-qPCR and enzymatic assay further corroborated the results. Current findings extend our understanding of host adaptation in conifer-feeding bark beetles. It also lays the foundation for future research on bark beetle adaptation to different conifer hosts and may facilitate the formulation of sustainable pest management strategies using RNAi (Gupta et al., 2023; Joga et al., 2021; Mogilicherla and Roy, 2023).

2. Materials and methods

2.1. Host-plant materials and bark beetles

Pinus sylvestris L. (Scots pine) and *Picea abies* L. (Norway spruce) trees were extensively collected the Kostelec nad Černými lesy (50°00′07.2″ N 14°50′56.3″ E) and Rouchovany (49°04′08.0″ N 16°06′15.4″ E) for beetle host switch experiments and metabolomic studies. Trees aged 80–100 years of each species (diameter 30–45 cm, length 38–42 cm) were collected and never treated with pesticides. *Ips sexdentatus* were collected from Kostelec nad Černými lesy within pine logs. They were reared in an insect climate chamber at 27 ± 1 °C under 70 ± 5% humidity and a 16:8-h light/dark (L:D) photoperiod for collection of adults (F_0) and set up for the next generation (called Lab reared F_1) on spruce and pine (Sellamuthu et al., 2021).

2.2. Gas chromatography-mass spectrometry (GC-MS) analysis for host-plant

The phloem samples were collected from pine and spruce logs from the forest. The samples were freeze-dried in liquid nitrogen and powdered with mortar and pestle. Approximately 200 mg of powdered samples were used to extract compounds in 1 ml hexane by sonication for 10 min. Separation and identification of extracted compound were made using GC-MS Agilent 7890B (Agilent Technologies, Palo Alto, CA, USA) employing time of flight mass analyser Pegasus 4D (LECO, St. Joseph, MI, USA) as described before with modifications (Ramakrishnan et al., 2022). One microliter of the extract was injected into a cold PTV injector (20C) in split mode (split ratio 1:10). After injection, the inlet was heated to 275 °C at a rate of 8 °C/s. Separation was conducted on the HP-5MS UI capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness from Agilent. The GC oven temperature program was as follows: 60 °C for 2 min; then ramped at a rate of 10 °C min^{-1} up to 330 °C and held for 4 min. The total GC run time was 33 min. Ions (ionisation energy at 70eV) were collected in a mass range of 35–400 Da with a frequency of 10 full spectra/s. The solvent delay was 240s. Automated spectral deconvolution and peak-finding algorithms extracted signals with a signal-to-noise ratio (S/N) higher than 50. The data were cleaned, normalized, and after CLR transformation , evaluated using principal component analysis (PCA) and partial least squares discriminant analysis (OPLS-DA) in SIMCA 17 software (Sartorius Stedim Data Analytics AB, Malmö, Sweden). The identity of compounds was based on spectral similarity, and further confirmation was performed by comparing the retention times of respective standards and literal retention indexes from National Institute of Standards and Technology (NIST, 2017).

2.3. Interspecific and intraspecific reciprocal transplant (no-choice bioassay)

2.3.1. Offspring performance and fecundity

To evaluate the differences in beetle performance, we compared the performance (left elytra length, weight and fecundity) of ISX after the interspecific transfer (ISX beetle transfer Pine to Spruce for next-generation [F_0 Pine to Spruce referred to as F_1 Spruce]) and intraspecific transfer (ISX beetle transfer Pine to Pine for next-generation [F_0 Pine to Pine referred to as F_1 Pine]). *I. sexdentatus* (F_0) adults (male and female equal ratio reared on *Pinus sylvestris*) were shifted to *Pinus sylvestris* (natal host, pine) and *Picea abies* (alternative host, spruce) logs in an insect climate chamber (Sellamuthu et al., 2021) for the next generation (F_1). Equal pairs (10 pairs) of males and females from F_0 ISX pine were placed on the respective host logs (pine, spruce) in F_1 to test the fecundity. Fecundity was calculated as the number of larval galleries (alternatively, the number of successfully hatched larvae) per square centimeter of phloem. The experimental setup was maintained according to Sellamuthu et al. (2021). The F_1 ISX adult body weight and left elytra length (n = 60) were measured to evaluate the performance.

2.3.2. RNA extraction, library preparation and illumina sequencing

To assess the transcriptome plasticity upon the host switch, we performed RNA sequencing with the gut tissues of ISX [F_0-Pine (hereafter called F_0Pi), F_1-Pine (hereafter called F_1Pi), F_1-Spruce (hereafter called F_1Sp)] after feeding on *P. sylvestris* and *P. abies,* respectively. The dissected ISX gut tissues from respective treatments were shock-frozen in liquid nitrogen, and the total RNA was extracted using PureLinkTM RNA Kit (Ambion, Invitrogen, USA). The RNA concentration was

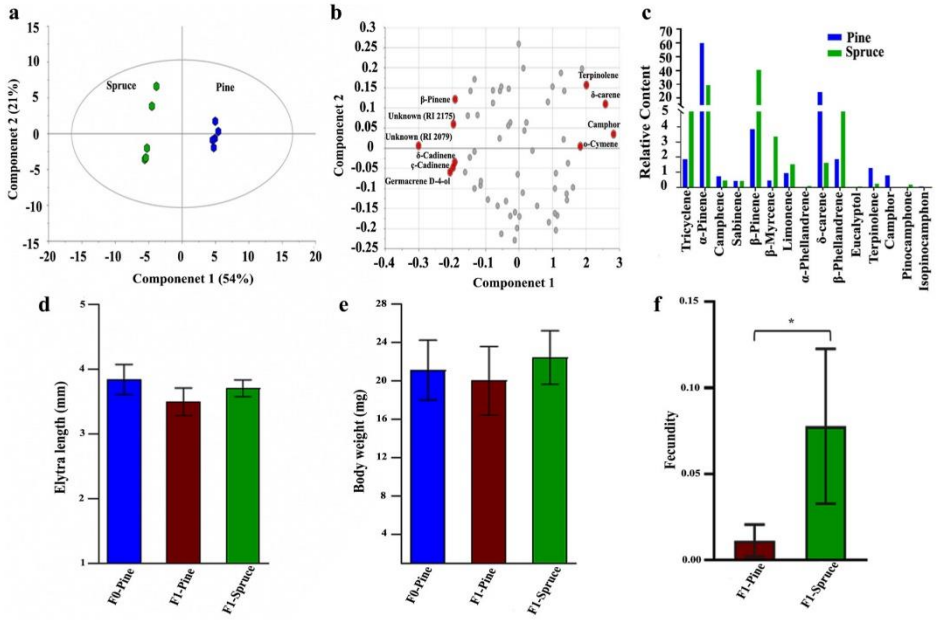

Fig. 1. Conifer tissue metabolomics and bark beetle bioassay. a) PCA scores plot, showing clear differentiation of spruce (blue) and pine (green) wood b) PCA loading plot, presenting volatile compounds influence for separation in Fig. 1a and highlighted in red are the ten most important compounds according to OPLS-DA VIP (variable importance) plot c) Relative abundance of typical terpenic compounds in conifers wood. Effects of ISX host tree species on percentage survival, mean adult weight, and fecundity (±SE) based on laboratory rearing of the ISX. d; Elytra length, e; Body weight and f; Fecundity.

measured with Qubit 2.0 Fluorometer (Life Technologies) and integrity with 2100 Bioanalyzer (Agilent, CA, USA). Five biological replicates (each with ten pooled ISX gut tissues) were used to obtain sufficient total RNA for downstream preparations. The pooling reduced individual heterogenicity among the samples and reduced the necessity of substantial sequencing efforts. RNA samples (1 μg) were used for library construction. RNA sequencing (PE150) was performed on an Illumina NovaSeq 6000 platform (Illumina, Novogene, China).

2.3.3. *De novo assembly, annotation and differential gene expression (DGE)*

The RNA-seq data analysis was performed using the OmicsBox transcriptomics module (ver 1.4.11) following the developer protocol. Generated Illumina reads per sample were merged into a single dataset, and the resulting FASTQ files (Illumina) were filtered to remove low-quality reads and reads containing adapter sequences (Andrews, 2010). FastQc and Trimmomatic were used to filter reads and remove low-quality reads. Subsequently, Q20, Q30, GC-content, and duplication were calculated after cleaning the data. These clean reads were *de novo* assembled without a reference genome using the TRINITY program (Grabherr et al., 2011; Langmead and Salzberg, 2012) in the OmicsBox platform (ver 1.4.11), applying the recommended setup with some modifications such as minimum coding length 350, Non-strand specific, pairs distance 500 to optimize the assembly. BUSCO evaluated the completeness of the assembly (Seppey et al., 2019). The coding region was predicted using Transdecoder (Haas and Papanicolaou, 2020). Transcript sequences were first blasted against the non-redundant protein sequences in NCBI using the BLASTX-fast search programme with an e-value cut-off of <1.0E-5 in the Blast2GO platform under the functional analysis module within OmicsBox (ver 1.4.11). The gene ontology (GO) annotation was performed in Blast2GO using the GO database (Consortium, 2004), and metabolic pathways from the Kyoto Encyclopedia of Genes and Genomics (KEGG) (Kanehisa et al., 2016), EggNOG and orthologous groups (COG) (Huerta-Cepas et al., 2019). The transcript levels of unigenes were calculated using the RSEM software package in OmicsBox (ver 1.4.11) (Langmead and Salzberg, 2012; Li and Dewey, 2011). The pairwise differential expression analysis (DGE) was conducted using the edgeR software package (Bioconductor project) (Robinson et al., 2010) with the following parameters as the false discovery rate (FDR) p-value <0.05 and fold change ±2 in OmicsBox (ver 1.4.11). The data analysis parameters are as follows: count per million (CPM) filter 1.0, Samples reaching CPM filter 5, and normalization method – weighted trimmed mean of M values (TMM), robust-true (estimation is strengthened against potential outlier genes). Hierarchical clustering was carried out using the average linkage approach with Euclidean distance based on log fold change data to depict the expression pattern using Cluster 3.0. (Eisen et al., 1998). Fisher extract test (Bullard et al., 2010) was performed for GO enrichment analysis on the DEGs (up and down) obtained from all comparisons.

2.3.4. *Real-time quantitative PCR (RT-qPCR) validation*

RT-qPCR was conducted to validate the expression level of differentially expressed genes (DEGs). Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Isolated RNA was further treated with DNasesI using a TURBO DNAase Kit (Ambion, USA) to remove trace DNA contamination. RNA quantity and quality were evaluated using 1.5% agarose gel, quantified by NanoDrop (2000)/2000c from Thermo Fisher Scientific® (Waltham, MA, USA), and stored at −80 °C. cDNA (first-strand) was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription kits (Applied Biosystems-Life Technologies) following the manufacturer's recommendations and stored at −20 °C. RT-qPCR experiments were performed using the Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems) with a reaction mix containing 5.0 μL of

SYBR® Green PCR Master Mix (Applied Biosystems), 1.0 μL of cDNA, 1.0 μL optimized concentrations of primers (Supple Table 1), and RNase-free water (Invitrogen) to a total volume of 10.0 μL. The reference gene was selected from the previous study on ISX (Sellamuthu et al., 2021). Amplification conditions were initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. To confirm the primer specificity, a melt curve analysis was performed using default parameters with a steady increase in temperature from 60 to 95 °C. The relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All RT-qPCR assays were carried out in four biological replicates, including two technical replicates.

2.3.5. Enzyme assay

Enzyme activities were measured for esterase (EST), glutathione S-transferase (GST) and NADPH–Cytochrome P450 Reductase after total protein extracted from ISX gut samples using the preoptimized protocol (Naseer et al., 2023). The enzyme activities were assessed following the published protocol with minor modifications (Bosch-Serra et al., 2021). The laboratory-reared ISX gut tissues from F_0 (pine-fed) and F_1 generation (pine and spruce-fed) were collected for the enzymatic analysis. Four individual replicates were prepared per treatment, and for each replicate, 10 guts were dissected and homogenized into 100 μl sodium phosphate buffer (50 mM, pH 7.0). The crude ISX gut protein extracts were prepared following our pre-optimized protocol (Naseer et al., 2023). Protein concentration was measured using the Bradford method (Bradford, 1976). The detailed enzymatic protocol was provided as Supplementary Protocol File 1.

2.3.6. Statistical analysis

The ISX body size and mass differences after different host feeding (no-choice bioassay) were determined using ANOVA. A paired *t*-test was performed to consider the significant differences in fecundity. The statistical analysis deployed for DGE data analysis is based on fitting negative binomial Generalized Linear Models (Likelihood Ratio Test, GLM). One-way ANOVA (Šídák's multiple comparisons test) was employed to test the significance of differences in RT-qPCR and enzymatic activities.

3. Results

3.1. Host metabolites

An untargeted metabolomics approach was carried out to analyse the metabolite variation between *P. sylvestris* and *P. abies* encountered by beetles (ISX). PCA (explained variance of data 75%) showed that two test groups were well separated with many differentiating metabolites (Fig. 1a). For further identification of the most discriminating compounds among *P. sylvestris* and *P. abies*, OPLS-DA (Orthogonal partial least squares-discrimination analysis) was used, and 10 most differentiating compounds according to VIP (variable importance plot) was obtained (Fig. 1b). Many of them are typical terpenoid compounds reported commonly in conifer species. Among the biologically relevant monoterpenes, β-Pinene, β-Phellandrene, β-Myrcene, and limonene are more abundant in the spruce as compared to pine wood. Similarly, δ-carene, Camphor, Terpinolene and α-Pinene are copious in pine wood (Fig. 1c; Supple Table 2).

3.2. Development and fecundity of ISX on host switch

The effect of host species on the growth and development of ISX was evaluated with sixty representative beetles from F0-Pine (F_0Pi), F1-Pine (F_1Pi) and F1-Spruce (F_1Sp) lab populations. No significant effect on their body weight and elytra length was found among different host-fed ISX lab populations (Fig. 1d and e). However, significantly higher fecundity was found in the F_1-spruce-fed population compared to the F_1-pine-fed population (Fig. 1f).

Table 1
Summary of *Ips sexdentatus* (ISX) gut transcriptome.

Features	Transcripts
Total transcripts	130,267
Total genes	55,395
GC%	40.05
Contig length N50 (bp)	2053
BUSCOs	
Complete	1190/87.05%
Single copy	82/6%
Coding Regions	
Complete ORF	18,598
5' partial	7197
3' partial	2231
Internal 6528	
Reference Genome Sequences 34,554	
Minimum Length	261
Maximum Length	24,726
Average Length	835
Total Length	28,852,761
EggNoG annotation	34554
Total input sequences	
No of GO annotated sequence	15858
Total number of GO annotations	125120
GOG annotations	
Information storage and processing	5421 (19.58%)
Cellular processes and signalling	8321 (30.06%)
Metabolisms	6073 (21.94%)

3.3. Transcriptome assembly, completeness, coding sequence and EggNOG annotation

RNA-seq of *I. sexdentatus* gut tissues resulted in approximately 485 million reads (Supple Table 3). De-novo assembly resulted in 130,267 transcripts (55,395 genes, N50- 2053 bp) with GC content of 40.05% (Table 1). BUSCO analysis based on insecta_obd10 database (curated-10/09/2020, including 75 species) showed a score of 93.05%, of which 87.05% complete single copy, 6% complete duplicated, 1.54% fragmented and 5.41% missing BUSCOs (Table 1 and Supple Fig. 1a). The coding region analysis of transcripts showed 18,598 complete, 6528 internal, 7197 partial (5′), and 2231 partial (3′) ORFs, respectively (Table 1 and Supple Fig. 1b). The EggNOG annotation results of orthologous groups (COG) analysis showed GO annotation to 15,858 transcripts, which are divided into 23 functional categories (Supple Fig. 2) under information storage & process (19.58%), cellular process & signalling (30.06%), and metabolisms (21.94%) (Table 1). The most well-represented functional categories are involved in transcription (1583 transcripts), translation, ribosomal structure and biogenesis (1479 transcripts), post-translational modification, protein turnover, chaperones (2691 transcripts), Signal transduction mechanisms (2654 transcripts): Cytoskeleton/Cell wall/membrane/envelope biogenesis (1124 transcripts), Carbohydrate transport and metabolism (1253 transcripts), Energy production and conversion (1010 transcripts), other transport and metabolism (3810 transcripts) (Supple Fig. 2 and Supple Table 4).

3.4. Gene expression analysis of DEGs

Different ISX gut tissue samples were compared after different host feeding (F_0Pi vs F_1Pi; F_0Pi vs F_1Sp; F_1Pi vs F_1Sp, respectively), deploying standard cut-offs (FDR corrected p-value $\leq$0.05, and fold change $\pm$2). Overall results were represented using the PCA plots and the volcano plots. PCA plots showed that different host-fed beetle gut samples clustered separately (Supple Fig. 3a–c) due to the influence of host and generation effects on gene expression, ensuring the reliability and rationality of the experimental data. Notably, host feeding causes a more prominent separation of samples than generation, which is understandable. Volcano plots represented the number of genes differentially expressed in different comparisons (Supple Fig. 3a–c). The overall gene

Table 2
Number of DEGs from ISX gut tissues respective host shift.

S. No	Comparison	Sample ID	Upregulated (FDR $p < 0.05$, Fold change ≥ 2)	Downregulated (FDR $p < 0.05$, Fold change ≤ -2)
1	F_0 Pine vs F_1 Pine	F_0Pi vs F_1Pi	186	113
2	F_0 Pine vs F_1 Spruce	F_0Pi vs F_1Sp	649	566
3	F_1 Pine vs F_1 Spruce	F_1Pi vs F_1Sp	929	616

expression differences between the three comparisons were presented as a hierarchical clustering heatmap (Supple Fig. 4a–c). The results showed 186 up-regulated and 113 down-regulated genes (Supple Tables 5 and 6) in the F_0Pi vs F_1Pi comparison, whereas 649 and 929 up-regulated (Supple Tables 7 and 8), and 566, 616 down-regulated (Supple Tables 9 and 10) genes were observed in F_0Pi vs F_1Sp; F_1Pi vs F_1Sp comparisons respectively (Table 2). DEGs were clustered separately, showing generation and host-switch effects, such as the conserved generation effect (cluster A) and conserved spruce diet switch effects (cluster B) (Fig. 2a). The Venn diagram summarized the uniquely up-regulated and downregulated genes within identified DEGs among three comparisons, i.e., 106, 293, and 650 genes were up-regulated, and 40, 310, and 416 genes were down-regulated in F_0Pi vs F_1Pi; F_0Pi vs F_1Sp; F_1Pi vs F_1Sp comparisons, respectively (Fig. 2b and c). Only 3 up-regulated and 17 downregulated genes were common in all comparisons. Genes associated with transmembrane transporter and hydrolase activity were highly abundant in the spruce-feeding-up cluster (Cluster B1; Fig. 2b; Supple Table 11). In contrast, oxidoreductase and peptidase activity were higher in the spruce-feeding-down cluster (Cluster B2; Fig. 2c; Supple Table 12). Genes associated with hydrolase, oxidoreductase and ATPase activities in ISX gut tissues were predominantly affected between generations (Cluster A1 and A2; Fig. 2b and c; Supple Tables 13 and 14).

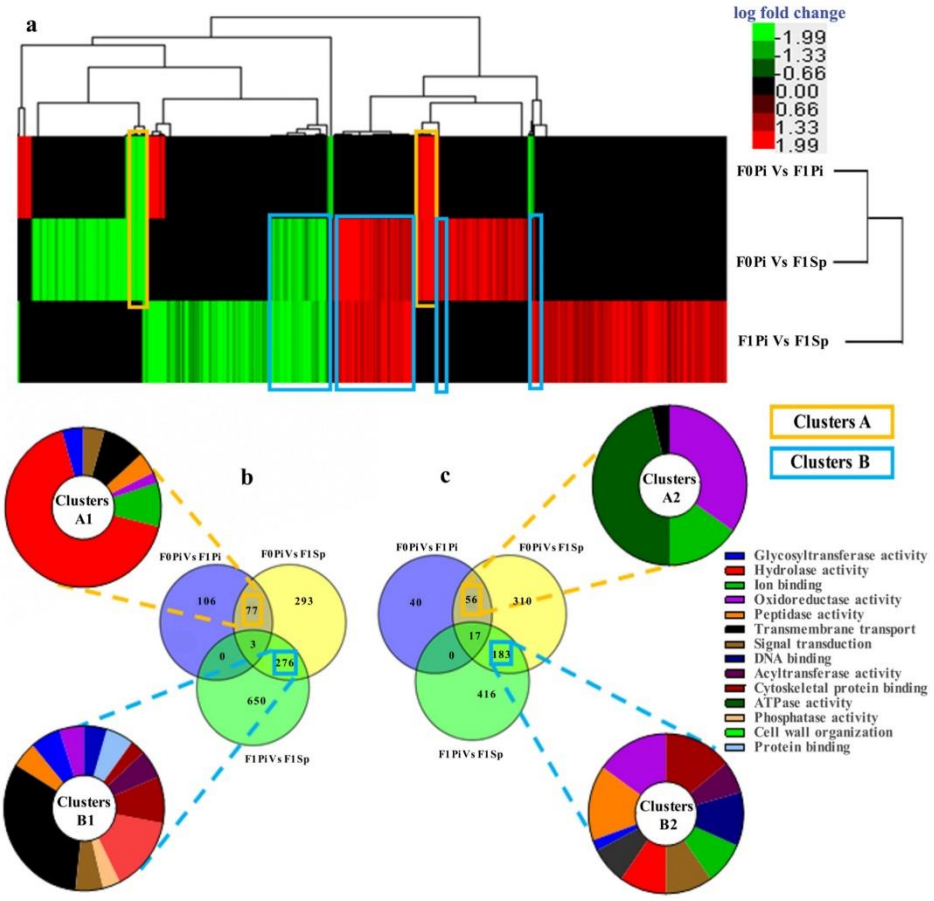

Fig. 2. a). The number of the up-regulated and down-regulated DEGs for the different host feeding of ISX. b). Venn diagram showing up-regulated genes in ISX on F0 Pi vs F1 Pi, F1 vs F1 Sp and F1 Pi vs F1 Sp (Supple Tables S5, S7, S8), (c) Venn diagram showing down-regulated genes in ISX on F0 Pi vs F1 Pi, F0Pi vs F1 Sp and F1 Pi vs F1 Sp. (more details in Supple Table S6, S9, S10). Heatmap representing differentially expressed genes between comparisons. Cluster B1 spruce-specific up-regulated DEGs (Supple Table 11) and Cluster B2 spruce-specific down-regulated DEGs (Supple Table 12). Cluster A1 generation effect up-regulated DEGs (Supple Table 13), and Cluster A2 generation effect down-regulated DEGs (Supple Table 14). The DEGs display a fold change of log2 ±1 with an FDR *p*-value of less than or equal to 0.05.

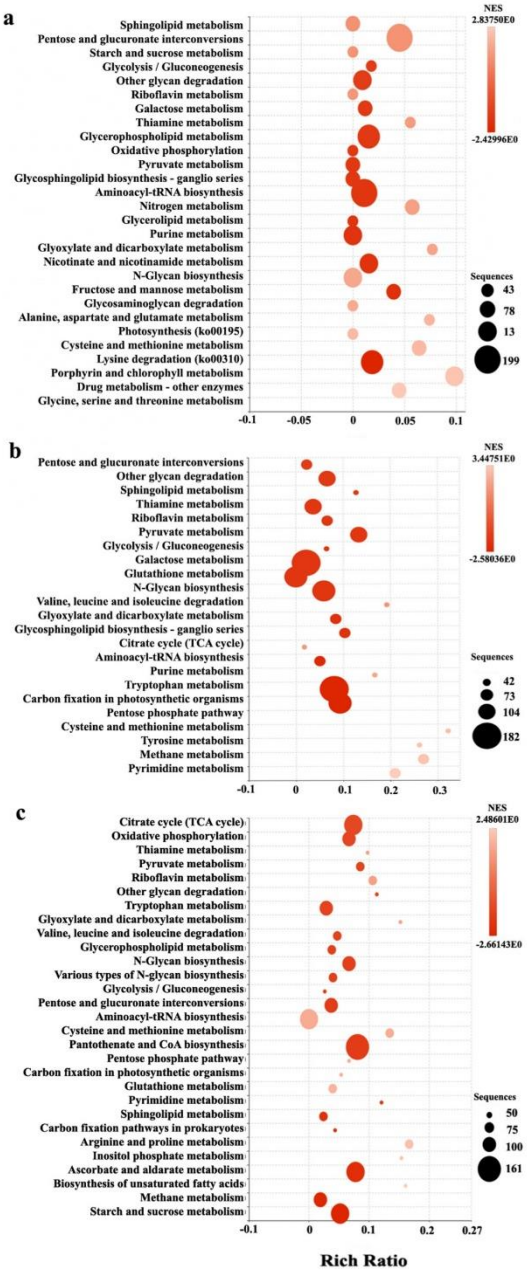

Fig. 3. KEGG pathway enrichment in response to host shift (a) F0 Pi vs F1 Pi; (b) F0Pi vs F1 Sp; and (c) F1 Pi vs F1 Sp (for details, refer to supple Table S16).

3.5. Enrichment analysis

To detect the putative biological functions of DEGs, the significant DEGs from three comparison groups were functionally categorized through enrichment analysis using the Fisher extract test (Supple Tables 15; Supple Fig. 5a–c) and pathway analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of specific host diet switches is shown in Fig. 3a–c (Supple Tables 16). Gene enrichment analysis (GSEA) was used to identify the pathways of functional importance in the present comparisons. Based on the threshold (fold change ±2 and FDR p-value <0.05), the top 30 enriched pathways were identified in response to generation and host shift (Pine to Spruce) (Fig. 3a–c, and Supple Table 16). Analogous functional enrichments were also found in all three comparisons after the Fisher extract test (Supple Table 15), such as carbohydrate metabolic process-GO:0005975; peptidase activity- GO:0008233; glycosyltransferase activity- GO:0016757: oxidoreductase activity-GO:0016491; ATP hydrolysis activity-GO:0016887, and phosphatase activity- GO:0016791.

3.6. Gene expression after spruce feeding

A transfer from the native host *P. sylvestris* to *P. abies* for one generation triggered the highest proportion of transcriptional changes (Fig. 2a) compared to the shift to the native host (*P. sylvestris*) in the second generation. Host shift (Pine to Spruce; F_1Pi vs F_1Sp) a total of 1066 (650 up and 416 down) unique DEGs were found specific to the spruce diet (Fig. 2b and c; Supple Tables 8 and 10) and identified significantly differentially regulated genes with digestion (22), detoxification (16), oxidative stress & signalling (38) and cellular transport (24), indicating the spruce diet effect (Fig. 4a–d; Supple Table 17). Furthermore, the common DGEs between F_0Pi vs F_1Sp and F_1Pi vs F_1Sp revealed the conserved effect of the spruce diet (Cluster B1; Cluster B2). A total of 459 DGEs (276 up and 183 down) were found to be shared between F_0Pi vs F_1Sp and F_1Pi vs F_1Sp (Fig. 2b,c; Supple Tables 11 and 12).

3.6.1. Digestion and detoxification

3.6.1.1. Diet effect (F_1Pi vs F_1Sp). A total of 22 DEGs associated with digestion were differentially expressed. Transcripts of lipase member H-A-like isoform X2, probable chitinase 3, membrane alanyl aminopeptidase-like, dipeptidase 1-like, brachyurin-like protein were up-regulated whereas chymotrypsin 4a, pancreatic triacylglycerol lipase, zinc carboxypeptidase-like, amylase B isoform 1, and acid phosphatase were downregulated (Fig. 4a and Supple Table 17). Similarly, 16 DEGs involved in the detoxification process were up-regulated, including UDP-glucose: glycoprotein glucosyltransferase, ABC transporter (ABCC and ABCB6), and glucose dehydrogenase [FAD, quinone] isoform X2. Alternatively, cytochrome P450, ABC transporter (ABCC4 and ABCG4), and dehydrogenase were downregulated in the ISX gut after spruce diet feeding (Fig. 4b and Supple Table 17).

3.6.1.2. Conserved diet effect (F_0Pi vs F_1Sp and F_1Pi vs F_1Sp). Some DGEs (15 up and 7 down-regulated) were spruce-specific and shared between F_0Pi vs F_1Sp and F_1Pi vs F_1Sp. Up-regulated spruce-specific DGEs involved in xenobiotic digestion included chymotrypsin-like, lipase member H-A-like isoform X1, probable serine hydrolase, lysosomal alpha-mannosidase-like, lysosomal acid phosphatase-like isoform X2, myrosinase 1-like (2), venom acid phosphatase Acph-1-like (5), and aminopeptidase N-like (4). Significantly down-regulated spruce-specific transcripts include antichymotrypsin-2-like isoform X8, digestive cysteine proteinase 2, chymotrypsin-like serine proteinase, and calcium-independent phospholipase A2-gamma-like (Fig. 5a & Supple Table 18).

There were 24 up and 4 down-regulated spruce-specific DGEs related to detoxification. Many key regulatory genes were highly up-regulated after spruce feeding, such as different cytochrome P450s (i.e., CYP4CV1, cytochrome P450 6a17), esterase FE4-like, UDP-glucuronosyltransferases (i.e., 2C1-like, 40AF2, 328B1), multidrug resistance-associated proteins, ATP-binding cassette sub-family B members, ABC

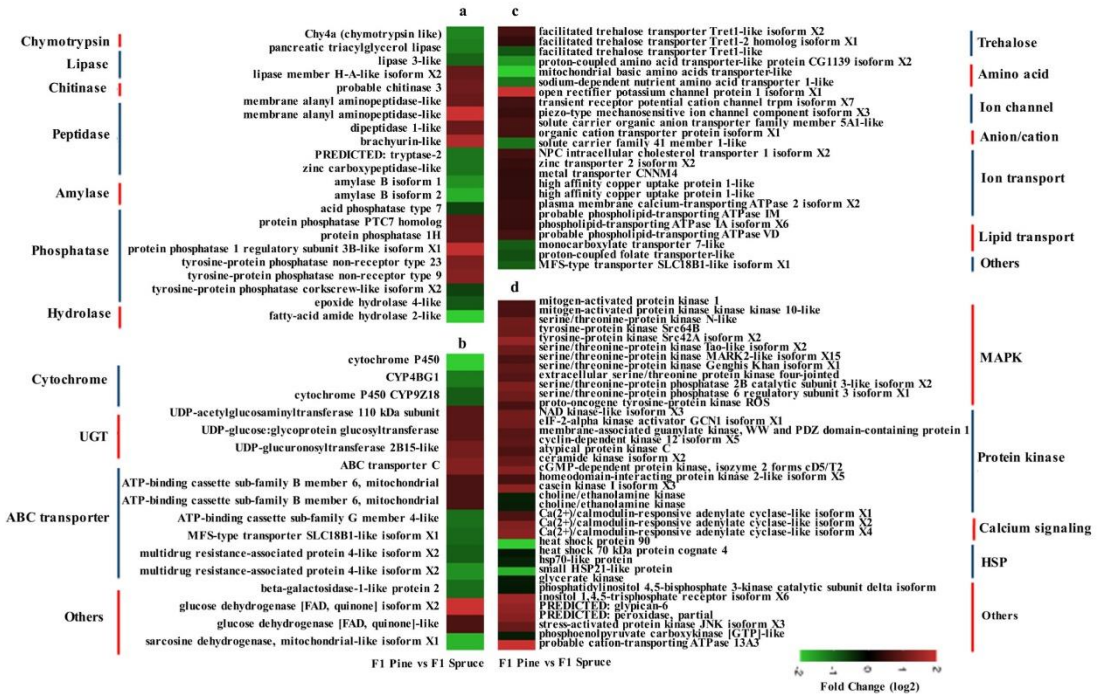

Fig. 4. Spruce diet influences the expression of ISX gut genes related to key physiological functions such as digestion and detoxification, transporter, oxidative stress, and signalling. Heatmap representing DGEs related to - a) digestion; b) detoxification; c) transporter; d) oxidative stress and signalling. The red colour indicates upregulated, whereas the green indicates downregulated gene expression. DGEs are determined by FDR corrected p-value <0.05 (more details in Supple Table 17).

transporter C, alanine–glyoxylate aminotransferase 2-like, estrogen sulfotransferase-like, etc. Alternatively, two UGTs (2B15-like, 2B10-like), esterase E4-like, and probable multidrug resistance-associated protein (isoform X2) were downregulated after spruce feeding (Fig. 5b & Supple Table 18).

3.6.2. Molecular transport, oxidative stress and signalling

3.6.2.1. Diet effect (F_1Pi vs F_1Sp). A total of 24 and 38 DEGs associated with transport and oxidative stress & signalling pathways, including trehalose transporters, ion channels and transporters, lipid transporters, mitogen-activated protein kinases, serine/threonine-protein kinases, Ca (2+)/calmodulin-responsive adenylate cyclase-like isoform X1, peroxidase and other kinase were up-regulated after spruce feeding (Fig. 4c and d and Supple Table 17). Alternatively, amino acid transporter (3), monocarboxylate transporter 7-like, proton-coupled folate transporter-like, MFS-type transporter SLC18B1-like isoform X1 and choline/ethanolamine kinases, and heat shock proteins were downregulated (Fig. 4c and d and Supple Table 17).

3.6.2.2. Conserved diet effect (F_0Pi vs F_1Sp and F_1Pi vs F_1Sp). A total of 29 transporters were highly up-regulated after feeding on the spruce host in both F_0Pi vs F_1Sp and F_1Pi vs F_1Sp comparisons, i.e., facilitated trehalose transporter Tret1-like, putative inorganic phosphate co-transporter isoforms, monocarboxylate transporters, sodium-coupled monocarboxylate transporter, organic solute transporter, cationic amino acid transporter, glucose transporter, amino acid transporter-like protein, chloride channel protein, etc. (Fig. 5c & Supple Table 18). However, only two transporters, i.e., trehalose transporter Tret1-like and potassium/sodium channel protein, were downregulated in both comparisons. A total of 24 (13 up and 11 down) oxidative stress and signalling-related DGEs were found common in between F_0Pi vs F_1Sp and F_1Pi vs F_1Sp ISX gut tissue comparisons, including serine/threonine-protein kinase, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase isoform X1, peroxisomal acyl-coenzyme A oxidase 3-like, glutamate decarboxylase isoform X1, etc. (Fig. 5d & Supple Table 18).

3.7. Gene expression differences between generations

When being exposed to the same host (pine) for the next generation (F_0Pi vs F_1Pi), the PREDICTED: endoglucanase-like, phospholipase DDHD2 isoform X2, lipase 3-like, amylase B isoform 1, PREDICTED: heparanase-like, glycoside hydrolase family protein 48, neuropathy target esterase sws isoform X6, cytochrome P450 9e2-like isoform X1, proton-coupled folate transporter-like, facilitated trehalose transporter Tret1-like, proton-coupled amino acid transporter-like protein CG1139 isoform X3, heat shock protein 90, small HSP21-like protein, ornithine aminotransferase was overrepresented in genes exclusively up-regulated in pine host. Similarly, heat shock 70 kDa protein cognate 3, glycine-rich cell wall structural protein, and monoacylglycerol lipase ABHD12-like were downregulated in pine hosts (Supple Fig. 6a & Supple Table 19).

Conserved generation effects were documented by the shared DGEs between F_0Pi vs F_1Pi and F_0Pi vs F_1Sp. A total of 133 (77 up and 56 down) common DEGs were differentially expressed in both comparisons (Supple Tables 13 and 14). Among them, pancreatic triacylglycerol lipase-like, chymotrypsin 1a, acidic mammalian chitinase-like, polygalacturonase-like, endoglucanase-like, lysosomal alpha-mannosidase-like, UDP-glucuronosyltransferases, glutathione S-transferase-like,

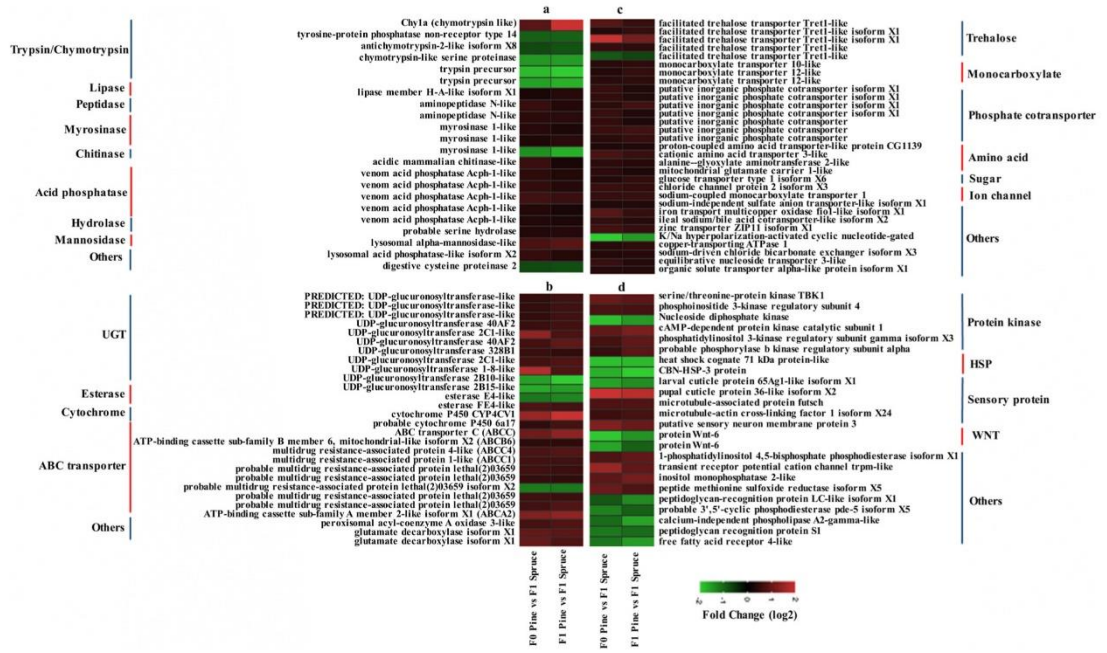

Fig. 5. Spruce diet effect (conserved). Heatmap displaying DGEs related to - a) digestion, b) detoxification, c) transporter, d) oxidative stress and signalling. The red colour indicates upregulation, whereas the green indicates downregulation of the respective genes. DGEs are determined by FDR corrected p-value <0.05 (more details in Supple Table 18).

venom carboxylesterase-6-like, cytochrome P450 9e2-like, and putative inorganic phosphate co-transporters were up-regulated. In contrast, putative cytochrome *b*5, NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, and many heat shock proteins were downregulated (Supple Fig. 6b & Supple Table 20) in both comparisons.

3.8. Validation of gene expression using RT-qPCR

RT-qPCR was used to quantify 20 selected genes involved in host detoxification and defences (Fig. 6). Among these 20 genes, most exhibit consistent expression patterns between RNA-seq and RT-qPCR (Supple Fig. 7). Specific to diet switch, 11 candidate genes were up-regulated in spruce-fed ISX gut tissues; for example, lipase member H-A-like (LipH-A), UDP-glucuronosyltransferase 40AF2, UDP-glucuronosyltransferase 1-8like (UGT1A1), UDP-glucuronosyltransferase 2B10 (UGT2B10), esterase-FE4 (EST-EF4), ABC transporter C (ABCC), trehalose transporter Tret1-like (Tret1-1), trehalose transporter Tret1-like isoform X1 (Tret1), glucose transporter (GLUT1), inorganic phosphate cotransporter (Picot), and glycerol-3-phosphate dehydrogenase (GPD1) genes were highly up-regulated in the spruce host as compared to pine host switch. In contrast, the expression levels of digestive cysteine proteinase −2 (LCP2), epoxide hydrolase (EHs), and cytochrome P450 (CYP4CV1) were downregulated in the spruce host switch compared to the pine host. This result endorsed the RNA-seq results further.

3.9. Effect of host switch on detoxifying enzyme activity at protein level

The host switches lead to temporal changes in the detoxifying enzymes EST, GST, and CRP activities in *I.sexdentatus* gut tissues (Fig. 7). A significant increase in EST and GST activities was observed after spruce feeding (Fig. 7a and b). The GST activity increased <3 folds during spruce feeding (F_0 and F_1). However, CRP activity was comparable during pine and spruce feeding (Fig. 7c).

4. Discussion

Host switch has been identified as a critical factor influencing population dynamics among phytophagous (Cunningham et al., 2001). The host quality is determined by individual genotypes and metabolites, which are also influenced by environmental factors and microclimate (Ballhorn et al., 2010; Bonte et al., 2010; Rostás et al., 2003). Host genetic variation influences their susceptibility to phytophagous insects (Smith et al., 2011; Stone et al., 2018). Alternatively, host shifts directly influence phytophagous insect physiology and its resident microbiome (Bernays and Minkenberg, 1997; Roy et al., 2016, 2023). The present study unravels the physiological impact of pine-feeding bark beetle (*I. sexdentatus,* shortly ISX) upon host shift (spruce-feeding). It is worth mentioning here that Turkey, Georgia, and Southern Russia have reported that *Picea orientalis* is the primary host for ISX. In contrast, *Pinus* spp is reported as the primary host in Europe (Schimitschek, 1939; Health et al., 2017; Cebeci and Baydemir, 2019; Knížek et al., 2022). Although *Picea* spp. was a less frequent host for ISX, we observed a significant positive effect on fecundity after spruce feeding in the current study (Fig. 1f). Similar results were documented in the pine needle scale (*Chionaspis pinifoliae*), which showed higher survival and fecundity in scot pine than in red pine (Glynn and Herms, 2004). Our primary observations and bioassay results suggested that ISX could thrive on spruce hosts quite well, which puzzled us with the low occurrence of ISX on spruce in our Czech forests. One of the reasons may be the high occurrence of *Ips typographus* and *Ips duplicatus* on spruce, making it a second choice for ISX to reduce inter-species competition.

We also observed differences in quantities of metabolites present in

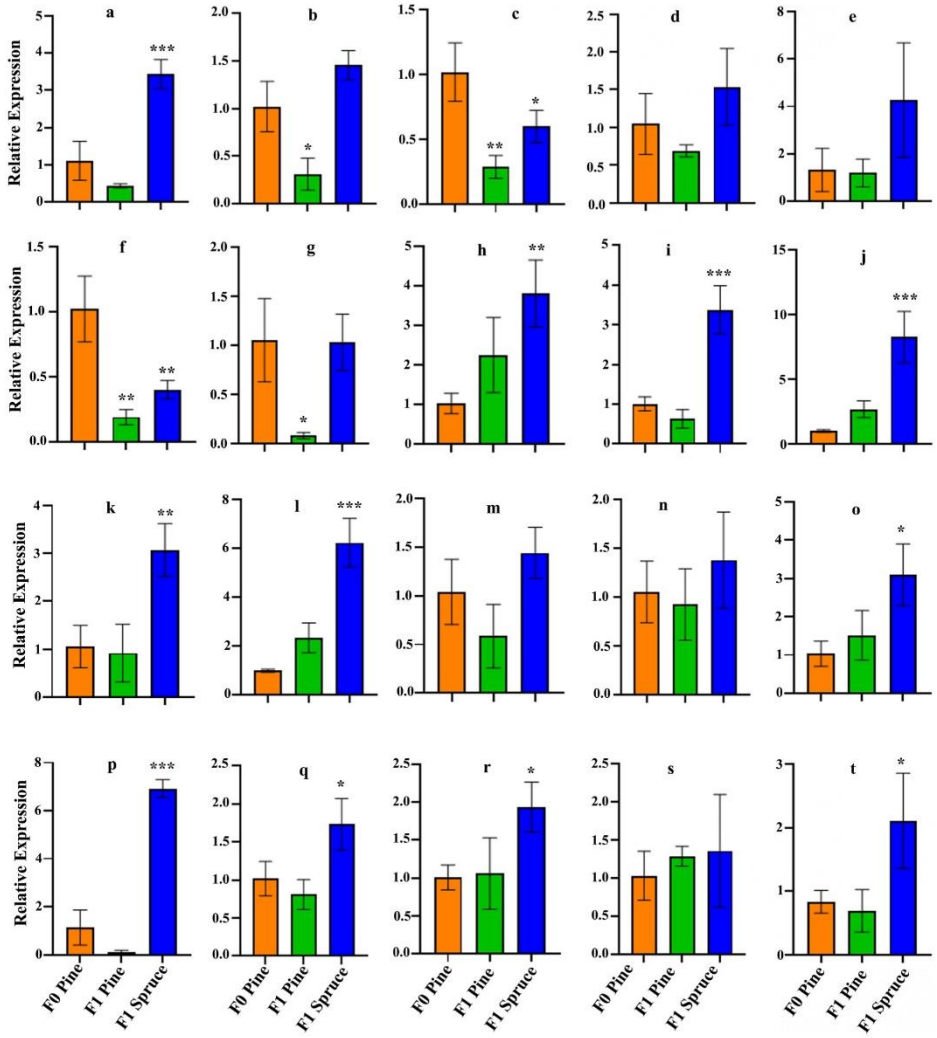

Fig. 6. Validation of differentially expressed genes (DEG) related to digestion, detoxification, transporter, and oxidative stress-related genes by RT-qPCR. The selected genes are as follows: a). Lipase member H-A-like; b). Amylase-B; c). Cysteine proteinase −2; d). Epoxide hydrolase; e). Venom acid phosphatase; f). Cytochrome P450 CYP4CV1; g). Cytochrome P450; h). UDP-glucuronosyltransferase 40AF2; i). UDP-glucuronosyltransferase 1-8like; j). UDP-glucuronosyltransferase 2B10; k). Esterase-FE4; l). ABC transporter C; m). MFS-type transporter; n). Multidrug resistance-associated protein 4; o). Trehalose transporter Tret1-like; p). Trehalose transporter Tret1-like isoform X1; q). Glucose transporter; r). Inorganic phosphate co-transporter; s). Glucose dehydrogenase; and t). Glycerol-3-phosphate dehydrogenase (Supple Table 1- primer list).

pine and spruce in our GC-MS analysis. The primary chemical defence system in conifers is oleoresin, which includes a complex mixture of different monoterpenes, diterpenoids and sesquiterpenes (Keeling and Bohlmann, 2006; Kolosova and Bohlmann, 2012; Zulak and Bohlmann, 2010). Oleoresins are produced constitutively, but the biosynthesis is induced by biotic stress, including beetle attack (Zulak et al., 2009). Monoterpenes are already known to be entomotoxic to diverse groups of insects, including bark beetles (Phillips et al., 2010; Smith, 1961, 1963; Stamopoulos et al., 2007; Tarelli et al., 2009). Interestingly, the terpenoid profile (monoterpene amounts) varies across conifer species during constitutive and induce defence responses (Clark et al., 2014; Keeling and Bohlmann, 2006), endorsing our current observation between pine and spruce (Fig. 1a,b,c).

Contrasting monoterpene profiles and host chemistry affect bark beetle host acceptance behaviour and mediate host range expansion (Erbilgin, 2019). For instance, a higher concentration of (−)-α-pinene can increase the probability of successful aggregation on Jackpine compared to Lodgepole pine for mountain pine beetle *D.ponderosae* (MPB) by facilitating the production of trans-verbenol, an oxygenated product of (−)-α-pinene (Libbey et al., 1985). Alternatively, a lower concentration of antifeedant or repellent monoterpenes (i.e., 3-carene, limonene, 4-allylanisole) in jack pine can facilitate MPB host entry for

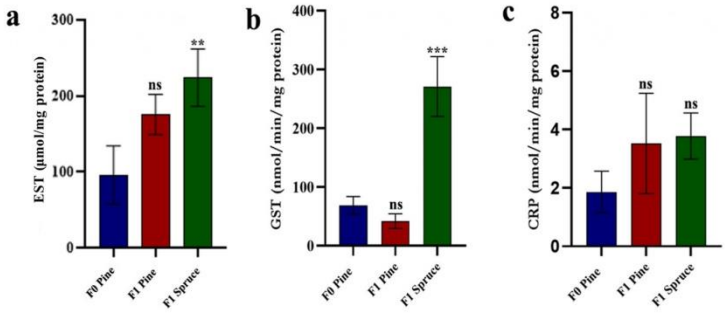

Fig. 7. Enzyme assays. a) Esterase (EST), b) glutathione S-transferase (GST) and c) cytochrome P450 (CRP) activities are assessed in ISX gut tissues after feeding one generation of pine (F0Pi, F1Pi) and spruce (F1Sp) hosts. The data are presented as the mean ± SE (n = 8). The asterisks indicate significant differences in the enzymatic activities between different ISX samples. Statistical significance is tested using t-tests (**$p < 0.001$; ***$p < 0.001$; NS- non-significant). EST is expressed as nmol/mg proteins, and GST and CRP activities are expressed as nmol/min/mg protein.

colonization (Chiu et al., 2017; Reid et al., 2017). It is worth mentioning here that (−)-α-pinene can act as an antifeedant at a higher concentration for MBP (Chiu et al., 2017). Artificial inoculation with MPB-associated fungi also leads to the production of the above-mentioned biologically relevant monoterpenes in pine trees within seven days of induction, endorsing its importance against biotic stress tolerance in conifers (Arango-Velez et al., 2016). Furthermore, monoterpenes such as α-pinene and myrcene caused ultrastructural alternations in the midgut of pine beetle, *Dendroctonus valens* (Fernanda López et al., 2011). An independent study on another pine beetle (*Dendroctonus armandi*) revealed that host allelochemical (α-pinene, 3-carene, limonene, and turpentine oil) exposure induced the expression of cytochrome P450 (CYPs) enzymes, which are known to metabolize host terpenes in MBP (Blomquist et al., 2021; Dai et al., 2016). Such findings link the impact of different host chemistry with the detoxification machinery of the bark beetles. Interestingly, many of these monoterpenes mentioned above were found in different amounts in spruce and pine (Fig. 1c); hence, it can be hypothesized that such metabolite differences may pose considerable challenges for ISX to thrive on them, leading to bark beetle gut gene expression differences. We followed up our initial bioassay and metabolomic results at the transcriptomics level using ISX gut tissues to understand the fine-tuning of gene expression after spruce host shift. We also observed some generation effects on ISX gut gene expression after feeding pine on consecutive generations (F_0 and F_1). However, it is worth mentioning here that the F_0 beetles are from the Scots pine logs collected from the forest, which may add additional variablities in ISX gut gene expression. Hence in the current study, we primarily focused on the beetle gut gene expression changes conserved on spruce feeding by mostly looking at those that are common in between F_0Pi vs F_1Sp and F_1Pi vs F_1Sp.

4.1. Transcriptional plasticity during novel host acclimation

Several Coleoptera species show transcriptional plasticity in response to host-specific secondary metabolites (Li et al., 2022a, 2022b; Müller et al., 2017), similar to other species of Cicadidae (Hou and Wei, 2019); Lepidoptera (Noriega et al., 2020); Diptera (De Panis et al., 2022; Ragland et al., 2015). The current study also found several plastic responses at the ISX gut gene expression level after spruce host feeding involving digestion, detoxification, transporter, oxidative stress, and signalling.

4.1.1. Xenobiotic digestive enzyme expression after spruce feeding

The digestive enzymes support the increasing growth and development by digesting the host food materials (Noriega et al., 2020). Digestive enzymes are hydrolases that break down a nutrient compound into small ones by inserting water molecules. The primary digestive enzyme produced by the phytophagous insect midgut tissues mainly consists of amylases, lipases, chitinase, and proteases, which are variable expression levels in the dietary source (Ferreira et al., 1988; Noriega et al., 2020; Sarate et al., 2012; Tamaki et al., 2014). Our results elucidated that several digestive metabolic enzymes were overexpressed after spruce feeding, including amylase, lipases, proteases, and chitinase (Figs. 4a and 5a). Chitinase and chymotrypsin digest host toxin materials, fungal substrates, and other substances (Erthal et al., 2007; Osman et al., 2015). Primary protein digestion in insects relies on trypsin and chymotrypsin (Srinivasan et al., 2006). Expression levels of *H. armigera* trypsin and chymotrypsin increase by ingesting soybean Kunitz trypsin inhibitor as an adaptive mechanism for reducing the deleterious effects of proteinase inhibitors and different diets (Bown et al., 1997; Chougule et al., 2008). Another report demonstrated the trypsin and chymotrypsin expression pattern in *H. armigera* in response to different host plants. It suggests that insects adapt to different hosts with different nutritional qualities by altering the expression of their digestive enzyme arsenal (Chikate et al., 2013; Chougule et al., 2005). A recent report also revealed the differential expression of serine protease and trypsin in *Subpsaltria yangi* during novel host interaction (Hou and Wei, 2019). Differential expressions of lipase- and triacylglycerol-related lipase-related transcripts were also observed during *Subpsaltria yangi* and its host interaction (Hou and Wei, 2019). Aminopeptidases and carboxypeptidases of *H. armigera* exhibited considerable differential expression in response to different host plant proteinase inhibitors (Chougule et al., 2005). Our study found functional genes: beta-galactosidase, triacylglycerol lipases, carboxypeptidase, aminopeptidase, venom acid phosphatase, and myrosinase-1 were highly up-regulated in ISX gut after spruce-feeding. Additionally, lipid metabolisms, carbohydrate metabolisms, amino acid metabolism, and energy metabolism DGEs were up-regulated after spruce feeding, which endorses higher plant tissue digestion and energy supply for ISX and indirectly justifies the observed body weight increase (Fig. 1e).

4.1.2. Xenobiotic detoxifying enzyme expression after spruce feeding

Host plants produce diverse allelochemicals or phytotoxins to defend against phytophagous insect pests (Kong et al., 2019). Insects have to deal with them for successful colonization. Phytophagous insects developed specialized mechanisms to counteract host toxins using detoxifying enzymes like Cytochrome P450 (CYPs), Esterase (ESTs), UDP-glucuronosyltransferase (UGTs), ABC transporters (ABCs), Alcohol dehydrogenase (ADHs), Glutathione transferases (GSTs) (De Panis et al., 2016; Hoang et al., 2015; Hou and Wei, 2019; Roy et al., 2016; Zheng et al., 2022). Cytochrome P450s (P450s) (CYP2, CYP3, CYP4, and mitochondrial) is one of the largest detoxification enzymes in insects

significantly induced by host toxins (Chandra et al., 2016; Dai et al., 2016; Li et al., 2022a, 2022b). CYP3 and CYP4 are mainly involved in the detoxification of xenobiotics, fatty acid metabolism, odorant degradation, Exo-Brevicomin biosynthesis, and inducible moulting hormones (ecdysone) (Davies et al., 2006; Song et al., 2014; Tiwari et al., 2011; Waxman, 1999). Our data indicated that CYP4 and CYP6 (CYP4CV1, CYP6a14, CYP4c3, and CYP6A1) were up-regulated in host shift to spruce (Supple Tables 7 and 8), endorsing their putative role during spruce feeding. Terpenoids as allelochemical inducers of P450s metabolism can be considered a pivotal adaptation to dealing with a different conifer host (Feyereisen, 2012; Müller et al., 2017). In addition, CYP4CV1, CYP4C, CYP6BW5, and CYP9AP1 were also found in sibling species of bark beetles *D. valens, D. rhizophagus*, and *D. armandi* involved in conifer oleoresin detoxification (Dai et al., 2015; Torres--Banda et al., 2022). Recently, camphor tree essential oil and synthetic D-Camphor (also present in spruce) stimulated the expression level of CYP6 and CYP9 in the monophagous weevil pest, *Pagiophloeus tsushimanus* (Li et al., 2022a). Our result CYP4CV1 was highly up-regulated with shifting the spruce host, suggesting cytochrome P450s as a crucial player during alternative host adaptation in bark beetles. However, such claims need further experimental endorsement.

Esterases/carboxylesterase (ESTs) have been major compounds metabolizing various endogenous and exogenous components of insect xenobiotics (Oakeshott et al., 2010; Torres-Banda et al., 2022; Wang et al., 2018; Wei et al., 2020). In our study, ESTs such as esterase-E4/EF4 were the more abundant after spruce feeding (Supple Table 11; Fig. 6k). Esterases are associated with developing cyhalothrin resistance in Bird cherry-oat aphid, *Rhopalosiphum padi* (Wang et al., 2018). The over-expression of carboxylesterase involved in insecticide resistance in various orders of pest insects such as *Bemisia tabaci* (Alon et al., 2008); *Nilaparvata lugens* (Small and Hemingway, 2000); *Helicoverpa armigera* (Wu et al., 2011); *Drosophila melanogaster* (Cui et al., 2015). It is already functionally proven that esterase involvement in malathion detoxification and insecticide resistance in *Liposcelis bostrychophila* (Wei et al., 2020); *Rhopalosiphum padi* (Wang et al., 2018); *Plutella xylostella* (Li et al., 2021; Ran et al., 2022); also oxidative stress resistance (Ma et al., 2018). Hence, higher EST expression and enzymatic activity in ISX gut tissues after spruce feeding may aid spruce allelochemical detoxification.

UDP-glycosyltransferase (UGTs) is a vital conjugation enzyme that catalyzes the transfer of glycosyl residues to lipophilic chemicals and plays a crucial role in detoxifying and eliminating excreting toxic xenobiotics compounds. Earlier studies reported that UGT is involved in insecticide resistance in Coleoptera insect *Leptinotarsa decemlineata* (Kaplanoglu et al., 2017) and other insect species from different orders, such as *Spodoptera* spp. (Hu et al., 2019); *Aphis gossypii* (Pan et al., 2018); *Anopheles sinensis* (Zhou et al., 2019); *Plutella xylostella* (Li et al., 2019); *Chilo suppressalis* (Zhao et al., 2019); *Diaphorina citri* (Tian et al., 2019). Similarly, UGTs have also been reported to detoxify host allelochemicals in *Manduca sexta* (Ahmad and Hopkins, 1993); *Myzus persicae* (Pan et al., 2019); *Heliothis virescens* (Krempl et al., 2016). Our study found that UDP-glucuronosyltransferase 1-8-like (UGT1A8) and UDP-glucuronosyltransferase-like, UDP-glucuronosyltransferase 40AF2, UDP-glucuronosyltransferase 2C1-like (Supple Table 11; Fig. 6h and i) genes were up-regulated during the spruce host shift, indicating their putative contribution to spruce allelochemical detoxification in bark beetles.

In addition, the ATP-binding cassette (ABC) transporters are involved in xenobiotic detoxification to efflux (Martinoia et al., 2002; Sun et al., 2017; Wu et al., 2019). Insecticide resistance mediated by ABC transporters reported in Coleoptera Colorado potato beetle, *Leptinotarsa decemlineata* (Gaddelapati et al., 2018); Lepidoptera, *Pectinophora gossypiella* (Mathew et al., 2018); *Chilo suppressalis* (Meng et al., 2020); and also in Diptera, *Anopheles stephensi* (Epis et al., 2014). Silencing the ABC transporters (ABCH1 and ABCA2) increased susceptibility against cry toxin in *Plutella xylostella* (Guo et al., 2015) and *Helicoverpa zea* (Fabrick et al., 2022). In the present study, ABCB6, ABCC4, ABCA2, ABCC, and ABCC1 were observed to be highly abundant in ISX gut tissue transcriptome after spruce feeding (Supple Table 11; Fig. 6l and n), revealing their putative role in spruce allelochemical excretion. However, such possibilities need to be functionally validated.

4.1.3. Transmembrane transporter activity after spruce feeding

The transmembrane proteins (TPs) or solute carrier (SLC) transporters in the herbivorous gut are associated mainly with the exchange of nutrient uptake, transfer of ions and metabolites (absorption, distribution, metabolism, and elimination of toxins) across biological membranes, and maintaining cellular homeostasis (Keogh et al., 2016; Lin et al., 2015; Qian et al., 2022; Schmidt et al., 2019; Sugahara et al., 2017; Woods et al., 2002). SLC catalyzes the specific exchange between cells and compartments by transporting many molecules, such as amino acids, glucose, ions, fatty acids, and neurotransmitters, using existing electrochemical gradients or facilitated diffusion (Denecke et al., 2020; Qian et al., 2022). In this study, inorganic phosphate co-transporters (SLC34), cationic amino acid transporters (SLC3 and SLC7), anion transporters (SLC26- chloride, bicarbonate, and sulfate), zinc transporter (SLC39), and transmembrane transport of sugars (such as SLC2-trehalose and glucose) (Supple Tables 17 and 18; Fig. 6o–r) were up-regulated during the host shift. The results of Schmidt et al. (2019) in *Chrysomela populi* suggest that the transporter genes are abundant in gut tissues during sequestration/absorption processes, which could explain the higher expression of some transporter genes identified in our data. The essential inorganic phosphate (Pi) crosstalk between intestinal phosphate absorption and the excretion through sodium/phosphate co-transporters are considered to be essential mediators of phosphate homeostasis and immediate response to dietary interventions for cell and biological processes, signal transduction, including skeletal development (Biber et al., 2013; Murer et al., 2001; Wubuli et al., 2019). Also, the phosphate sensing activates a MAPK signalling network (Bergwitz et al., 2012). In our data, inorganic phosphate co-transporters (SLC34) were highly abundant after the spruce host switch and also increased MAPK expression, suggesting that cellular uptake and intracellular transport of phosphate are required for the signalling MAPK for spruce feeding and adaptation. The family SLC7 (cationic amino acid transporters -CATs) is necessary for all living cells and organisms, including insects, to reproduce and supplement nutrients for egg development. An amino acid-rich diet regulates the reproductive potential and population of *Cyrtorhinus lividipennis* by the target rapamycin (TOR) pathway (Zhu et al., 2020). RNAi-mediated knockdown of an amino acid transporter gene in *Nilaparvata lugens* strongly inhibited fecundity and oviposition (Yue et al., 2021). Hence, we can assume that SLC7 family genes might help nutrient transport for the developmental process and improve fecundity after spruce feeding in ISX. It is worth mentioning here that these findings generate testable hypotheses that can be functionally validated in the future.

4.1.4. Energy metabolism and stress response after spruce feeding

Energy metabolism is essential for ensuring all organism's growth and development and environmental survival potential (Bretscher and O'Connor, 2020). Glucose and trehalose sugars act primarily as energy and carbon sources for insect growth, development, and reproduction (Kikawada et al., 2007; Li et al., 2020b). Sugar transporters were significantly expressed when ISX was shifted to spruce logs (Figs. 4c and 5c; Supple Tables 17 and 18). Sugar transporters are involved in blood sugar homeostasis, and trehalose is the primary hemolymph sugar in most insects. The excess glucose from the diet is transferred to the fat body through the glucose transporter (Glut1) and stored as trehalose by TPP (trehalose-6-phosphate phosphatases) (Ge et al., 2015; Price and Gatehouse, 2014; Tang et al., 2017). Stored trehalose is an energy source for flight and development through hydrolysis by trehalase (TREH) in the fat body and released into the hemolymph (Becker et al., 1996; Kanamori et al., 2010). It can directly transform the fat body to the

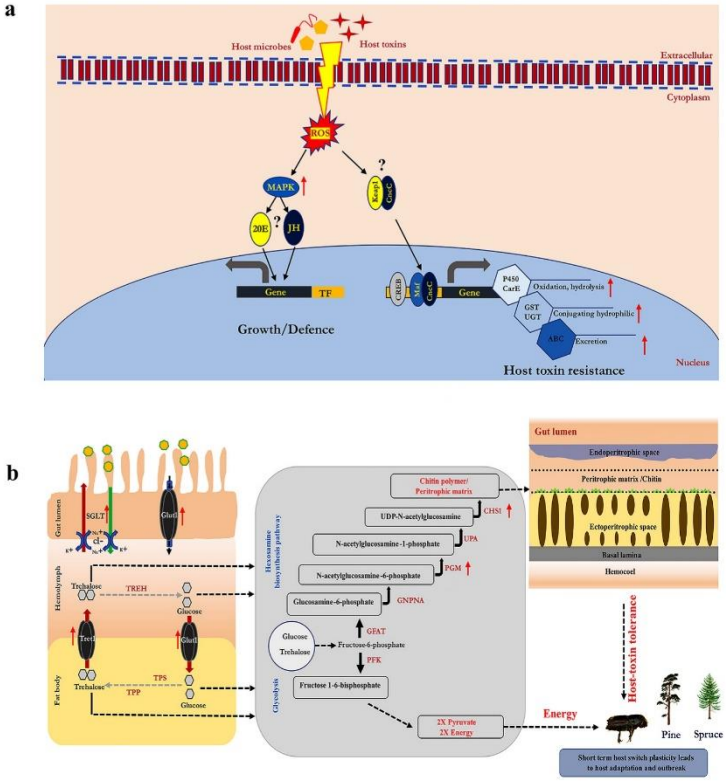

Fig. 8. Putative mechanisms underlying host terpene tolerance and adaptation. (a) MAPKs phosphorylate extracellular signals to major insect hormones 20-hydroxyecdysone (20E) and juvenile hormone (JH) to elicit growth and defence. CncC/Keap1 pathway CncC translocates to the nucleus and initiates transcription of detoxification pathway genes (i.e., Cytp450, EST, GST, UGT, ABC transporters) as indicated by arrows (↑). However, the upstream regulatory aspects related to MAPKs or CncC/Keap1 pathway need to be functionally validated and indicated by the question marks (?). (b) Overexpression of the transporter genes was highlighted in red arrows (↑) during the spruce host switch. TRET1 and GLUT over-expression possibly maintain ISX gut peritrophic membrane integrity, making host monoterpenes impermeable to beetle gut epithelial cells. (Supple figure 8).
TREH: trehalase, TPS: Trehalose-6-phosphate synthase, TPP:trehalose-6-phosphate phosphatases, CHS1: chitin synthase 1, UPA:UDP-N-acetylglucosamine pyrophosphorylase, PGM: phosphoacetylglucosamine mutase, GNPNA: glucosamine-phosphate N-acetyltransferase, GFAT:fructose-6-phosphate transaminase and PFK: phosphofructokinase.

hemolymph and back to the fat body by reabsorption in the cell membrane through a trehalose transporter (Tert1) (Kanamori et al., 2010; Kikawada et al., 2007). Knockout Tret1a and Tret1b lead to long-term diapause in *Colaphellus bowringi* (Li et al., 2020a). Trehalose was reportedly involved in environmental stress and insecticide/xenobiotic protection through chitin metabolisms (Elbein et al., 2003; Tang et al., 2017). Knockout of Tret1 reduced the adaptability to extreme temperatures (Zhou et al., 2022) and sensitivity to stress (Liu et al., 2013). In our data, Tert1 and Glut1 were highly abundant in ISX gut tissues after spruce feeding, which may reflect the extra initiative of enhancing energy storage for preparing the flight to search for a suitable host and reduce sensitivity to the imposed stress by alternative host (spruce) feeding. Furthermore, glucose and trehalose addition in an artificial diet impacts insect growth, development, and fecundity. Increased reproduction of *Harmonia axyridis* with sugar consumption and accumulation in the early stages of development (Li et al., 2020b). It supports our observation of the increase in ISX fecundity after spruce feeding.

During chitin synthesis of insects, trehalose is considered a major substrate for the exoskeleton for proper growth and development (Merzendorfer and Zimoch, 2003) of the peritrophic matrix that (PM) actively protects the gut from pathogens and toxins (Campbell et al., 2008). During chitin biosynthesis, the primary precursor trehalose is converted to glucose in the hexosamine pathway (Doucet and Retnakaran, 2012; Wang et al., 2021). In the present study, we identified chitin biosynthesis pathway genes were up-regulated, particularly chitin synthase 2 (CHS2) and GFAT (Glutamine fructose-6-phosphate amidotransferase) (Supple Fig. 8) after spruce host shift, which might protect ISX gut from spruce associated pathogens. Earlier studies reported that CHS2 is expressed in late pupal and adult stages and primarily utilized to synthesise peritrophic membrane-associated chitin in the midgut (Arakane et al., 2004, 2005). The gut-specific CHS2 expression protects from mechanical damage and neutralization of ingested toxins by peritrophic matrix proteins mediate matrix barrier (Agrawal et al., 2014; Hegedus et al., 2009; Kelkenberg et al., 2015). Similarly, in stored grain pest and model beetle, *Tribolium castaneum* proved that CSH2-specific knockdown reduced the chitin content required to maintain the midgut PM essential for adult feeding and survival (Arakane et al., 2005). Trehalose-6-phosphate synthase (TPS) and Glutamine: fructose-6-phosphate aminotransferase (GFAT) knockdown increased mortality rates with misregulation of chitin metabolism in the rice

brown planthopper, *Nilaparvata lugens* (Xu et al., 2021; Yang et al., 2017); *Leptinotarsa decemlineata* (Shi et al., 2016); *Acyrthosiphon pisum* (Wang et al., 2021). Trehalase inactivation disrupts the trehalose and chitin biosynthesis pathways in *N. lugens* (Tang et al., 2017). Hence, the upregulation of chitin synthase 2 (CHS2) during spruce feeding in ISX may play multiple crucial physiological roles, which are worth following up on for a better understanding of spruce adaptation by pine-feeding beetles.

5. Conclusion

The current study documented fecundity and transcriptional changes in response to a short-term host switch on spruce in pine beetle *I.sexdentatus* (ISX). Host metabolomic analysis showed considerable differences in the abundance of the biologically relevant terpenoids (i.e., β-Pinene, β-Phellandrene, β-Myrcene, limonene, δ-carene, Camphor, Terpinolene and α-Pinene) between spruce and pine wood. Transcriptomics study discovered changes in gene expression known to be involved in the detoxification process of terpenes (i.e., GST, ESTs, CYP4, CYP9, CYP6a, UGT1, ABC transporters) in ISX after host switch. Higher detoxification enzymatic activities (i.e., GST, ESTs) were also documented in beetles after spruce feeding, suggesting ISX gut tissue cells enhance the key enzyme activities to deal with altered amounts of host metabolites in spruce (Fig. 8a). Additionally, the overexpression of trehalose transporter genes and other transporters (i.e., Glut1 transporters) probably holds ISX gut peritrophic membrane integrity, making host monoterpenes impermeable (Fig. 8b). Such adaptive mechanisms under the influence of varied levels of terpenes can be functionally validated in future bark beetle research endeavours and aid in bark beetle management (Joga et al., 2021). Understanding how these beetles perceive the chemical cues from pine and spruce will also be exciting, which may open new research avenues and management strategies.

CRediT authorship contribution statement

Gothandapani Sellamuthu: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Aisha Naseer:** Data curation, Methodology. **Jaromír Hradecký:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Amrita Chakraborty:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Jirí Synek:** Data curation, Formal analysis, Investigation, Methodology. **Roman Modlinger:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Amit Roy:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

We have submitted the data to NCBI under Bioproject. The data will be released to after acceptance of the manuscript. the data availability statement added in the manuscript.

Acknowledgements

The "EXTEMIT-K" and Internal Grant Agency (IGA) from the Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, funded the project. Infrastructural support was obtained from the grant "EXTEMIT-K," No. CZ.02.1.01/0.0/0.0/15_003/0000433 and "EVA 4.0", No. CZ.02.1.01/0.0/0.0/16_019/0000803 financed by OP RDE. Additional financial support was obtained from the "Excellent Team Grants" to AR, AC, and GS by FLD, CZU. We also acknowledge Prof. Ladislav Kokoška, FTZ, CZU, for providing the instrument facility for enzymatic studies.The authors would also acknowledge the anonymous peer reviewers who gave valuable comments on the earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.104061.

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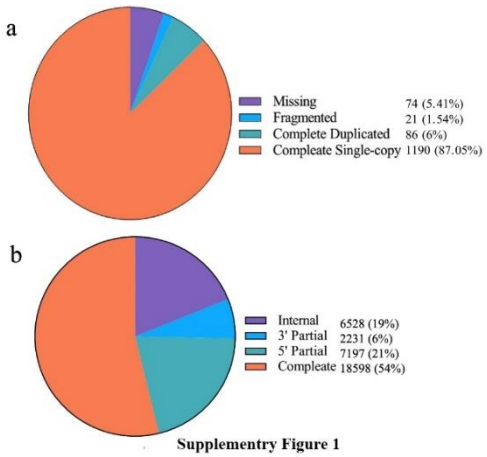
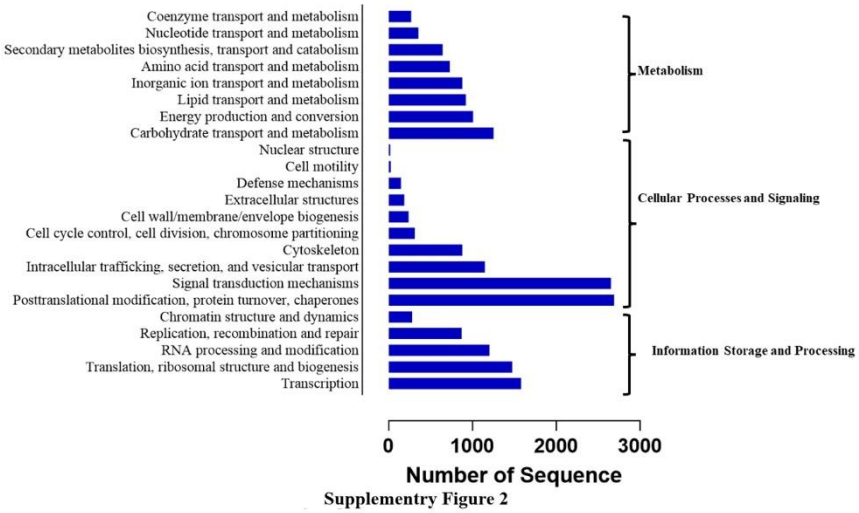
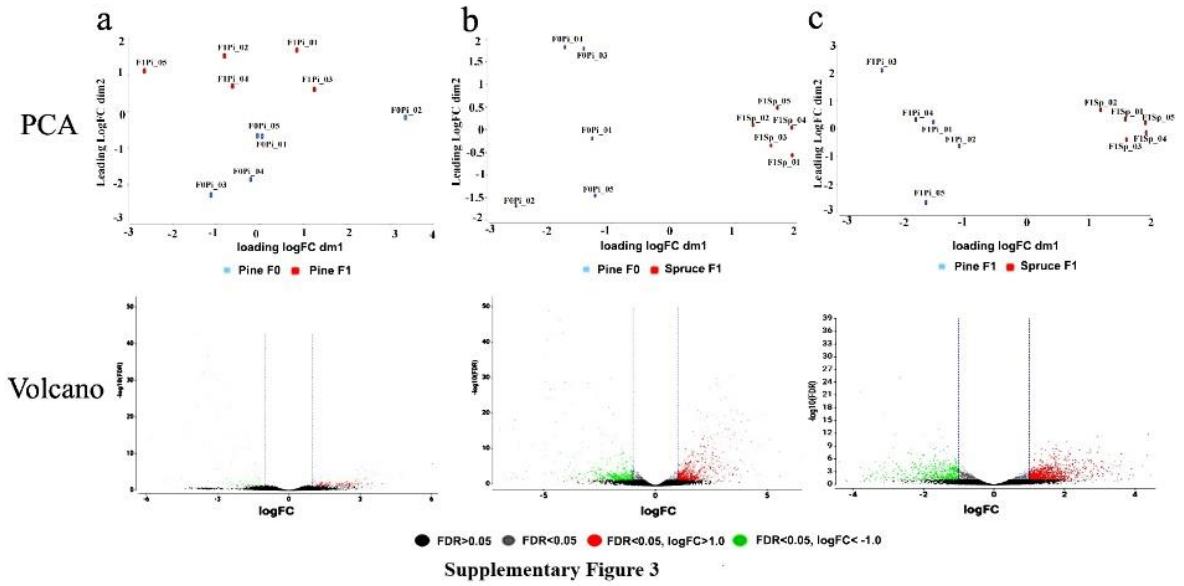


Supplementary Figure 1: Transcriptome evaluation. a) BUSCO analysis; b) The coding region prediction analysis of the total transcripts using Transdecoder.

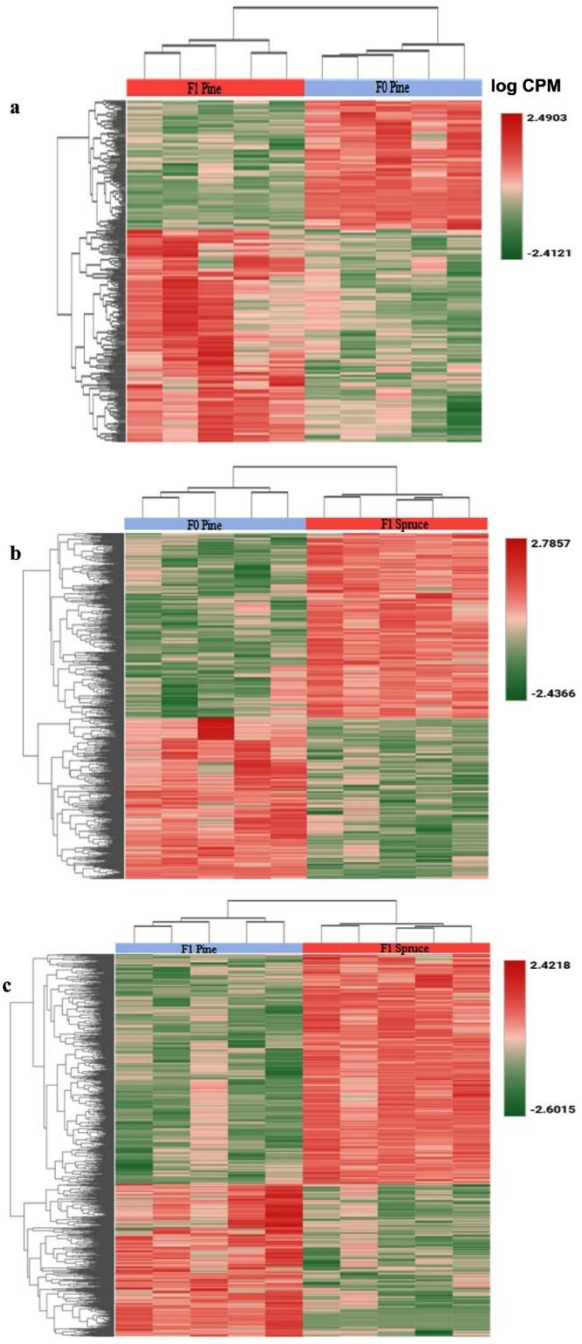


Supplementary Figure 2: COG and EggNOG analysis. The annotation of the transcriptome was classified into three major groups: information storage and processing, cellular processes and signalling, and metabolism.



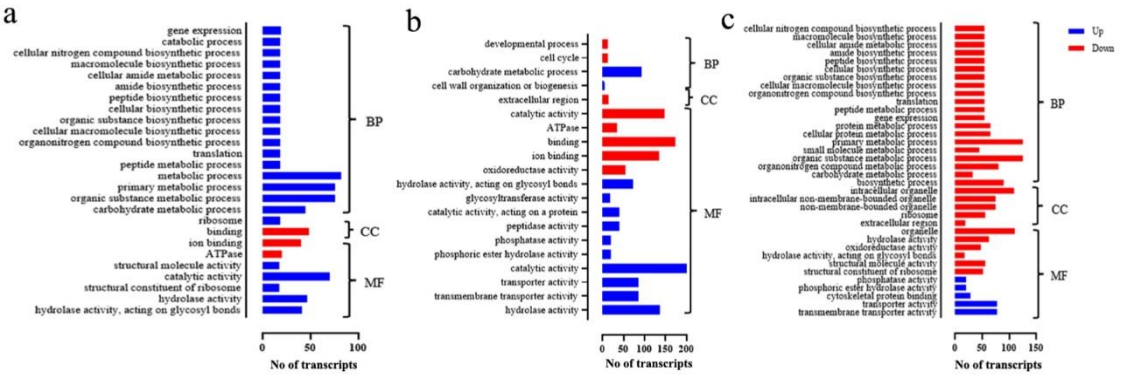
Supplementary Figure 3

Supplementary Figure 3: Evaluation of different treatment data sets. PCA and the volcano plots show overall gene expression patterns in different sample groups, such as a). F0 Pine vs F1 Pine (F0Pi vs F1Pi); b). F0 Pine vs F1 Spruce (F0Pi vs F1Sp); c). F1 Pine vs F1 Spruce (F1Pi vs F1Sp).



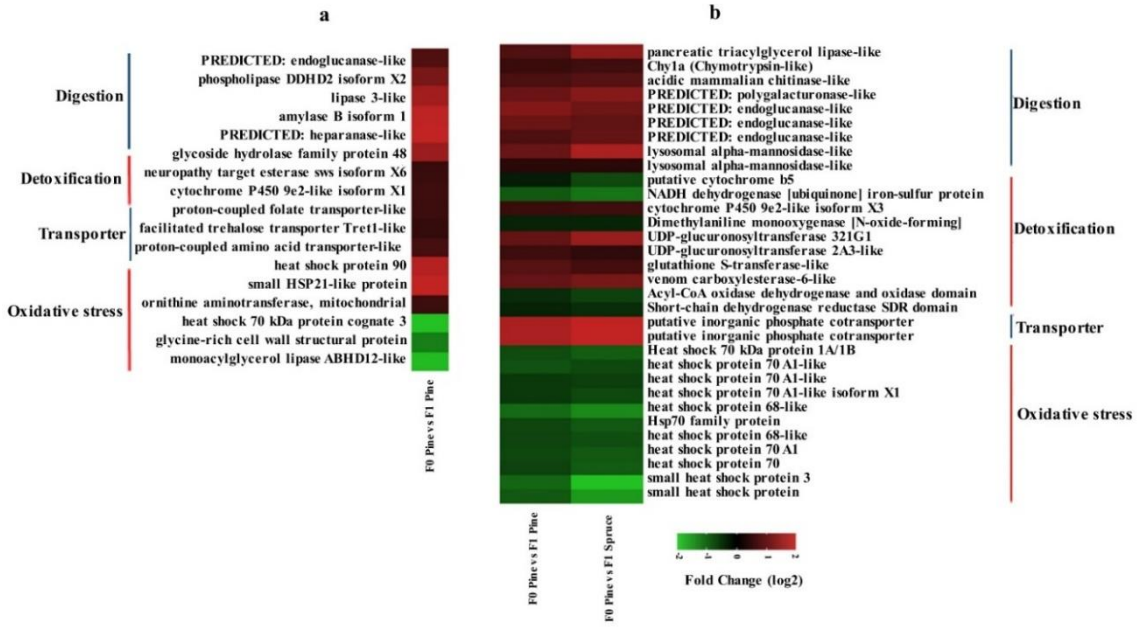
Supplementary Figure 4

Supplementary Figure 4: Hierarchical clustering of the differentially expressed genes with all biological replicates. a). F0 Pine vs F1 Pine (F0Pi vs F1Pi); b). F0 Pine vs F1 Spruce (F0Pi vs F1Sp); c). F1 Pine vs F1 Spruce (F1Pi vs F1Sp).



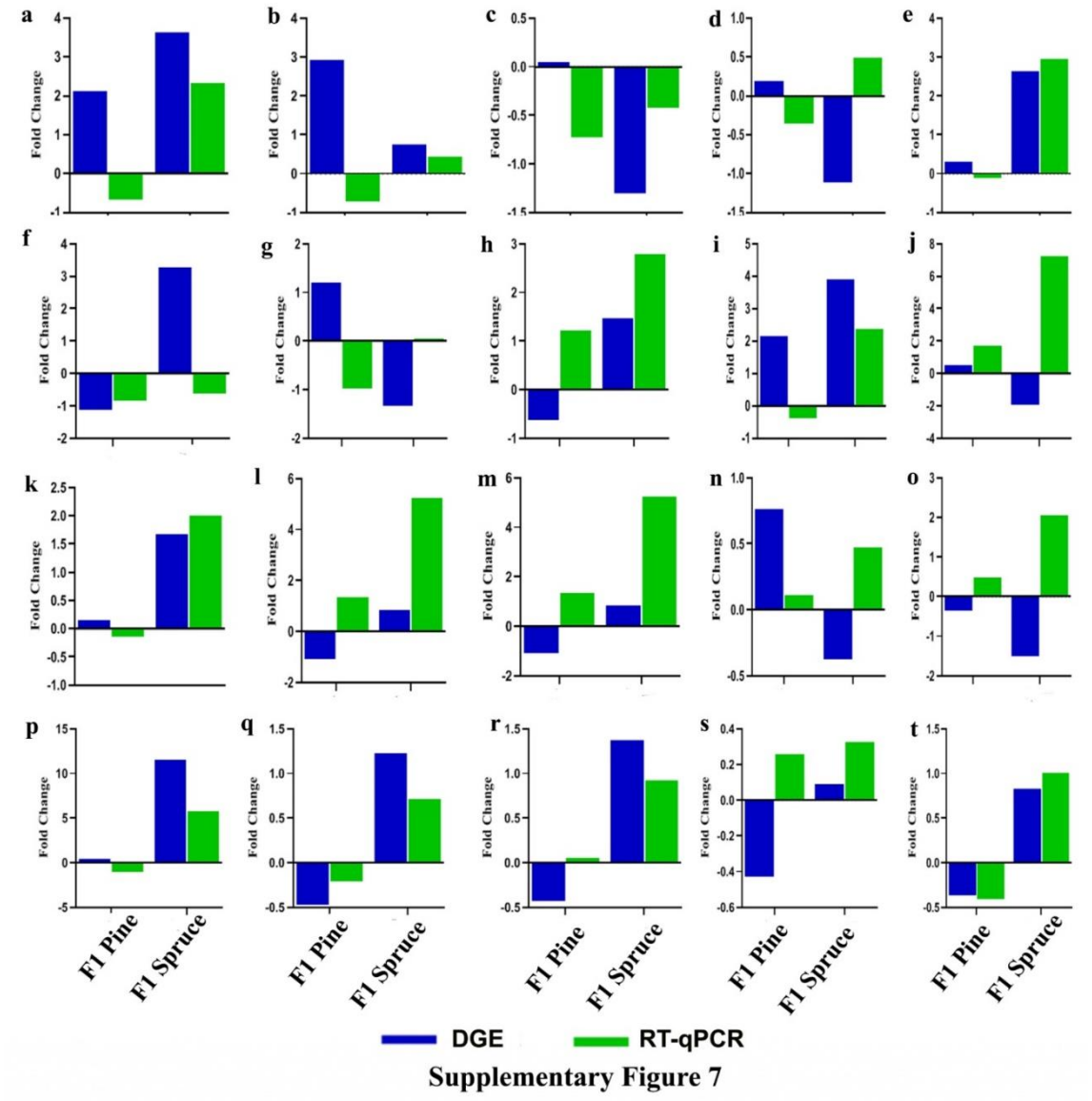
Supplementary Figure 5

Supplementary Figure 5: Gene Ontology (GO) analysis. Differentially expressed transcripts were annotated and clustered into cellular components and biological process molecular function categories from the following comparisons: a). F0 Pine vs F1 Pine (F0Pi vs F1Pi); b). F0 Pine vs F1 Spruce (F0Pi vs F1Sp); c). F1 Pine vs F1 Spruce (F1Pi vs F1Sp).

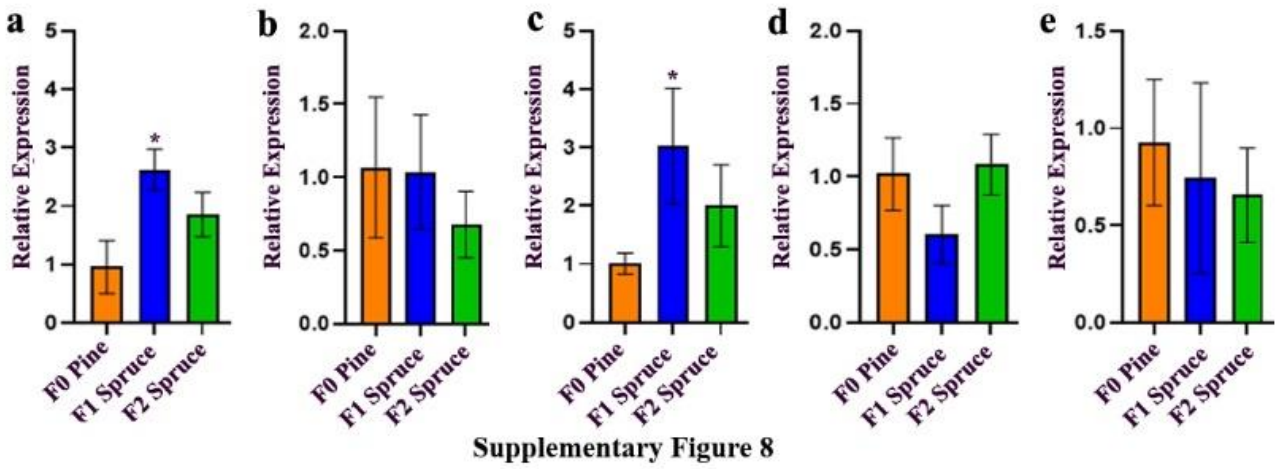


Supplementary Figure 6

Supplementary Figure 6: Influence of generation on beetle gut gene expression. a) host-specific (Pine), and b) generation effect (conserved). Differentially expressed genes are related to digestion, detoxification, transporter, oxidative stress, and signalling. Statistical significance of differentially expressed genes represented in the heatmap. The red colour indicates upregulated, whereas the green colour indicates downregulated expression. DGEs are determined by an FDR corrected p-value < 0.05 (more details in Supple Tables 19 and 20).



Supplementary Figure 7: Correlation of expression patterns between RNA-seq and RT-qPCR.



Supplementary Figure 8: Expression patterns of hexaomine pathway genes in different generations of spruce feeding. A). glutamine-fructose-6-phosphate aminotransferase (GFAT); B). glucosamine-6-phosphate N-acetyltransferase (GNPNA); C). phosphoacetylglucosamine mutase (PGM); D). UDP-N-acetylglucosamine pyrophosphorylase (UPA) and E). Chitin synthase 2 (CSH2).

Gene expression plasticity facilitates different host feeding in *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae)

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Supplementary Protocol File 1

1. Enzyme activity

1.1 Esterase activity:

For EST activity measurement, the reaction contained 20 µl of enzymatic source along with 160µl of 50mM of sodium phosphate buffer and 20µl of 1mM substrate solution (in distilled water with 1% acetone) in a 96-well transparent microplate. The sample mixture was incubated at 30°C for 60 minutes. The enzymatic reaction was terminated by adding 20µl of a solution comprising SDS (35mg/ml) and Fast Garnet (10 mM) in distilled water. The optical density was estimated at 550nm in a microplate reader. The esterase activity was calculated as a nmol hydrolyzed substrate per minute per mg of protein (Bosch-Serra et al., 2021).

1.2 Glutathione S-transferase (GST) activity:

For the measurement of GST activity, the reaction contained 20 µl of enzymatic source along with 170µl of GSH in a 50mM sodium phosphate buffer solution as a substrate conjugation cofactor. To initiate the reaction, 10µl of CDNB was added. Immediately, the GST activity was measured at 340nm intervals of 1 to 10 min at 30°C in a microplate reader. Enzyme activity was expressed as a nmol substrate conjugated per min per mg protein using CDNB molar extinction coefficient of 9.6mM-dm-1 (Bosch-Serra et al., 2021; Habig et al., 1974).

1.3 NADPH–Cytochrome P450 Reductase:

For measurement of CPR activity, the reaction comprised 20 µl of enzymatic source along with 160 µl of 50Mm sodium phosphate buffer solution consisting of an NADPH generating system as a cofactor necessary to the conjugate substrate. The mixture was incubated for 10 minutes at 30°C and Cytochrome P450 activity by adding 20 µl of cytochrome C solution in sodium phosphate buffer (50 mM, pH 7). Immediately, the CRP activity was measured at 550nm intervals of 1 to 10 min at 30°C in a microplate reader. Cytochrome P450 a ctivity was expressed as nmol substrate reduced per min per mg of protein, using the molar extinction coefficient for the reduced form of cytochrome C of 27.6 $mM^{-1} \cdot cm^{-1}$ (Bosch-Serra et al., 2021; Margoliash and Frohwirt, 1959).

References:

Bosch-Serra, D., Rodríguez, M.A., Avilla, J., Sarasúa, M.J., Miarnau, X., 2021. Esterase, Glutathione S-Transferase and NADPH-Cytochrome P450 Reductase Activity Evaluation in Cacopsylla pyri L.(Hemiptera: Psyllidae) Individual Adults. Insects 12, 329.

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6. Discussion

The beetles have existed for ages in the boreal forests; however, the last few decades have seen a massive shift in the host-pest interaction and ecology owing to climate change and human interventions in the ecosystem. Human involvement, such as promoting monoculture forests and excessive timber harvesting, have disrupted the ecological balance. This has not only altered the carbon cycle but also triggered severe outbreaks in the central Eurasian boreal forests. This thesis, comprising four research articles, explores *I. typographus* ecology and its interaction with its host, *P. abies,* Norway spruce. The chapters analyse how various abiotic and biotic factors contribute to bark beetle outbreaks and highlight novel emerging methods to mitigate these impacts on forest ecosystems (Chapter 5.1), followed by the molecular mechanisms the beetles use to survive the host defences in different stages and tissue-specific way (Objective I, Chapter 5.2). The beetle's life stages that develop and emerge from the bark of the spruce face a blend of allelochemicals that elicit a conserved detoxification response. Such a response may differ when the insects are exposed to individual monoterpenes (Objective II) , as discussed in Chapter 5.3. This study extends our understanding of the conserved detoxification gene expression mechanism of ESBB in response to selected monoterpene and the disruption of development mechanisms. Such mechanisms result from years of co-evolution that enable ESBB to survive and coordinate mass attacks. Although such co-evolution helps overcome a regular host, in times of unavailability of the potential regular host or if the primary host is already occupied by the existing kins (higher competition), an alternate host may be attacked. The new alternate host provides less competition and poses higher stress to adapt and overcome new challenges of host defences. In Chapter 5.4, one such host-shift was studied using eight-tooth bark beetle *Ips sexdentatus* (ISx) to understand the underlying molecular mechanism for such new and rapid adaptation. The key findings of each study are discussed further in this chapter.

Chapter 5.1 examines the significant impacts of climate change on *Ips typographus* outbreaks, focusing on the interplay between environmental factors such as temperature, drought, and forest structure while also exploring novel pest management strategies. Over the recent decades, rising global temperatures have drastically altered forest ecosystems, creating favourable conditions for bark beetle outbreaks. Increased temperatures have resulted in more frequent and intense droughts, which weaken tree defences, making them more susceptible to beetle infestations. While many studies have previously reviewed various aspects of host pest interaction between Norway spruce and ESBB —such as host defence mechanisms (Krokene, 2015; Huang et al., 2020), ecological dynamics (Biedermann et al., 2019; Hlásny et al., 2021), drought and fungal symbiosis (Netherer et al., 2021), and early detection (Marvasti-Zadeh et al., 2023; Trubin et al., 2023; Kautz et al., 2024). This study uniquely brings these elements together in a comprehensive framework. It elaborates current understanding of bark beetle outbreak dynamics at stand and landscape levels while explaining primary, secondary, and mass attack mechanisms. It further emphasizes the role of major abiotic drivers and how they compromise a tree's ability to defend itself and facilitate the success of bark beetle attacks.

The study further explains how drought stress compromises tree defence, particularly through reduced resin production, which is crucial for repelling beetle attacks. Prolonged droughts deplete non-structural carbohydrates (NSCs), further weakening trees and making them more vulnerable to beetle colonisation. Warmer temperatures exacerbate the problem by enabling faster beetle dispersal, range expansion, and shorter development cycles, leading to multiple generations (polyvoltine life cycles) in a single year. This rapid population growth creates cascading effects, causing widespread tree mortality and significant disruption to forest ecosystems.

In addition to climate factors, the study examines how forest structure and elevation gradient play a significant role in bark beetle outbreaks. Dense monoculture plantations of Norway spruce are particularly vulnerable to beetle attacks, likely due to competition effects. These monoculture plantations lack diversity in age class and species richness, making them susceptible

to biotic and abiotic disturbances, thus limiting the trees' collective resilience to stressors like drought, windthrow, and insect infestations. High-density stands experience increased competition for resources, leading to weakened trees that are more susceptible to beetle infestation, providing ideal conditions for beetle population build-up. The chapter further explores the efficiency of current and future novel management strategies for containment and identification of infestation. It highlights recent advancements in remote sensing and canine detection for early identification of infested trees. It synthesizes the state of knowledge and potential of cutting-edge molecular approaches, including RNAi, sterile insect techniques, and CRISPR/Cas9 in managing bark beetle outbreaks.

Chapter 5.2 explored into the intricate gene expression dynamics of the Eurasian spruce bark beetle (ESBB), *Ips typographus*, and highlights the beetle's multi-phase detoxification system, which includes three critical stages of enzymatic breakdown and excretion. Phase I involves lipophilic attacking enzymes, such as CYPs, esterases, hydrolases, and dehydrogenases, which initiate the breakdown of allelochemicals. Phase II includes conjugation reactions, where enzymes such as GSTs and UGTs convert the intermediate products into more water-soluble forms. Lastly, Phase III involves the transport of these less harmful substances out of the cells via ABC transporters, and finally out of the beetle's body through body fluids. The study primarily focuses on the gene expression patterns in different life stages and tissues of ESBB, exploring how these patterns are linked to detoxification, digestion, and transport processes essential for the beetle's survival and adaptation to its host environment. Through this study, we reported that various gene families associated with these critical processes were differentially expressed across the beetle's life stages—larvae, pupae, callow adults, and sclerotised adults—as well as between male and female tissues, including the gut, and fat body. For instance, the expression of CYPs of CYP4, CYP6, and CYP9 family, GST1, and UGT2B family genes fluctuated depending on the beetle's developmental stage. Host allelochemicals has been reported to elicit several P450s (family 4, 6, and 9), UGTs,

in other insects. For instance, GST23, CYP6AE14, CYP6B8, CYP6B6, CYP4L11, CYP6AB9, CYP9A12, CYP9A14, and CYP9A17 in *Helicoverpa armigera* (Celorio-Mancera et al., 2011; Celorio-Mancera et al., 2012; Jin et al., 2019; Tian et al., 2019). On gossypol feeding, UGT41B3 and UGT40D1 are expressed leading to gossypol metabolism via glycosylation (Krempl et al., 2016). UGTs are reported to be involved also insecticide resistance, for example, UGT2B17 in diamond moth (Li et al., 2017), and UGT2B20 malondialdehyde resistance in Asiatic honeybee (Cui et al., 2020). In *Spodoptera exigua,* CYP6AB14, CYP9A10 and CYP9A98 provides insecticidal resistance towards deltamethrin (Hafeez et al., 2019; Hafeez et al., 2020), and in *S. frugiperda,* CYP6B39 is overexpressed after xanthotoxin feeding (Giraudo et al., 2015), and UGT33F28 is utilised to metabolise maize defence compounds (Israni et al., 2020). In agricultural pests, GSTs are mainly reported for their insecticidal resistance, like GST1-1, GSTd2, and GSTE1 in brown plant hopper (Yang et al., 2021), GSTS6 in red flour beetle (Song et al., 2022), and GSTD9 in oriental fruit fly (Meng et al., 2020). Although there are fewer reports on bark beetle's detoxification gene characterisation, Gao et al. (2020) identified sixteen full-length GSTs in Chinese white pine beetles. Similar to our study, whole genome transcript analysis in *Dendroctonus ponderosae* revealed several P450s, GSTs, and esterases putatively involved in the detoxification present in the midgut and fat body (Robert et al., 2013). Among these, CYP6DJ1, CYP6BW1, and CYP6BW3 were characterised for pine monoterpene and diterpene detoxification (Chiu et al., 2019). In *D. armandi,* CYP6CR2 and CYP6DE5 were characterised to metabolise host phloem tissue and important pine monoterpenes like α -pinene, β-pinene, (+)-3-carene, (±)-limonene and turpentine (Liu et al., 2022). In our study, we reported high expression of CYP6CR2 in adult beetle's midgut denoting their involvement in detoxification. This differential gene expression is particularly pronounced during the feeding stages, where larvae and callow adults show heightened expression of these detoxification genes, while pupae, being a non-feeding stage, show a marked downregulation. Similar gene expression due to feeding in the early stages of development was also reported in *D. rhizophagus* (Sarabia et al., 2019). This suggests that detoxification is energetically costly and is tightly regulated to occur primarily during stages when the beetle is exposed to the host's chemical defences. Our results are in accordance with previously reported

life-stage dependent gene expression of detoxification genes in *Dendroctonus* species, a close relative of ESBB (Dai et al., 2015; Gao et al., 2020).

Additionally, the study also highlights sex-specific differences in gene expression, particularly in the context of pheromone biosynthesis, which is crucial for attracting conspecifics and coordinating mass attacks on new host trees. We found that certain genes involved in pheromone production, such as those encoding juvenile hormone esterases and epoxide hydrolases, were differentially expressed in the fat bodies of male and female beetles, with males generally showing higher expression levels. This supports the idea that males, as the pioneering sex, are more actively involved in initiating and coordinating mass attacks through pheromone signalling, as previously reported in *I. typographus* by Ramakrishnan et al. (2022a, 2024), and in *I. paraconfusus* by Huber (2007).

In summary, this study showed that the variation in gene expression suggests that the beetle's detoxification system is highly dynamic, adjusting to the varying levels of toxic exposure that the beetle encounters throughout its life cycle and offers valuable insights into the molecular underpinnings of ESBB's adaptation to its environment, emphasizing the importance of life stage, tissue type, and sex in shaping the gene expression landscape of this destructive forest pest.

Although the bark beetles feed on the host tissues and undergo varying degrees of gene expression of their detoxification machinery, the effect of individual monoterpene, which constitutes the major proportion of spruce allelochemicals, may vary. Chapter 5.3 was aimed to understand the effect of individual host monoterpene on ESBB and unveil the genes involved in their individual detoxification pathways. This study utilises fumigation bioassay, a well-studied and proven method to study host-pest adaptation in-vitro (Fernanda López et al., 2011; Huang et al., 2018; Huang et al., 2019). Previous studies have shown that the monoterpenes may vary in concentration in the host and their toxicity toward the bark beetle (Everaerts et al., 1988; Chiu et al., 2019), which may render differential levels of resistance to the beetles. Chapter 5.3 discusses

the ability of ESBB to detoxify important monoterpene, and its metabolic adaptations to survive and thrive despite the host tree's chemical defences. Fumigation assays demonstrated that the tested monoterpenes cause significant toxicity, with sabiene emerging as the most toxic followed by *α*-pinene, *R*-limonene, and myrcene. Our results go in accordance with a similar study by Everaerts et al. (1998) indicating the quantitative and qualitative importance of the individual monoterpenes in terms of their toxicity to fend-off the pest. These results indicate that, at higher concentrations, sabiene and R-limonene could serve as effective natural deterrents to beetle infestation, disrupting the beetles' ability to establish colonies on spruce trees. In this study, *α*-pinene was more toxic to ESBB than *R*-limonene, suggesting even though *α*-pinene and myrcene are kairomones and serve as attractants, they possess inhibitory effect at higher concentration (Erbilgin et al., 2003; Sandstrom et al., 2006; Blomquist et al., 2010; Song et al., 2013). *R*- limonene had been previously reported to be more toxic than *α*-pinene in Chinese white pine beetle (Dai et al., 2015), however, the low LC_{70} of sabiene, which was not evaluated before, suggests its effectiveness as a fumigant.

The RNA-seq study followed with sabiene and R-limonene fumigation further revealed many essential previously reported detoxification genes indicating a dynamic and inducible defence mechanism that allows the beetle to rapidly adapt to varying levels of host allelochemicals depending on the host physiology (Amezian et al., 2021; Dai et al., 2021). For instance, cytochrome P450 family members CYP4C1 and CYP6A2 were the most upregulated in both the treatments. The former has been reported to breakdown synthetic insecticides and provide cold tolerance and heat resistance in *Bemisia tabaci* (Shen et al., 2021), and the latter has been reported to be associated with insecticide metabolism, mainly DDT in *Drosophila* and imidacloprid in *Aphidius gifuensis* (Wan et al., 2014; Kang et al., 2018; Lien et al., 2019). Xenobiotic associated gene ST1B1 (Chen et al., 2015), and UGT2B17, associated with Chlorantraniliprole resistance (Li et al., 2017) in particular, were identified as a key gene as upregulated in the detoxification of monoterpenes with RNA-seq data and all the chemical treatment in RT-qPCR in the detoxification of monoterpenes. GSTT1, which is associated peroxidase activity and is involved

with protein binding for organophosphates (Wongtrakul et al., 2014), which was not highly expressed in study I (Chapter 5.2) but consistently high across different treatments used in study II and III (Chapter 5.3 and 5.4), suggesting these enzymes are part of *Ips* beetle's induced defence against the excessive host toxin and are used under higher oxidative stresses. These genes can be impressive potential targets for functional studies in ESBB detoxification pathways in the future. Furthermore, the increased enzymatic activity of EST, GST, and CPR, aligns with the observed gene expression changes, confirming that *I. typographus* enhances its detoxification machinery in response to these allelochemicals. These findings suggest that ESBB can survive exposure to toxic monoterpenes and thrive in environments where these compounds are prevalent, contributing to its success as a notorious forest pest.

Interestingly, the genes related to development and pupation, eggshell formation, and diapause initiation were consistently downregulated in RNA-seq data. For instance, CYP306A1, CYP3015A1 which are associated with moulting and ecdysone biosynthesis (Niwa et al., 2004; Zhang et al., 2022), peroxiredoxin-6 associated with diapause induction, and chorion peroxidase associated with eggshell formation (Li et al., 2004). This suppression of developmental genes and the upregulation of detoxification genes indicates a trade-off that *I. typographus* employs during high toxin exposure: prioritizing survival over development. This dynamic allows the beetles to respond rapidly to the chemical defences of their host trees.

The RT-qPCR with four chemicals on wild beetles (F0) and laboratory-reared beetles (F1) ESBB revealed that the tested detoxification genes were highly expressed in wild ESBB than the F1. Such higher tolerance to monoterpenes likely occurred due to their pre-exposure to these compounds in natural environments, and a genetic memory or readiness which may have induced the rapid expression of detoxification genes. The F0 beetles demonstrated higher enzyme activity and lower mortality rates compared to the F1 beetles, emphasising the role of environmental exposure in enhancing detoxification capabilities.

The results of Chapters 5.2 and 5.3 reveal that the detoxification mechanism is cost-efficient, and the expression of detoxification genes is elicited upon exposure to the natural defence of the host during infesting a host tree or by exogenous exposure of the host allelochemicals. Such complex enzyme systems result from co-evolution between a host and its specialist pest. However, in the absence of a suitable host, host-switch may be required to survive. The new host may offer lesser competition from the same species; however, it poses a strong selection pressure to adapt quickly to the new host defence systems. Chapter 5.4 was dedicated 'to understand the quantitative and qualitative effect of host and host chemical defences in *Ips* beetles and understand host shift.' *I. sexdentatus* (ISx) is a major bark beetle in Eurasia, that attacks Scots pine (*Pinus sylvestris*) which is weakened by climatic or anthropogenic stressors like drought, windstorms, wildfires, and mass felling (Fernández, 2006; Pineau et al., 2017; Hlávková and Doležal, 2022; Knížek et al., 2022). The study emphasizes the physiological and molecular mechanisms that facilitate host adaptation in ISx upon a host shift from Scots pine to Norway spruce. The study's findings highlight that host shifts, which are common in herbivorous insects, necessitate significant adjustments in gene expression and physiological processes. This adaptation is crucial for the beetles to thrive in a chemically different environment, driven by the defensive compounds of the new host. ISx feeds on a wide variety of pine hosts; however, it is predominantly associated with Scots pine in Europe, although it has been occasionally found also on spruce logs in Czechia (Pfeffer, 1995; Health et al., 2017; Knížek et al., 2022). This geographic variation in host preference could be related to the competitive landscape in different environments, where the prevalence of other bark beetles, such as *Ips typographus* and *Ips duplicatus*, on spruce may drive ISx to specialise in pine in certain regions. When reared on the alternate host, in a non-choice experiment, ISx exhibited a notable increase in growth rate and fecundity, suggesting that the host shift positively affected reproductive success. The monoterpene profiles differ across the tree species and during the constitutive and induced defence state (Keeling and Bohlmann, 2006; Clark et al., 2014), which might be the reason for the host specificity of these beetles as depicted differences in the untargeted metabolomics of the spruce and pine wood. Comparative transcriptomic analyses between the F0-pine ISx, F1-pine ISx,

and F1-spruce ISx revealed less generational difference in gene expression in F0 vs F1 beetles reared on pine, instead, a higher effect of spruce feeding on the genes related to digestion, detoxification, and transporter activities. The higher expression of digestive metabolic enzymes like amylase, trypsin, chymotrypsin, serine protease, lipase, and peptidases when shifting to spruce host show an adaptive mechanism to reduce the deleterious effects of host diet by altering their expression. Such adaptations are also reported in other insects such as *H. armigera*, *Mamestra brassicae*, and *Subpsaltria yangi* (Bown et al., 1997; Chougule et al., 2005; Chougule et al., 2008; Chikate et al., 2013; Hou and Wei, 2019). Important detoxification genes, like CYP4 and CYP6 (CYP4CV1, CYP6A14, CYP4C3, and CYP6A1), which were up regulated during fumigation (study II), were key overexpressed CYPs during host shift to spruce showing their conserved behaviour against spruce allelochemicals. These genes are previously reported in *D. valens*, *D. rhizophagus*, and *D. armandi*, which are involved in conifer oleoresin detoxification (Dai et al., 2015; Torres-Banda et al., 2022). In this study, ESTs such as esterase-E4/EF4 and carboxylesterases were the more abundant after spruce feeding which was also reported in various other species for insecticide resistance like *Bemisia tabaci* (Alon et al., 2008); *Nilaparvata lugens* (Small and Hemingway, 2000); *Helicoverpa armigera* (Wu et al., 2011); *Drosophila melanogaster* (Cui et al., 2015). Some UGT enzyme (like, UDP-glucuronosyltransferase 1-8-like (UGT1A8), UDP-glucuronosyltransferase-like, and UDP-glucuronosyltransferase 2C1-like), and members of ABC sub-family B members and multidrug resistance-associated proteins (MDPs), were reported in our study I (Chapter 5.2) to be highly abundant during the feeding stages of ESBB (Naseer et al., 2023). These results suggest that the beetles activate a suite of molecular mechanisms associated with digestion, detoxification, oxidative stress & signalling and cellular transport to overcome the chemical defences of spruce. The assessment of generation on gene reveals the plasticity of ISx and the intense survival pressure on arriving on a new host.

7. Conclusion and Recommendation

7.1. Conclusion

The presented studies collectively provide a deeper understanding of bark beetle ecology, adaptive mechanisms, and pest management challenges in the face of climate change. The findings from Chapters 5.2 and 5.3 demonstrate that the ESBB, *I. typographus,* employs efficient and inducible detoxification mechanisms revealing differential expression of key detoxification genes, including cytochrome P450s (CYPs), glutathione S-transferases (GSTs), and UDP-glucuronosyltransferases (UGTs) in response to host tree defences and allelochemicals. Similar upregulation of detoxification genes has been observed in other species like *Dendroctonus* and *Helicoverpa*. This study provides a comprehensive catalogue of differentially expressed genes in ESBB, which could serve as potential targets for RNAi-based pest management strategies. By disrupting these critical genes, it may be possible to impair the beetle's ability to detoxify host chemicals, digest plant tissues, or synthesize pheromones, thereby reducing its survival and reproductive success. Such a targeted approach could mitigate the ecological and economic impacts of this beetle on European spruce forests.

Furthermore, Chapters 5.1 and 5.4 highlight the intricate interplay between climate change, forest structure, and bark beetle outbreaks, emphasizing the role of rising temperatures and drought stress in weakening tree defences and facilitating rapid beetle population growth. The vulnerability of dense monoculture plantations underscores the necessity for greater species diversity and structural complexity in forest management to enhance ecosystem resilience. The research also explores the molecular mechanisms of host shifts in other *Ips* species, particularly *Ips sexdentatus*, revealing the beetle's capacity to rapidly upregulate detoxification and transporter genes when adapting to new hosts. This adaptability underscores the evolutionary flexibility of bark beetles and points to the potential for innovative control methods, such as RNAi

or CRISPR/Cas9, to limit outbreaks. Overall, the presented research significantly contributes to the understanding of bark beetle-host interactions and the broader ecological impacts of climate change, advocating for integrative pest management strategies that combine advanced molecular tools with sustainable forestry practices.

7.2. Study Limitations

Despite providing extensive insights into the resistance mechanisms of *I. typographus* against conifer chemical defences, this study has certain limitations. A key constraint is its reliance on laboratory-based experiments, which may not fully capture the complex, dynamic interactions occurring in natural ecosystems. In Chapter 5.2, while RNA-seq has significantly advanced insect physiology research, transcriptomics alone may not fully capture translational-level (protein-level) changes. While fumigation bioassays (Chapter 5.3), and gene expression analyses yield valuable data on detoxification pathways and host-pest dynamics, these findings may not accurately reflect beetle adaptation in natural environments influenced by variables such as climate fluctuations, host diversity, and interspecies competition. The focus on specific detoxification genes also leaves other important genetic and epigenetic factors, including secondary metabolic pathways and immune responses, insufficiently explored. Chapter 5.3 face limitations due to the reliance on a single RNA-seq replicate. Increasing the number of biological replicates in future research would enhance the robustness of the RNA-seq analysis. Furthermore, host-shift experiments performed in non-choice settings limit the capacity to study natural behaviours and host preferences, potentially omitting factors such as predation and competition in mixed-species forests. Furthermore, the study did not include long-term ecological consequences or potential evolutionary adaptations in response to long-term host changes. We also acknowledge variability in gut gene expression, as bark beetles collected from forests may show differences across generations.

To mitigate the limitations related to transcriptomics used in Chapters 5.2 to 5.4, the studies validated key differentially expressed genes (DEGs) using RT-qPCR and enzyme assays.

While these approaches offered useful information on gene expression, this study is limited by functional validation of candidates required for key genes identified in the study. Additionally, further research is essential to develop molecular biopesticides, using CRISPR/Cas9 and RNAi techniques, to better understand the functions of detoxification, host adaptability genes, and target invasive pests without harming non-target species, ensuring a balanced ecosystem approach. Future studies should incorporate field surveys, wider genomic analysis, and functional genomics to confirm gene functions and their influence on adaptation to new or stressed hosts.

7.2. Practical Applications and Recommendations for Future Research

The findings of this thesis highlight the critical need for practical forest management and pest control strategies, especially given the changing ecological conditions. Integrating advanced pest management techniques, including molecular tools like RNA interference and CRISPR/Cas9, can help target specific detoxification genes in bark beetles. Investigating the functional roles of detoxification genes through gene editing will offer deeper insights into beetle biology and support the development of sustainable forest management practices. Future research should focus on long-term field studies to better understand how climate change affects host-pest interactions, while expanding genomic studies to reveal broader adaptive mechanisms in bark beetles.

In summary, we recommend-

1- Including samples from various geographical locations and climatic conditions to enhance the identification of genes.
2- Use of more biological replicates, to reduce heterogeneity.
3- Future gene validation and characterisation using gene knockdown and in-*vitro* expression studies.
4- Use of strong biostatistical tools for genome-wide analysis to mitigate the overlapping of target sequences between target and non-target species.

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