Effects of Changing Climate on Interactions Among Mountain Pine Beetle, Host Tree, and Microorganisms

by

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Abstract

Environmental factors such as elevated levels of CO_2 and O_3 are increasingly affecting forest trees globally. Changes in climate have led to shifts in the geographic distribution of pests and pathogens associated with forests, with predictions of more native and invasive pests in the future. In North America, the mountain pine beetle (*Dendroctonous ponderosae*, (Hopkins), Coleoptera: Curculionidae, Scolytinae; MPB) poses a significant threat to lodgepole pine (*Pinus contorta* var. *latifolia*) forests, yet research on the impacts of climate change on MPB and its symbiotic organisms remains limited. Understanding the mechanisms involved in interactions between plants, herbivores, and pathogens is crucial for assessing the effects of invasive species in a changing climate. First, the study investigated how the fungal symbionts associated with MPB can detoxify host monoterpenes in live lodgepole pine trees and logs. Analysis revealed an increase in terpenes and oxygenated monoterpene concentrations following the fungal infection, suggesting a potential role of fungal symbionts in altering host defense chemistry and attracting MPB. Subsequent research explored the effects of two pathogenic fungal species, including *Atropellis piniphila,* causal agent of Atropellis canker, *Cronartium* harknessii, the causal agent of western gall rust, and three ophiostomatoid fungi (*Grosmannia clavigera*, *Ophiostoma montium*, and *Leptographium longiclavatum*) associated with MPB on the defense chemistry of mature lodgepole pine trees, highlighting distinct alterations in terpene chemistry and the importance of considering specific biotic stress agents in understanding tree susceptibility to insect attacks, particularly by MPB under changing environment. Furthermore, the study examined the impact of elevated ozone (eO_3) concentrations on MPB physiology and behaviour, revealing a dose-dependent effect on mating success and offspring morphology and behaviour. To my surprise, while adaptive responses to oxidative stress were observed, including reduced respiration and

heightened locomotor activity, mating success was compromised due to the degradation of pheromone components. Intriguingly, transgenerational plasticity in offspring morphology and behaviour suggested potential stress resistance mechanisms, underscoring the sensitivity of MPB to eO_3 concentrations and providing crucial insights into the ecological implications of such changes on insect populations. Additionally, controlled climate chamber experiments manipulating CO_2, O_3, and humidity levels revealed climate change-induced alterations in MPB reproduction, fungal growth, and immune responses, highlighting the significance of understanding the evolving relationships between forest pests and their symbiotic partners in a changing environment. Overall, these results contribute to understanding the complex interactions between bark beetles, fungi, host trees, and the changing climate. They emphasize the importance of considering both biotic and abiotic factors in developing effective strategies for resilient forest ecosystems amidst ongoing environmental changes.

Preface

The central chapters of this thesis are based on the articles that are either published or currently under review. A version of Chapters 2, 3, and 5 has been published (Zaman et al., 2024, 2023b, 2023a), and Chapters 4 was written based on the article currently under review.

1. **Chapter 2**: Zaman, R., May, C., Ullah, A., & Erbilgin, N. (2023). **Bark beetles utilize ophiostomatoid fungi to circumvent host tree defenses.** *Metabolites*, 13(2), 239. https://doi.org/10.3390/metabo13020239. RZ and NE conceived and designed the experiments. RZ and AU analyzed the results. RZ and CM performed the experiments. RZ completed the original draft. RZ, AU, and NE reviewed and edited the manuscript. NE supervised the study and acquired the funds. All authors have read and agreed to the published version of the manuscript.
2. **Chapter 3**: Zaman, R., Antonioli, F., Shah, A., Ullah, A., May, C., Klutsch, J. G., & Erbilgin, N. (2023). **A pine in distress: how infection by different pathogenic fungi affects lodgepole pine chemical defenses**. *Microbial Ecology*, 1, 1–8. https://doi.org/10.1007/s00248-023-02272-0. RZ, FA, and NE designed the experiment; RZ, FA, CM, JGK and NE developed protocols and conducted the experiment; RZ, AS, and AU analyzed the data; RZ & NE wrote the manuscript; all authors contributed to editing. NE supervised the study and acquired the funds. All authors have read and agreed to the published version of the manuscript.
3. **Chapter 4**: Zaman, R., Shah, A., Ishangulyyeva, G., & Erbilgin, N. (2024). **Exploring behavioural and physiological adaptations in mountain pine beetle in response to elevated ozone concentrations**. Submitted for review in a peer-reviewed journal. RZ and NE designed the study and developed protocols. RZ, AS, and GI conducted the experiment;

RZ analyzed the data; RZ & NE wrote the manuscript; all authors contributed to editing. NE supervised the study and acquired the funds. All authors have read and agreed to the published version of the manuscript.

4. **Chapter 5**: Zaman, R., Shah, A., Shah, A., Ullah, A., Ishangulyyeva, G., & Erbilgin, N. (2024). **Unraveling the multifaceted effects of climatic factors on mountain pine beetle and its interaction with fungal symbionts**. *Global Change Biology*, *30 (3)*, e17207, https://doi.org/10.1111/gcb.17207**.** RZ and NE designed the experiment; RZ, GI, and NE developed protocols. RZ, AS, AS, GI, and AU conducted the experiment; RZ, and AU analyzed the data; RZ & NE wrote the manuscript; all authors contributed to editing. NE supervised the study and acquired the funds. All authors have read and agreed to the published version of the manuscript.

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Chapter 1: Thesis Introduction

1.1 Mountain pine beetles in Canadian boreal forest

Studying the behaviour and ecology of eruptive herbivore species can yield valuable insights into critical ecological processes, including coevolutionary selection pressures, symbiotic relationships, multitrophic interactions, ecosystem disturbances, and chemical signalling. Furthermore, it serves as a valuable system for examining the adverse impacts of human activities on forest ecosystems (Raffa et al., 2008). Mountain pine beetles (MPB) (Coleoptera, Curculinonidae, Scolytinae), an integral component of western North American pine-dominated ecosystems, are recognized as initiators of wood decay in natural forest ecosystems. Acting as agents of natural forest regeneration and modifying species composition, they promote fungal, insect, and plant diversity (Wood, 1982). The MPBs have responded to climate change over the past four decades and expanded their geographical range to higher altitudes and northeastern pine (*Pinus*) forests in western North America. In 2008, MPBs breached the Rocky Mountains and colonized lodgepole pine (*Pinus contorta* Dougl.) trees in Alberta, affecting millions of hectares of forest lands (Bentz et al., 2010; Kurz et al., 2008; Raffa et al., 2008). Apart from their co-evolved historical host, lodgepole pine, MPBs also infest other pine species, including sugar pine (*Pinus lambertiana* Dougl.), western white pine (*Pinus monticola* Dougl. Ed. D. Don), whitebark pine (*Pinus albicaulis* Engelmann), and ponderosa pine (*Pinus ponderosa*, P. Laws. Ex C. Laws) (Wood, 1982). Recently, MPBs have extended their host range into pure jack pine (*Pinus banksiana* Lamb.) forests. Jack pine forests extend from the Alberta range across the boreal forest to the Atlantic Provinces in eastern Canada (Cullingham et al., 2011). There is a growing ecological and economic concern about the further spread of MPB to eastern jack pine forests.

1.2 The biology of MPB

The mountain pine beetle shows two density-dependent host selection behaviours. In the endemic stage, beetles colonize stressed or old trees, which are more susceptible to beetle attacks due to their weakened defenses (Safranyik and Carroll, 2006) (**Figure 1.1**). Upon successful

entry into the host tree, the host monoterpene α-pinene, is transformed into aggregation and anti-aggregation pheromones, *trans*-verbenol and verbenone, respectively (Wood, 1982). Additionally, frontalin and *exo*-brevicomin subsequently coordinate the MPB host colonization and maintain attack density. *exo*-Brevicomin, released by males at low concentrations, acts as an attractant for females, while at higher concentrations, both frontalin and *exo*-brevicomin act as anti-attractants (Pureswaran et al., 2000). In western Canada, MPB completes its lifecycle in a year. It has four developmental stages egg, larva, pupa, and adult. From late July to mid-August, mating, oviposition, emergence, dispersion, host selection, and colonization of new trees take place. Females mate under the bark and excavate oviposition tunnels, depositing oval, pearly white to cream-coloured eggs with an average diameter of 1mm. Larvae hatch within about a week and feed on phloem tissues infected by symbiotic fungi introduced by their parents within the tree's phloem. Before the onset of cold weather, larvae typically reach the third or early fourth instar. Winter temperature is considered the most significant source of MPB mortality despite MPBs acquiring cold tolerance through the accumulation of glycerol in the hemolymph. With the arrival of warmer spring temperatures, larvae resume feeding and complete development, transforming into pupae around June. By late June to early July, the development cycle concludes with the emergence of adult beetles, which begin colonizing new host trees (Safranyik and Carroll, 2006).

1.3 The role of symbiotic fungi in MPB biology

All bark beetle species have symbiotic relationships with multiple species of microorganisms, where their partners assist the beetles in responding to environmental changes, resource availability, and predator pressure (Hofstetter et al., 2015). Among these symbiotic partners, mutualistic fungi play a crucial role in enhancing the beetle's fitness by providing nutrients (Ayres et al., 2000), protection (Six, 2003), aiding communication (Brand et al., 1977), detoxifying host chemicals (DiGuistini et al., 2011), as well as influencing interactions with other organisms (Cardoza et al., 2008; Hofstetter and Moser, 2014). The biology of MPB is significantly influenced by several fungal species, which also determine the success of host colonization (Safranyik and Carroll, 2006). The majority of fungi associated with bark beetles are saprophytes that inhabit wood and inner bark, often in association with coniferous bark-

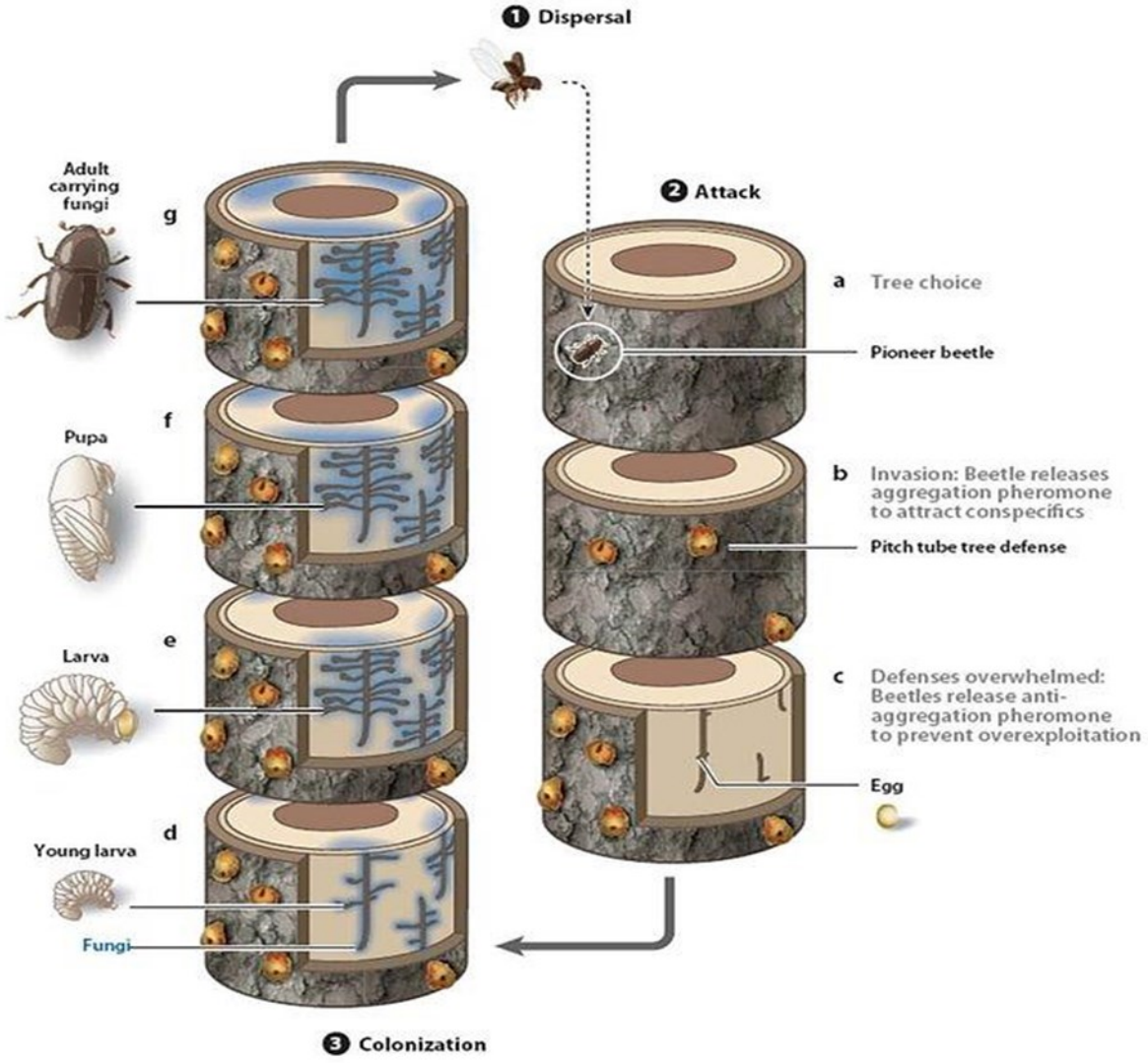

Figure 1.1: The life cycle of mountain pine beetles and their symbiosis with mutualistic fungi. (1) Dispersal of adult beetles under favourable conditions. (2) Attacking period: (a) pioneer female beetle chooses a suitable tree, (b) beetles enter into the tree and release aggregation pheromones to attract conspecifics of both sexes to induce a mass attack, which exhaust the tree defense system, (c) after mating and egg laying, beetles release an anti-aggregation pheromone to avoid over-exploitation, (3) colonization period: (d) parent beetles construct vertical egg galleries, lay eggs and inoculate fungi, hatched larvae start to develop, (e) the developing larvae tunnel away from the oviposition gallery, feed on fungal spores and phloem tissues, (f) later they excavate pupal chambers and remain there during pupation, (g) after becoming adults they emerge carrying fungal spores on the exoskeleton. Adapted from (Six and Wingfield, 2011).

beetles, earning them the name blue-stain fungi due to their melanistic hyphae and their tendency to discolour infected wood blue-gray or black (Harrington, 2005). These fungi are vectored in mycangia by MPB. The symbiotic relationship between MPB and blue-stain fungi offers several advantages. Firstly, these fungi protect beetle broods from antagonistic fungal species; secondly, they enhance the moisture content of phloem for larvae; and thirdly, they provide essential nutrients for larval development (Safranyik and Carroll, 2006). Among the commonly observed fungal symbionts with MPBs are ophiostomatoid fungi (Ophiostomatales, Ascomycota), including *Grosmannia* clavigera (Robinson-Jeffery and Davidson) Zipfel, de Beer, and Wing., *Ophiostoma montium* (Rumford) von Arx, and *Leptographium longiclavatum* Lee, Kim, and Breuil. These symbionts contribute to successful host tree colonization by influencing feeding behaviour, nutrient acquisition, and detoxification of host tree secondary metabolites (Agbulu et al., 2022; DiGuistini et al., 2011; Goodsman et al., 2012; Guevara-Rozo et al., 2020; Zaman et al., 2023b). Larvae rely on these fungi to enzymatically break down complex secondary metabolites in the phloem into more digestible compounds (Ayres et al., 2000; Bleiker and Six, 2007). *Grosmannia clavigerum* is primarily acquired and spread *via* mycangia, while the adhesive spores of *O. montium* are predominantly transported phoretically on the beetle's exoskeletons (Six, 2003). This symbiotic relationship enhances the beetles' access to essential nutrients and creates a favourable environment for larval feeding. Various studies have highlighted that climate fluctuations in different regions may affect the presence, pathogenicity, and virulence of these fungal symbionts (Moore and Six, 2015; Rice et al., 2008; Six and Bentz, 2007). Yeasts (Kingdom Fungi), well-known endosymbionts of insects, appear present throughout most bark beetle life stages (Six, 2003). Additionally, MPB colonization renders host trees susceptible to infections by saprophytic fungi such as *Trichoderma* spp. and *Aspergillus* spp., leading to a significant reduction in beetle egg-laying and larval survival (Therrien et al., 2015).

1.4 Defense mechanism in conifer host tress

Conifers have evolved sophisticated defense mechanisms comprising various physical, chemical, and histological mechanisms to protect themselves against bark beetles (Bohlmann et al., 2000; Franceschi et al., 2005). These defenses maintain the integrity of the tree by safeguarding crucial components such as the nutrient-rich phloem, vascular cambium, and sapwood, which are vital

for transpiration and nutrient transportation. Conifer defenses consist of constitutive and inducible phases. Constitutive defense involves both mechanical and chemical processes. Mechanically, trees deter intruders by reinforcing tissues with lignin and suberin polymers, enhancing resistance to penetration, breakdown, or ingestion (Franceschi et al., 2005). Primary mechanisms include polyphenolic parenchyma cells, calcium oxalate crystals, and sclerenchyma cells comprising the secondary phloem. Calcium oxalate crystals and sclerenchyma cells may serve as mechanical barriers to deter beetles. Chemical defenses include the production of toxic compounds such as secondary metabolites, proteins, enzymes, and chemical reservoirs like resins, which can physically entrap or repel insects (Franceschi et al., 2005; Keeling and Bohlmann, 2006). Polyphenolic parenchyma cells contain phenolic bodies, offering chemical defense against fungi (Franceschi et al., 2005). Additionally, resin cells produce terpene-containing resins stored under pressure in extracellular lumens. These resins, when released, form sticky substances that can repel or trap insects damaging the bark (Bohlmann, 2008).

Inducible structural and chemical processes constitute the second phase of the defense mechanism. Shortly after bark beetle attacks, conifers initiate an induced defense response. Until this point, the bark beetle infestation is hindered by constitutive defenses. Inducible chemical processes involve protein-based and non-protein-based compounds, such as terpenoid resins, alkaloids, and phenolics. Protein-based compounds include lectins, enzymes like glucanases or chitinases, and enzyme inhibitors like proteinases or amylase inhibitors. Enzymes like chitinases can lignify tree cell walls, hindering digestion, while enzyme inhibitors impede the invasive organism’s functionality (Bohlmann et al., 2000; Felicijan et al., 2016; Franceschi et al., 2005). Hypersensitive responses at the attack sites trigger the formation of reactive oxygen species and rapid cell death (Bleiker and Uzunovic, 2004). The periderm formation around the wound restricts its area and limits the supply of nutrients to the injured tissues (Franceschi et al., 2005). By binding with amino acids and proteins, phenolics diminish the nutrient value of tissues and hinder digestion by binding to the digestive gut enzymes of beetles. Furthermore, resin flow, with its altered composition of terpenes, may obstruct the beetle's advancement into the tree by blocking and masking the release of pheromones from the entry site (Franceschi et al., 2005; Schiebe et al., 2012; Zhao et al., 2011). The conifer defense mechanism against bark beetles can be attributed to four sequential steps. The first stage involves employing effective constitutive protection to deter or impede attacks. If the initial defense is ineffective, the second step is

eliminating or containing the bark beetles using induced defense mechanisms. Repairing the injured area constitutes the third line of defense against opportunistic fungi that may cause secondary infections. Finally, locally and systemically produced acquired resistance mechanisms may be developed to enhance adaptation to future challenges (Eyles et al., 2010; Franceschi et al., 2005; Krokene, 2015).

1.5 Chemical interactions in MPB-host tree-fungal symbionts

Chemical communication remains a fundamental means of information transmission within and between species (Bergström, 2008; Steiger et al., 2011). Chemical signals, originating from a sender and recognized by a receiver, evolved from pre-existing cues when they enhance the fitness of one or both partners (Steiger et al., 2011). For efficient short- and long-range intra- and inter-specific interactions, these signals often consist of lipophilic volatile molecules with low molecular weights (< 300 Da) and high vapour pressures (Insam and Seewald, 2010; Kanchiswamy et al., 2015; Nevo et al., 2018). When occurring between species, these molecules are called allelochemicals, whereas, within a species, they are referred to as pheromones. The receiver's olfactory system processes chemical information, including lipophilic volatiles, *via* odorant-specific olfactory receptors, while contact chemoreception processes substances with larger masses and polarities, such as proteins, lipids, carbohydrates, and toxins (Chapman, 2003; Pelosi et al., 2006). The intricate relationships between microorganisms, plants, insects, and more complex situations involving multiple trophic levels highlight microbial volatile compounds (mVOCs) as significant contributors (Davis et al., 2013; Whitehead et al., 2022). In reality, mVOCs are mixtures of various chemical classes, including nitrogen and sulfur-containing molecules, alcohols, aldehydes, acids, esters, ketones, lactones, and other compounds. Often, they are waste products or byproducts of biosynthesis (Lemfack et al., 2018).

Bark beetles utilize terpenes as cues for locating and colonizing host trees and as formidable obstacles against these herbivores and their symbionts (Raffa, 2014). Mountain pine beetles enhance their orientation toward host trees by utilizing volatile cues during host location. They also utilize low concentrations of certain monoterpenes as feeding stimulants and cues for host recognition upon landing. In contrast, oxygenated terpenes derived from both hosts and beetles serve as pheromones to attract mates. Additionally, various physiological reactions driven by

terpenoid juvenile hormones play crucial roles at different life stages of MPB (Blomquist et al., 2010; Pureswaran et al., 2000).

The primary aggregation pheromone in MPB, *trans*-verbenol, is derived from the hydroxylation of the host tree secondary metabolite α-pinene, which can further be oxidized to verbenone by both MPB and their fungal symbionts (Cale et al., 2019a; Tittiger and Blomquist, 2017). Following initial attacks by females and the release of *trans*-verbenol to attract more conspecifics, arriving males release low concentrations of *exo*-brevicomin, which attract female mates. *exo*-Brevicomin released by females synergizes with the aggregation power of *trans*-verbenol, while male-produced frontalin signals to incoming males that certain female galleries are already occupied. Verbenone serves dual roles for incoming beetles and phloem-infected fungi. For beetles, it signals saturation of attack density on a particular tree, regulating spatial attacks and indicating the availability of limited fresh resources. For fungi, it indicates the occupied boundaries utilized by specific fungi. A combination of multiple volatile cues such as verbenone, frontalin, 2-phenylethanol, cymene, and nonanal regulates anti-aggregation in MPB. 2-Phenyl ethanol, produced by paired males and females, and nonanal, produced by MPB, act as synomones for *Ips pini* (Say), signaling resource partitioning in attacked trees by MPB (Pureswaran et al., 2000; Tittiger and Blomquist, 2017). Host pine monoterpenes such as myrcene, terpinolene, and 3-carene act as aggregation synergists in MPB (Borden et al., 2008).

Apart from the pheromones of MPB and allomones of host trees, compounds produced by symbiotic fungi can provide information to MPB regarding the quality of host substrate, food sources, and toxicity levels of hosts (Brand et al., 1977; Cale et al., 2016; DiGuistini et al., 2011; Lieutier et al., 2009). Beyond these advantages, this thesis explores further potential functions of fungal symbionts as providers of semiochemicals or as symbionts that provide sustenance or detoxification of host metabolites for MPB.

1.6 Climate change impacts on terrestrial ecosystem

The escalating concentrations of atmospheric greenhouse gases have significantly contributed to climate change, impacting various environmental factors (Calvin et al., 2023). Examples of these changes include heightened precipitation, increased frequency of natural catastrophes, elevated levels of greenhouse gases, rising sea levels, increasing Earth's surface temperature, and altered

relative humidity (Boullis et al., 2016, 2015). Sustainable forest management is influenced by many global change issues (Ramsfield et al., 2016). Climate change may exacerbate invasions of forest pests and pathogens, leading to further ramifications for native ecosystems. For instance, it may facilitate the range extension of native and exotic pests and pathogens or impact tree tolerance to pests (Ramsfield et al., 2016).

Among the frequently studied global change factors affecting forest ecosystems (plants, insects, pathogens) are temperature, drought, carbon dioxide (CO_2), and ozone (O_3). Global temperatures have reportedly risen by 2-4°C since industrialization. CO_2 and O_3 levels have surged several folds and currently stand at around 420 ppm and 50 ppb, respectively, with predictions of doubling by the end of this century (Calvin et al., 2023; Price et al., 2013). Preliminary studies suggest that CO_2 and O_3 will likely continue altering ecosystem processes, feedback, and terrestrial biodiversity across different trophic levels (Fuhrer et al., 2016; Roggatz et al., 2022).

High oxidative stress induced by increased ozone has led to significant reductions in plants physiological functions including stomatal conductance, carboxylation efficiency, and leaf chlorophyll content. This can damage leaf cells and reduce the availability of glucose precursors (Ainsworth et al., 2012; Ashmore, 2005). Conversely, elevated CO_2 can positively affect plants by enhancing photosynthesis and total plant tissue biomass (Xia et al., 2021). However, the direct effects of CO_2 can have positive and negative consequences on insect and fungal biology, depending on the context. Conversely, elevated O_3 predominantly negatively impacts insect and fungal biology and their chemical communication. Increased O_3 may induce the production of secondary defense metabolites in plants due to oxidative stress, negatively affecting insects (Agathokleous et al., 2020). Additionally, numerous studies have demonstrated that increased ozone results in the breakdown of semiochemicals, including plant-released volatiles and insect pheromones (Démares et al., 2024; Jiang et al., 2023; Saunier et al., 2023; Venkateswaran et al., 2023). This breakdown may result in intermediate products, rendering insects unable to perceive the disrupted signals (**Figure 1.2**). However, CO_2 is reported to elicit varied responses in the chemical communication and biology of insects (Roggatz et al., 2022).

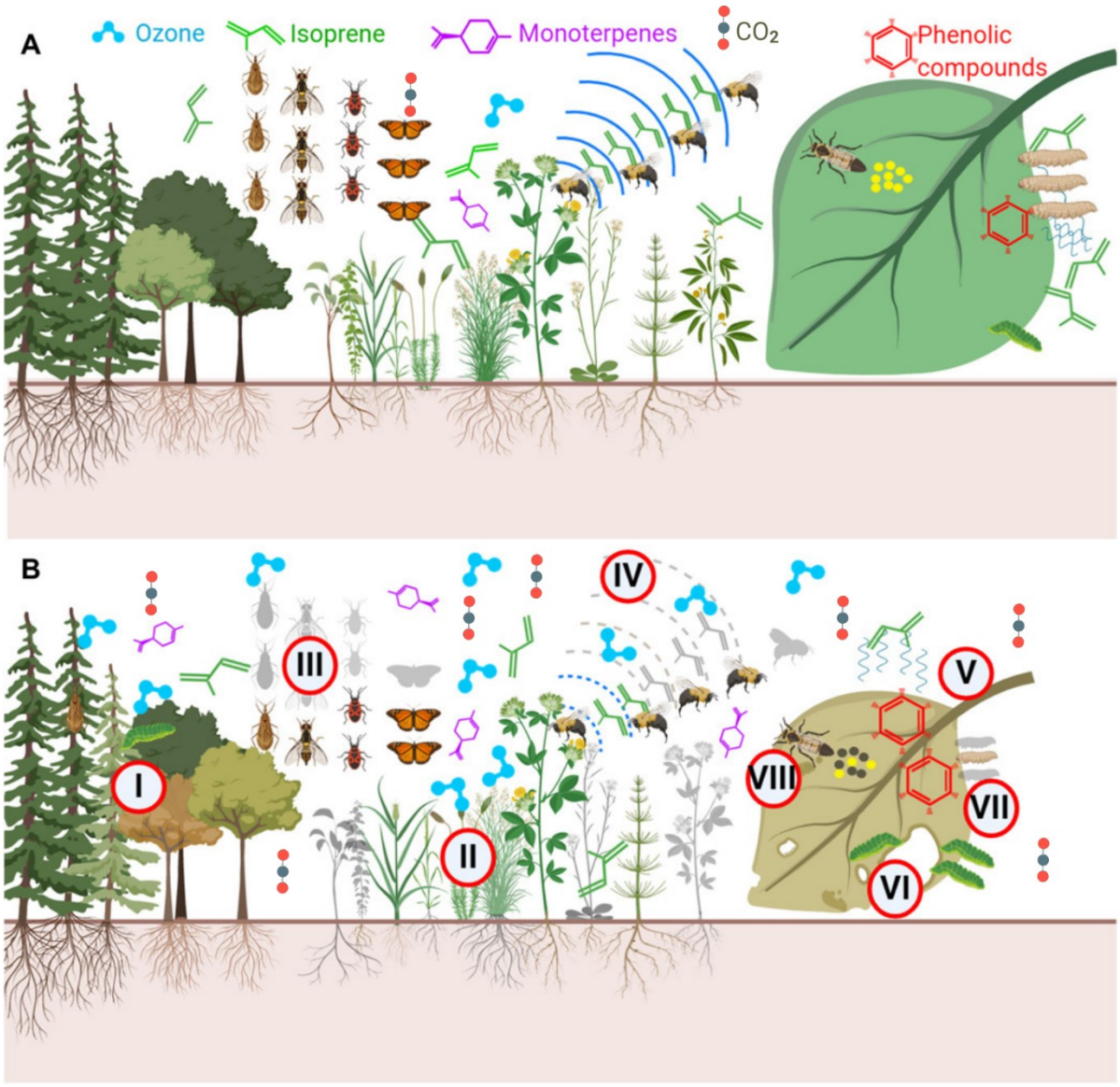

Figure 1.2: Increased O3, CO2 and their effects on aboveground ecological processes.

Multitropic organismal interactions at the foliar and ecosystem levels in an ambient environment **(A)** as opposed to one that is altered by elevated O_3 and CO_2 levels **(B)**. The decline in the variety of plants or insects is denoted by gray icons. The growth rate and biomass of plants, especially forest trees, are lowered by O_3 and increased by CO_2 **(I)**. O_3 may also change the makeup of communities and decrease the abundance of plant species, while CO_2 can increase **(II)**. O_3 lowers the number of insect species, but CO_2 increases species richness in forest environments due to shorter generation time and selective pressure **(III)**. Volatile organic compounds are broken down by O_3, which hinders plant-pollinator communication **(IV)**.

Interactions between CO_2 and O_3-plants and insects may be very intricate and species-specific. O_3 decreases foliar size and causes foliage immaturity. In contrast, CO_2 can cause the opposite (I, V), boost monoterpene emissions intolerant and evergreen species, and make plants more vulnerable to insects and diseases **(I, VI)**. In other situations, O_3 causes phenolic compounds to accumulate in leaves, discouraging insects from feeding (lowering insect abundance), increasing insect mortality, and preventing insects from growing larger bodies. At the same time, CO_2 alters the C:N ratio by enhancing C-containing compound production **(VII)**. Additionally, O_3 modifies foliar phytochemistry, which hinders insect oviposition **(VIII)**. Adapted from (Agathokleous et al., 2020; Roggatz et al., 2022; Xia et al., 2021).

1.7 Thesis Aims

The primary objective of my thesis is to gain deeper insights into the intricate relationship among host trees, insects, and fungi under shifting climate conditions. Specifically, I have utilized the lodgepole pine- MPB-symbiotic ophiostomatoid fungi triad as a model system to investigate the influence of changing environmental factors, such as elevated CO_2, O_3, and relative humidity, on their interactions and biological processes. Additionally, I have incorporated native phytopathogens of lodgepole pine, including *Atropellis piniphila* and *Cronartium harknessii*, and saprophytic fungi, such as *Aspergillus* and *Trichoderma*, into this model system. This integration aims to evaluate their virulence and impact on the susceptibility of host trees to MPB infestation. Overall, this thesis contributes to the existing literature by offering a comprehensive understanding of the effects of elevated CO_2 and O_3 on MPB biology within the context of the boreal forest ecosystem. Furthermore, it sheds light on factors influencing MPB range expansion and the potential implications of these changes under future climate scenarios.

1.8 Research Questions

The study attempted to respond to the research questions below:

1. How does the symbiotic relationship between fungi and the mountain pine beetle (MPB) facilitate successful host colonization?
2. Can native phytopathogens *A. piniphila* and *C. harknessii* influence the susceptibility of MPB to host trees, considering the changing climate?
3. How does elevated tropospheric ozone affect MPB's chemical communication?

4. What is the anticipated impact of predicted climate change effects on the likelihood of bark beetle outbreaks and tree mortality in the next century?

1.9 Research Hypothesis

The research hypotheses of this study are:

1. Symbiotic fungi enhance MPB's host colonization success by altering and metabolizing host tree terpene defenses and attracting beetles towards fungal volatiles that may signal favourable breeding host substrates.
2. Native phytopathogens may increase MPB's susceptibility to host trees by altering the tree terpene defenses to a similar extent to MPB's fungal symbionts and changing climatic factors may further enhance the virulence of the phytopathogens.
3. Elevated O_3 can disrupt mating success by degrading pheromone components and inducing oxidative stress in MPB.
4. Under predicted and anticipated effects of climate change, including elevated CO_2, O_3, and reduced humidity, further bark beetle outbreaks and the resulting tree deaths will likely occur in the next century.

1.10 Thesis layout and contribution

This thesis comprises six chapters, including an introduction and general discussion, with each core study chapter focusing on different aspects of the main objective, contributing to a comprehensive understanding of the broader context. The following is a breakdown of each chapter:

Chapter 2 explores the beneficial role of ophiostomatoid fungi in symbiosis with the mountain pine beetle (MPB) and investigates how these fungal symbionts aid host chemical detoxification and facilitate chemical communication with MPB. Specifically, it examines alterations in the terpene profile of host trees due to fungal infection and the contributions of each symbiont to the detoxification process. **Chapter 3** assesses the chemical profile similarities and dissimilarities between ophiostomatoid fungal symbionts of MPB and other phytopathogens such as *Atropellis piniphila* and *Cronartium harknessii*. It also investigates the virulence of various phytopathogens compared to fungal symbionts of MPB under ambient and elevated CO_2 and O_3 conditions. **Chapter 4** examines the effect of elevated O_3 on the physiology and olfaction of MPB. It also

explores the impact of elevated O_3 on MPB mating efficiency, resulting in significant reductions in mating success and apparent changes in offspring morphology and pheromone production capacity. It also investigates whether parental treatment affects offspring reproduction and whether elevated O_3 degrades pheromone components. **Chapter 5** investigates the effects of elevated CO_2 on MPB reproduction and compares the effects of elevated CO_2 with the impacts of O_3 from Chapter 4. It also evaluates the impacts of humidity on MPB reproduction separately. The study will extend to assess the effects of these environmental variables on the fungal symbionts of MPB. **Chapter 6** (General Discussion) compiles all the findings from the individual studies to generate a cohesive narrative predicting the dynamics of MPB under climate change contexts. It discusses the implications of the research findings and potential future directions in the field.

Overall, each chapter contributes essential insights into understanding the complex interactions among host trees, insects, and fungi under changing environmental conditions, providing valuable contributions to the broader understanding of forest ecosystem dynamics in the face of climate change. My research revealed that fungal symbionts could detoxify host tree defense monoterpenes with varying efficiency. Each symbiont metabolized monoterpenes differently, producing distinct compounds that attract and facilitate MPBs to recognize their symbionts. Diseases caused by *C. harknessii*, excluding *A. piniphila*, can increase susceptibility to subsequent MPB attacks by reducing the host tree's defense chemicals. Elevated CO_2, but not O_3, enhances the virulence of these pathogens. Despite elevated O_3 disrupting MPB mating success, their offspring exhibit resistance plasticity and enhanced immunity. Conversely, elevated CO_2 accelerates larval development, while low humidity enhances it. Furthermore, low humidity promotes the growth of symbiotic fungi, outcompeting other saprophytic species. Throughout my research, I identified several monoterpene biomarkers that could aid in diagnosing resistant and diseased host trees. The chemical profiles of diseased trees and fungal volatiles generated from this work can be field-tested to assess low-density MPB populations. Moreover, my research introduces novel methodological approaches for evaluating insect biology and behaviour under changing environmental conditions, paving the way for future research.

Chapter 2: Bark beetles utilize ophiostomatoid fungi to circumvent host tree defenses

2.1 Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are subcortical insects that primarily feed on host tree phloem. These species play critical roles in maintaining the ecosystem function, including nutrient cycling, by killing defensively compromised trees (stressed, diseased, etc.). Host tree colonization starts with the release of aggregation pheromones by the pioneering beetles that attract conspecifics after locating a potentially suitable host tree. The aggregation pheromone is produced by bark beetles either de novo or using the host chemicals as precursors. During host colonization, beetles also introduce their symbiotic ophiostomatoid fungi into the host trees. All bark beetles are associated with one or several species of fungi from the genera Ophiostoma, Ceratocystiopsis, Grosmannia, and Ceratocystis (Frago et al., 2012). These fungal symbionts are critical components of successful host tree colonization by bark beetles (Blomquist et al., 2010; Vite and Pitman, 1968; Zhao et al., 2015). After mating, female beetles excavate oviposition galleries and lay eggs. The newly hatched larvae make their galleries where they feed on phloem tissues infected with the fungal symbionts (Safranyik and Wilson, 2006; Six, 2013, 2012). Due to their widespread associations (Hammerbacher et al., 2013; Lehenberger et al., 2021; Wadke et al., 2016; Wang et al., 2014; Zhao et al., 2019), there is growing literature on bark beetle-fungal interactions; however, fungal-host tree interactions have received relatively less attention. In particular, how fungal infection alters the production of host secondary metabolites over time and their role in assisting beetles in overcoming metabolite toxicity require additional studies. Furthermore, ophiostomatoid fungi produce a diversity of fungal volatile organic compounds or FVOC (Cale et al., 2016; Kandasamy et al., 2019, 2016). In a few species of bark beetles, the role of FVOCs in bark beetle attraction was reported (Jirošová et al., 2022; Kandasamy et al., 2019; Pureswaran et al., 2000; Six, 2012).

Several species of bark beetles can also attack healthy trees once their populations reach a certain threshold density (Bentz et al., 2010; Raffa et al., 2008; Wermelinger, 2004). Such attacks usually lead to landscape-level tree mortality. However, coniferous trees have developed

sophisticated defenses against bark beetles-fungi complexes that comprise physical, chemical, and histological mechanisms that can be expressed both constitutively and induced. The main constitutive response is located in the secondary phloem, which contains cells that act as mechanical barriers against attacking beetles. In particular, the resin cells produce oleoresin that contains terpenes that provide chemical protection against the bark beetle-fungal complexes (Bohlmann et al., 2000). At the induced phase, attacks induce resinosis and additional traumatic resin duct formation, auto-necrosis, and biosynthesis of structurally diverse terpenoids through the methylerythritol phosphate pathway (Bohlmann, 2008; Franceschi et al., 2005; Keeling and Bohlmann, 2006). Oleoresins are toxic to both beetles and fungi and also physically entrap invading bark beetles (Klepzig et al., 1996; Krokene, 2015; Raffa and Berryman, 1983; Ullah et al., 2021). However, some monoterpenes are utilized by bark beetles as precursors for pheromone production during host colonization (Erbilgin et al., 2007; Raffa et al., 2016; Raffa and Berryman, 1983). For instance, the Norway spruce beetle, *Ips typographus*, can oxidize host monoterpene α-pinene to *cis*-verbenol, which is then used as an aggregation pheromone by the same beetles in combination with fungal-produced volatile, 2-methyl-3-buten-2-ol (Klimetzek and Francke, 1980; Lanne et al., 1989).

The mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) is an eruptive native bark beetle species in western North America and has killed millions of pines, mainly lodgepole pine (*Pinus contorta* Douglas), during the last outbreak (Bentz et al., 2010). Three main symbiotic fungi associated with MPB include *Grosmannia clavigera* (Robinson-Jeffery and Davidson) Zipfel, de Beer, and Wing., *Ophiostoma montium* (Rumford) von Arx, and *Leptographium longiclavatum* Lee, Kim, and Breuril (Bleiker et al., 2009; Lee et al., 2006a; Roe et al., 2011). Toxic terpenes such as monoterpenes and diterpene resin acids are produced in response to MPB attacks (Erbilgin, 2019; Ullah et al., 2021).

The interaction between fungi growing in beetle-colonized hosts and the host defense chemistry may result in the production of metabolites that can be attractants to bark beetles (Kandasamy et al., 2023, 2019). Determining whether fungi can emit bark beetle-attractive compounds would promote our understanding of the sources of semiochemical (behavior modifying specific compounds) landscape that bark beetles encounter during host-tree colonization. Although several studies have shown the ability of symbiotic fungi to modify host defense chemistry, the

mechanism of how bark beetles surpass host defenses in the MPB-symbiotic fungal complex is still not clear. Such understanding can help us to determine the symbiotic fungi's role in aiding beetles to overcome the host defenses. We hypothesize that symbiotic fungi improve beetles' successful host colonization (1) by modifying terpene defenses of trees and (2) by attracting beetles towards fungal volatiles that may signal favorable breeding-host substrates.

Several studies have investigated the role of FVOCs in fungus-tree, fungus-beetle, and fungus-fungus interactions (Agbulu et al., 2022; Cale et al., 2016; Guevara-Rozo et al., 2020; Hulcr et al., 2011; Kandasamy et al., 2016; Liu et al., 2021; Pureswaran et al., 2000; Wang et al., 2020). These studies have reported that (1) host defense chemistry, mainly monoterpenes, can affect the production of FVOCs; (2) different fungal species have similar FVOC profiles, but the abundance of specific compounds varied by the fungal species; (3) competition among different species of fungi can affect both composition and concentration of FVOCs; (4) fungi can produce volatile compounds that can be attractive or inhibitive to bark beetles; (5) some symbiotic fungi are capable of transforming the primary MPB aggregation pheromone *trans*-verbenol into its anti-aggregation pheromone, verbenone; (6) tree chemical defenses affect host suitability to bark beetles through influencing their fungal symbionts; and (7) different species of fungal symbionts respond differently to host defense metabolites.

Our research objectives are (1) to investigate the host terpene detoxification by MPBs' fungal symbionts (Erbilgin et al., 2014; Kandasamy et al., 2023; Nones et al., 2022); (2) to determine the benefits of maintaining multiple species of fungal symbionts to MPBs; (3) to test whether MPB elicits behavioral responses to FVOCs produced by its fungal symbionts. Here, we inoculated the mature lodgepole pine trees in a forest stand with three fungal species (*G. clavigera*, *L. longiclavatum*, and *O. montium*) of MPB. To complement this field study, we inoculated the same fungal species on lodgepole pine logs in the laboratory. While live trees allow us to measure the time-specific interaction between trees and fungi, the log experiment allows measuring the host metabolites degradation process driven by the fungi. By collecting and analyzing the fungal-infected phloem samples, we identified and quantified the terpenes (monoterpenes, sesquiterpenes, and diterpenes) and the FVOC profile of each fungus. We then conducted an olfactometer assay to determine whether MPB is attracted to FVOCs associated with its symbiotic fungi through olfaction.

2.2 Methods

2.2.1 Field phloem sample collection

We carried out a field experiment in lodgepole pine forests to characterize how different species of fungal symbionts of MPB alter the terpene chemistry of host phloem over time. We selected 10 healthy (asymptomatic) lodgepole pine trees (DBH = 25.05 ± 0.78 cm) at 22 km North-East of Hinton (Alberta; 53°30′50.7″ N 117°17′31.2″ W). On each tree, we made 4 holes of 20 mm in size in four cardinal directions equidistant from each other at breast height (1.40 m) along the tree stem. We placed one 2 cm-sized plug of fungal mycelium (one of three fungal species) on each hole and 1 agar plug without fungal mycelium as control. The fungal plugs were taken from the edges of 10-day-old fungal cultures on potato dextrose agar media. Then, the wounds were covered with saran wraps. Phloem samples (from the fungal-infected and immediate upper part of the initial inoculation point, at different locations along the tree stems, i.e., 5–6 cm above the earlier sample) were collected after every 2 weeks for a total of 6 weeks, stored in dry ice in the field, brought to the laboratory, and stored at −40° C until analysis. The tissues were processed and extracted based on the method described earlier (Erbilgin et al., 2014). The following fungi were used in this experiment; *G. clavigera* (EL004), *O. montium* (EL 031), and *L. longiclavatum* (EL002). Fungal cultures were obtained from different sources: *G. clavigera* was originally isolated from MPB in Fox Creek (Alberta) and provided by AV Rice (Northern Forestry Centre, Canadian Forest Service, Edmonton, Alberta), *L. longiclavatum* (NOF 3100) was provided by the Northern Forestry Centre Culture Collection, and *O. montium* (UAMH 4838) was provided by the University of Alberta Microfungus Collection and Herbarium (Edmonton).

2.2.2 Laboratory experiment

The preliminary results from the above field study showed the highest induced terpene production in the phloem occurred at week 2; hence we further conducted a complementary laboratory experiment using logs of lodgepole pine trees. This study enabled us to better understand the host metabolite degradation process by fungi as well as to collect FVOCs. A total of 10 logs (21 × 30 cm: diameter × height) were selected. A 10 mm-sized plug of three fungi (as mentioned above) and 1 control (agar without fungal mycelium) were randomly inoculated on four cardinal directions of each log. Phloem samples were collected on day zero, during fungal

inoculations, and 14 days post-inoculation and stored at −40° C until analysis. After 14 days, fungal growth margins were traced, photographed, and used to quantify the culture area using ImageJ software version Java 1.8.0-172 (National Institutes of Health, Bethesda, MD, USA) (Schindelin et al., 2012).

Headspace volatiles from fungal-infected phloem samples were collected according to the method described in Cale et al. (2016). Briefly, infected tissues excised from logs were placed into a volatile collection chamber consisting of a 473 mL glass jar with Teflon tape on its threading and fitted with a metal cap. The jar was attached with a vacuum/pressure pump (Cole-Parmer Canada Inc., Montreal, QC, CAN). Constant airflow through chamber lines was set to 450 mL min^{-1} using a flowmeter. A Teflon tube filled with activated carbon (450 mg; 6–14 mesh, Fisher Sci., Hampton, NH, USA) fixed in place with glass wool was used to collect headspace volatiles from the jar for 6 h, after which time the carbon-filled tubes were removed from the collection apparatus. Volatiles were extracted by adding the activated carbon to a microtube containing 1 mL of dichloro-methane with tridecane as the internal standard (0.002%). This mixture was vortexed for 30 sec, sonicated for 10 min, and centrifuged (at 18,213 rcf) for 30 min before the extract was collected and transferred to a 2 ml glass gas chromatography (GC) vial. This procedure was repeated a second time (Cale et al., 2019a). Phloem samples were processed as above and stored at −40 °C until further analysis (Erbilgin et al., 2014).

2.2.3 Chemical analysis

All extracts were analyzed using a GC fitted with a DB-5MS UI column (30 m × 0.25 mm ID × 0.25 μm film, product: 122-5532UI; Agilent Tech, Santa Clara, CA, USA) and coupled to a mass spectrometer (GC-MS; GC: 7890A, MS: 5062C, Agilent Tech.). Helium was used as a carrier gas flowing at 1 mL min^{-1} with a temperature program beginning at 45–50 °C (held for 2 min), followed by an increase of 3 °C min^{-1} to 70 °C, then 5 °C min^{-1} to 130 °C, after that 12 °C min^{-1} to 170 °C, and finally the column temperature was brought to 300 °C (held 2 min) at a rate of 30 °C min^{-1}. A 1 μL sample injection volume was used; the injector temperature was 250 °C, and samples were run in splitless mode. The Sim and Scan acquisition mode was conducted simultaneously; while Sim mode allows us to acquire low traces of VOC and terpene compounds, Scan mode is performed for identification purposes. The NIST 2017 Mass Spectral library version 2.3 was used for the verification of all compounds. All compounds were

quantified based on the following standards availability: Monoterpenes: limonene (Chem Purity: >99%, racemic mixture), β-pinene (CP: >99%, RM), β-myrcene (CP: 90%), α-pinene (CP: 98%, RM), β-phellandrene (CP: 96%, RM), α-phellandrene (CP: 95%), p-cymene (CP: >99%), terpinolene (CP: 90%), 3-carene (CP: 98.5%, RM), camphene (CP: 90%, RM), α-terpinene (CP: 85%), γ-terpinene (CP: 97%), ocimene (CP: 90%), Oxygenated monoterpenes: (-)-borneol (>99%), camphor (CP: 95%), α-terpineol (CP: 90%, RM), linalool (CP: 97%), *cis*-grandisol (CP: >95%), verbenone (CP: >99%), Phenylpropenes: 4-allylanisole (CP: 98.5%), Sesquiterpenes: (+) aromadendrene (CP: 97%), caryophyllene oxide (CP: 95%), β-caryophyllene (CP: 80%), Aliphatics/others: iso-butanol (CP: >99%), phenethyl alcohol (CP: >99%), 2-methyl-1-butanol (CP: >99%), phenethyl acetate (CP: >98%), 3-methyl-1-butanol (CP: >98%), iso-amyl acetate (CP: >97%), acetoin (CP: >96%),. All standards were obtained from Sigma-Aldrich (Oakville, ON, CAN), except β-phellandrene from TRC Canada (Toronto, CAN). For the quantitation of some sesquiterpene compounds, due to their unavailability in the market, we used some of the above-mentioned standards to quantify based on hydrocarbon groups along with unique ion masses.

2.2.4 Two-choice olfactometer test

We prepared a plant-based media as described earlier (Kandasamy et al., 2023, 2019): 7% lodgepole pine phloem powder and 4% bactoagar were mixed in 100 mL of distilled water. The powder supplemented fungal growth on the agar and made the diet palatable for adult MPBs. All three symbiotic fungi were grown on the media for 5 days. We developed a novel olfactometer setup that contained a 55 mm round Petri dish connected with 2×10 cm polyvinyl chloride tubes from opposite sides. The 2 tubes were further distally connected with two 15 mL falcon tubes (attached through the lid). The petri dish and the tubes were masked with vinyl electrical tape to make the experimental environment dark. As MPBs are positively phototactic insects, the whole setup was placed under a light source that was visible to beetles through the falcon tubes. A 6 mm fungal plug was placed inside either of the 2 falcon tubes. A media plug without fungus was inserted inside the other falcon tube that served as a control. A single adult female beetle was placed inside the Petri dish, and after 20 min, the beetle choice was recorded. Thus, a total of 20 beetles were tested for each fungal treatment. A beetle that did not respond to either treatment after 20 min was discarded from the experiment and replaced with another beetle. We used

beetles that emerged from our mountain pine beetle colony in our bioassays; these beetles were reared on lodgepole pine logs.

2.2.5 Statistical analysis

Data from fungal growth were transformed to Ln (Y) of original values to assure normality (Kolmogorov–Smirnov test). Then, Welch's ANOVA test was done in GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com, accessed on 15 July 2022). Dunnett's T3 multiple comparison test was done to compare the means between different treatments ($p < 0.05$).

The final data matrices of chemical concentration from the field and laboratory studies were imported into the metabolomics data analysis platform Metaboanalyst 5.0 for data exploration, visualization, and multivariate statistical analysis (http://www.metaboanalyst.ca, accessed on 1 September 2022) (Xia et al., 2009). Missing inputs were replaced by values calculated as half of the minimum positive values in the original data. The data were log2 transformed and auto-scaled to obtain a normal distribution.

The field study data were used for repeated measure analysis in live trees and were processed using the "Time-Series/One-factor" option. Principle component analysis (PCA) was applied to inspect the variation with averages of ten replicates from each treatment and each week. PERMANOVA test was performed to ascertain significant differences among different weeks, different treatment samples, and interaction between week and treatment, followed by Tukey's multiple comparison test. Two-way repeated measure ANOVA, ANOVA-Simultaneous Component Analysis (ASCA) and multivariate empirical Bayes (MEBA) time-series analysis were performed to determine potential biomarkers that significantly regulated the host tree-fungal interaction.

To understand the dimensionality of the overall terpene chemical profile from the laboratory inoculations, we subjected the whole compound concentration dataset to Non-Metric Multidimensional Scaling (NMDS) indirect gradient analysis. PERMANOVA test was performed to ascertain the significant impact of treatments on terpene chemistry. Then for each fungal treatment, we performed fold change analysis coupled with one-way ANOVA to estimate the individual compounds that showed at least 2-fold upregulation or downregulation compared to

the control treatment. After that, multiple comparisons between different treatments for the specific compound were done by Fisher's protected least significant difference (LSD). Correlations between fungal treatments and VOCs were calculated with the parametric test Pearson's correlation.

Data from the 2-choice olfactometer assay were subjected to a 2-sample t-test. Significant differences were determined at $p < 0.01$. All the data were tested for normality assumption and homogeneity of variance before performing the statistical tests.

2.3 Results

2.3.1 Metabolic profiles of live *P. contorta* trees inoculated with symbiotic fungi

We quantified 20 metabolites: monoterpenes (β-phellandrene, β-ocimene, 3-carene, terpinolene, limonene, β-pinene, camphene, β-myrcene, bornyl acetate, α-pinene, p-cymene, and geranyl acetate), sesquiterpenes (germacrene-d-4-ol, β-caryophyllene), diterpene (epi-13-manool), and oxygenated monoterpenes (α-terpineol, γ-terpinene, terpinen-4-ol, borneol, camphor). The metabolic profile of trees over 6 weeks was visualized by a heat map using the actual concentration of metabolites. The heat map provided interesting findings, including (1) inoculations with all fungal symbionts led to the induction of all of the host monoterpenes, relative to the control treatment; (2) the highest metabolite induction occurred at week 2 and thereafter, the concentrations decreased; (3) the concentrations of oxygenated monoterpenes increased by several folds following fungal inoculations compared to the control at weeks 2 and 4 (**Figure 2.1**; Appendix A: **Table A2**–A5).

We performed PCA combined with PERMANOVA to investigate the effects of different explanatory variables on the changes in metabolite concentrations over time. The PCA 1 and 2 explained a total of 71% variance in the metabolite concentrations (**Figure 2.** ***2***). While concentrations in control and fungal treatments at week 0 clustered closely, treatment clusters separated away from each other by weeks 2–6. Furthermore, the PERMANOVA test revealed significant differences in the metabolite concentrations over time ($R^2 = 0.33$, $F_3 = 28.41$, $p = 0.001$). Treatment and treatment × time interaction were also significant ($F_3 = 4.66$, $R^2 = 0.05$, $p = 0.001$ and $F_9 = 1.48$, $R^2 = 0.05$; $p = 0.04$ respectively; **Figure 2. 2**).

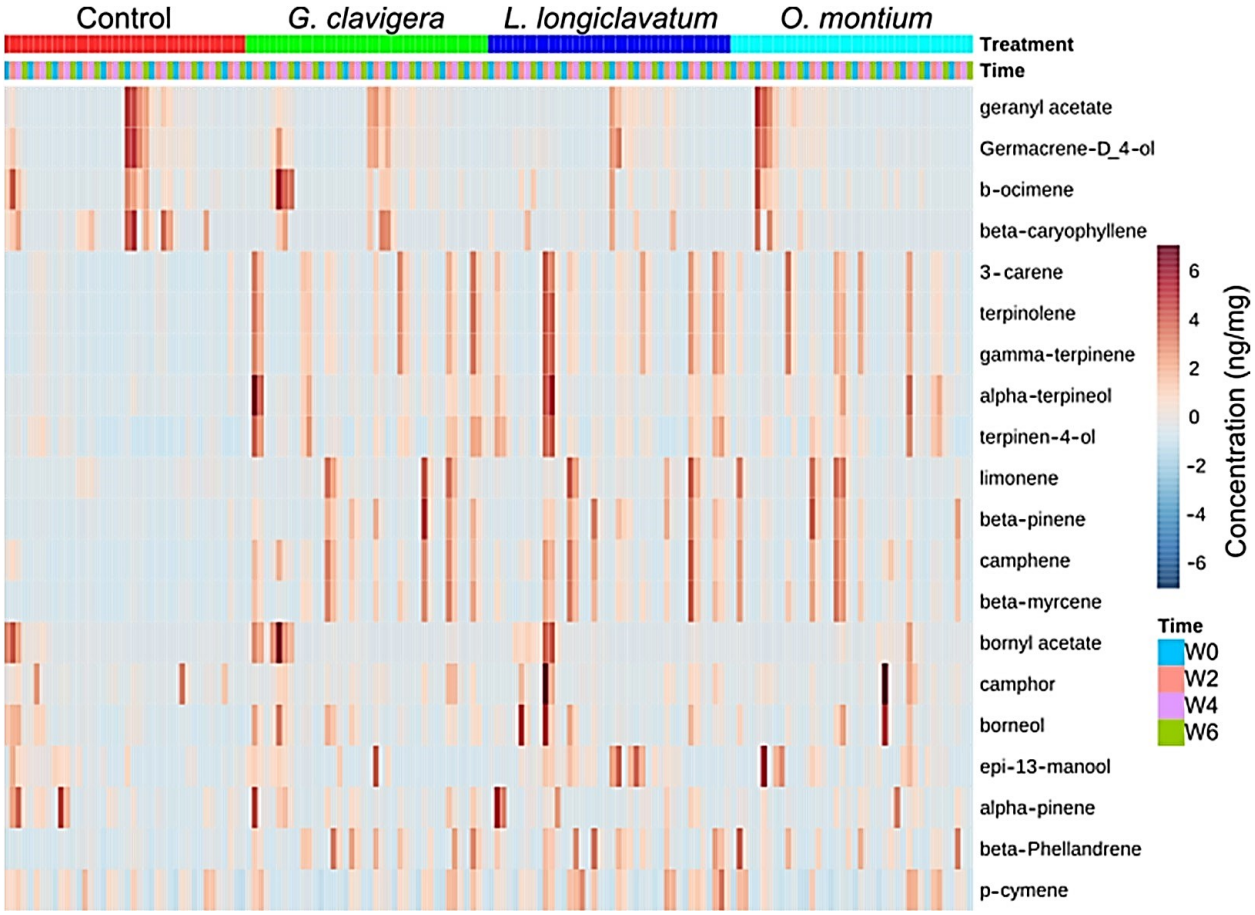

Figure 2.1: Heat map analysis of the secondary metabolites in *Pinus contorta* var. *latifolia* phloem (treatment and control groups) during the period of weeks 0 to 6 (n = 10). The color gradient panel on the right represents the highest to lowest concentrations from the darkest red to the darkest blue, respectively. Legends for time factor denoted by W0 = week 0, W2 = week 2, W4 = week 4 and W6 = week 6. Here, the distance was measured by the Euclidean method.

We also conducted two-way repeated measure ANOVA (within subjects) to analyze which factors (time, treatment, and their interaction) caused differences among metabolites. Out of 20 metabolites, the concentrations of 5, 17, and 8 metabolites were affected by treatments, time, and their interaction, respectively (**Table 2.1**).

Furthermore, we performed ASCA to ascertain the trends associated with different treatments, time, and their interactions (He et al., 2019). The score plot for the time factor with component 1 (98.07% of variation explained) of the corresponding model showed a decrease in scores from week 0 to week 2, then increased at weeks 4 and 6 (**Figure 2. 3**).

Table 2.1: The metabolite profile of *Pinus contorta* var. *latifolia* phloem inoculated with the fungal symbionts of *Dendroctonus ponderosae*. Biomarkers are selected based on statistical significance in two-way ANOVA and well-modeled with Hotelling's T2 value > 10.

Metabolites	Treatment			Time			Interaction			Hotelling-T^2		
	F	*p**	*pˆ*	F	*p**	*pˆ*	F	*p**	*pˆ*	Gc	Ll	Om
β-Myrcene	6.333	0.001	0.026	80.864	1.69×10^{-27}	3.39×10^{-26}	6.085	6.93×10^{-7}	1.39×10^{-5}	27.102	17.5	15.052
β-Pinene	5.311	0.004	0.026	43.314	1.86×10^{-18}	5.31×10^{-18}	4.387	6.72×10^{-5}	0.001	23.415	21.095	13.926
Camphene	5.077	0.005	0.026	71.673	1.39×10^{-25}	1.39×10^{-24}	4.9301	1.52×10^{-5}	0.000	8.676	5.779	5.2164
β-Phellandrene	5.043	0.005	0.026	30.098	3.20×10^{-14}	6.41×10^{-14}	2.994	0.003	0.010	61.257	25.006	44.444
γ-Terpinene	3.172	0.036	0.143	57.656	2.49×10^{-22}	1.14×10^{-21}	3.653	0.001	0.003	1.309	0.8404	0.616
3-Carene	2.831	0.052	0.173	57.416	2.86×10^{-22}	1.14×10^{-21}	3.348	0.001	0.005	23.153	18.273	13.56
Terpinolene	2.654	0.063	0.181	49.326	3.69×10^{-20}	1.23×10^{-19}	2.975	0.003	0.010	16.078	11.555	9.421
Terpinen-4-ol	2.083	0.120	0.299	34.55	9.82×10^{-16}	2.46×10^{-15}	2.196	0.028	0.055	0.039	0.025	0.019
Limonene	1.924	0.143	0.318	22.144	2.99×10^{-11}	5.43×10^{-11}	2.533	0.011	0.025	10.265	8.910	9.106
Borneol	1.769	0.171	0.341	34.235	1.25×10^{-15}	2.77×10^{-15}	2.675	0.008	0.019	0.014	0.014	0.009
p-Cymene	1.349	0.274	0.456	68.208	8.04×10^{-25}	5.36×10^{-24}	1.217	0.292	0.450	0.021	0.103	0.021
α-Pinene	1.206	0.321	0.494	15.264	2.39×10^{-8}	3.67×10^{-8}	1.842	0.069	0.125	11.495	8.122	4.828
α-Terpineol	1.036	0.388	0.537	18.548	8.90×10^{-10}	1.48×10^{-9}	1.040	0.413	0.551	0.180	0.078	0.063
Camphor	0.959	0.423	0.537	14.371	6.05×10^{-8}	8.64×10^{-8}	1.130	0.348	0.498	0.001	0.001	0.001
epi-13-Manool	0.944	0.430	0.537	3.202	0.026	0.031	1.359	0.221	0.368	3.295	4.982	1.629
Germacrene-D-4-ol	0.284	0.837	0.877	5.457	0.002	0.002	0.581	0.811	0.897	3.313	1.627	1.228
Bornyl acetate	0.227	0.877	0.877	4.914	0.003	0.004	0.528	0.852	0.897	1.369	1.187	0.709

*p**: raw *p*-value; *pˆ*: adjusted *p*-value; Gc: *Grosmannia clavigera*, Ll: *Leptographium longiclavatum*, Om: *Ophiostoma montium*.

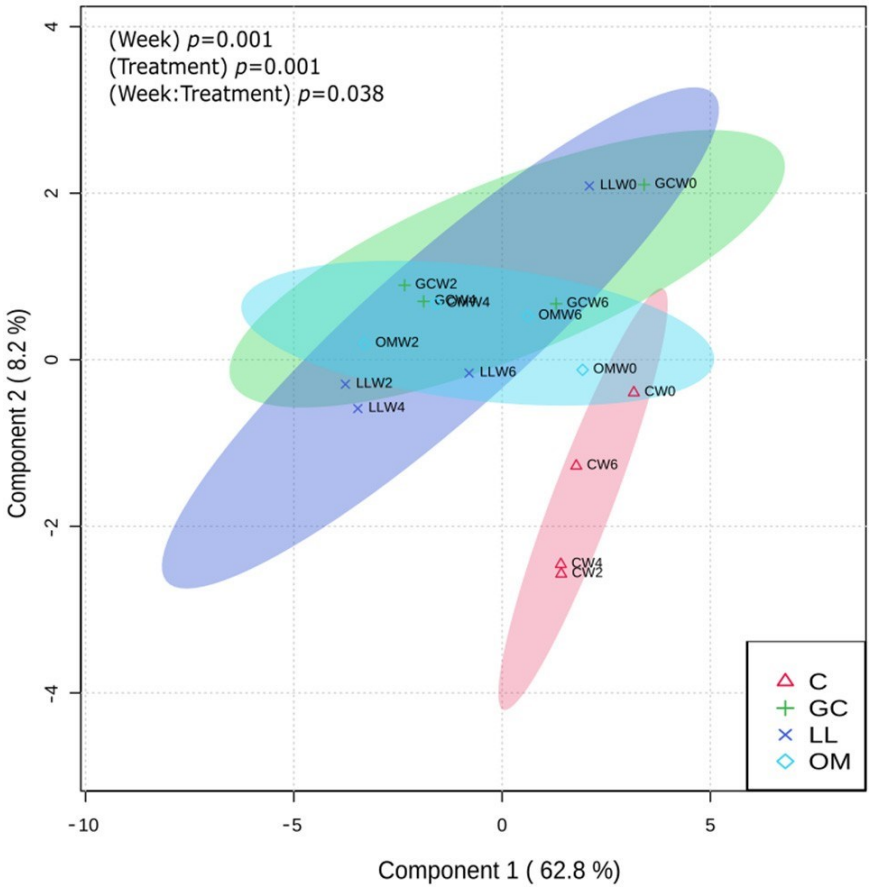

Figure 2. 2*:* Principal component analysis of secondary metabolites of *Pinus contorta* var. *latifolia* inoculated with the three fungal symbionts of *Dendroctonus ponderosae.* Here, C = control, GC = *Grosmannia clavigera*, LL = *Leptographium longiclavatum*, OM = *Ophiostoma montium*. The clusters of different treatments were denoted with different colors and 95% confidence interval eclipses. Significant differences among treatments were determined by perMANOVA.

The score plot for the treatment factor showed that treatment types differed in their PC1 scores; the control treatment score was higher than those of fungal treatments, with *L. longiclavatum* having the lowest score (**Figure 2. 3**). The interaction effect score plot exhibited clear opposite trends at week 2 between treatments and control (**Figure 2. 3**). To correlate metabolic features with explanatory variables, we further constructed Leverage/squared prediction error (SPE) plots. Leverage assesses the importance of metabolites to the model, and SPE tests the model's

fitness for a particular metabolite. Well-modeled metabolites were selected based on high-leverage SPEs that contribute significantly to the model.

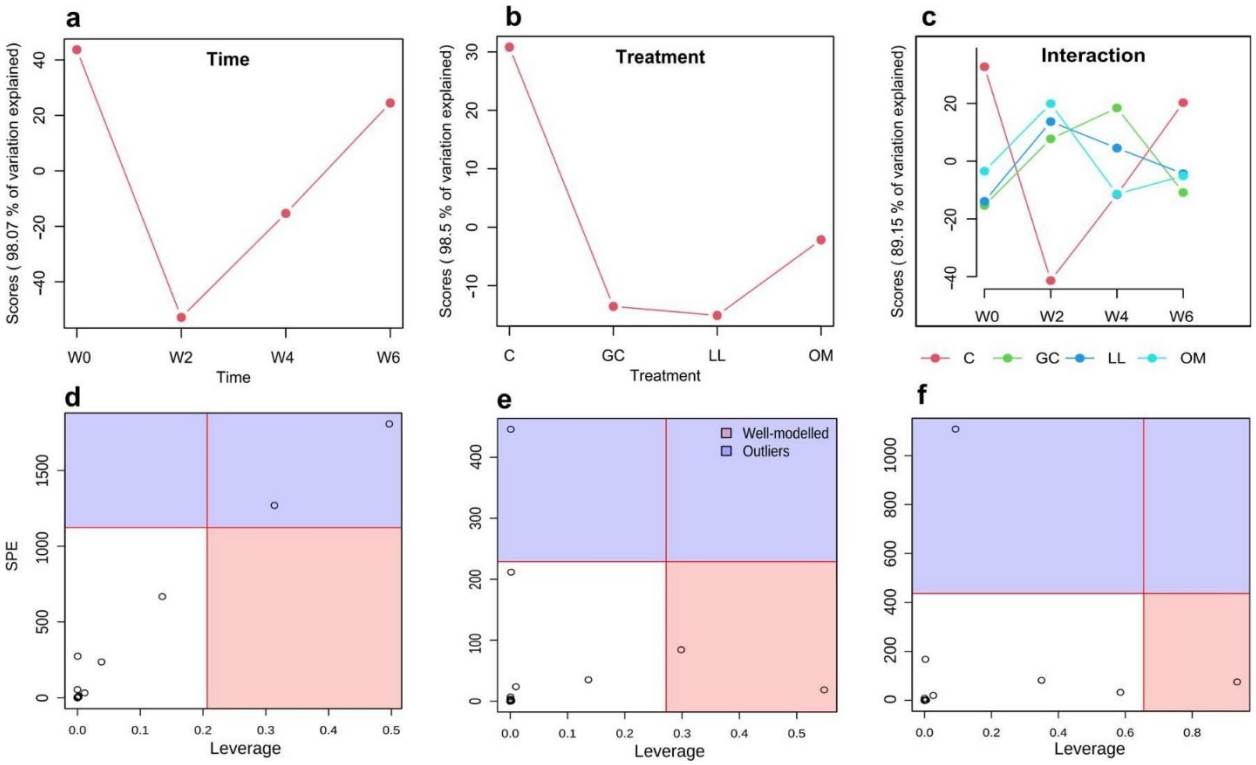

Figure 2. 3: ANOVA-simultaneous component analysis (ASCA) of the induced secondary metabolites of *Pinus contorta* var. *latifolia* phloem following fungal inoculations of trees from weeks 0 to 6. (**a**–**c**) Major pattern related to time, treatments, and interaction between them; (**d**–**f**) important variables (metabolites) selected by ASCA related to time, treatments and their interaction respectively calculated by leverage/SPE analysis.

The dots in the red area of **Figure 2. 3**e correspond to β-myrcene, β-pinene while the dot in Fig. 2.3f shows β-phellandrene. We then further conducted multivariate empirical Bayes (MEBA) time-series analysis to specify metabolic biomarkers that significantly (Hotelling T^2 value > 10) shifted in comparison to the control (**Table 2.*1***). Analyzing both models, four metabolites were selected out of 20, which were considered potential biomarkers (β-phellandrene, β-myrcene, β-pinene, and 3-carene; **Figure 2.4**, **Table 2.1**).

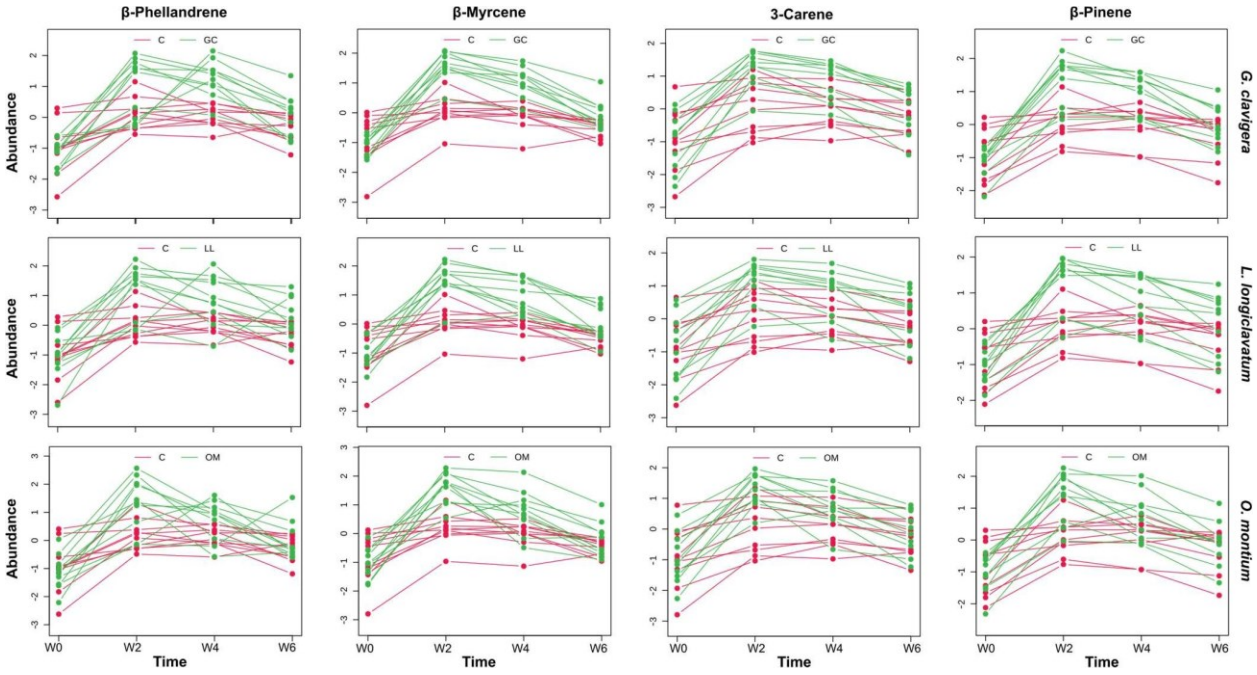

Figure 2.4: Multivariate empirical Bayes time-series analysis of *Pinus contorta* var. *latifolia* metabolites following inoculations by the fungal symbionts of *Dendroctonus ponderosae* selected based on two-way ANOVA, well-modeled by SPE and Hotelling's T2 value > 10. Here, C = control; GC = *Grosmannia clavigera*; LL = *Leptographium longiclavatum*; OM = *Ophiostoma montium*.

2.3.2 Kinetic metabolic pattern of potential biomarkers following fungal infection

Following two-way ANOVA and ASCA, the four metabolites that were affected by either time or treatments or their interactions were further analyzed by MEBA to see the kinetic pattern change over time. Week 2 was the critical time point when the abundance of all four metabolites increased sharply (**Figure 2.4**). By weeks 4 and 6, the abundance of metabolites decreased gradually. In response to all three fungal inoculations, the abundance of β-phellandrene was increased up to four-fold at week 2 compared to the control (Hotelling's T^2 value > 20; F = 30.10, $p < 0.05$; **Figure 2.4**, **Table 2.1**). The abundance of β-myrcene in all fungal treatments significantly increased up to five-fold by week 2 com-pared to the control (time: F = 80.86, $p < 0.001$; treatment: F = 6.33, $p < 0.05$). Similarly, 3-carene was also significantly upregulated up to two-fold by week 2 compared to the control in all three fungal treatments (time: F = 57.41, *p*

<0.001). The abundance of β-pinene was affected by both time and treatment (time: F = 43.31, p < 0.001; treatment: F = 5.31, p < 0.05; Fig. 2.4, Table 2.1).

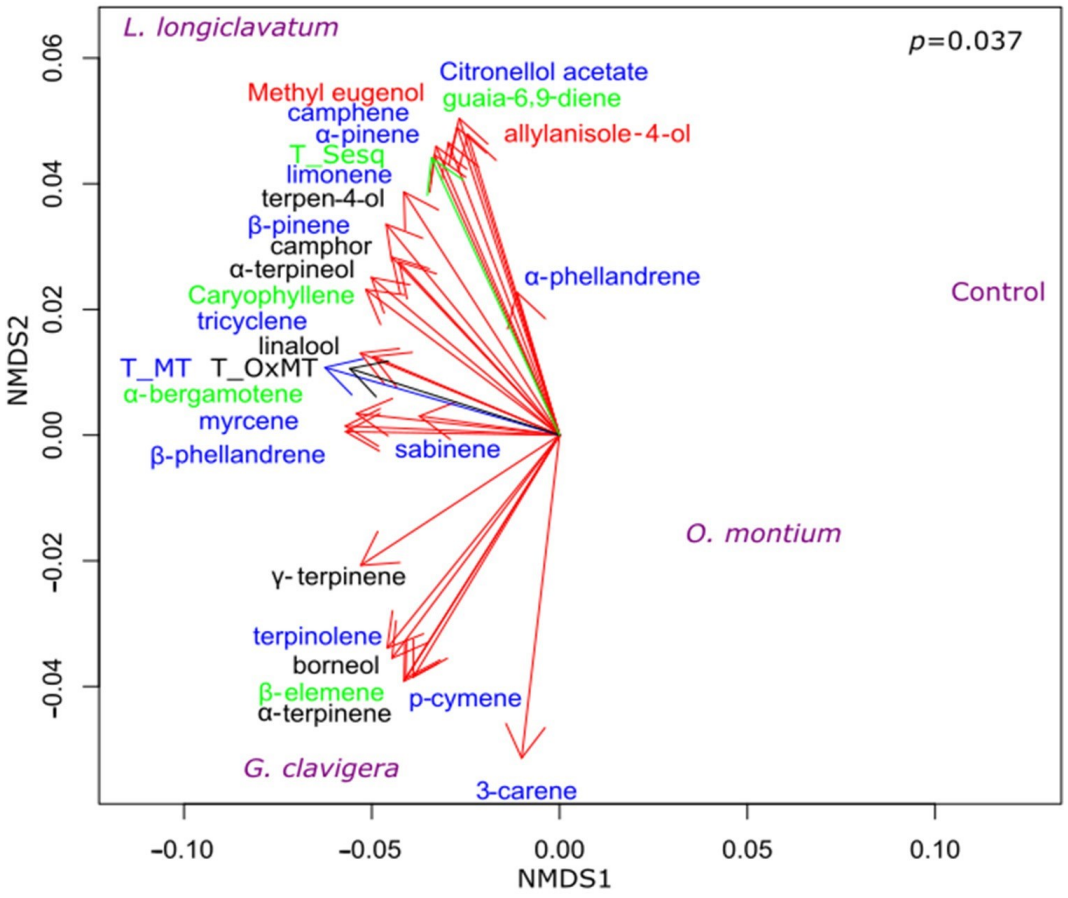

Figure 2.5: Effects of the inoculations by the fungal symbionts of *Dendroctonus ponderosae* on the terpene concentration of *Pinus contorta* var. *latifolia* phloem (logs). Individual terpene concentrations (ng mg^{-1} DW) were used in the analysis. Data were analyzed using NMDS gradient analysis. Treatments, monoterpenes, oxygenated monoterpenes, sesquiterpenes, and phenylpropanes were represented in violet, blue, black, green, and red, respectively (all red vectors). Significant differences between treatments were determined by PERMANOVA at p < 0.05. T_MT = Total Monoterpenes (blue vector), T_OxMT = Total Oxygenated Monoterpenes (black vector), T_sesq = Total Sesquiterpenes (green vector).

2.3.3 Effect of fungal inoculations on chemotypic traits of *P. contorta* logs

We performed NMDS on the metabolites collected from infected and non-infected phloem tissues of logs. The analysis was combined with the PERMANOVA test to further investigate the

significance of treatment effects. We found that fungal inoculations significantly altered the metabolite concentrations (F = 4.66, $p < 0.05$; **Figure 2.5**). The total monoterpene and the total oxygenated monoterpene concentrations were correlated with both *G. clavigera* and *L. longiclavatum*, whereas the total sesquiterpene concentration was correlated with *L. longiclavatum*. Phenylpropenes such as methyl eugenol and allylanisole-4-ol were correlated with *L. longiclavatum* (**Figure 2.5**).

Symbiotic fungi also differed in their virulence (F_3, 14.24 = 135.50). Overall, *G. clavigera* induced the largest lesion (total fungal infected area on phloem) area compared *to L. longiclavatum* ($p < 0.001$), *O. montium* ($p < 0.001$), and control ($p < 0.001$) treatments. Both *L. longiclavatum* and *O. montium* had similar lesion areas ($p > 0.99$) while larger than the control ($p < 0.001$; **Figure 2.6**a–d). In pre-fungal inoculation samples, we detected a total of 23 compounds: α-pinene, camphene, β-myrcene, 3-carene, limonene, α-terpinene, p-cymene, γ-terpinene, terpinolene, linalool, β-phellandrene, α-phellandrene, β-pinene, α-terpineol, bornyl acetate, aromadendrene, allylanisole-4-ol, germacrene-d-4-ol, δ-cadinol, γ-cadinene, α-muurolene, guaia-6,9-diene, δ-cadinene. In 14 days post fungal inoculated samples, we detected additional 24 compounds that were not detected constitutively: tricyclene, borneol, camphor, terpen-4-ol, methyl eugenol, caryophyllene, α-bergamotene, β-elemene, citronellol acetate, acetoin, grandisol, isobutanol, phenethyl alcohol, verbe-none, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-2-butanol, 2,4-dimethyl-1-heptene, 4-methyl-octane, 4-methylheptane, 1-butanol, 3-methyl-2-butanone, 2-ethyl-1-butanol, 3,4-dimethoxyphenol (Appendix A: **Table A1**).

2.3.4 Symbiotic fungi influence the proportion of oxygenated monoterpenes

To investigate whether the fungal inoculations can alter the concentration of oxygenated monoterpenes, we conducted a fold change analysis combined with a parametric t-test. Interestingly, all three fungi significantly upregulated borneol at least two-fold compared to the control (**Figure 2.6**a–c). *Leptographium longiclavatum* significantly upregulated α-terpineol up to four-fold (FC = 4.78, $p < 0.001$), and *G. clavigera* was upregulated two-fold (FC = 2.16, $p < 0.05$) relative to the control. Both *G. clavigera* and *L. longiclavatum* significantly increased the concentration of terpinen-4-ol compared to the control and *O. montium* (FC = 2.53 and 6.77, respectively; $p < 0.05$).

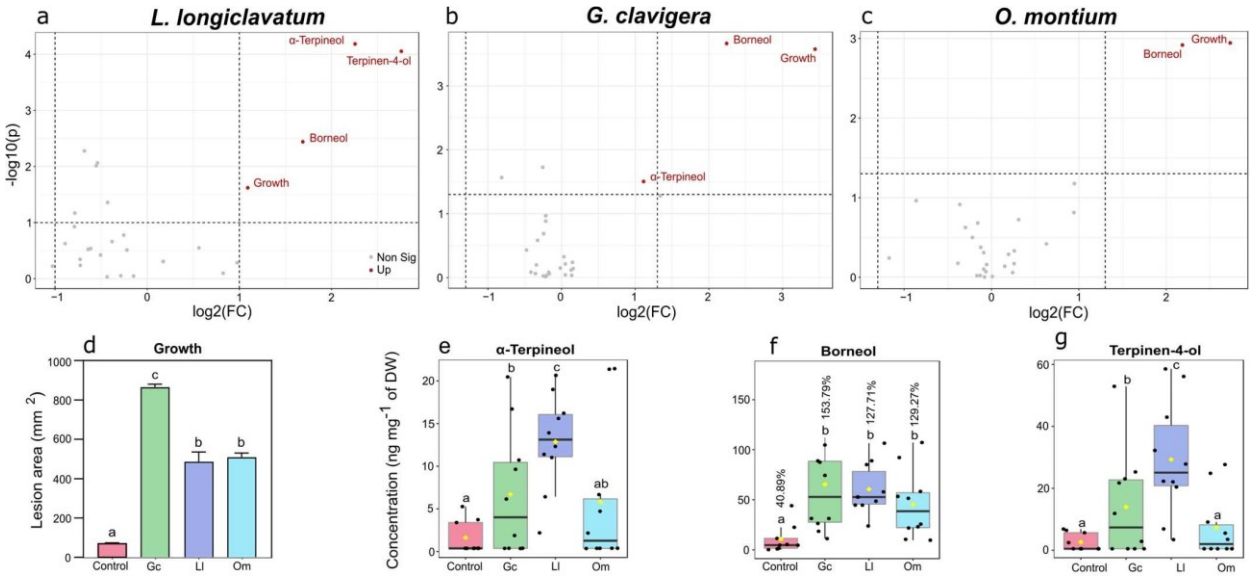

Figure 2.6: Upregulation of oxygenated monoterpenes in *Pinus contorta* var. *latifolia* phloem following inoculations by the fungal symbionts of *Dendroctonus ponderosae*. (**a**–**c**) Volcano plots show the fold change analysis of oxygenated monoterpenes combined with t-test (**a**) *Leptographium longiclavatum*, (**b**) *Grosmannia clavigera*, (**c**) *Ophiostoma montium* (Fold change analysis in X-axis, T-test in Y-axis; $p < 0.05$), (**d**) compar- ison of symbiotic fungal growth on the phloem, (**e**–**g**) upregulation of oxygenated monoterpenes such as (**e**) α-terpineol, (**f**) borneol, and (**g**) terpinen-4-ol by fungal infection (One-way ANOVA; followed Fisher's LSD; $p < 0.05$), (**f**) proportional increment of borneol to bornyl acetate in the phloem tissues inoculated with symbiotic fungi compared to control after 14 days (One-way ANOVA followed by Fisher's LSD test at $p < 0.05$). Significant differences (**d**–**g**) between treatments are denoted by small letters.

Logs inoculated with all three fungi caused a stronger proportional increment of borneol to bornyl acetate compared with the control, corresponding to an over three-fold increase (**Figure 2.6**f). Moreover, in the log experiment, we detected bornyl acetate at day 0 only before the fungal inoculations but not borneol and camphor, which were only detected on day 14 post-inoculation. In addition, there was a positive correlation between fungi-induced lesions and borneol ($R^2 = 0.5253$, Spearman's correlation, $p < 0.001$). Altogether, the oxygenated monoterpene concentration increased several folds post-fungal treatments (Appendix A: **Figure A1**).

2.3.5 Mountain pine beetles were attracted to their symbiotic fungi

Two-choice olfactometer assay revealed significant results between control and fungal symbionts (**Figure 2.*7***). Here, both *G. clavigera* and *O. montium* attracted 80% of tested beetles as compared to the control ($p < 0.01$). In contrast, *L. longiclavatum* only attracted 25% of the tested beetle as compared to the control, while the remaining 75% showed attraction towards the control treatment ($p < 0.01$). Interestingly, concentrations of most of the FVOCs were comparatively higher in *L. longiclavatum* (**Figure 2.*8***). The FVOC identified were as follows acetoin, grandisol, isobutanol, phenethyl alcohol, verbenone, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-2-butanol, 2,4-dimethyl-1-heptene, 4-methyl-octane, 4-methylheptane, 1-butanol, 3-methyl-2-butanone, 2-ethyl-1-butanol, and 3,4-dimethoxyphenol (**Figure 2.*8***, Appendix A: **Table A1**).

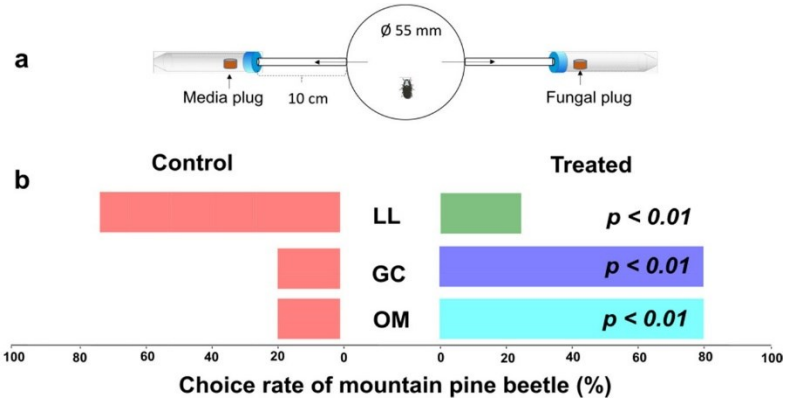

Figure 2.7: Behavioral responses of female *Dendroctonus ponderosae* to different fungal symbionts. (**a**) Experimental setup (described in methods). (**b**) The right bar graph shows beetle responses to fungal treatments, and the left bar graph shows beetle responses to controls, *p*-values show a significant difference at the 0.05 level by conducting a two-sample t-test. Here, LL = *Leptographium longiclavatum*, GC = *Grosmannia clavigera*, OM = *Ophiostoma montium*.

2.4 Discussion

We clearly show that MPB fungal symbionts can upregulate tree terpene defenses both in mature trees and logs and modify host monoterpenes to oxygenated derivatives in logs. Time seems to be a crucial factor in tree-induced responses, as the highest induction occurred two weeks after inoculations over the six-week duration of the experiment. Furthermore, the three fungal symbionts differed in their virulence as evidenced by differences in lesion lengths, the

conversion efficiency of monoterpenes to oxygenated monoterpenes, and attraction to MPB via FVOCs. Together, these results demonstrate that MPB fungal symbionts play crucial roles during host colonization by bark beetles, including assisting beetles in the alteration of host tree defenses and likely increasing beetle attraction via the production of oxygenated monoterpenes and FVOCs (Frago et al., 2012; Lieutier et al., 2009; Westrick et al., 2021). Furthermore, both field and laboratory experiments provide complementary information that cannot be achieved by either alone.

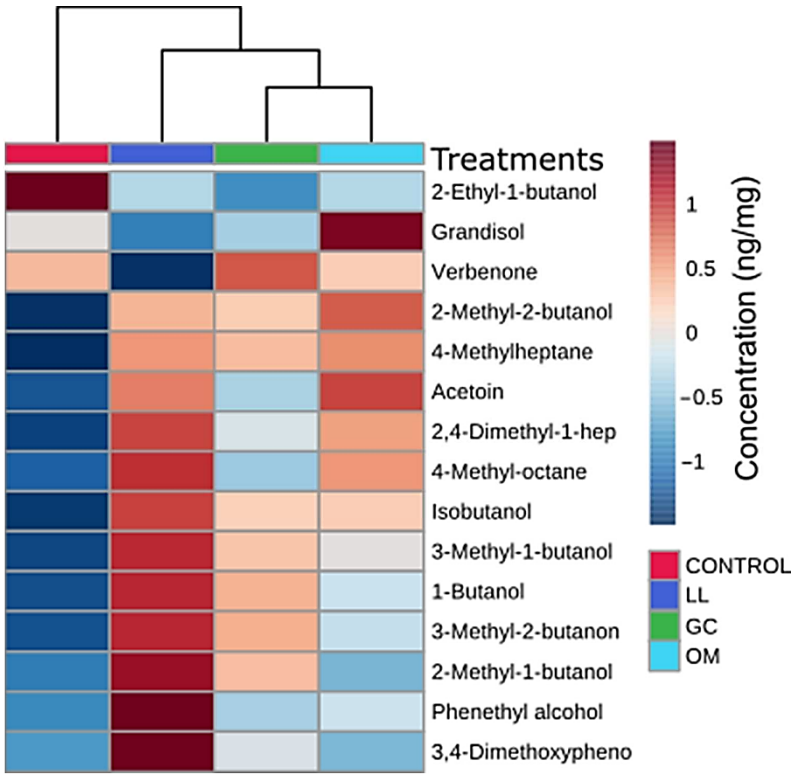

Figure 2.8: Heat map analysis, combined with hierarchical cluster analysis (HCA) of the fungal volatile organic compounds in *Pinus contorta* var. *latifolia* a phloem (treatment and control groups) after 14 days (n = 10). The color gradient panel on the right represents metabolic abundance from the darkest red (high) to the darkest blue (low). Legends for treatment factor denoted by GC = *Grosmannia clavigera*, LL = *Leptographium longiclavatum*, OM = *Ophiostoma montium*. Here, the distance was measured by the Euclidean method and clusters were prepared by the Ward clustering algorithm method.

2.4.1 Several monoterpene biomarkers are associated with tree responses to fungal inoculations

We show that low-density fungal inoculations can upregulate defense metabolites of lodgepole pine trees as fast as two weeks following inoculations, in agreement with earlier investigations in this (Kim et al., 2008; Plattner et al., 2008; Rice et al., 2007) and other (Ben Jamaa et al., 2007; Brignolas et al., 1995; Zhao et al., 2011) study systems. However, not all terpenes were similarly upregulated as the concentrations of some of the monoterpenes and diterpenes were increased, while concentrations of all sesquiterpenes identified remained similar over the period of 6 weeks. Among monoterpenes, concentrations of β-phellandrene, β-myrcene, β-pinene, and 3-carene were several fold greater in the fungal inoculated trees, relative to the control, parallel to the results of other studies (Cale et al., 2019b; Ullah et al., 2021). Some of these monoterpenes are reported to be highly toxic to MPB (Chiu et al., 2017), supporting their importance in tree resistance. Among diterpenes, we found upregulation of 13-epi-manool which can suppress the reproduction and growth of fungal pathogens (Cheng et al., 2012). This is the first report of this labdane diterpenoid in response to the fungal inoculations in lodgepole pine. These results suggest that β-phellandrene, β-myrcene, β-pinene, 3-carene, and 13-epi-manool can be potential biomarkers and important components of the host chemical defenses against the fungal infection.

2.4.2 Conversion of monoterpenes to oxygenated derivatives appears to be a common strategy to reduce the toxicity among bark beetles

Conifer monoterpenes are toxic to several species of bark beetles, including MPB (Chiu et al., 2017; Coats et al., 1991; Everaerts et al., 1988; Regnault-Roger and Hamraoui, 1995; Reid et al., 2017; Scalerandi et al., 2018; Werner, 1995), and their conversion to oxygenated derivatives may lessen their toxicity. For example, α-pinene and myrcene were reported to be more toxic than their oxygenated derivatives, bornyl acetate and linalool, respectively, to bark beetles (Chiu et al., 2017; Davis, 2020; Everaerts et al., 1988). Therefore, the conversion of monoterpenes to their less toxic oxygenated derivatives by fungal symbionts could reduce the extent of monoterpene toxicity to MPB. Earlier studies reported a similar detoxification mechanism by fungal symbionts of several bark beetle species (Hammerbacher et al., 2013; Nones et al., 2022). Auto-oxidation of monoterpenes can also occur as the resin encounters air; however, in this study, we found the concentrations of several oxygenated monoterpenes such as borneol and terpinene-4-ol

were multiple fold higher in the fungal treated tissues compared to the control, suggesting the possible role of fungi in the detoxification process. In support of this, Wang et al. (2014) reported that *G. clavigera* contains genes encoding cytochromes P450 and several other oxidative enzymes that can degrade and utilize monoterpenes, such as limonene. Interestingly, some of the oxygenated monoterpenes, such as borneol, were reported to elicit attraction in MPB (Pureswaran et al., 2000).

2.4.3 Fungal-produced volatile organic compounds serve as attractant cues for beetles

Fungal symbionts of bark beetles are reported are reported to synthesize VOCs de novo which act as attractants for several species of bark beetles (Cale et al., 2016; Kandasamy et al., 2019, 2016). Here, we demonstrated a close-range attraction of MPBs to VOCs of their fungal symbionts. Among them, phenethyl alcohol was reported as an attractant for MPB in field tests (Pureswaran et al., 2000). These results complement the vast literature on the attractiveness of FVOCs in other bark beetle species (Christiaens et al., 2014; Hulcr et al., 2011; Luna et al., 2014; Saerens et al., 2010). For instance, the fungal volatiles 2-phenylethyl acetate and 3-methyl-1-butyl acetate increased the attraction of *D. frontalis* to its pheromone blend (Brand et al., 1977). Similarly, *I. typographus* utilizes the fungal volatile 2-methyl-3-buten-2-ol as aggregation pheromone (Kandasamy et al., 2019; Raffa et al., 2016); other FVOCs released from the same fungal symbionts improved the attraction of *I. typographus* to its pheromone (Jirošová et al., 2022; Kandasamy et al., 2023). Furthermore, the walnut twig beetle, *Pityophthorus juglandis*, was attracted to the FVOCs produced by its primary bark fungi (Davis et al., 2013). In the current study, we did not detect all FVOCs reported in our earlier studies (Cale et al., 2019a, 2016; Wang et al., 2020), probably due to differences in the timing of volatile collection between studies. Nevertheless, our olfactometer experiment reveals that MPB can recognize their fungal symbionts by detecting their FVOCs. Interestingly, not all three fungal species tested were attractive to MPB, as 80% of adult MPB tested were attracted to *G. clavigera* and *O. montium*, but only 25% of beetles were attracted to *L. longiclavatum*. Such differences in attraction may be attributed to the greater abundance of most of the FVOCs associated with *L. longiclavatum* relative to those of *G. clavigera* and *O. montium*.

2.5 Conclusions

We demonstrate that fungal symbionts of MPB can upregulate host tree defense metabolites and convert monoterpenes to oxygenated derivatives. Through this mechanism, fungi can help beetles to exhaust and deplete terpene defenses, enabling beetles to overcome host resistance and making host substrates suitable for larval growth (Kim et al., 2008). Maintaining multiple symbionts provides the beetles with a variety of benefits, including nutritional supplementation, protection, and many other complementary benefits. We further propose that *de novo* synthesized FVOCs volatiles and oxygenated monoterpenes may improve the attraction of bark beetles to trees during host colonization. However, FVOCs and oxygenated monoterpenes can be attractive or repellent to beetles depending on their specific concentrations. Whether FVOCs elicit behavioral responses in bark beetles should be verified in the field experiment. Nevertheless, FVOCs and oxygenated monoterpenes can be potential components in integrated pest management strategies to control the bark beetle population (Davis et al., 2013; Jirošová et al., 2022; Kandasamy et al., 2016). The potential tree resistance biomarkers we have identified in our study can be used in tree breeding through genomic approaches to generate more resistant trees to beetle-fungal attack.

Chapter 3: A pine in distress: how infection by different pathogenic fungi affect lodgepole pine chemical defenses

3.1 Introduction

Global climate change has increased the frequency of pest outbreaks in many forest ecosystems, resulting in significant tree mortality and decline in biodiversity (Kausrud et al., 2012; Teshome et al., 2020). As one of the most widespread plant communities, coniferous forest trees encounter a vast diversity of insect pests and pathogens with attacks by some organisms becoming more frequent and long-lasting as the climatic conditions change. However, conifers are equipped with a biosynthetic machinery that strategically repels, and/or resists herbivores (Celedon and Bohlmann, 2019; Franceschi et al., 2005). Plant secondary metabolites, also called defense metabolites, such as terpenes constitute chemical defense machinery of conifers against an array of biotic stressors. Conifers have high chemical and structural diversity of terpenes (Franceschi et al., 2005; Holopainen et al., 2018). In general, plant secondary metabolites are expressed constitutively or induced. The preformed constitutive defenses constitute the first line of defense against colonizers. Continuous attacks trigger the induced defenses which may differ from the constitutive defenses qualitatively and/or quantitatively and are usually long lasting (Erbilgin et al., 2017b; Franceschi et al., 2005; Holopainen et al., 2018). The expression of plant induced defenses substantially varies with the species of organisms colonizing the same host tree (Kandasamy et al., 2023; Pichersky and Raguso, 2018; Zaman et al., 2023b). In many plant systems, we lack studies comparing defense responses of plants to multiple pathogenic organisms. A clear understanding of differences in plants responses to the different biotic stressors may explain, for instance, why some pest attacks may make plant resistant to the subsequent attacks by the same or different organisms (Mertens et al., 2021).

Lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm) is one of the most abundant coniferous species in western North America. In western Canada, lodgepole pine forests represent approximately 35% of the forested lands in British Columbia and Alberta (Critchfield, 1985). Lodgepole pines are affected by several pathogenic fungal diseases.

Atropellis canker, *Atropellis piniphila* (NOF3174), and western gall rust, *Cronartium harknessii* (J.P. Moore) E. Meinecke are two of the most common fungal pathogens affecting lodgepole trees in western Canada (Van der Kamp, 1994). It is unknown how infection by such pathogens alters lodgepole pine susceptibility to further disease and insect colonisations.

Atropellis piniphila (Helotiales; Ascomycetes) infects the cambium layer of lodgepole pine and the infection creates highly visible signs on the stems of infected trees. Typically, the pathogen enters the tree through branch nodes or wounds on the lower stem created by fire or any other animal damage. Such infection possibly affects the cambium hindering the water and nutrient flow between roots and canopy. Fruiting body formation may take up to 15 years on large grown trees. The infection eventually results in stunted tree growth, reduced photosynthesis, carbon and nutrient storage (Achotegui-Castells et al., 2016; Hopkins, 1963; Hossain et al., 2018; Muthuchelian et al., 2005). The infection can also elevate defense-related metabolites (Wallis et al., 2008). *Cronartium harknessii* (Uredinales; Basiodiomycetes) is characterized by the formation of galls on branches and stems of the infected pine trees. During spring, spores are released from the infected pine trees and carried by wind and infect the emerging shoots and cones on other pine trees. The lower portion of the crown is more susceptible to infection due to its ability to retain moisture. Within the cambial tissues, the fungus begins to grow. As a result, round swellings known as galls are formed on branches, twigs, and stems. Over time, these galls constrict and eventually cause the death of the affected branch or stem (Hoffman and Hagle, 2011). Currently, how *C. harknessii* affects lodgepole pine defense chemistry is unknown but trees infected with another species of gall rust (*Uromycladium falcatarium*) have been found to have increased production of secondary metabolites, particularly, phenolics and terpenes (Rahmawati et al., 2019).

We also included infection by three phytopathogenic fungal associates (Ophiostomatales; Ascomycetes) of the invasive mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB; Coleoptera: Curculionidae, Scolytinae), *Grosmannia clavigera* (Robinson-Jeffery and Davidson) Zipfel, de Beer, and Wing., *Ophiostoma montium* (Rumford) von Arx, and *Leptographium longiclavatum* Lee, Kim, and Breuil. The mountain pine beetle has a profound impact on lodgepole pine forests in western Canada. MPB infestations lead to the widespread death of lodgepole pines, resulting in significant changes to forest composition and structure. These

infestations disrupt the natural succession of tree species and can lead to an increased risk of wildfires (Cale et al., 2019b; DiGuistini et al., 2011; Erbilgin, 2019; Lee et al., 2006b). Symbiotic fungi play a crucial role in the biology of the mountain pine beetle. The fungi colonize the tree, breaking down its defenses and facilitating beetle reproduction. Additionally, the fungi provide essential nutrients to the beetles, aiding in their development and survival. This symbiotic relationship between MPB and fungal symbionts highlights the intricate ecological dynamics at play during infestations and emphasizes the interdependence between organisms in forest ecosystems (Erbilgin, 2019; Lieutier et al., 2009; Raffa et al., 2008).

Bark beetle attacks initiate with boring holes in the bark and gallery formation within the phloem while introducing the fungal symbionts simultaneously. Both beetle infestation and fungal introduction stimulate tree defense in phloem and sapwood, aiding beetle establishment by overcoming tree resistance and defense exhaustion which further facilitates the fungal establishment in the galleries (DiGuistini et al., 2011). Fungal associates of bark beetles are commonly used for their combined effects on the defense responses of conifer trees attacked by bark beetles (Frago et al., 2012; Lieutier et al., 2009). Field studies commonly employ field inoculations with live fungal cultures and measure the changes in tree secondary metabolites in response to the inoculations. Knowledge in this area can help inform how bark-beetles utilize ophiostomatoid fungi to overcome host tree defenses (Kandasamy et al., 2023; Zaman et al., 2023b; Zhao et al., 2019). For instance, our recent study has revealed that fungal symbionts of MPB alter host defense chemistry and transform toxic monoterpenes to less toxic oxygenated monoterpenes, suggesting their potential role in making trees suitable for beetle development (Zaman et al., 2023b).

In this study, we have examined lodgepole pine defense response to infection by the five phytopathogenic fungi (*A. piniphila*, *C. harknessii*, three symbiotic fungi of MPB). We analyzed and compared the monoterpenes of lodgepole pine phloem infected by these fungi and uninfected host phloem tissues. Since these pathogenic fungi have the capacity to kill lodgepole pine, we hypothesized that both *A. piniphila* and *C. harknessii* alter the concentration of the host toxic monoterpenes in a similar capacity to that of ophiostomatoid fungi.

3.2 Methods

3.2.1 Sampling

We conducted a series of field experiments to investigate how five fungal pathogens alter the terpene chemistry of lodgepole pine phloem. To sample we located 29 mature lodgepole pine infected by *E. harknessii* in north-central Alberta (54°24' N, 115°34'W) (Ullah et al., 2021). We collected 2 phloem samples from each infected tree. These samples were taken at ca. 1 m up the stem on the north-facing side using a 2 cm dia. Punch (n=58). We also collected samples from 50 healthy pine trees in the same stand (4 samples from each tree; n=200). Similarly, eight lodgepole pine trees infected by *A. piniphila* were sampled in another lodgepole pine forest in Hinton, Alberta (53°30'50.7"N 117°17'31.2"W) (Zaman et al., 2023b). We collected at least six samples from each infected tree using a 2 cm dia. punch. Samples were collected near the canker infection site where trees showed advance canker development which was evident with the typical stem cankers located along pine stems. The number of *A. piniphila*-infected trees was low because of the scarcity of such trees in the field. All samples were collected in the months of July and August.

In the same forest stand, we located ten healthy lodgepole pine trees and inoculated them with three fungal associates of mountain pine beetle, *G. clavigera* (EL004), *O. montium* (EL031), and *L. longiclavatum* (EL002) (Zaman et al., 2023b). These fungal isolates were collected from MPB infected tissues and identified by Dr. Nadir Erbilgin from different locations during 2015-2016 (EL004 from Banff, Alberta; EL002 from Graham fire base, Alberta; EL031 from Westcastle, Alberta). The virulence of these isolates was determined earlier (Zaman et al., 2023b). We inoculated fungi by creating four cardinal holes on each tree at breast height and placed one fungal mycelium plug (2 cm) (one from each of three fungal species) in each hole, along with a control plug of agar (without fungal mycelium). Phloem samples were taken from the fungal-infected and upper parts of the inoculation point along the tree stems after two weeks (n=40). We used the week-2 data because the concentrations of monoterpenes and oxygenated monoterpenes were highest (Zaman et al., 2023b). In all cases, we wrapped all samples in aluminum foil, put them in dry ice, transported them to University of Alberta, and stored at –40 °C freezer in the laboratory until analysis.

3.2.2 Chemical extraction and analysis

Following the procedures described earlier (Erbilgin et al., 2017a) all samples were ground in liquid nitrogen using a pestle and mortar. We took a 100 mg of the ground tissues, extracted twice in 0.5 mL hexane with 0.004% pentadecane as an internal standard in 2 mL centrifuge tubes. After adding the solvent, the extracts were vortexed for 30 sec at 3,000 rpm, sonicated for 10 min, centrifuged at 15,000 rpm for 15 min at 4 °C, and then transferred into gas chromatography 1.5 mL vials, and finally placed in a –40 °C freezer until further analysis.

We transferred the extracts into gas chromatograph-mass spectrometer (GC-MS; GC:7890A, MS: 5975C, Agilent Tech, Santa Clara, CA, USA), equipped with a DB-5MS UI column (30 m x 0.25 mm ID x 0.25 um, Agilent Tech). The gas flow rate was at 1.1 mL min^{-1} with helium as a carrier gas. The initial temperature of the program was set up at 50 °C, held for 1 min and at 10 °C min^{-1} which increased up to 300 °C (held for 7 min). A 1 μL injection volume of sample was used with 250 °C inlet temperature along with split mode. The NIST 2017 Mass Spectral library version 2.3 was applied for verification of all compounds. All compounds we quantified were based on the following standards availability from commercial suppliers; Monoterpenes: limonene (Chemical Purity: 99%) , β-pinene (CP: 98%), β-myrcene (CP: 96%), α-pinene (CP: 98%), β-phellandrene (CP: 99%), ocimene (CP: 90%), β-thujone (CP: 92%), α-phellandrene (CP: 98%), p-cymene (CP: 99%), geranyl acetate (CP: 97%), terpinolene (CP: 94%), 3-carene (CP: 98%), camphene (CP: 90%),, α-terpinene (CP: 95%), γ-terpinene (CP: 97%), Sabinene hydrate (CP: 95%), Oxygenated monoterpenes: (-)-borneol (CP: 99%), camphor (CP: 99%), α-terpineol (CP: 99%), linalool (CP: 99%), Phenylpropenes: 4-allylanisole (CP: 99%), bornyl acetate (CP: 97%), Sesquiterpenes: (+) aromadendrene (CP: 97%), caryophyllene oxide (CP: 95%), Farnesol (CP: 95%), β-caryophyllene (CP: 80%), and Diterpenes: dehydroabietal (CP: 96%), abietic acid (CP: >94%), dehydroabietic acid (CP: 99%), sandarocopimaric acid (CP: 90%), isopimaric acid (CP: 99%), levopimaric acid (CP: 96%), neoabietic acid (CP: 99%), and palustric acid (CP: 91%). All standards were obtained from Sigma-Aldrich (Oakville, ON, CAN), except β-phellandrene from TRC Canada (Toronto, ON, CAN). For the quantification of some of the compounds, due to their unavailability in the market, we used some of the above-mentioned standards based on hydrocarbon groups along with unique ion masses. We identified compounds by comparing their retention times and mass spectra with those of the commercial

standards. Then calibration curves were generated using the standard compounds by combining a serial of three dilutions of known concentrations and calculated as "μg mg^{-1}" of fresh tissue.

3.2.3 Statistical analysis

The chemical concentration data matrices obtained from phloem samples were imported into the Metaboanalyst 5.0 platform for data exploration, visualization, and multivariate statistical analysis (http://www.metaboanalyst.ca, accessed on 1 April 2023). Any missing inputs (4 inputs) were replaced by values calculated as half of the minimum positive values present in the original data. To achieve a normal distribution, the data were transformed to square root of original values, normalized by median and auto scaled by centering the mean and dividing by the standard deviation of each variable. We initially selected 15 compounds that were present in all the treatments to compare the similarity and dissimilarity of chemical profiles. Then, we conducted Non-Metric Multidimensional Scaling (NMDS) indirect gradient analysis on the entire dataset of selected compounds' concentration to understand the dimensionality of the overall chemical profile from the phloem samples. To determine the significant influence of treatments on monoterpene and oxygenated monoterpene chemistry, PERMANOVA test was used. We then performed partial least squares discriminant analysis (PLS-DA). Then data were subjected to one-way ANOVA to test effect of treatments and represented in heatmap followed by Fisher's least significant difference method for multiple comparisons among the various treatments. All the figures were constructed using Inkscape (ver. 1.1.2, https://inkscape.org).

3.3 Results

3.3.1 Pathogen infection altered the lodgepole pine defense chemistry

We identified a total of 66 compounds (Appendix B: **Table B1**), including 15 monoterpenes (β-phellandrene, α-pinene, camphene, β-pinene, myrcene, 3-carene, limonene, thujene, β-ocimene, tricyclene, α-terpinene, p-cymene, γ-terpinene, terpinolene), 15 oxygenated monoterpenes (geranyl acetate, α-terpineol, p-cymen-8-ol, terpinen-4-ol, ascaridole, bornyl acetate, α-terpinyl acetate, camphor, borneol, linalool, cuminal, *endo*-borneol, car-3-en-5-one, pinocarvone, citronellyl acetate, sabinene), 20 sesquiterpenes (α-farnesene, germacrene D, *cis*-muurola- 3.5-diene, α-muurolene, β-curcumene, aromadendrene, β-elemene, γ-elemene, γ-cadinene, cubenene, β-farnesene, β-*trans*-farnesene, α-bisabolol, nerolidol, tau-cadinol, tau-muurolol, α-cadinol,

germacrene D4-ol, farnesol, β-caryophyllene), 11 diterpenes (13-epi-manool, agathadiol, cembrene, levopimaral, abietal, sandaracopimaral, kaurenal, isopimaral, isopimarol, manoyl oxide, torulosol), five other chemicals that are in different classifications (pinosylvin dimethyl ether, linalyl cinnamate, 4-allylanisole, β-sitosterol, 4-ethoxy ethylbenzoate). For the statistical analysis, we only considered 15 compounds (monoterpenes and oxygenated monoterpenes) that were present in all infected trees and because of their known roles in the MPB biology (Erbilgin, 2019).

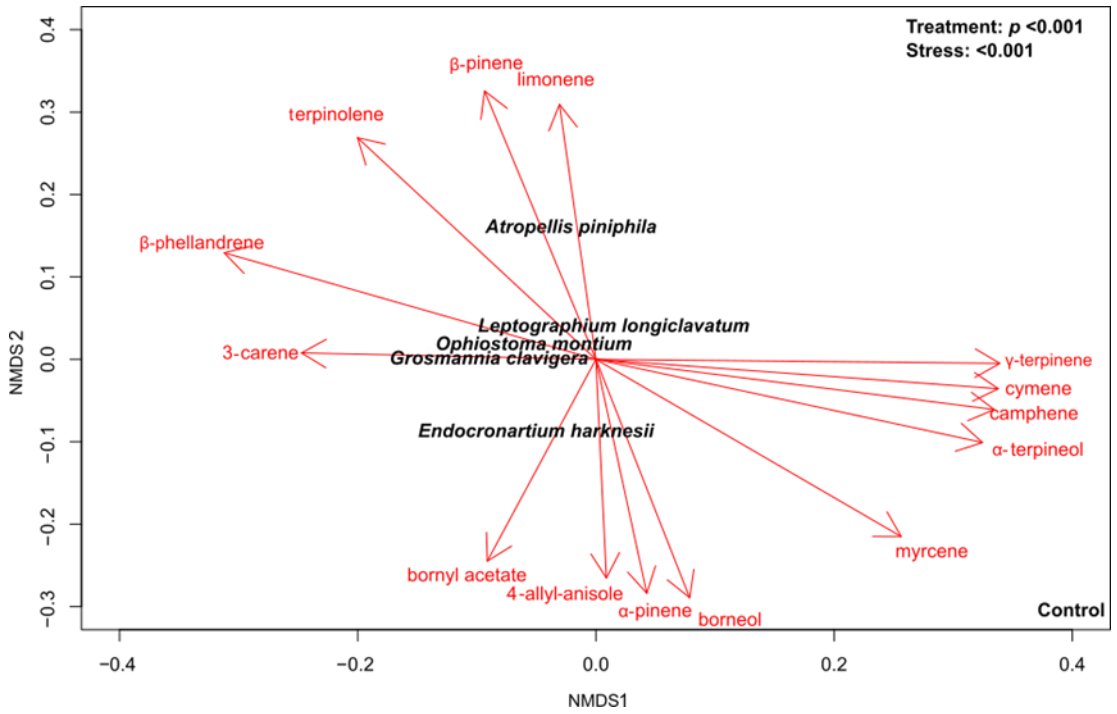

Figure 3.1: Non-Metric Multidimensional Scaling (NMDS) representation of similarities and differences in phloem chemical profiles of lodgepole pine (*Pinus contorta* var. *latifolia*) infected by various pathogenic fungal species. The concentrations of individual compounds (ng mg^{-1} DW) were used in the analysis. Red vectors represent different compounds. Different fungal species were shown in black. perMANOVA test was carried out to denote the significant differences among different treatments at $p < 0.05$.

We used NMDS (stress: < 0.001) for visualizing similarities and differences among samples based on multivariate data (**Figure 3.1**). We observed a large variation of defense chemical profiles between healthy and infected lodgepole pine trees (PERMANOVA; $F_9 = 113.7$, $R^2 = 0.71$; $p < 0.001$) (**Figure 3.1**). Gradient analysis using the concentrations of 15 compounds

revealed that the proportions of γ-terpinene, cymene, camphene, α-terpineol, and myrcene were highly correlated with healthy uninfected pine trees. Furthermore, infection by different fungal species altered the proportions of individual and total monoterpenes. Especially, *C. harknessii*, *A. piniphila*, *G. clavigera*, *O. montium*, and *L. longiclavatum* infections were highly correlated with β-phellandrene. Bornyl acetate, 4-allylanisole, α-pinene and borneol were correlated with *C. harknessii*. α-Terpinene was excluded from the NMDS since it was absent in some of the major treatments.

3.3.2 Different fungal pathogens differentially altered the monoterpene profiles of lodgepole pine

Since NMDS analysis showed that specific chemicals were correlated with a specific fungal species (**Figure 3.1**), we further analyzed how each fungal infection alters the monoterpene and oxygenated monoterpene profiles of lodgepole pine trees and whether the effects of fungal pathogens on the monoterpene proportions vary with the species. We carried partial least squares discriminant analysis (PLS-DA) as a downstream analysis using monoterpene and oxygenated monoterpene concentrations to visually characterize the distribution of all the five pathogen species in reduced dimensional space (**Figure 3.2**, Appendix B: **Figure B1**). PLS-DA model with six components held a total of 65.1% variation. Here, we observed a pattern of segregation between control and fungal infection clusters described by component 1 (31.6% variance). The component 2 further separates *C. harknessii* from the rest of the fungal species (9.6% variance). We further cross-validated the model with the Leave-one-out cross-validation method to estimate the ability of the model to predict the outcomes of our data. The model predicted the data with 84% accuracy (R^2 =0.18, Q^2 =0.083) (Appendix B: **Figure B2**), indicating good clustering and distinction among the six treatment groups including control.

For simple explanation and representation, we showed the PLS-DA results as synchronized 3D plots with the first three components (components 1-3) (**Figure 3.2**a). The complete model is shown in **Figure B1** (Appendix B). The loadings plot illustrated the level of contribution of each variable or feature to the classification of the model. It also showed the correlation between the original variables in the X matrix and the latent variables or components in the PLS-DA model (**Figure 3.2**b). Limonene, α-terpinene, β-phellandrene, 4-allylanisole, terpineol, and α-pinene showed high variation among treatments in the model (**Figure 3.2**b).

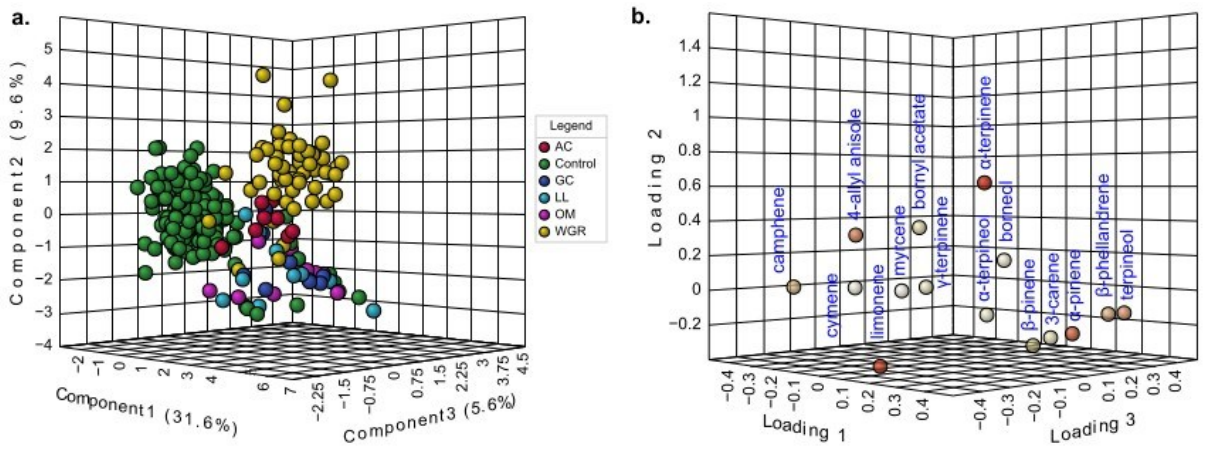

Figure 3.2: Partial Least Squares Discriminant Analysis (PLS-DA) analysis of metabolite profiles of lodgepole pine (*Pinus contorta* var. *latifolia*) phloem in response to infection by different pathogenic fungal species. (a) A 3D score plot of the overall samples, **(b)** a 3D loading plot of the variable (metabolites). Variables with a significant effect on the separation between classes were positioned farther away from the origin on the plot, whereas variables having a minor impact on the separation were closer to the origin. WGR = *Cronartium harknessii*, AC = *Atropellis piniphila*, GC= *Grosmannia clavigera*, LL= *Leptographium longiclavatum*, OM = *Ophiostoma montium*, and Control= non-infected samples.

We further calculated the coefficient score to measure the strength and direction of the association between each variable and the PLS-DA scores. Positive coefficients signified that a higher value of a variable is correlated with higher scores for a specific class, while negative coefficients indicated the opposite. Here, we used coefficient scores to rank the variables (compounds) based on their significance and to identify the variables that have the strongest correlation with separation among classes (treatments) (Appendix B: **Figure B3**, **Table B3**).

3.3.3 Monoterpene profiles of lodgepole pine trees varied among different fungal pathogens

Based on the coefficient scores from PLS-DA analysis, we further investigated to determine differences in individual monoterpenes among different fungal species and the control treatment (**Figure 3.3**, **Table B2**). We found significant differences in monoterpene concentrations across fungal species. β-Phellandrene, terpinolene, and β-pinene were higher (F=130.01, 63.11, 82.78

respectively; p <0.001) and camphene, γ-terpinene, cymene, myrcene, and α-terpineol were lower (F= 97.02, 35.74, 27.11, 22.29 respectively; p <0.001) in all infections.

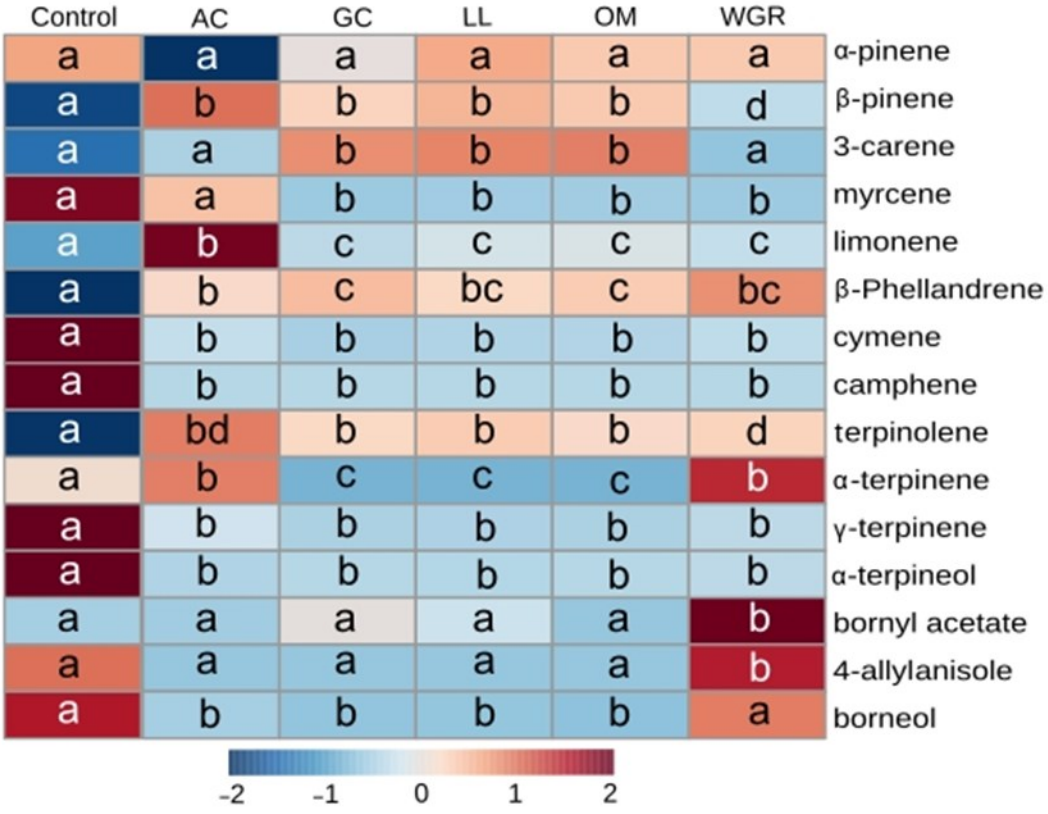

Figure 3.3: Heatmap representation of monoterpene profiles of lodgepole pine (*Pinus contorta* var. *latifolia*) in response to infection by different pathogenic fungal species. Columns and rows indicate individual agents and monoterpenes, respectively. The colour scale on the bottom ranges from -2 (low) to 2 (high) representing the concentrations of monoterpenes as the proportion of the total monoterpenes. Different small letters on each row denote significant differences in monoterpene concentrations among treatments (p < 0.01; One-way ANOVA; Fisher's LSD post hoc multiple comparisons). WGR = *Cronartium harknessii*, AC = *Atropellis piniphila*, GC= *Grosmannia clavigera*, LL= *Leptographium longiclavatum*, OM = *Ophiostoma montium*, and Control= non-infected samples.

Interestingly, infection by *C. harknessii* significantly increased the concentration of some oxygenated monoterpenes such as α-terpinene, bornyl acetate, borneol, and 4-allylanisole when compared with the other pathogens (F= 38.94, 8.47, 4.38, 3.97 respectively; p <0.001).

Conversely, *A. piniphilla* significantly increased the concentrations of limonene and myrcene more than other pathogens (F= 16.34 and 22.29 respectively; p <0.001) while 3-carene was significantly induced only in the trees infected with the fungal symbionts of MPB (F= 8.10, p <0.001).

3.4 Discussion

This is the first report on the phloem monoterpene composition of lodgepole pine trees infected by *A. piniphila* and *C. harknessii*. Our results highlight the complex host tree responses to different species of fungal pathogens. Furthermore, the identification of 66 compounds, including monoterpenes, oxygenated monoterpenes, sesquiterpenes, diterpenes, and other chemicals, demonstrates the diverse secondary metabolites associated with the infection of lodgepole pine by *A. piniphila, C. harknessii* and different fungal symbionts of MPB. Together, the observed large variation in the terpene profiles of lodgepole pine trees in response to the infection by different fungi underscores the importance of species-specific plant chemical defense responses to pathogenic organisms. Information on differential responses can inform us about the plant evolutionary processes in a multispecies pest environment. According to the 'interaction diversity theory', plants develop a variety of chemicals as a result of their simultaneous interactions with various other organisms. Since different pathogen and herbivore species differ in their vulnerability to various molecular pathways of bioactivity, one metabolite or a single class of metabolites cannot protect plants against all enemies (Whitehead et al., 2021).

Based on our results, three inferences can be drawn. As expected, the overall terpene profiles and concentrations of monoterpenes and oxygenated monoterpenes of lodgepole pine, when infected by fungal pathogens, substantially varied from those of the healthy trees. For instance, when the symbiotic fungi of MPB were inoculated, lodgepole pine trees showed a well-known induced defense response; however, concentrations of some monoterpenes were different among the fungal species, supporting the results of previous studies in our study system as well as in others (Agbulu et al., 2022; Cale et al., 2017; Erbilgin et al., 2017b; Kandasamy et al., 2023; Zaman et al., 2023b). In comparison to the control, the fungal symbionts upregulated some monoterpenes, such as 3-carene and terpinolene, but not all. Upregulation is likely an integral part of the tree defense response to stop the spread of the pathogen infection. For instance, it was reported that upregulation of 3-carene can restrict the growth of *G. clavigera* (Ullah et al., 2021). Conversely,

upregulation of terpinolene may increase MPB attraction to its aggregation pheromone (Klutsch et al., 2017). Thus, upregulation of host tree monoterpenes can sometimes be seen as a defense strategy while sometimes it may be beneficial to bark beetles, suggesting that we need to consider the subsequent effects of individual host tree defense compounds on the bark beetle-fungal symbiont complexes (Raffa et al., 2008; Raffa, 2014; Ullah et al., 2021; Zaman et al., 2023b).

Second, we showed that *A. piniphila* infected trees can increase the concentrations of several compounds such as limonene and myrcene. Furthermore, compared to other pathogens, *A. piniphila*-infected trees contained a much higher diversity of sesqui- and diterpenes that were usually absent or in lower quantities in trees infected by the other fungal species. Since pines produce antifungal diterpenes at levels that correspond to the virulence of pathogens as a form of defense (Cale et al., 2019b; Kushalappa et al., 2016), our results suggest that *A. piniphila* seems to be more virulent than the others used in the current study and diterpenes seem to be the primary metabolites directly involved in the pine defense against this particular pathogen (Klepzig et al., 1996; Kopper et al., 2005). In agreement with a previous report, we also found a large numbers of alcoholic derivatives of several mono- and sesquiterpenes in *A. piniphila* infected tissues (Hunt and Kuechler, 1970). Currently, we do not know what roles any of these terpenes play in tree defense against *A. piniphila*. However, our results demonstrate that tree induced defense response to combat the fungal infection varied among species based on their virulence (Hunt and Kuechler, 1970; Klepzig et al., 1996; Raffa and Smalley, 1995).

Third, the profile and concentration of individual terpenes varied based on the different fungal species (Kopaczyk et al., 2020). Among five fungal species investigated, the profile of lodgepole pine induced defense response to the infection by *C. harknessii* was different from the other four pathogens. In particular, *C. harknessii* infection upregulated bornyl acetate, 4-allylanisole, α-terpinene, and borneol production compared to the others. Some of these were oxygenated monoterpenes such as bornyl acetate and borneol. Similarly, a previous study on *Pinus sylvestris* infected with *Cronartium pini* rust reported upregulation of bornyl acetate (Kaitera et al., 2021). This indicates the ability of *C. harknessii* to biotransform monoterpenes to their oxygenated derivatives (Nones et al., 2022; Zaman et al., 2023b). In general, oxygenated monoterpenes are known to be more toxic to fungi than their parent hydrocarbon derivative compounds (Agwunobi

et al., 2022; Scariot et al., 2020). However, it is unknown whether fungi directly benefit from the oxygenated monoterpene biotransformation. Of note, production of oxygenated monoterpenes (allelopathy) may benefits specific fungal species to compete against other competitors to limit their growth (Boddy and Hiscox, 2016; Hung et al., 2015).

The current study captured the terpene composition of trees after sampling once each tree in a single stand. Naturally, the results do not represent the natural variation in defense profiles of lodgepole pine trees across the season and neither between sites. Hence, the readers should consider this shortcoming of the current study. Nevertheless, our study provides the first step towards understanding the changes in tree secondary metabolites in response to infection by different pathogenic organisms. Therefore, it is reasonable to compare the chemical profiles of infected trees by different pathogenic organisms. Overall, our results confirm that a pathogen infection can alter defense chemical profiles of mature lodgepole pine trees. However, depending on the pathogen species, we found substantial variation in lodgepole pine responses to the individual fungal species. We suspect that such changes in defense chemistry can alter the lodgepole pine suitability to subsequent insect attacks such as by MPB (Colgan and Erbilgin, 2011, 2010; Dooley and Six, 2015). For instance, *A. piniphilla* is the only pathogen that increased the production of most of the toxic terpenes (to the MPB) such as limonene, 3-carene, β-pinene and likely make lodgepole pine trees less suitable to the subsequent mountain pine attacks (Chiu et al., 2017; Erbilgin et al., 2017a). In contrast, *C. harknessii* and the three symbiotic fungi of MPB reduced the production of compounds toxic to the MPBs, suggesting that these trees may be suitable to subsequent colonization by MPBs. Currently, additional studies are needed to determine whether lodgepole pine trees infected by pathogens affect MPB host colonization behaviour. Nevertheless, our study highlights the importance of understanding the impact of individual biotic stressors on the defense chemical composition of trees.

Chapter 4: Exploring Behavioural and Physiological Adaptations in Mountain Pine Beetle in Response to Elevated Ozone Concentrations

4.1 Introduction

Air quality and climate are significantly impacted by pollution, which is linked to increasing anthropogenic activities (WHO, 2021). For instance, ozone (O_3), an atmospheric pollutant with a substantial impact on global warming, has been steadily increasing over the last four decades and has been projected to increase by 2-4 times in the next decades (Cooper et al., 2014; Gaudel et al., 2018; Yeung et al., 2019). As a secondary pollutant, O_3 is formed by the reaction of volatile organic compounds (VOCs) and nitrogen oxides (NO_x) under sunlight (Agathokleous et al., 2020). It is a strong oxidant and can damage subcellular DNA, lipids, and proteins, creating an imbalance in antioxidant systems and oxidative stress in many species (Chaitanya et al., 2016). One effect of O_3 that has received particular attention is its negative impact on pollinating insects. Studies have demonstrated that it can substantially reduce pollination (Cook et al., 2020; Farré-Armengol et al., 2016; Fuentes et al., 2013) by interfering with intraspecific communication *via* pheromones (Mcfrederick et al., 2008), foraging behaviour (Saunier et al., 2023), and olfactory memory (Démares et al., 2024). Similarly, pheromones, intraspecific chemical signals within a species, produced by several other insect species are degraded by O_3 (Arndt, 1995; Blande, 2021; Farré-Armengol et al., 2016; Fuentes et al., 2013; Jiang et al., 2023). Hence, elevated O_3 concentrations may affect biodiversity and ecosystem function, including above- and below-ground trophic interactions (Agathokleous et al., 2020). We currently do not know whether elevated O_3 concentrations affect major forest insect species such as bark beetles (Coleoptera: Curculionidae, Scolytinae), as this group of beetles contain some of the most economically and ecologically important species globally and rely heavily on pheromones for mating, successful host colonization, and reproduction.

Due to their ability to kill several pine species during intermittent outbreaks, mountain pine beetles (MPB) (*Dendroctonus ponderosae* Hopkins) (Coleoptera: Curculionidae, Scolytinae) are considered a major forest pest in western North America (Bentz et al., 2010; Logan et al., 2010;

Pureswaran et al., 2018; Raffa et al., 2008). Mountain pine beetles coordinate their mass attacks through the production of aggregation and anti-aggregation pheromones such as *trans*-verbenol, *exo*-brevicomin, frontalin, and verbenone (Erbilgin, 2019; Pureswaran et al., 2000; Safranyik and Carroll, 2006; Wood, 1982). After initial attacks, female beetles release aggregation pheromones to attract conspecific beetles to initiate mass colonization on host trees. After mating, beetles construct oviposition galleries where female beetles lay eggs along the gallery walls. Emerging larvae feed on the host phloem and the ophiostomoid fungi (Ophiostomatales, Ascomycota) inoculated during the host colonization by beetles (Safranyik and Carroll, 2006). The fungi grown on the phloem provide nutrients such as nitrogen to beetle larvae, helping their growth and development (Agbulu et al., 2022; Goodsman et al., 2012; Guevara-Rozo et al., 2020; Ishangulyyeva et al., 2016). More recently, studies have shown that female beetles are attracted to volatiles produced by the symbiotic fungi at short distances (Zaman et al., 2023b).

Pheromones are crucial for mating, successful host colonization, and reproduction in bark beetles and thus, any interruption can potentially disrupt their host aggregation, mating, and reproduction (Frühbrodt et al., 2024; Huber et al., 2021; Payne et al., 1992; Schlyter et al., 1989). This can ultimately affect beetle survival and population dynamics (Knaden et al., 2022). Currently, how elevated O_3 (eO_3) affects the physiology, behaviour, and reproduction of MPB is unknown. Investigating the effects of anthropogenic pollutants such as O_3 on these biologically important species is highly relevant and timely because MPB has been expanding its geographical and host species range in western North America (Jactel et al., 2019; Knaden et al., 2022; Pureswaran et al., 2018).

This study aimed to investigate the effects of eO_3 concentrations on the reproduction, physiology, and behaviour of MPB. We asked the following questions: (1) Does eO_3 exposure affect MPB physiology (respiration, survival, locomotion, and olfaction)? (2) Does eO_3 impair the mating success of MPB? (3) Does eO_3 exposure to parent beetles affect their broods? (4) Does eO_3 degrade MPB's pheromones? We hypothesize that eO_3 would alter the respiration, locomotion, and olfaction of MPB and degrade its pheromones, impair mating success, and reduce the fitness of its broods. To test our hypothesis, we conducted a series of *in vivo* experiments. First, we subjected MPB to different O_3 concentrations and estimated their survival, respiration, locomotion, and olfaction. We then continuously reared broods from parent MPBs exposed to

eO_3 for two generations to investigate the cascading effects of eO_3 on their brood production and development. Finally, we exposed the MPB pheromones to eO_3 to determine if eO_3 can degrade the concentrations of their pheromones.

4.2 Materials and Methods

4.2.1 MPB collection and sex differentiation

One mature lodgepole pine (*Pinus contorta*) tree (diameter at breast height: 30 cm) infested by MPB was felled from Minnow Lake, Alberta, Canada, in July 2022 (53º 21.767'N, -116º 02.943'W) and cut into smaller logs (50 cm ht). Logs were placed in rearing containers at room temperature at the University of Alberta. Emerging beetles were identified and sexed under a stereo microscope based on a highly sclerotized plectrum on the seventh abdominal tergite in adults and the male stridulation sound (Safranyik and Carroll, 2006). Male and female beetles were kept separately in 50 mL plastic falcon tubes with dampened paper towels at 4 °C until the bioassays were conducted.

4.2.2 O_3 treatment and concentrations

A novel bioassay apparatus was developed to expose beetles to different O_3 concentrations. The apparatus consisted of a 4L cylindrical glass chamber with an opening on either side. One of the openings was connected to the O_3 generator *via* a Teflon tube. The O_3 generator that can produce up to 450 mg hr^{-1} O_3 (DC PRO 450 Ozone Generator, Langley, BC, CAN) was connected to a controller unit (EcoZone™ Model EZ-1X, Newark, CA, USA) to control the production of O_3 concentration by the generator. Inside the chamber, we exposed 30 female beetles in a Petri dish (diameter ⌀13 cm) to one of the four O_3 concentrations, ambient (control), 100 ppb, 150 ppb, and 200 ppb for 30 min. A moistened paper towel was kept on the bottom of the chamber to prevent beetles from getting desiccated. Depending on the concentrations tested, a steady O_3 concentration was maintained inside the chamber. For the control treatment, we provided clean air (with 15±1 ppb of O_3) passed through the charcoal filter. In all cases, the airflow rate was maintained at approximately 450 ml min^{-1} using a flowmeter.

4.2.3 Estimation of beetle CO_2 respiration

CO_2 concentration inside the same bioassay chamber was recorded for the first 10 min with a portable CO_2 analyzer (Temtop CO_2 Monitor Portable Carbon Dioxide Meter, Elitech Technology, San Jose, CA, USA) for baseline correction. Following O_3 exposure, beetles were rested for 30 min inside the respective chambers at room conditions, as suggested by Sousa et al. (2012). Afterward, the CO_2 concentration exhaled by beetles was recorded for 60 min at 10 min intervals. For each time point, the amount of CO_2 respired by beetles (n=30) was calculated as: Amount of CO_2 produced (ppm)=Final Concentration-Initial Concentration (baseline).

4.2.4 Locomotion and Olfactory behaviour assessment test

Female beetles exposed to eO_3 were tested for their walking behaviour using a novel locomotion bioassay apparatus developed for this project (Fig. 4.1a). The apparatus consisted of a 15 cm long Teflon tube. One end of the tube was attached to a 2 ml transparent glass gas chromatography (GC) vial adjacent to a light source and the other end was connected to a 2 ml amber GC vial away from the light source, representing darkness. The tube from the transparent vial end was divided into three checkpoints every 5 cm. The first 5 cm was considered the stress phase since beetles inside the tube tend to pass it quickly, reflecting an avoidance behaviour. The middle 5 cm was considered the acclimatization phase since beetles spend more time passing this phase than the first checkpoint. The last 5 cm was regarded as the assessment phase because beetles spent the most time here. A single beetle exposed to eO_3 or ambient air was placed inside the transparent vial to assess whether eO_3 exposure induces stress in beetles. For each beetle, the time taken to pass through each checkpoint was recorded in seconds. We excluded the beetles from the test if they did not walk through the tube within 5 min.

To determine whether O_3 exposure alters the olfaction of beetles, we subjected the eO_3 or control beetles to the locomotion bioassay (Fig. 4.1b). We used the VOCs of *G. clavigera* as they are known to attract MPB in short distance (Agbulu et al., 2022; Zaman et al., 2023b). A 5 mm filter paper impregnated with 2 µL of fungal VOC mixture was inserted inside the amber vial. A treated beetle was allowed to walk through the tube towards the volatile source, and the time required between checkpoints was recorded in seconds. Beetles that did not respond to the source within 5 min were discarded from the experiment.

4.2.5 Survival probability test

The beetles used in the locomotion and CO_2 respiration tests were kept at normal laboratory conditions in Petri dishes containing moistened paper towel to prevent desiccation. The paper towel was re-watered every day with 1ml of tap water. The number of dead and live beetles counted every day until all beetles died. Beetles were considered dead when we observed no movement for some time.

4.2.6 Assessing mating success, reproductive behaviour, and brood fitness

To determine the impact of eO_3 treatment on MPB reproduction and the fitness of its broods, we reared beetles on recently cut logs obtained from the same location described above. We used 10 logs to rear beetles for each ambient (control) and eO_3 treatments (Erbilgin et al., 2020). Briefly, five pairs of male and female MPBs were introduced to a single log, covered in nylon mesh, and placed inside one of the two grow tents (Lumo-X [5'x5'] Mylar Hydroponic Grow Tent; Canbridge Tech Ltd., Markham, ON, CAN). One tent was connected to the O_3 generator with a controller, and the other was used as a control treatment with ambient condition (O_3=18.58±0.59). We maintained O_3 at a constant level of 100 ppb for 30 d (Jiang et al., 2023; Masui et al., 2023; Pinto et al., 2007a). The eO_3 concentration was selected based on the expected and frequently tested amount in the literature (Xia et al., 2021). All other parameters in both tents were kept constant: 16-h light and 8-h dark cycle, 23±1°C, and 65% relative humidity (RH) with continuous air circulation. After 30 d, all environmental parameters were reverted to ambient settings and remained constant until all beetle broods emerged from logs. Emerging broods were collected daily. We randomly selected 25 newly emerged female beetles from each treatment group and measured their pronotum width at its broadest point using a slide caliper (to the nearest 0.01 mm) and weight using an analytical balance (to the nearest 0.01 mg).

After brood emergence ceased, beetle oviposition galleries were analyzed by removing the outer bark. We considered mating successful if the oviposition galleries contained at least one larval gallery. We randomly selected two oviposition galleries per log, charted the gallery patterns into transparent plastic sheets, scanned the sheets, and measured gallery lengths using the Image J software (Version Java 1.8.0-172, National Institutes of Health, Bethesda, MD, USA) (Abràmoff

et al., 2004). A group of 20 first-generation female beetles emerged were subjected to the survival probability test as described earlier.

4.2.7 Pheromone collection and mating success assessment first-generation of broods

For collecting pheromones from the first-generation beetles, three pairs of 2-3 d old beetles were introduced onto fresh logs for each treatment group as described earlier (Erbilgin et al., 2020, 2014). Briefly, we drilled a hole in the outer bark of the logs and introduced a female beetle. The hole is covered with one-half of a gelatin capsule. Once the female constructed a gallery, i.e., frass inside the capsule was visible and beetles were no longer visible on the introduction point, a male was introduced to the same hole. A new male was introduced within 24 h when female beetles rejected the male beetles. Pheromones emitted from female and male beetles were continuously collected from the same holes at 12, 24, 36, 60, 84, and 108 h after the initial introduction of female beetles. To collect pheromones, we placed a small glass funnel (2.5 cm in dia of mouth) above each beetle entrance hole and a charcoal filter (Honeywell, Southborough, MA, USA) at the gap between the bark and the mouth of the funnel. Each funnel was attached to a vacuum pump (Cole-Parmer Canada Inc., Montreal, QC, CAN) with a Teflon tube and an adsorbent cartridge (Porapak Q, OD, 6 mm; length, 110 mm; adsorbent: front layer, 150 mg; back up layer, 75 mg; separated by glass wool, SKC Inc., Eighty-Four, PA, USA) was inserted in the tube between the pump and the funnel. Pheromones emitted from individual entrance holes were trapped in the adsorbent cartridges for 4 h. The flow rate (100 ml min^{-1}) of the pumps was kept constant during the pheromone collection. Additional pheromone collections were made from the same entrance holes using the same collection method but with a new adsorbent cartridge. After each collection, the adsorbent cartridges were capped and stored at -40°C until extraction and further analysis. During pheromone collection, the bolts were kept at room temperature (23±1°C and 65% RH).

After the pheromone collection, we used the same logs to evaluate how eO_3 exposure of parent beetles affected their broods' mating and colonization behaviour. The logs were placed in individual rearing containers for 8-10 wks at room temperature to allow beetle larvae to complete their development. After the emergence of beetles, we removed the bark from the logs and analyzed the gallery patterns, as described above.

4.2.8 O_3-pheromone interaction assessment

We built three air-tight plexiglass boxes of approximately 4 L volume (30 cm L, ×20 cm W, ×20 cm H). Each box contained five holes (5 mm in dia) on the top. We followed the method described earlier to test whether O_3 can degrade MPB pheromones (Arndt, 1995) with some modifications. Each box held one 2mL open GC vial containing 2 µL of synthetic pheromone. We placed one Porapak Q adsorbent cartridge at each of the five holes, and each cartridge was connected to a pump to absorb pheromones fumigated inside the box. Each box received 100 ppb O_3, which was allowed to react with pheromone for 2 h. A second box was used as a negative control, receiving only O_3 without pheromone. A third box was a positive control, receiving ambient air and pheromone inside. We tested four MPB pheromones, including *cis*-/*trans*-verbenol, *exo*-brevicomin, frontalin, and verbenone separately. After pheromone collection, the cartridges were sealed and stored at -40°C until extraction and analysis. The room conditions were 23–25°C with 65% RH throughout the experiment.

4.2.9 Pheromone analysis

We followed a method described earlier to extract and analyze the pheromones (Erbilgin et al., 2020). Briefly, we extracted pheromones with 1 ml of dichloromethane containing 0.0010% heptyl acetate (Sigma-Aldrich) as an internal standard. The extracts were injected into a GC/Mass Spectrometry (Agilent 7890A/5975C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP-Chiral-20β column (I.D. 0.25 mm, L 30 m) (Agilent Tech.) in split-less mode with helium as the carrier gas at a flow rate of 1.2 ml min^{-1}. The temperature setting was as follows: starting at 50 ºC for 2 min, increased to 90 ºC by 45 ºC min^{-1} and held for 2 min, increased to 155 ºC by 6 ºC min^{-1} and held for 1 min, and then to 230 ºC by 25 ºC min^{-1} and held for 3 min. The MS was set to both SCAN and SIM mode with the following ion masses and time program: ion masses 72, 85, 100, and 114 starting at 3.5 min, ion mass 43 starting at 16.5 min, ion mass 170 starting at 18.0 min, and ion masses 107 and 109 starting at 21.7 min, respectively. The standard curves were constructed from four dilutions (10x, 100x, 1000x, 10,000x) prepared using analytical standards of specific pheromones. Pheromone concentrations were quantified using these standard curves. The pheromones standard used were *cis*-/*trans*-verbenol, *exo*-brevicomin, frontalin, and verbenone, with chemical purity greater than 85% (Contech Enterprise Inc. BC, CAN).

4.2.10 Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 10.1.0 for Windows (GraphPad Software, San Diego, CAL, USA, www.graphpad.com). First, the data were checked for the assumptions of homoscedasticity using Bartlett's test and normality with the Shapiro–Wilk test. A natural log transformation was used before analysis if data did not meet the criteria for normality.

The following statistical methods were employed for experiments involving different ozone conditions. Since the data from mating success (percentage), oviposition gallery length (cm), average number of broods emerged, beetle weight (mg), and pronotum size (mm) were normally distributed with equal variances, we used unpaired T-tests to analyze the data. We analyzed using the Mann-Whitney test for non-normally distributed data with similar variances after the natural log transformation. The amount of pheromones released was analyzed by a two-way ANOVA repeated measures mixed effects model with treatments and time as fixed factors and individual beetles as random factors, followed by Fisher's LSD test for multiple comparisons.

CO_2 respiration data were analyzed using repeated measures of one-way ANOVA with Geisser-Greenhouse correction for unequal variances, followed by Tukey's multiple comparison test with individual variances computed for each comparison. The relationship between the production of CO_2 and time was analyzed using a linear regression. Intercepts and slopes of the regression lines for different O_3 concentrations were compared by analysis of covariance. Beetle survival data was analyzed by Kaplan-Meier's simple survival analysis. Curves generated from the survival data were compared by the Mantel-Cox log-rank test. Time data from the locomotion test were analyzed by the Kruskal-Wallis test due to the non-normality of the data followed by Dunn's multiple comparison test. Pheromone degradation data were analyzed by independent unpaired t-test. Figures were constructed using the non-transformed data.

4.3 Results

4.3.1 Does O_3 exposure affect MPB's physiology?

4.3.1.1 Locomotion

The locomotion test results revealed significant differences in all three phases (stress, acclimatization, and assessment) tested (**Figure 4.1**c-e). At the stress phase, the Kruskal-Wallis test showed significant differences among O_3 treatments ($H_3 = 9.1$, $p = 0.03$). Dunn's multiple comparisons indicated that the ambient and 200ppb were highly different from one another ($p = 0.01$) (**Figure 4.1**c). There were no other differences between treatments. Similar results were obtained during the acclimatization and assessment phases with significant Kruskal-Wallis statistics (Acclimation: $H_3 = 20.6$, $p < 0.001$; Assessment: $H_3 = 32.0$, $p < 0.001$) (**Figure 4.1**d & e).

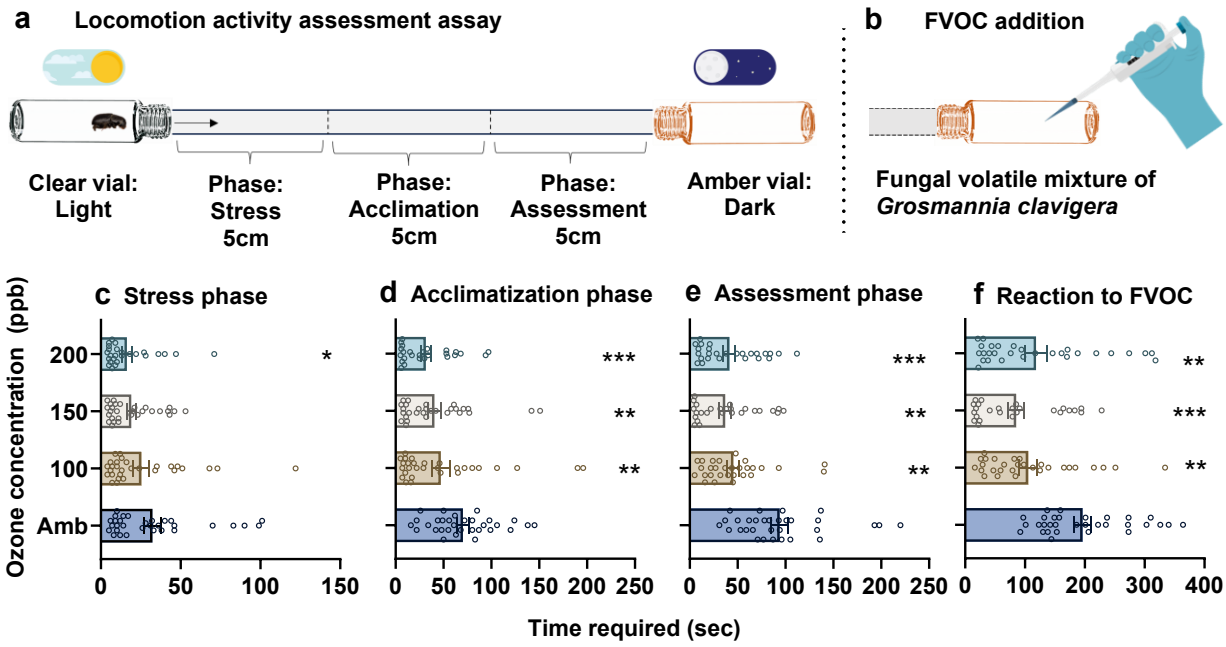

Figure 4.1: Effects of different ozone concentrations on the locomotion and olfaction of mountain pine beetles. (a) Schematic representation of locomotion activity assay and **(b)** addition of a mixture of fungal volatile organic compounds (FVOC). **(c-e)** Time required for beetles under different phases when subjected to different concentrations of ozone (100, 150, and 200 ppb); Amb denotes ambient ozone concentration, and **(f)** time required for beetles to reach the FVOC source under different ozone concentrations. Error bars are the standard errors for means. Statistical significance at p <0.05, <0.01, and <0.001 are denoted by (*), (**), and (***), respectively.

4.3.1.2 Olfaction

Different O_3 concentrations profoundly impacted the response time of beetles to the fungal VOCs. Compared to the control beetles, the total time taken to reach the volatile source by O_3-treated beetles was reduced by at least 40%. Overall, the treated beetles spent less time to reach the volatile source than the control beetles (**Figure 4.1**f).

4.3.1.3 CO_2 respiration

Repeated measures of one-way ANOVA revealed a significant effect of O_3 on the CO_2 respiration of beetles ($F_{1.517,\ 7.583} = 42.57$, $p = 0.0001$). Notably, the mean respiration level decreased with increasing O_3 concentrations. Post-hoc analysis revealed that the most significant mean difference was between the control and 200 ppb treatments (mean difference=15.6±1.8, p= 0.0013) (**Figure 4.2**a).

Table 4.1 shows the results of regression analyses for CO_2 production by MPB in different O_3 treatments. There were significant differences in the slopes across O_3 treatments ($F_{3,\ 16} = 13.79$; $p< 0.001$). Hypothetically, if the slopes were identical, there was a 0.01061% chance of randomly choosing data points with different slopes. **Table 4.1** indicates that the differences between slopes were highly significant. In particular, the regressions under control, 100 ppb, 150 ppb, and 200 ppb of O_3, produced strong relationships between treatment time and CO_2 production.

Table 4.1: Regression analyses for CO_2 respiration in mountain pine beetles treated with different ozone concentrations.

Ozone treatments (ppb)	Parameters							
	Slopes	Std. error	Y-intercept	Std. error	R^2	$F_{1,4}$ values	p-values	Equations
Ambient	0.4207	0.04656	6.317	1.813	0.9533	81.64	<0.001	Y = 0.4207×X+6.317
100	0.4250	0.02378	2.333	0.9261	0.9876	319.4	<0.001	Y = 0.4250×X+2.333
150	0.4979	0.02042	-4.717	0.7953	0.9933	594.4	<0.001	Y = 0.4979×X-4.717
200	0.2286	0.02647	-2.583	1.031	0.9491	74.56	<0.001	Y = 0.2286×X-2.583

4.3.1.4 Survival

There were differences in the median survival times across O_3 treatments (**Figure 4.2**b). The median survival was four days for the control and 100ppb, six days for the 150ppb, and seven days for the 200ppb treatments. We then compared the survival curves using the Log-Rank (Mantel-Cox) test. The results showed highly significant differences among the survival curves (df: 3, χ^2=40.57, p:<0.001).

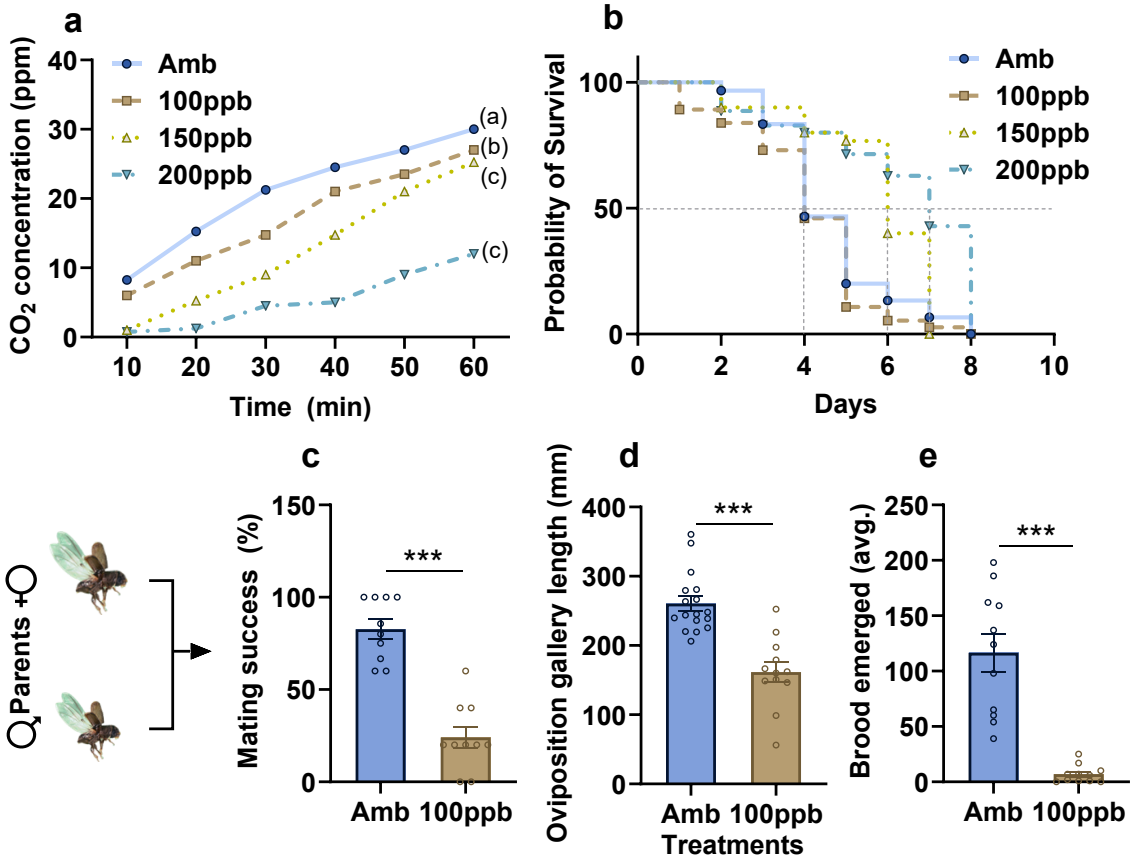

Figure 4.2: Effects of ozone on the biology of parent mountain pine beetles. (a) The concentration of CO_2 respired by the beetles. **(b)** The survival probability of beetles in different ozone treatments. **(c,d)** The mating behaviour of ozone-exposed beetles. **(e)** The average number of broods produced by adult beetles subjected to different ozone treatments. Different small letters denote significant differences at p <0.05. Error bars are the standard errors for means. Statistical significance at p <0.05, <0.01, and <0.001 are denoted by (*), (**), and (***), respectively.

4.3.2 Does eO_3 impair mating success of MPB?

Exposure to eO_3 resulted in significant differences in MPB reproduction. Particularly, eO_3 treatment of beetles reduced their mating success relative to the control beetles (t_{18}= 7.45; p<0.001) (**Figure 4.2**c). Furthermore, eO_3-treated beetles excavated shorter galleries than the control beetles (t_{27}= 5.62; p<0.001) (**Figure 4.2**d). Brood emergence was also reduced in the eO_3-treated compared to the control beetles ($t_{6.37}$= 10.39; p<0.001) (**Figure 4.2**e).

4.3.3 Does eO_3 exposure to parent beetle affect their broods?

4.3.3.1 Morphology, mating success and survival

O_3 exposure of parent beetles affected the brood morphology, with reduced pronotum size (t_{48}=3.48, p< 0.001) (**Figure 4.3**a) and weight (t_{48}= 2.65, p< 0.01) (**Figure 4.3**b) compared to those from the control beetles.

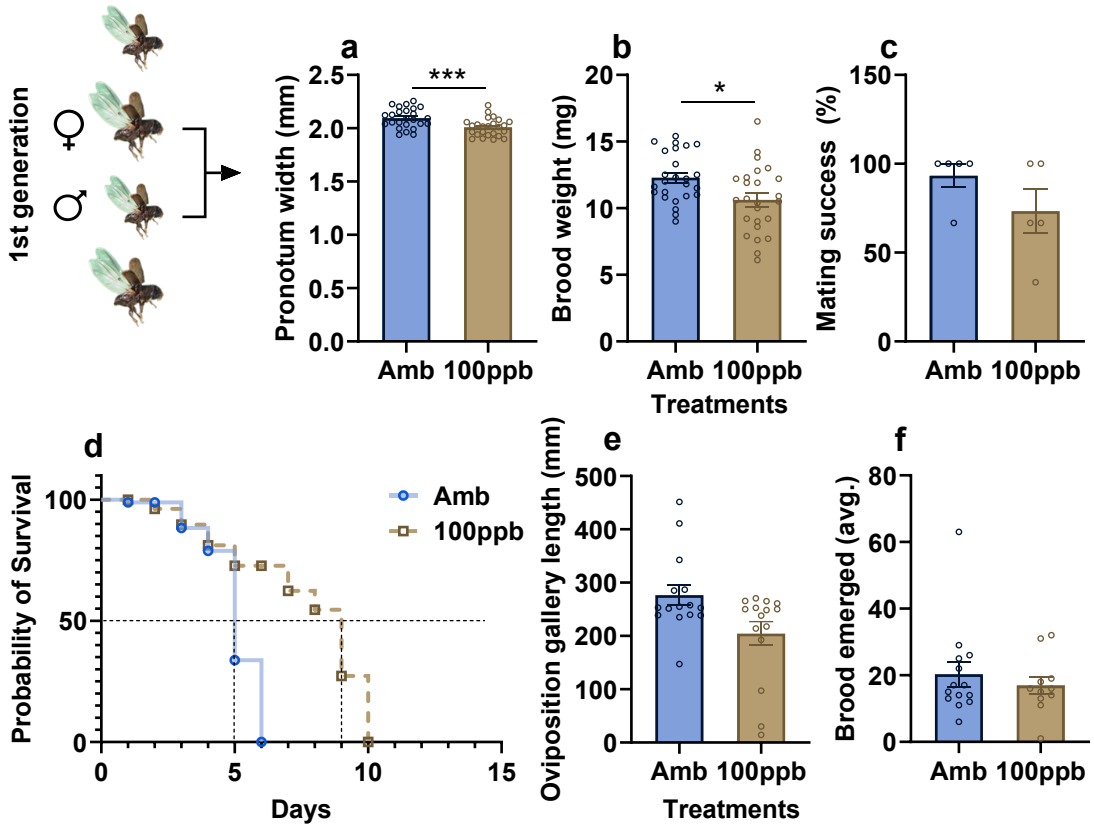

Figure 4.3: Transgenerational effects of ambient and elevated ozone concentrations on the biology of first-generation offspring of mountain pine beetles. (a, b) The pronotum width and

weight of broods emerged from the ozone-exposed parent beetles. **(c)** The mating success of the first-generation brood. **(d)** The survival probability of the broods. **(e-f)** The length of the oviposition gallery constructed by the first-generation brood and the average number of broods they produced (Second generation). Error bars are the standard errors for means. Statistical significance at p <0.05 and <0.001 are denoted by (*) and (***), respectively.

However, mating success in the eO_3 brood was similar between the treated and control parents (**Figure 4.3**c). We did not observe any differences in maternal gallery length and the number of the second generation of broods that emerged between the O_3-treated and control parents (**Figure 4.3**e & f). Similar to the improved survival probability of their parents, the brood from the eO_3 treated-parents had a considerably higher survival probability than the control parents (Log-rank test: χ^2_1= 8.2; p<0.05) (**Figure 4.3**d). In particular, 50% of the beetles from the eO_3 treated parents died after nine days, compared to half of the control group died on day five.

4.3.3.2 Pheromone production

O_3 exposure of parent beetles affected the pheromone production of their broods across treatments and time points. *cis*/*trans*-Verbenol production was increased in the eO_3-broods at 60 h (p=0.04) and 84 h (p= 0.02) (**Figure 4.4**a). Frontalin was decreased at 36 h (p=0.01) and increased at 60 h (p=0.02) (**Figure 4.4**d).

4.3.4 Does eO_3 degrade MPB's pheromones?

In all cases, pheromone concentrations in the presence of O_3 were reduced (**Figure 4.4**e-h). In the presence of 100 ppb O_3, the concentration of *cis*/*trans*-verbenol, verbenone, frontalin, and *exo*-brevicomin was decreased significantly by approximately 64% (Welch corrected $t_{4.78}$= 7.002; p= 0.001), 34% (t_6= 4.74; p= 0.002), 19% (t_8= 1.932; p= 0.09), and 15% (t_8= 3.53; p= 0.008) in comparison to their concentrations in the absence of O_3, respectively.

4.4 Discussion

Our study provides empirical evidence that O_3 exposure had long-lasting impacts on MPB behaviour and physiology, resulting in reduced respiration, impaired locomotion behaviour, altered response time to volatile cues, and improved survival probability due to oxidative stress. We also demonstrated that eO_3 concentrations may degrade the concentrations of MPB

pheromones. Overall, these results have several implications in MPB biology and population dynamics, particularly intraspecific pheromonal communication, host selection and aggregation, and transgenerational impact on their brood biology.

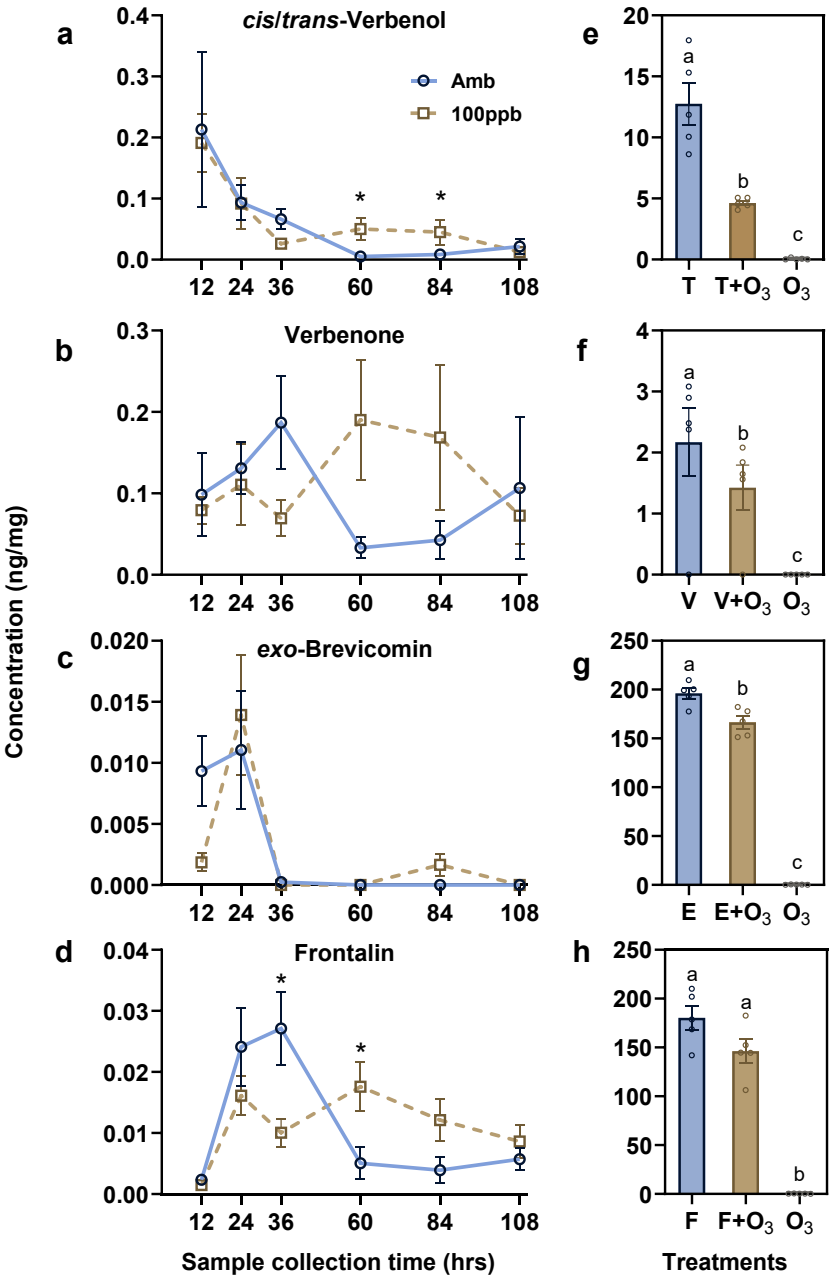

Figure 4.4: Chemical analysis of pheromones produced by broods of parent mountain pine beetles exposed to ambient and elevated ozone concentrations. (a-d) Major pheromones emitted by the first-generation offspring over 108 hrs. **(e-h)** Degradation of pheromones in

response to ozone exposure at 100 ppb. Error bars are the standard errors for means. Statistical significance at $p < 0.05$ is denoted by (*). Different small letters on top of error bars **(e-h)** denote statistical differences. T= *cis/trans*-verbenol, V= verbenone, E= *exo*-brevicomin, F= frontalin, and O_3= 100ppb of ozone.

4.4.1 Disruption of bark beetle pheromone communication under elevated ozone

Bark beetles rely heavily on detecting and responding to specific volatile cues to navigate their mating and foraging activities. The ability to discriminate their own pheromone signals is pivotal for their survival. Degradation of pheromone components or alteration in their quality and quantity may therefore be detrimental for the successful communication between conspecifics. We showed that eO_3 concentrations degraded MPB pheromones, potentially impairing the beetles' ability to detect and respond to conspecific pheromones. The exposure to the eO_3 has been observed to alter olfactory sensory environments in several insect species, leading to disruptions in their olfactory behaviour (Agathokleous et al., 2017; Masui et al., 2020; Saunier et al., 2023; Vanderplanck et al., 2021; Zaman et al., 2024).

Mountain pine beetles use aggregation and anti-aggregation pheromones to coordinate mass attacks on host trees. Hundreds of beetles can arrive within only 3-4 days of the aggregation pheromone production by both sexes. Such coordinated mass attacks are critical to exhaust host tree defenses and to secure host mortality which enables beetles to establish and to reproduce. Given that bark beetles are confronted with volatiles from multiple sources of plants and animals, and must maintain intraspecific communications despite this background emission, any reduction in their pheromone quality and quantity likely result in failure to aggregate on hosts, which result in failure to mate and reproduce. Our findings align with previous studies demonstrating the disruptive effects of eO_3 on reproduction and olfaction in other insect species (Démares et al., 2022; Farré-Armengol et al., 2016; Fuentes et al., 2016; Zaman et al., 2024).

4.4.2 Adapting to eO_3 stress: implications for beetle metabolism and behavioral responses

We showed a significant reduction in the CO_2 respiration levels of beetles following exposure to eO_3 concentrations. This result suggests potential changes in the metabolic processes in beetles, likely influenced by oxidative stress. Insects exhibit one or a combination of three respiratory patterns: (1) continuous, (2) cyclic, and (3) discontinuous gas-exchange cycle (DGC) (Contreras

and Bradley, 2009; Matthews, 2018). Of these, DGC is an important respiratory pattern for the coleopteran insects such as bark beetles and it consists of three phases: (1) closed, (2) flutter, and (3) open (Oladipupo et al., 2022). During the DGC pattern, bark beetles are able to accumulate a load of CO_2 in the hemolymph with less O_2 intake during the first two phases and release it during the last phase (Chown and Nicolson, 2004). According to oxidative damage hypothesis (Hetz and Bradley, 2005), insects exhibiting DGC pattern to avoid oxidative stress such as caused by eO_3 exposure, control spiracle valves to filter particulate matters and avoid toxic gases (Tan et al., 2018), resulting in lower their metabolic rate (Contreras and Bradley, 2009) and hence CO_2 respiration. Together all these results highlight the ecological relevance of O_3 across taxonomically different insect species including *Apis mellifera* (Démares et al., 2024), in *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst), *Rhyzopertha dominica* (F.) (Lu et al., 2009), *Sitophilus zeamais* (Sousa et al., 2016, 2012), and *Agelastica coerulea* (Baly) (Abu ElEla et al., 2018). Furthermore, all these studies suggest that insects can adapt to changing environmental conditions by adjusting their metabolic rates (Chevin and Hoffmann, 2017; Contreras and Bradley, 2009; Fusco and Minelli, 2010; Sommer, 2020).

Interestingly, changes at the metabolic levels also affect insect behaviours as we found an increased locomotion activity and attraction to VOC stimuli in response to eO_3 exposure in MPB, potentially reflecting changes in their energy usage tactics (Pimentel, 1994). Such behavioural adaptations can have broader implications for MPB biology and ecology, influencing host colonization and mating behaviours, similar to other insect species. For instance, eO_3 concentrations negatively affected the striped cucumber beetle (*Acalymma vittatum*) to locate flowers of its host plant (Fuentes et al., 2013), disrupted the sexual recognition in many drosophilid species (Jiang et al., 2023), and also masked the floral volatile interaction of tobacco hawkmoth *Manduca sexta* (Cook et al., 2020).

4.4.3 Unforeseen benefits: transgenerational adaptations of MPB to eO_3 stress

We showed an improved survival probability in both parents and the subsequent generations of MPB. Contrary to expectations, we observed higher survival rates at eO_3 concentrations. In addition, in our previous study, we reported that eO_3 exposure to parents triggered an improved immunity in their broods when challenged against an entomopathogenic fungus (*Beauveria bassiana*) (Zaman et al., 2024). This prompts a question whether exposure of beetles to eO_3

triggers a “stress resistance” response, bolstering their overall resilience and survival capabilities. Interestingly, while parent MPBs exposed to eO_3 concentrations exhibited reduced mating success and shorter oviposition galleries, their broods displayed improved mating success and excavated similar length of oviposition galleries compared to the broods from the control beetles. This phenomenon aligns with previous observations of transgenerational heritable phenotypes, resulting from environmental stressors in various insect species, attributed to heritable epigenetic modulation of gene expression (Bueno et al., 2023; Schausberger and Rendon, 2022). In general, insects employ diverse strategies to adapt to changing environmental conditions, including phenotypically plastic alterations and evolutionary responses (Dukes et al., 2009; Garnas, 2018). Notably, defense-related genes such as growth-blocking peptides, heat shock proteins, cytochrome P450s, ABCs, AchEs, and GSTs may be overexpressed in this adaptive process (Kliot and Ghanim, 2012; Qi et al., 2017; Qian et al., 2017; Shears and Hayakawa, 2019; Sinclair et al., 2013; Sørensen et al., 2003). O_3 exposure has also been linked to enhanced total antioxidant activity and reduced α- and β-esterase activities, indicative of increased stress resistance (Abu ElEla et al., 2018; Wool and Greenberg, 1990).

The study presented here faced some constraints. While we used varied concentrations of O_3 to examine the physiological effects on beetles, we opted for a concentration of 100 ppb to assess reproductive success and reflect real-world conditions. Additionally, the adverse impacts of eO_3 on beetle reproduction may also be attributed to its adverse effects on the MPB fungal symbionts, potentially leading to nutritional deficiencies in broods (Agathokleous et al., 2020; Hibben and Stotzky, 1969; Korzun et al., 2008; Zaman et al., 2024). Furthermore, while our study was conducted under controlled laboratory conditions, extrapolating these effects to natural settings may pose some challenges due to the complexity of ecological interactions involving multiple mutualistic and antagonistic organisms associated with MPB, along with various environmental factors. To address these challenges effectively in future research, additional studies should focus on dose-response relationship across a range of O_3 concentrations while considering multiple ecologically relevant partners. Finally, we also recommend investigating the effects of eO_3 on the olfactory sensory activities of beetles towards their host tree blends using GC-Electroantennographic Detection methods and assessing the degradation of host tree VOC blends by eO_3 concentrations. Moreover, exploring differential developmental gene expression in

beetles exposed to eO_3 concentrations would be crucial for comprehending potential transgenerational effects.

4.5 Conclusion

Our research demonstrates that eO_3 concentrations can interfere with the mating behavior of MPB by degrading their pheromones and disrupting their normal physiological functions. This highlights the complex process of ecological adaptation to changing environments, underscoring how climate change scenarios could profoundly impact the population dynamics of bark beetles. The anticipated rise in atmospheric O_3 concentrations may have significant repercussions, influencing interactions across multiple levels and resulting in severe economic and ecological implications. The next phase of our work involves developing a more comprehensive understanding of the repercussions on forest ecosystem functioning and the expansion of the MPB range.

Chapter 5: Unraveling the multifaceted effects of climatic factors on mountain pine beetle and its interaction with fungal symbionts

5.1 Introduction

The delicate balance of ecosystems often hinges on the interplay of numerous environmental factors, and any disruption to this equilibrium can have far-reaching consequences. In recent years, climate change has emerged as a global challenge, with increasing concentrations of carbon dioxide (CO_2) and ozone (O_3) being among the most prominent causes and consequences (Calvin et al., 2023; Harvey et al., 2023). In fact, the current global challenge of climate change has manifested in rising concentrations of CO_2 and O_3, reaching 421 ppm, which is substantially higher than the pre-industrial level of 280 ppm (Calvin et al., 2023) and 40 ppb (historically <10 ppb) (Wittig et al., 2009), respectively, and the possibility of doubling by the end of the century (Calvin et al., 2023). These changes in atmospheric composition and precipitation patterns are contributing to the current climate crisis, further exacerbating the conditions which can profoundly affect plant and insect populations and interspecies interactions (Jactel et al., 2019; Lindroth, 2010). Periodic insect outbreaks are significant drivers of forest mortality and shifts in forest ecosystems worldwide (Hartmann et al., 2022; Pureswaran et al., 2018). In North America, projected climate conditions indicate winter warming at high latitudes, reduced summer precipitation in mid-latitudes, and increased extreme weather events such as storms and droughts (Calvin et al., 2023). These climate variations could result in the population growth of forest insect herbivores as climate conditions become more favourable and the availability of suitable host trees for breeding becomes more abundant (Price et al., 2013; Pureswaran et al., 2018; Ramsfield et al., 2016). However, there is currently a lack of empirical data exploring the impact of elevated CO_2 (eCO_2) and O_3 (eO_3), along with reduced relative humidity (RH), on the biology of tree-killing forest insect herbivores.

Current research has predominantly focused on how changes in tree characteristics, driven by eCO_2 and eO_3, mediate insect responses (Couture and Lindroth, 2012; Jactel et al., 2019; Lindroth, 2010). These studies have reported that eCO_2 generally leads to reduced nitrogen

content, resulting in a higher carbon-to-nitrogen ratio, and the development of carbon-based defenses that are detrimental to defoliating insect herbivores (Jactel et al., 2019; Lindroth, 2010). Conversely, eO_3 negatively affects plant growth by inhibiting various plant cellular and metabolic functions (Lindroth, 2010; Wittig et al., 2009). Furthermore, the impacts of eCO_2 and eO_3 on insects studied to date are context-dependent and species-specific (Lindroth, 2010), making it challenging to establish a general pattern of their impacts on forest insect herbivores. In addition, reduced RH can affect plant chemical composition by influencing plant physiology (Lysenko et al., 2023; Pagadala Damodaram et al., 2021) while improving the chemoreception and flight activity of several insect species (Bassett et al., 2011; Jaworski and Hilszczański, 2013; Suárez-Hernández et al., 2023). However, comparatively little attention has been paid to the direct impact of eCO_2, eO_3, and reduced RH on forest insect herbivores. Investigating the effects of these major climatic factors is equally crucial for understanding the phenology of insect herbivores under projected climate conditions (Jactel et al., 2019).

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB) (Coleoptera: Curculionidae, Scolytinae), a phloem-feeding bark beetle species, is a component of natural forest process and succession. It is also considered a major forest pest due to its ability to kill several pine species during intermittent outbreaks and its significant role in shaping forest species composition and ecosystem function across western North America (Bentz et al., 2010; Jactel et al., 2019; Logan et al., 2010; Pureswaran et al., 2018; Raffa et al., 2008; Robbins et al., 2022; Zaman, et al., 2023). The host tree colonization of MPB is primarily driven by its ability to aggregate on host trees and its interactions with microbial symbionts (Paine et al., 1997; Safranyik and Carroll, 2006; Wood, 1982). Mountain pine beetle uses aggregation and anti-aggregation pheromones to keep conspecifics in sync and facilitate host tree colonization. Its major aggregation pheromones are *trans*-verbenol, *exo*-brevicomin, and frontalin, and the anti-aggregation pheromone is verbenone (Safranyik & Carroll, 2006). Once mated under bark, female beetles excavate oviposition galleries and deposit eggs within these galleries. After hatching, larvae develop within the inner bark of the tree, feeding on phloem tissues infected with symbiotic fungi inoculated by their parents. At the end of the larval stage, the pupal stage unfolds discreetly within these galleries. The completion of this cycle marks the emergence of adult beetles and the initiation of new host tree colonization by the emerging brood (Safranyik & Carroll, 2006).

Different fungal species play a crucial role in shaping the biology and determining the success of host colonization for the MPB. For instance, symbiotic ophiostomatoid fungi (Ophiostomatales, Ascomycota), including *Grosmannia clavigera*, *Leptographium longiclavatum*, and *Ophiostoma montium*, can contribute to successful host tree colonization by acquiring nutrients and detoxifying host tree secondary metabolites (Agbulu et al., 2022; DiGuistini et al., 2011; Goodsman et al., 2012; Guevara-Rozo et al., 2020; Lieutier et al., 2009; Ojeda Alayon et al., 2017; Six & Wingfield, 2011; Six, 2012; Wang et al., 2014; Zaman et al., 2023). In particular, as larvae feed on the phloem, they depend on these fungi to breakdown the complex secondary metabolites into simpler digestible forms (Ayres et al., 2000; Bleiker and Six, 2007). This mutualistic interaction improves the beetle's access to essential nutrients and fosters an environment favourable to larval feeding. Several studies have highlighted that climatic variations across different geographic regions can potentially influence the availability and pathogenicity of these fungal symbionts (Moore & Six, 2015; Rice et al., 2008; Six & Bentz, 2007). Furthermore, MPB colonization also makes the host tree available for attacks by saprophytic fungi *Trichoderma* sp. and *Aspergillus* sp. which can substantially reduce beetle-oviposition and larval survival (Therrien et al., 2015). In addition to these fungal communities, MPB interacts with an entomopathogenic fungus *Beauveria bassiana*. This fungus can kill MPB along with many other bark beetle species (Rosana et al., 2021) and may provide a viable biocontrol tool to reduce MPB (Fernandez et al., 2023).

Despite prior research efforts that made significant discoveries revealing the effects of eCO_2 and eO_3 levels on various insect species, there remains a notable gap in our understanding of how these atmospheric pollutants directly impact the reproductive dynamics of ecologically significant bark beetle species like MPB (Jactel et al., 2019; Lindroth, 2010; Pureswaran et al., 2018). This research gap is particularly relevant when considering the MPB's dual role as a keystone species within coniferous ecosystems. Therefore, our study aims to bridge this gap and enhance our understanding of ecological responses to changing atmospheric conditions by investigating the impacts of eCO_2 and eO_3 concentrations and reduced RH on the reproduction of the MPB and its fungal symbionts (Fig. 1). Our objectives were to determine the effects of eCO_2 and eO_3 and reduced RH on the reproduction and fitness of MPB and to assess how the same environmental factors influence the growth of its symbiotic fungi. We hypothesize that the reproduction and fitness of both MPB and its fungal symbionts would exhibit positive responses

to eCO_2 and lower RH conditions. Conversely, we anticipated adverse effects on MPB reproduction and fungal growth under eO_3 condition.

5.2 Methods

We incorporated our workflow with a conceptual visualization illustrating the impact of climate change on the complex web of ecological interactions between MPBs and their symbiotic fungi in **Figure 5.1** (Steps I-VI).

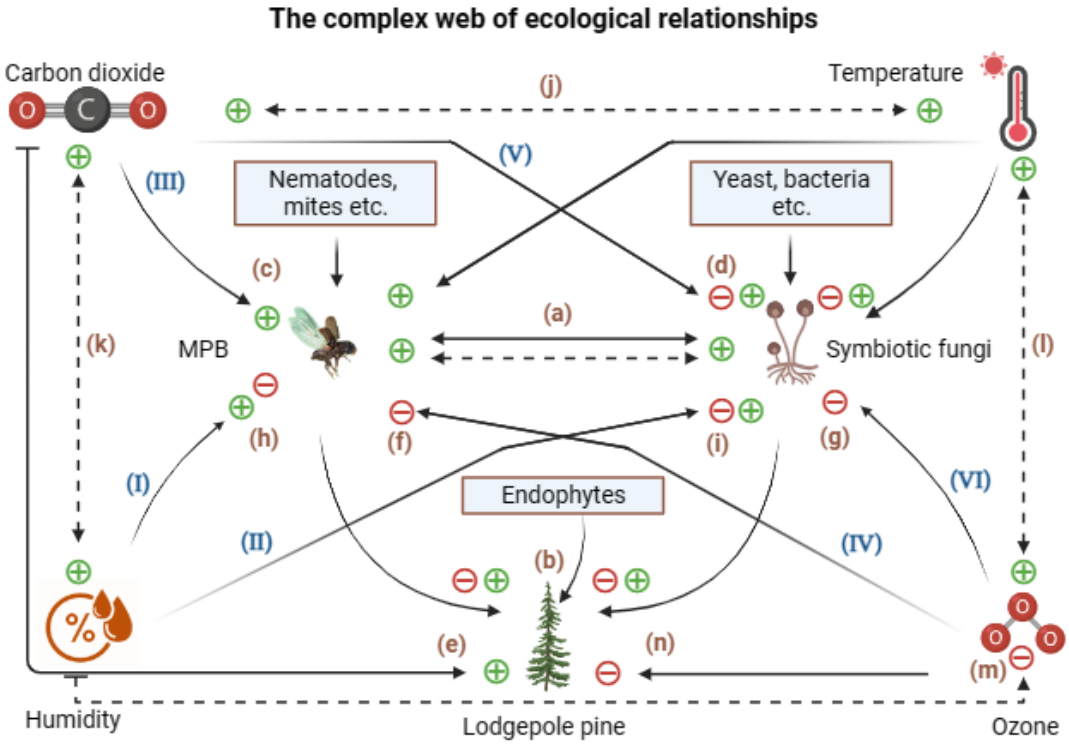

Figure 5.1: Impact of climatic variables on the functional network of ecological interactions among the mountain pine beetle, their host tree (lodgepole pine) and symbiotic fungi. Solid and dashed lines represent direct and indirect effects, respectively. (+) and (–) signs represent positive and negative effects, respectively. Roman numerals **(I-VI)** correspond to the specific research questions outlined in Methods. Specific interactions were shown from "a" to "n." **(a)** Beetles and fungi have both direct and indirect positive effects on each other where the beetle vectors the fungi to a host tree, and the fungi benefit the beetle by providing essential nutrients and detoxifying toxic plant metabolites. **(b)** Both beetles and fungi contribute to forest

regeneration by accelerating the mortality of stressed trees (positive effect), while at high population density, they can kill a large number of healthy trees (negative effect). **(c-e)** Elevated CO_2 can accelerate beetle development (positive effect on beetle), whereas it can have positive and negative impacts on specific fungal symbionts depending on their isolates. It can positively affect plant growth. **(f-g)** Elevated O_3 has detrimental effects on both beetles and fungi. **(h-i)** Lower humidity improves symbiotic fungal development, which benefits beetle fitness; the ambient humidity level improves saprophytic fungal growth. **(j-k)** CO_2, temperature, and humidity can have a positive relationship. **(l-m)** While temperature has a positive relationship with ozone concentration, higher humidity tends to impact ozone concentration negatively. **(n)** Elevated ozone concentrations induce oxidative stress on the host trees, significantly decreasing plant productivity.

Our study is organized around a series of pertinent questions designed to understand individual environmental variables and their impact on either MPB or their symbiotic fungi. (1) Does low RH affect the MPB reproduction (**Figure 5.1**, I)? If it does, (2) is this impact attributed to the varied effects of RH on the competition between MPB's fungal symbionts and saprophytes (**Figure 5.1**, II)? (3) Do eCO_2 and eO_3 affect the MPB reproduction (**Figure 5.1**, III & V respectively)? If it does, (4) what are the mechanisms by which eCO_2 and eO_3 affect symbiotic fungal biology (**Figure 5.1**, IV & VI, respectively)? As we found significant differences in pheromone production by the F_1 generation of beetles from eCO_2 and eO_3 exposed parents, we further asked, (5) do these effects persist in the F_2 generation (**Figure 5.1**, V & VI, respectively)? All the experimental parameters measured for each question and organism were presented in **Table 5.1** and Appendix C: **Figure C1**.

5.2.1 Rearing beetles under different humidity conditions

Live MPB were collected from naturally infested lodgepole pine (*Pinus contorta*) trees in the Minnow Lake, Alberta, Canada, in July 2021 (53º 21.767'N, -116º 02.943'W). We felled infested trees, cut them into 30 cm long logs, and placed them in rearing containers at room temperature. The beetles emerging from these logs were collected and stored in 50 mL plastic falcon tubes with dampened paper towels. Additionally, we felled five healthy lodgepole pine trees, cut them into logs, treated both ends with paraffin wax to prevent desiccation, and introduced live MPBs into logs, following Erbilgin et al. (2020). These 20 logs were divided equally into two groups

and placed in two plant growth chambers (E8 Reach-In Plant Growth Chambers, Conviron, Winnipeg, MB, CAN) at the University of Alberta.

Table 5.1: Comprehensive overview of experimental design and measured parameters for mountain pine beetle and their fungal symbionts under various environmental conditions including 33% and 65% relative humidity, ambient CO_2 and O_3, and elevated CO_2 and O_3.

Organisms tested	Parameters measured	RH: 65%		RH: 33%		Amb CO_2 & O_3		eCO_2		eO_3	
		P	F_1	P	F_1	P	F_1	P	F_1	P	F_1
	Sample size (log)	10	5	10	5	10	5	10	5	10	5
Beetles	Reproduction success	√		√		√	√	√	√	√	√
	Oviposition gallery length	√		√		√	√	√	√	√	√
	Larval gallery density	√		√		√	√	√	√	√	√
	Average beetles emerged	√		√		√	√	√	√	√	√
	Beetle emergence pattern per day	√		√		√		√		√	
	Females' weight	√		√		√		√		√	
	Females' pronotum width	√		√		√		√		√	
	Pheromone concentration		√		√		√		√		√
	Beetles' survival by feeding on *Beauveria bassiana*						√		√		√
Fungi	Growth area						√		√		√
	Conidiospore density						√		√		√
	Ergosterol concentration						√		√		√
	Growth competition among major symbionts		√		√		√		√		√
	Growth competition: symbionts vs. saprophytes		√		√						

Here, RH: relative humidity; Amb: Ambient; e: elevated; P: parents; F_1: F_1 generation

The experiment tested two relative humidity (RH) levels: 33% RH (low) (based on average RH values during the summer of 2017-2020 collected from Dorothy 1200 MST FWI weather station) ("Environment and Climate Change Canada," 2023) and 65% RH (ambient) (based on the average RH data (1994-2020) obtained from Jasper Warden and Willow Creek weather station) ("Environment and Climate Change Canada," 2023) for 30 d under a 16-h day (lighting-white) and 8-h night (dark) cycle, at 24°C, with continuous air circulation. Afterward, the logs were

moved to a laboratory environment in ambient conditions where individual logs were placed in a rectangular plastic container, equipped with glass jars. Emerging beetles were collected daily, with the number of broods recorded, and then stored at 4°C for subsequent analysis (Appendix C: **Figure C1**).

5.2.2 Rearing beetles under elevated CO_2 and O_3

The selection of eCO_2 and eO_3 concentrations was based on the prediction and observation reported from 810 peer-reviewed publications (Xia et al., 2021). For this experiment, we divided 30 logs from the same five trees equally into three groups: eCO_2, eO_3, and control logs under ambient conditions. Using three grow tents (Lumo-X [5'x5'] Mylar Hydroponic Grow Tent; Canbridge Tech Ltd., Markham, ON, CAN), live beetles were introduced to logs covered with nylon mesh. One set of 10 logs for each treatment was placed in its respective tent. The first tent, with a CO_2 generator (Two Burners Carbon Dioxide Generator LP, Titan control, Vancouver, WA, U.S.A) and a controller (GZ US CO_2 Controller, QC, CAN), maintained 1,000ppm of CO_2 (eCO_2) (Coviella and Trumble, 1999; Stiling and Cornelissen, 2007). The CO_2 generator was connected to a liquefied petroleum gas tank outside the tent. The second tent with an O_3 generator (the DC PRO 450 Ozone Generator, Langley, BC, CAN) and a controller (EcoZone™ Model EZ-1X, Newark, CA, USA) equipped with a timer, maintained 100ppb of O_3 (eO_3) (Jiang et al., 2023; Masui et al., 2023; Pinto et al., 2007b). The third tent served as the control under ambient conditions (Mean±SE; CO_2= 485.14±8.74, O_3=18.58±0.59). All three tents had constant environmental parameters, including a 16-h day and 8-h night cycle, at 24°C, and a relative humidity of 65%, with continuous air circulation. Beetles were exposed to eCO_2 or eO_3 for 30 d, after which climatic parameters were returned to ambient conditions, and emerging broods were collected daily (Appendix C: **Figure C1**).

5.2.3 Assessment of MPB reproduction

Following the cessation of beetle emergence in both experiments, we removed the tree bark to examine the bark beetle galleries. We considered reproduction was successful when at least one larval gallery was present per oviposition gallery, and reproduction failed when oviposition galleries lacked larval galleries. To obtain reproductive information, we randomly selected two oviposition galleries from each log in the successful reproduction category. These oviposition

galleries selected were then mapped onto transparent plastic sheets, which were subsequently scanned and gallery measurements were obtained from images using Image J software version Java 1.8.0-172 (National Institutes of Health, Bethesda, MD, USA) (Abràmoff et al., 2004). To gain further insight into the reproduction, behaviour, and physical characteristics of the MPB population under different experimental conditions, additional measurements and observations were made. We recorded the daily total number of broods emerged, the length of emergence (cumulative number of days over which beetle emergence occurred), the length of a maternal oviposition gallery, and larval gallery density (number of larval galleries per cm of a maternal oviposition gallery). Furthermore, we randomly selected 25 beetles from each treatment group and measured their weight (to the nearest 0.01 mg) using an analytical balance and pronotum width (the width of the pronotum at its widest part) using a slide caliper (to the nearest 0.01 mm).

5.2.4 Pheromone Collection

To test whether modified environmental conditions could influence the pheromone production of MPB, we collected pheromones produced by broods emerging from the five different treatment groups (ambient and reduced RH, eCO_2, eO_3, and ambient conditions). Specifically, we introduced three pairs of 2-3 d old beetles onto fresh logs for each treatment group (Erbilgin et al., 2014). If the male was rejected by the female, a new male was introduced within a 24 h window. Pheromones were continuously collected from the holes for four h.

To assess the transgenerational effects of eCO_2 and eO_3 on the brood, beetle-inoculated logs were retained in individual rearing containers after pheromone collection for two additional months at room temperature to complete their development. After beetle emergence, we removed the bark from the logs and analyzed the gallery patterns, as previously described.

Pheromone collection was conducted using a similar method described in Erbilgin et al. (2020). Briefly, a charcoal filter (Honeywell, Southborough, MA, USA) was placed between the bark and the mouth of a small Teflon funnel, which had a diameter of 2.5 cm at the mouth. Each funnel was attached directly above the entrance hole used by the beetles and connected to a vacuum pump (Cole Parmer Canada Inc., Montreal, QC, CAN) *via* Teflon tubes. A Porapak Q adsorbent cartridge (OD, 6 mm; length, 110 mm; adsorbent: front layer, 150 mg; back up layer, 75 mg; separated by glass wool), [by SKC Inc., Eighty-Four, PA, USA] was inserted into the

tube between the pump and the funnel. Pheromones were collected continuously for 4 h from each beetle entrance hole. Throughout the collection period, the flow rate of the pumps was maintained at a constant rate of 100 ml min^{-1}. Pheromones were collected at specific time intervals, including 12, 24, 36, 60, 84, and 108 h after the initial introduction of the female beetle. Each collection was taken from the same entrance hole and the same collection technique but with a new adsorbent cartridge. Once used for pheromone collection, the adsorbent cartridges were sealed and stored at a temperature of -40°C until extraction and further analysis. Throughout the process of pheromone collection, the logs were kept at room temperature, with conditions maintained at 23–25°C and 65% RH.

5.2.5 Pheromone analysis

To extract and analyze the pheromones, we followed a method described by Erbilgin et al. (2020) with slight modifications. Briefly, pheromones were extracted using 1 ml of dichloromethane as the solvent, adding an internal standard at a concentration of 0.0010% heptyl acetate (Sigma-Aldrich). The extracted pheromone samples were injected into a GC/MS system (Agilent 7890A/5975C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP-Chiral-20β column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) in split-less mode and helium was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. The temperature setting was as follows: starting at 50 ºC for 2 min, increased to 90 ºC by 45 ºC min^{-1} and held for 2 min, increased to 155 ºC by 6 ºC min^{-1} and held for 1 min, and then to 230 ºC by 25 ºC min^{-1} and held for 3 min. The MS was set to both SCAN and SIM mode with the following ion masses and time program: ion masses 72, 85, 100, and 114 starting at 3.5 min, ion mass 43 starting at 16.5 min, ion mass 170 starting at 18.0 min, and ion masses 107 and 109 starting at 21.7 min. Standard curves were generated using a series of four dilutions (10x, 100x, 1000x, 10,000x) prepared from analytical standards of specific pheromone compounds to quantify the pheromone components. These compounds included *cis*-/*trans*-verbenol, *exo*-brevicomin, frontalin, and verbenone (Contech Enterprise Inc. BC, CAN). The chemical purity of these standards was greater than 85%.

5.2.6 Offspring mortality assay using entomopathogenic fungus

To determine how exposure to eCO_2 and eO_3 treatments influenced the susceptibility of F_1 brood to an entomopathogenic fungus, we conducted a feeding mortality assay, as described in Agbulu

et al. (2022). Entomopathogenic fungus has previously been used to test beetle mortality (Fernandez et al., 2023; Mann and Davis, 2021). The entomopathogenic fungus *Beauveria bassiana* (NOF 3221, provided by Dr. Tod Ramsfield, Northern Forestry Centre, AB, CAN) was used in this assay. Briefly, *B. bassiana* was cultured for five days in Petri dishes (55 mm) containing Potato-Dextrose agar (PDA), and female beetles were introduced to these fungal cultures and allowed to feed until they died. We randomly selected 20 beetles from ambient conditions, eCO_2 or eO_3 treatments and placed individual beetles in separate Petri dishes containing *B. bassiana* cultures. All experiments were conducted under a laminar airflow bench in a dark and aseptic environment at 23±1°C with constant airflow. Survival of the beetles was monitored daily for 10 d using a stereomicroscope (Fisher Scientific, ON, CAN).

5.2.7 Testing the effect of elevated CO_2 and O_3 on MPB symbiotic fungi

Three symbiotic fungi, *G. clavigera*, *O. montium*, and *L. longiclavatum*, were utilized. Three different isolates were employed for each fungal species: *G. clavigera* isolates EL004, EL034, and EL035; *L. longiclavatum* isolates EL002, EL038, and EL037; and *O. montium* isolates EL030, EL031, and EL032. All isolates were initially obtained from naturally MPB-infected galleries in Alberta and were identified by Dr. Nadir Erbilgin (Appendix C: **Table C1**).

We initially sub-cultured these isolates from the master culture into PDA and allowed them to grow for 10 d at 22°C in complete darkness. Following this, a second sub-culturing was conducted on Petri dishes (55×15 mm), each containing 10 ml of PDA. Each isolate was replicated ten times for each treatment (30 replications for each fungal species). The sub-culturing process involved taking a 5 mm diameter plug from the actively growing margins of the 10-d-old culture and placing them upside down on the new plates. To create suitable experimental conditions, two glass cabinets (104 cm×46 cm) were constructed, featuring eight holes in the upper section to facilitate air passage and minimize condensation within the cabinets. Cabinets were thoroughly disinfected with 70% ethanol and 20% bleach before usage. Within each glass cabinet, the fungal plates were positioned on the upper shelf, accompanied by either a CO_2 or O_3 generator and their respective controllers, placed at the bottom of the cabinet. A second cabinet was employed to control ambient conditions without gas exposure. The temperature was maintained at 23±1°C throughout the entire duration of the experiment.

The fungi were exposed to either 1,000 ppm of CO_2 or 100 ppb of O_3 for five days. Following this exposure period, we assessed fungal growth using ImageJ software, quantified spore production, and estimated ergosterol concentrations to estimate fungal biomass as described below. Based on the results of this initial assay, we identified the dominant isolates for each fungal species as follows: *G. clavigera* EL035, *L. longiclavatum* EL038, and *O. montium* EL030. Consequently, these three isolates were chosen for further experimentation to investigate the impact of eCO_2 and eO_3 on interspecies competition among the three fungal species. To assess interspecies competition, we divided a Petri dish (90×55 mm diameter) into three equal sections and sub-cultured the isolates by placing 5 mm diameter fungal plugs from the actively growing margin of 10-day-old growth. These plugs were positioned equidistant from each other and allowed to grow for five days under eCO_2, eO_3, and ambient conditions. Subsequently, we measured their growth area and assessed the resources captured by each species.

The quantification of conidiospores produced followed a previously established method described by Cale et al. (2016). Briefly, a 1 mm tall section was excised from the inoculation plug, originally 5 mm in diameter, which was initially used for inoculating the plates. This plug was submerged in a microtube containing 1 mL of a 0.5% Tween 20 solution and then subjected to vortexing for 30 s. Subsequently, a 10 µL aliquot of the resulting spore suspension was loaded into a hemocytometer to determine the conidia concentration accurately. The conidia concentrations were standardized to ensure precision using the total fungal culture area within the respective plates.

5.2.8 Ergosterol analysis

To quantify the concentration of ergosterol produced by fungi, we extracted, identified, and quantified ergosterol following an established method with slight modifications (Rodriguez-Ramos et al., 2021; Sterkenburg et al., 2015). The entire fungal culture from the plates was freeze-dried (Labconco Corp., Kansas City, MO, USA). Ergosterol was then extracted from the dried samples. This extraction process involved suspending the dried pieces in 2.5 mL of a 10% w/v KOH solution. The mixture was vortexed for 2 min, sonicated for 15 min, and incubated for 60 min in a water bath at 70°C. After incubation, 0.5 mL of distilled water and 1.5 mL of cyclohexane were added to the mixture, which was then vortexed for 1 min. Subsequently, the mixture was centrifuged for 5 min at 3600 rpm. The non-polar layer from the centrifuged

mixture was carefully transferred into a new 20 mL glass tube. In the original tube, 1 mL of cyclohexane was added, followed by centrifugation as before. The upper layer from this second centrifugation was also collected, and the resulting extracts from both steps were pooled. The cyclohexane in each tube was evaporated using a 40°C heating block under a flow of N_2 gas until dry. The dried samples were resuspended in 600 µL of methanol and incubated in a water bath for 15 min at 40°C. Subsequently, the samples were sonicated for 5 min. Finally, the extracts were filtered through glass wool.

For analysis, a 20 µL injection volume of the extract was used with an Ultra-High-Performance Liquid Chromatograph (1290 Infinity; Agilent Tech.) equipped with a Poroshell 120 EC-C18 column (2.1 mm × 150 mm, 2.7 µm; Agilent Tech). The mobile phase consisted of an isocratic binary system comprising 25% methanol (HPLC-grade) and 75% acetonitrile, with a flow rate of 0.4 ml min^{-1} for 10 min. Detection of the ergosterol peak was achieved using UV/VIS at 282 nm, and concentrations (µg mg^{-1} fungal mass) were determined by constructing a concentration standard curve using serial dilutions of an ergosterol standard (≥ 95%; Sigma-Aldrich).

5.2.9 MPB symbionts and saprophyte competition assay under different RH conditions

While rearing beetles under varying RH conditions, we observed the presence of two saprophytic fungi, *Aspergillus* sp. and *Trichoderma* sp. These findings prompted us to conduct an assay to assess the competitive interactions between these saprophytes and MPB symbionts, specifically *Aspergillus* sp. EL050 and *Trichoderma* sp. EL051 (both of which were initially isolated from a bark beetle gallery by Dr. Nadir Erbilgin; Table S1). As previously described, the assay involved the following steps: we randomly positioned and sub-cultured *Aspergillus* sp., *Trichoderma* sp., *G. clavigera* (EL035), *L. longiclavatum* (EL038), and *O. montium* (EL030) in a Petri dish (150×20 mm diameter). Placing these Petri dishes in two separate growth chambers, each maintained at distinct RH conditions (33% or 65%). The growth chambers were kept in complete darkness at 24°C, with ten replicates for each condition for 10 d. After the 10-d incubation period, we analyzed the growth of all fungal species to assess their competitive interactions and outcomes under the varying RH conditions.

5.2.10 Statistical analyses

All statistical analyses were conducted using GraphPad Prism version 9.5.0 for Windows (GraphPad Software, San Diego, CAL, USA, www.graphpad.com). Before analysis, the data were checked for the assumptions of homoscedasticity and normality. Bartlett's test assessed homoscedasticity, while the Shapiro–Wilk test was used to evaluate normality. In cases where the data did not meet the criteria for normality, a log transformation (+1) was used before analysis.

For experiments involving different RH conditions, normally distributed data with equal variances were analyzed using unpaired T-tests. In contrast, non-normally distributed data (after transformation) with similar variances were analyzed using the Mann-Whitney test. Growth competition data between symbionts and saprophytes under different RH treatments were subjected to two-way ANOVA.

For comparisons among treatments involving eCO_2, eO_3, and ambient conditions, normally distributed data with equal variances were analyzed using an ordinary one-way ANOVA, while normally distributed data with unequal variances were analyzed using Welch's ANOVA and non-normally distributed data (after transformation) with unequal variances were analyzed using the Kruskal-Wallis test. The number of beetles emerging per day and pheromone concentrations were analyzed using a repeated-measures mixed effects model, with treatments and time as fixed factors and individual beetles as random factors. The survival probability of beetles exposed to entomopathogenic fungi was assessed using the Mantel-Cox log-rank test. Data from fungal growth, conidiospore production, ergosterol concentration, and interspecies competition were analyzed using two-way ANOVA or multiple Mann-Whitney tests, depending on whether the data exhibited a normal or non-normal distribution, respectively.

5.3 Results

5.3.1 Ozone exposure impairs beetle reproduction, while CO_2 accelerates brood emergence

Elevated O_3 treatment significantly reduced reproduction success compared to ambient conditions ($F_{2, 17.84}$= 14.8; p<0.001) with a mean reproduction success of 24±5.8 % (mean ± SE) in comparison to ambient (82.7±5.3 %) (**Figure 5.2**a). In contrast, eCO_2-exposed beetles exhibited reproduction success of 67.7±11.3% which was not different from the ambient condition (p= 0.43). Furthermore, eO_3-exposed beetles excavated significantly shorter galleries

(161.5±14.7 mm) than the beetles in ambient (260.4±10.5 mm) ($F_{2, 28.18}$= 10.2; p<0.001) (**Figure 5.2**b).

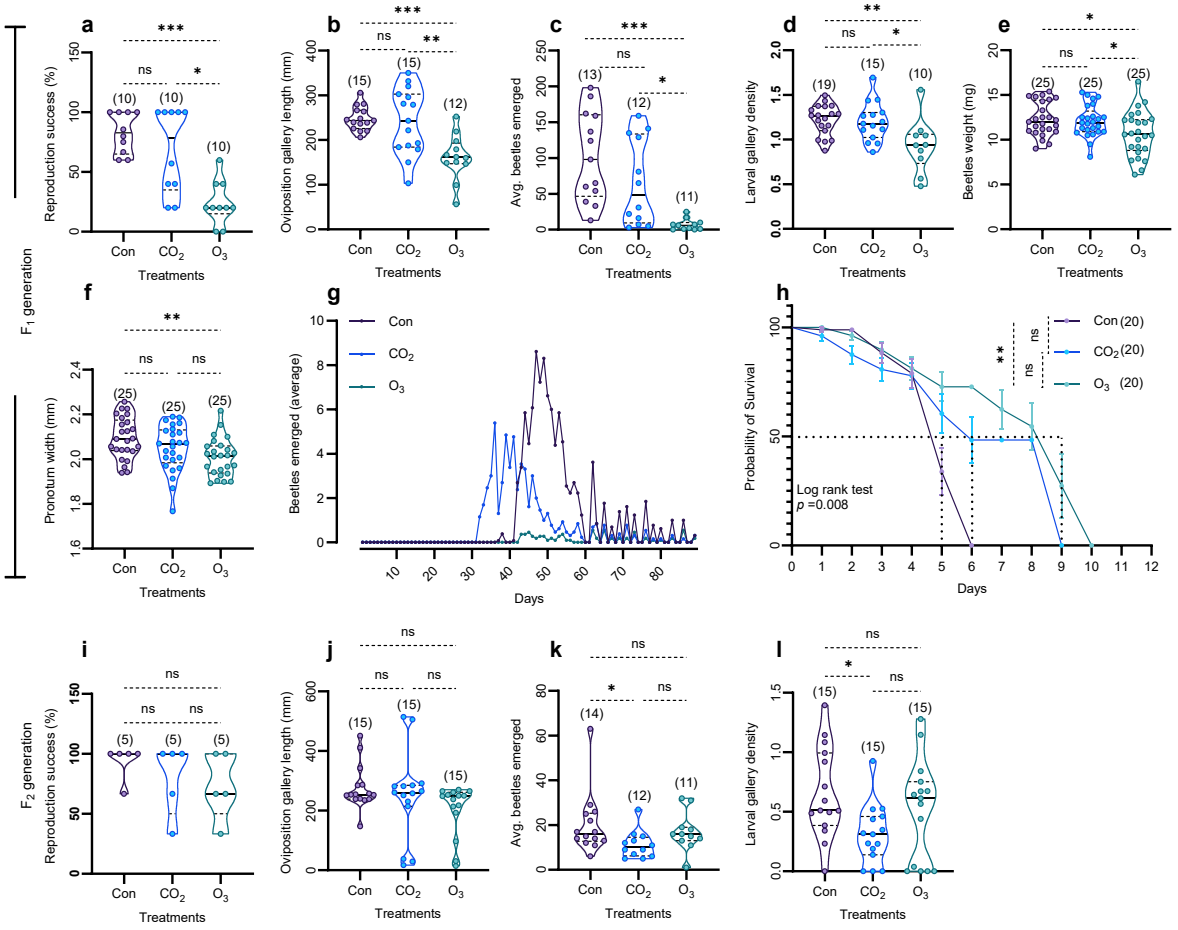

Figure 5.2: The effects of elevated CO_2, O_3, and ambient conditions on the reproductive biology of the mountain pine beetle. Panel **(a-f)** shows various aspects of gallery pattern and beetle fitness in the F_1 generation, following treatment of their parent generation with elevated CO_2, elevated O_3, and ambient conditions (sample sizes indicated in parentheses). Statistical analysis involved one-way ANOVA for multiple comparisons against the control group (ambient). Panel **(g)** presents the emergence of F_1 generation beetles per day, analyzed using a repeated measures mixed effects model. Panel **(h)** displays the survival probability of the F_1 generation when exposed to entomopathogenic fungi, assessed through the Mantel-Cox log-rank test. Panels **(i-l)** provide insights into the gallery and beetle emergence patterns in the F_2 generation, statistical analysis employing one-way ANOVA for multiple comparisons against the

control group. Notably, groups showing significant differences from the control group are denoted with asterisks, where *p<0.05, **p<0.01, ***p<0.001, and ns=not significant.

eCO_2-treated beetles excavated oviposition galleries similar in length to the ambient conditions, with a mean length of 241.5±19.1 mm. Brood emergence was reduced in eO_3 treatment compared to eCO_2 and ambient treatments ($F_{2, 25.37}$= 16.7; p<0.001) (**Figure 5.2**c). The larval gallery density per cm of oviposition gallery was also reduced in eO_3 treatment (1.1±0.3 gallery cm^{-1} oviposition gallery) compared to ambient (1.8±0.2 gallery cm^{-1} oviposition gallery) ($F_{2, 41}$= 4.1; p<0.05) (**Figure 5.2**d). eCO_2 exposure caused the brood to emerge earlier than both ambient and eO_3 treatments. Broods emerged at around 32 d from the first day of parent beetle exposed to eCO_2, while those in ambient conditions emerged at 37 d, and those in eO_3 treatments emerged at 43 d (Days × Treatments: $F_{176, 2992}$= 8.9; p< 0.001) (**Figure 5.2**g).

5.3.2 Cohort-specific responses of brood by elevated conditions: fitness, survival, and pheromone production

Since eCO_2 exposure accelerated offspring emergence, we asked whether exposure to eCO_2 or to eO_3 would affect brood fitness and survival. We measured the female brood's pronotum size and weight. Broods that emerged from the eCO_2 treatment did not show differences in weight (p=0.91) or pronotum size (p=0.15) compared to the brood from the ambient treatment. In contrast, exposure of the parent beetles to eO_3 significantly affected brood fitness. Broods from the eO_3-exposed parent beetles had significantly reduced weight ($F_{2, 72}$= 4.7, p< 0.05) and pronotum size ($F_{2, 72}$= 5.3, p< 0.01) compared to broods from the ambient condition (**Figure 5.2**e and 2f, respectively). We also investigated the probability of survival of the brood by exposing them to *B. bassiana* using the Kaplan-Meier survival curve (**Figure 5.2**h). The survival probability of brood from the eO_3 treatment when they were exposed to *B. bassiana* increased significantly compared to control offspring (Log-rank test: χ^2_1= 8.2; p<0.05). Specifically, by day 5, half of the control beetles had died, while it took nine days for 50% of the beetles from the eO_3 treatment to die.

We further investigated whether eCO_2 or eO_3 would affect brood pheromone production. Pheromone production was different across the treatments. Although brood from all treatments produced the four main pheromone components, *cis*-/*trans*-verbenol, verbenone, frontalin, and

exo-brevicomin (Appendix C: **Figure C3**), the concentration of these pheromone components varied across the different treatments and time points (**Figure 5.3**).

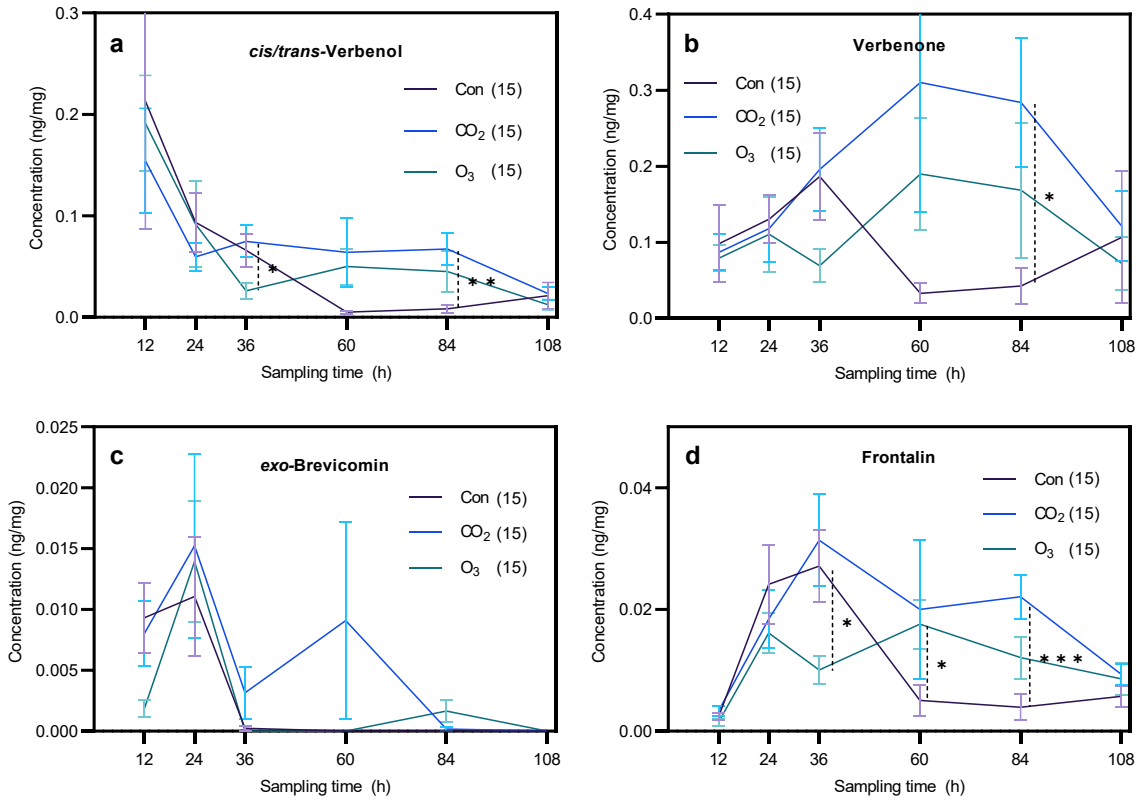

Figure 5.3: The effects of elevated CO2 and O3 treatments on the pheromone production of the mountain pine beetle offspring. Four distinct pheromones, **(a)** *cis*/*trans*-verbenol, **(b)** verbenone, **(c)** *exo*-brevicomin, and **(d)** frontalin, produced by the F1 generation were collected at various time points denoted in the X-axis, and their concentrations (on Y-axis) were quantified as ng mg^{-1}. Data analysis involved a repeated measures mixed effects model, and multiple comparisons were conducted using the Tukey test. Sample sizes are indicated within brackets, and groups exhibiting statistically significant differences are denoted as *p<0.05, **p<0.01, and ***p<0.001.

Overall, the eCO_2 treatment had a positive effect on the brood pheromone production. Specifically, the brood from the eCO_2 treatment produced significantly more *cis*-/*trans*-verbenol than those from the ambient treatment at 84 h (p= 0.04) (**Figure 5.3**a). Verbenone production was also significantly higher in the brood from the eCO_2 (Appendix C: **Table C2**) treatment

compared to the ambient treatment at 84 h (**Figure 5.3**b). Frontalin production showed differences between treatments at different time points: it was decreased at 36 h, increased at 60 h in the eO_3 treatment, and increased at 84 h in the eCO_2 treatment (**Figure 5.3**d).

5.3.3 Generational cascading effect of eCO_2 and eO_3 on the MPB brood

When the F_1 generation brood was allowed to mate and reproduce, the reproduction success was similar (χ^2_1= 2.1; p<0.15) (**Figure 5.2**i) among the F_1 generation offspring from different treatments. There were no differences among the oviposition galleries constructed by F_1 beetles across other treatments ($F_{2, 34}$= 0.6, p= 0.6) (**Figure 5.2**j). However, the larval gallery density per cm of oviposition gallery was significantly reduced for the F_2 descendants of eCO_2-treated parents ($F_{2, 42}$= 1.5, p< 0.05) (**Figure 5.2**k). Likewise, the F_1 descendants of eCO_2-treated parents produced a significantly lower number of offspring (11.3±1.8) compared to F_1 descendants of ambient parents (20.2±3.7) (H= 7.1, p< 0.05) (**Figure 5.2**l).

5.3.4 Reduced relative humidity improved MPB reproduction, offspring fitness, and the growth of its fungal symbionts

The MPB reproduction success was significantly improved under low RH treatment (86.4±2.9%) compared to ambient RH (59.9± 5.0%) (p< 0.001) (**Figure 5.4**a), leading to increased daily brood emergence (p< 0.01) (Days × Treatments: $F_{81, 1458}$= 1.7; p< 0.001) (**Figure 5.4**d & g). The larval gallery density cm^{-1} of the oviposition gallery was also increased in low RH compared to ambient RH (p< 0.05) (Fig. 5.4c). Consequently, low RH improved the fitness of brood females in terms of weight gain and pronotum size (weight: p< 0.001, pronotum size: p< 0.001) (**Figure 5.4**e & f).

All four primary pheromones of MPB were detected in ambient and low RH treatments (Appendix C: **Figure C2**, **Figure C3**). The emission pattern for *cis*-/*trans*-verbenol and *exo*-brevicomin were similar for both treatments, with emission peaks highest at specific time points (e.g., *cis*-/*trans*-verbenol emission peak at 12 h, *exo*-brevicomin emission peak at 24 h) (Appendix C: **Table C*2***, **Figure C2**a & c). In low RH, the highest peak for verbenone and frontalin was observed at 36 h, while in ambient RH, it was found at 12 h and 84 h, respectively (Appendix C: **Figure C2**b & d). On average, the concentration of all pheromone components was similar for both treatments, except for the difference in the emission periods (*cis*-/*trans*-

verbenol: $F_{1, 16}$= 0.015, p= 0.91; verbenone: $F_{1, 16}$= 0.02, p= 0.88; frontalin: $F_{1, 16}$= 0.33, p= 0.57; *exo*-brevicomin: $F_{1, 16}$=2.8, p= 0.11).

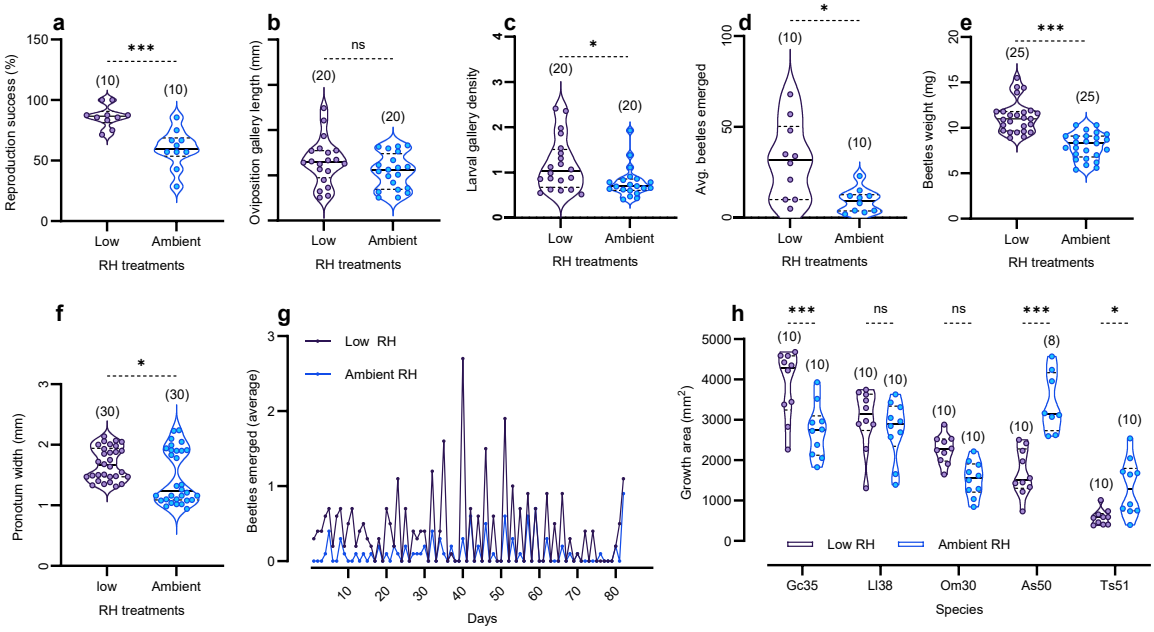

Figure 5.4: The effects of varying relative humidity (RH), specifically low (33%) and ambient (65%) RH, on the reproductive biology of the mountain pine beetle and its fungal symbionts. The F_1 generation's characteristics related to gallery pattern and beetle fitness **(panels a-g)** were assessed after subjecting their parents to these RH conditions. Data analysis employed unpaired T-tests or Mann-Whitney tests for certain parameters, while beetle emergence data **(panel g)** were analyzed using a repeated measures mixed effects model. The study examined interspecies competition between fungal symbionts and saprophytes under different RH conditions **(panel h)**, with statistical analysis conducted using a two-way ANOVA. Significantly different results between the low RH and ambient RH groups are indicated as follows: *p<0.05, **p<0.01, ***p<0.001, and ns=not significant. Here, Gc35= *Grosmannia clavigera* EL035, Ll38= *Leptographium longiclavatum* EL038, Om30= *Ophiostoma montium* EL030, As50= *Aspergillus* sp. EL050, and Ts51= *Trichoderma* sp. EL051.

We observed significant results from studies investigating the competition between the fungal symbionts and saprophytes under different RH conditions (**Figure 5.4**h). Among the fungal symbionts and saprophytes, *G. clavigera* EL035 and *Aspergillus* sp. EL050 were the most

dominant in terms of growth under both low and ambient RH conditions. *Grosmannia clavigera* EL035 showed better growth under low RH conditions (3872.9±267.4 mm^2) compared to ambient RH (2721.2±208.1 mm^2) ($p< 0.001$). In contrast, *Aspergillus* sp. EL050 grew better under ambient RH conditions (3413.4±263.2 mm^2) than under low RH (1682.5±181.9 mm^2) ($p< 0.001$). Under ambient RH conditions, both *G. clavigera* EL035 and *Aspergillus* sp. EL050 had similar growth ($p= 0.22$), suggesting neither had a clear advantage. However, under low RH conditions, *G. clavigera* EL035 outperformed *Aspergillus* sp. EL050 ($p<0.001$), indicating that low RH gave *G. clavigera* EL035 a competitive growth advantage.

5.3.5 Elevated ozone negatively affected the reproduction biology of MPB fungal symbionts

Exposure to eO_3 significantly reduced the overall growth of all fungal isolates, regardless of species (treatments: $F_{1, 161}= 1609$, $p< 0.001$) (**Figure 5.5**a). Both *G. clavigera* EL035 and *L. longiclavatum* EL038 had similar growth in both ambient and eO_3 conditions. The highest and lowest growth reduction was observed for *L. longiclavatum* EL037 (93.02%) and *G. clavigera* EL035 (47.97%) between ambient and eO_3 conditions. Exposure to eO_3 led to a reduction in spore production in almost half of the tested fungal isolates, except for *L. longiclavatum* EL037, which significantly increased spore production ($p< 0.01$) (**Figure 5.5**b). Exposure to eO_3 also reduced the total fungal biomass, as indicated by ergosterol concentration, in *G. clavigera* EL04 and *O. montium* EL031 (treatments: $F_{6, 52}= 13.9$, $p< 0.001$) in *G. clavigera* EL04 and *O. montium* EL031 (**Figure 5.5**c).

With the eCO_2 treatment context, our analysis revealed significant effects of treatment, isolates and their interaction (interaction: $F_{8, 158}= 58.4$, $p< 0.001$; isolates: $F_{8, 158}= 582.2$, $p< 0.001$; treatments: $F_{1, 158}= 16.4$, $p< 0.001$) (**Figure 5.5**d). The eCO_2 treatment did not exert significant impact on the growth of dominant isolates of *G. clavigera* EL035 and *L. longiclavatum* EL038. However, it significantly inhibits the growth of *G. clavigera* EL004 and *O. montium* EL032 while concurrently promoting the growth of *L. longiclavatum* EL002. Additionally, our observations indicated divergent effects on spore production. In particular, the eCO_2 treatment significantly increased spore production for *G. clavigera* EL004, whereas it had the opposite effect, leading to a reduction for *L. longiclavatum* EL002 (**Figure 5.5**e). While no significance was found, ergosterol concentration slightly increased under eCO_2 for *G. clavigera* EL035, EL034, *L. longiclavatum* EL037, and *O. montium* EL031 and EL 032 (**Figure 5.5**f).

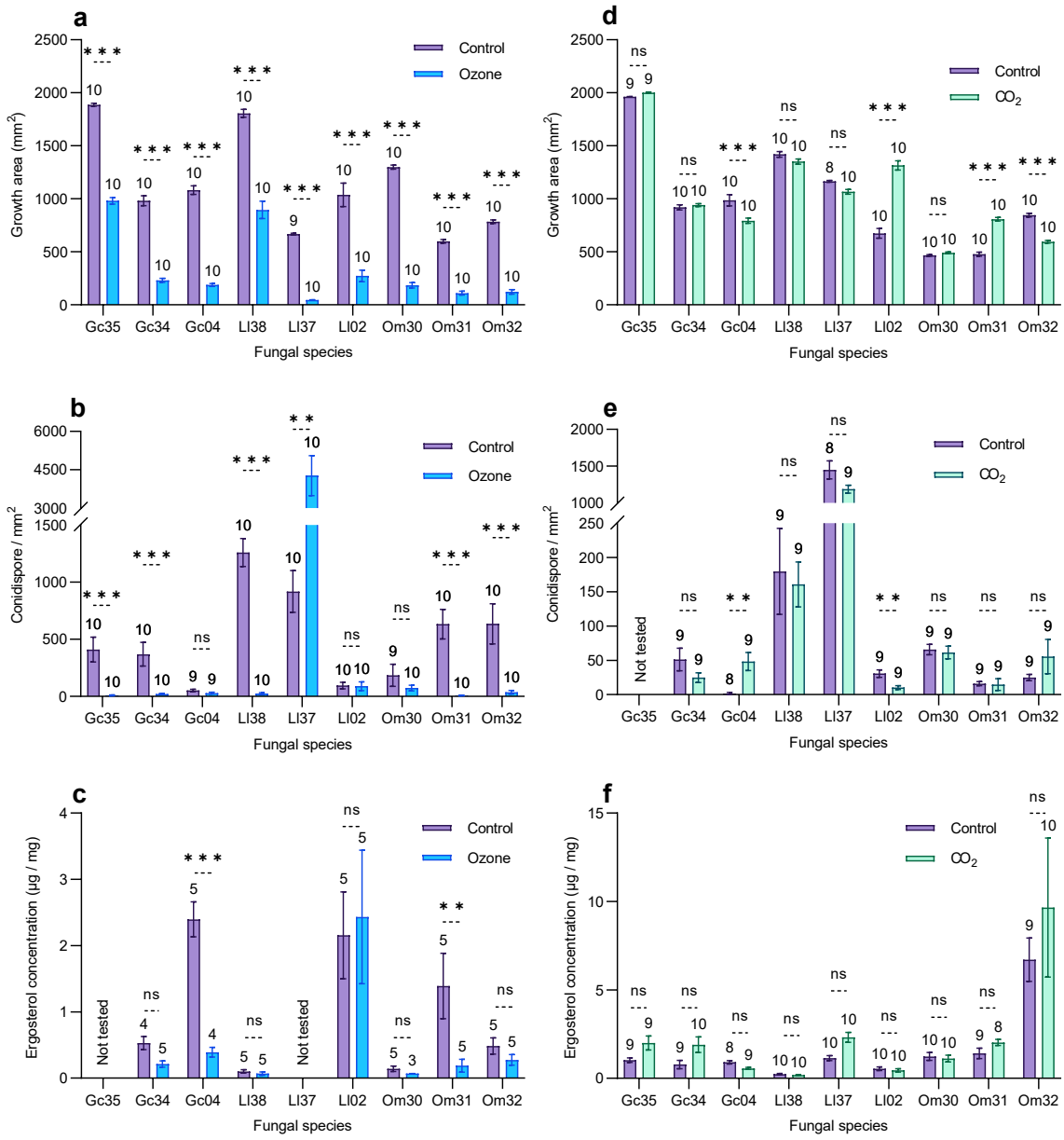

Figure 5.5: Effects of elevated CO_2 and O_3 on the reproduction of fungal symbionts of the mountain pine beetle. (a-c) Effects on the growth area, conidiospore production, and ergosterol concentration of fungal species and isolates when exposed to control (ambient) and elevated O_3 conditions for five days. **(d-f)** Effect on the growth area, conidiospore production, and ergosterol concentration of fungal species and isolates exposed to control (ambient) and elevated CO_2 conditions for five days. Data analysis utilized two-way ANOVA or multiple Mann-Whitney tests. Statistically significant differences between the treatment group and control are indicated as follows: *p<0.05, **p<0.01, ***p<0.001, and ns= not significant. In the data labels,

Gc=*Grosmannia clavigera*, Om=*Ophiostoma montium*, Ll=*Leptographium longiclavatum*, and numbers following Gc, Om, or Ll represent isolate numbers, with the sample numbers indicated on top of the bars.

Furthermore, we pooled the dominant isolates from each symbiont species and examined the effect of eCO_2 and eO_3 on the interspecies competition. As expected, eO_3 treatment significantly reduced the growth of all three fungal species (**Figure 5. 6**a).

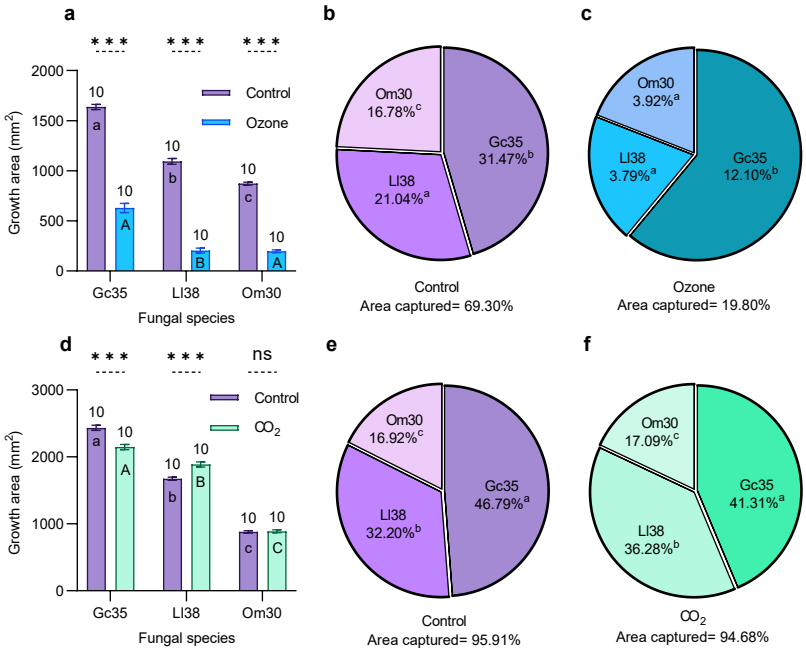

Figure 5. 6: Elevated levels of CO_2 and O_3 can impact the interactions between the dominant fungal symbionts of the mountain pine beetle. (a-c): Elevated O_3 and control conditions (ambient) on interspecies competition among co-cultured fungal species: **(a)** Growth of different fungal species. **(b)** Resource allocation under control conditions. **(c)** Resource allocation under elevated O_3 conditions. **(d-f)** Effects of elevated CO_2 and control conditions (ambient) on interspecies competition among co-cultured fungal species: **(d)** Compares the growth of different fungal species. **(e)** Resource allocation under control conditions. **(f)** Resource allocation under elevated CO_2 conditions. Different lowercase and uppercase letters in **(a)** and **(d)**, respectively, indicate significant differences among the various fungal species under control conditions and elevated O_3 or CO_2 conditions. Significant differences between the control and

different treatments are marked as ***p<0.001, with statistical analysis conducted using Two-way ANOVA and Tukey's multiple comparison test. In the data labels, Gc=*Grosmannia clavigera*, Om=*Ophiostoma montium*, Ll=*Leptographium longiclavatum*. Numbers following fungal species indicate isolate numbers, with the sample numbers indicated on top of the bars. Percentages in **(b)**, **(c)**, **(e)**, and **(f)** denote the area covered by each fungus, and lowercase letters signify significant differences among fungal species.

Particularly, *G. clavigera* EL035 was the most dominant species among the three, both in ambient and eO_3 treatments (species: $F_{2, 56}$= 164.5, p< 0.001; treatment: $F_{1, 56}$= 900.9, p< 0.001) in terms of both total growth and area captured (**Figure 5. 6**b & c). eCO_2 exposure had varying effects on different fungal species. While it caused an 11.71% reduction in growth for *G. clavigera* EL035 (p< 0.001), it resulted in a 12.66% increase in growth for *L. longiclavatum* EL038 (p< 0.001). It did not affect the growth of *O. montium* EL030 (**Figure 5. 6**d). Overall, *G. clavigera* EL035 was the most dominant species and captured the largest area, regardless of eCO_2 exposure (46.79% in ambient and 41.31% in eCO_2) (**Figure 5. 6**e, f).

5.4 Discussion

Our research sought to shed light on the complex responses of the MPB and its associated fungal symbionts to eO_3, eCO_2 and reduced RH. Through our investigation, we showed a complex web of interdependencies that underlines the dynamics between these environmental variables and the biology of the MPB-fungal complex (**Figure 5.1**). In light of our findings, we discuss these novel findings, their ecological ramifications, and the future directions for understanding the effects of climate change on insect herbivore-microbial symbioses complexes.

5.4.1 Generation-specific responses to eCO_2 and eO_3: accelerated development and diminished reproduction

One of the primary findings of our study was the distinct impact of eCO_2 exposure on the emergence timing of the F_1 generation of MPB (**Figure 5.1**c). This acceleration in development aligns with a growing body of research indicating that eCO_2 levels exert selective pressure on insect growth. Ectothermic insects, like MPB, adapt to these stressors by potentially modifying their metabolic rates (Deutsch et al., 2018; Hill et al., 2011; Hoffmann & Sgró, 2011; Hoffmann, 2017; Ziska et al., 2018). The ramifications of this adaptation extend beyond mere

developmental changes, to influencing insect life history traits and population dynamics. This is consistent with observations in several lepidopteran species, where eCO_2 exposure reduced larval duration and doubled the population growth rate (Akbar et al., 2016; Lu et al., 2022; Qi et al., 2017; Qian et al., 2017). Thus, eCO_2 concentration may significantly impact MPB biology, ultimately affecting the related ecological and evolutionary processes with their host plants and microbial symbionts (Zavala et al., 2013).

Conversely, eO_3 exposure unveiled a quite different narrative. The F_1 generation exhibited reduced oviposition behaviour, longer development time, and decreased brood fitness while demonstrating improved survival probability (**Figure 5.1**f). Importantly, it led to alterations in pheromone emission patterns, particularly in the case of frontalin. This shift in reproductive behaviour is consistent with established knowledge that eO_3 can disrupt insect pheromonal communication through oxidative stress and the breakdown of pheromone compounds (Li-Byarlay et al., 2016; Tasaki et al., 2017). Our findings are further supported by previous research, which emphasized the harmful impact of eO_3 on pheromonal communication (Arndt, 1995; Jiang et al., 2023; Vanderplanck et al., 2021). This disruption has implications for the population dynamics and interactions of MPB, illustrating the profound effects that eO_3 can have on insect behaviour and reproduction.

5.4.2 Enhanced survival in the face of compromised development: eO_3's surprising twist

Despite a lower number of broods produced, the brood of eO_3-exposed parents exhibited enhanced survival when challenged with an entomopathogenic fungus, *B. bassiana*. This unexpected resilience might be attributed to the smaller pronotum size and reduced weight of the exposed beetles. This size reduction is closely linked to larval diet, notably their consumption of mutualistic fungi, and is positively correlated with larger adult size in beetles (Six, 2012). Our findings suggest that eO_3 hinders symbiotic fungal growth within MPB galleries, potentially affecting the larval diet, which, in turn, influences offspring size and weight. In response to changing environmental conditions, insects employ various mechanisms, including phenotypically plastic changes and evolutionary responses (Garnas, 2018). This may involve the upregulation of defense-related genes such as growth-blocking peptides, Cytochrome P450s, ABCs, AchEs, and GSTs, with associated energy consumption costs (Qi et al., 2017; Qian et al., 2017; Shears and Hayakawa, 2019; Sinclair et al., 2013). Our observations align with the theory

of differential resource allocation, where resources usually allocated to reproduction are redirected to brood survival under stress conditions (Boggs, 2009).

5.4.3 Pheromone production: a response to environmental stress

Both eCO_2 and eO_3 treatments had apparent impacts on the brood pheromone production patterns. The variations in pheromone production may represent an adaptive response to environmental stress, with the potential for transgenerational phenotypic inheritance. This adaptation may enhance or disrupt mating success under stress. Although the observed alteration in specific pheromone production could be a stress adaptation (Berenbaum & Liao, 2019; Gowri & Monteiro, 2021; Verhoeven & van Gurp, 2012), questions remains if the beetles are dependent on the synchrony of the pheromone components release in a specific ratio over that time period. While these findings emphasize the intricacies of MPB's responses to changing environmental conditions, further studies are needed to fully elucidate these aspects and their implications for beetle mating success under challenging conditions.

5.4.4 Symbiotic fungi: a key player in MPB biology

The complex interaction between MPB and its fungal symbionts under the changing climate is a subject of paramount concern. Our research demonstrates the significant impacts of eCO_2 and eO_3 concentrations on the growth of these fungal symbionts (**Figure 5.1**d & g). Changes in species composition within this symbiotic relationship have the potential to reverberate throughout the ecological web (Åkesson et al., 2021). Under eO_3 conditions, we observed a substantial decline in symbiotic fungal growth and spore formation, highlighting the capacity of climatic factors to modify species interactions. Our previous studies have shown how these symbionts can enhance MPB’s foraging behaviours in artificial diets (Agbulu et al., 2022; Zaman et al., 2023). Any hindrance in symbiont growth and spore production could, in turn, adversely affect the fitness of the mutualist beetles, ultimately diminishing their reproductive success (Douglas, 2009; Six, 2012).

However, the responses of different fungal symbiont species to eCO_2 exposure were not straightforward. Some exhibited enhanced growth, while others displayed a reduction. This complexity underscores the multifaceted relationship between fungal species and eCO_2 concentrations, necessitating further investigation to clarify these interactions. Our findings also

raise questions about how climate change might disrupt the delicate balance among symbiotic fungal species, reshaping interactions within these microbial ecosystems. For example, *G. clavigera* EL035 emerged as the dominant species in both ambient settings and under elevated conditions, indicating the potential for a significant shift in the competitive balance. Currently, our knowledge is lacking regarding whether fungal symbionts of MPB demonstrate heritable plasticity and exhibit persistent relationships across multiple generations of MPB, or if a shift in the beetle-fungi symbiosis occurs within the context of climate change. Consequently, further exploration into the heritability, plasticity, and persistence of fungal symbionts within the MPB-fungi symbiosis under the influence of climate change will enhance our broader understanding of ecological interactions and evolutionary processes. Moreover, it will play a pivotal role in the development of well-informed strategies for conservation and management, addressing environmental challenges more effectively.

5.4.5 Saprophytic fungi: adding a layer of complexity

The presence of saprophytic fungi, such as *Aspergillus* sp. and *Trichoderma* sp., within MPB galleries further complicates our understanding of MPB-fungal interactions (Cardoza et al., 2006; Fox et al., 1992). The saprophytic fungi thrive in conditions of high RH, even at lower temperatures. Our study reveals that reduced RH enhances MPB reproduction success and brood development (Fig. 1h). This poses the question whether variable RH can modulate competition between symbionts and saprophytes (Åkesson et al., 2021; Van Der Putten et al., 2007). Our observations demonstrate that variable RH can alter this competition with *Aspergillus* sp. outcompeting and occupying a larger area than the fungal symbionts under ambient humidity condition. This finding explains some of the variability in offspring fitness traits.

5.4.6 The complex web of ecological relationships

Our research has unravelled the intricate and multifaceted interactions between MPB, its fungal symbionts, and changing environmental conditions. As illustrated in **Figure 5.1**, the impact of climatic variables involving eCO_2, eO_3, and lower humidity levels on the functional network of ecological interactions among bark beetles, their fungal symbionts, and host trees (a-b) will be complex—and will become more complex when we incorporate other organisms such as non-fungal phoretic symbionts (nematodes, mites, yeast, bacteria etc. with both MPB and

ophiostomatoid fungi) and endophytes (with host tree) in the network—and encompasses several key relationships (c-n). These environmental variables not only alter the host colonization behaviour and population dynamics of bark beetles but could also intensify intra-and inter-specific competition among symbionts, influencing the host physiology (both tree and beetles) and host colonization success of beetles (a-b). Moreover, changes in relative humidity may affect beetle and fungal responses, as well as the physiological responses of the host tree, thereby influencing the dynamics of the host-pest-pathogen interactions (h). Furthermore, the same environmental variables also affect one another and (j-m). For instance, it was reported that eCO_2 can counteract the harmful effects of eO_3 on the host tree physiology (Xia et al., 2021). In light of these expectations and the projected climate change, the potential increased bark beetle outbreaks and associated tree mortality in the coming century appears to be high (Bentz et al., 2010).

Our research emphasizes the importance of conducting biologically relevant studies for important forest insect species to understand the potential impacts of climate change on various aspects of mutualistic organisms. From individual physiology to interactions between species, the ripple effects are significant and warrant further investigation. As we look ahead, future research must focus on the physiological, genetic, and biochemical factors underlying the diverse responses observed across generations. For instance, the MPB symbiotic fungi not only contribute to the overall complexity but also exhibit spatial community and population structures, potentially influencing how the MPB adapts within the ecosystem. Thus, additional research is needed to elucidate these spatial community and population structures of the fungal symbionts of MPB in the context of climate change adaptation. By exploring these dynamics, we can better understand the connection within the ecosystem network and the potential cascading effects on the adaptation and behaviour of key species like the MPB. Establishing connections between the biology of insect hosts under various climatic conditions and gaining insights into how climate change affects microbial ecology, particularly in the context of fungal symbionts and non-fungal phoretic symbionts, will be of paramount importance. The ultimate goal is to integrate these findings for broader applications at the landscape and ecosystem levels, considering species-specific interactions.

While we recognize that our experimental approach, which involves symbiotic and entomopathogenic fungi, introduces a distinct microhabitat compared to the complex natural environment of MPB, and the absence of interactions with other microbial species, for instance, may have implications for the interpretation of our results, this methodology was the only feasible way to address the impact of future environmental conditions on the complex biological interactions. Nevertheless, our study highlights the complex nature of ecological responses to changing environments, demonstrating how future climate change scenarios, including eCO_2 and eO_3 concentrations and reduced RH, could significantly impact the bark beetle population dynamics and their interactions with fungal symbionts. Our collective responsibility is to continue unravelling this complexity and implement strategies that protect and preserve the delicate balance of insect herbivores and their interaction with microbial symbionts.

Chapter 6: Thesis Discussion

My studies have revealed that many factors can influence the interactions among host trees, insects, and their symbiotic fungi. Under normal environmental conditions, the symbiotic fungi of the mountain pine beetle (MPB) can metabolize the toxic host tree monoterpenes. This process generates oxidized derivatives of monoterpenes, facilitating successful host colonization by MPB. Furthermore, the production of fungal volatile organic compounds serves as a signal to the beetles, indicating suitable host breeding conditions. Host tree infections by other phytopathogens, such as *Cronartium harknessii*, can similarly induce the production of oxygenated monoterpenes, which are less toxic to MPB. However, infections by *A. piniphila* may trigger the production of toxic monoterpenes, such as limonene, indicating host tree resistance to MPB. Notably, elevated CO_2 enhances the virulence of symbiotic fungi like *Grosmannia clavigera* and *Leptographium longiclavatum*, while elevated O_3 suppresses their growth. Elevated O_3 also impacts MPB physiology and reproduction by degrading pheromone components, reducing mating efficiency, decreasing brood size, and producing smaller broods. Conversely, elevated CO_2 accelerates the development of the brood, resulting in earlier emergence. Both elevated O_3 and CO_2 alter pheromone production in the brood, with higher concentrations observed in CO_2-affected broods. However, broods emerging from elevated O_3 treatments exhibit developed resistance plasticity and enhanced immunity, as evidenced by their similar reproductive success to that of broods affected by ambient and elevated CO_2. Overall, my investigation underscores the complexity of forest ecology, wherein insect behaviour, symbiotic relationships, and tree health are intricately intertwined. The dynamics of the entire system can be significantly altered by the pervasive force of climate change, which impacts every component within the ecosystem.

6.1 Monoterpene metabolism: a vital mechanism in beetle-fungi symbiosis to battle tree defenses

I found that fungal symbionts can enhance or weaken tree defenses to bark beetle attacks (Zaman et al., 2023a, 2023b). My research reported the previously unknown conversion of monoterpenes into oxygenated derivatives by fungal symbionts. Furthermore, I reported the efficiency of this conversion process and the overall biomass production of these symbionts under specific

monoterpene treatments (Appendix A: **Figure A1**-A8). Notably, α-pinene was consistently produced in significant quantities by all fungal species when exposed to various monoterpene supplements, while certain fungi predominantly utilized limonene to enhance their biomass production. Overall, these results are not surpising as fungal symbionts can modify host-specific molecules, such as monoterpenes, thereby influencing the host tree defense mechanisms during bark beetle infestation (Kandasamy et al., 2023; Nones et al., 2022). For instance, it was reported that *G. clavigera* possesses gene clusters such as cytochrome P450s, P530s, and P630s monooxygenases and reductases, indicating specialized metabolic pathways evolved to tolerate host defense compounds (DiGuistini et al., 2011; Hesse-Orce et al., 2010). Additionally, I found that variations in the pathogenicity of a particular fungal symbiont can impact the composition and concentration of monoterpenes, supporting the outcome of earlier work (Plattner et al., 2008). In general, the breakdown of monoterpenes into oxygenated monoterpenes by fungal symbionts is crucial in determining the outcome of tree colonization by bark beetles (DiGuistini et al., 2011). This process reduces the toxicity of monoterpenes to bark beetles, thereby improving beetles' survival during host colonization (Lah et al., 2013).

6.2 Ecological synergy between MPB and phytopathogens

I investigated how commonly found pathogens of lodgepole pine trees affect their defense chemistry. I focused on phloem terpene metabolites alteration by the infection of *A. piniphila* and *C. harknessii* compared to MPB's fungal symbionts. I found that both MPB's fungal symbionts and *C. harknessii* similarly modulated the terpene constituents while *A. piniphila* exhibited the opposite trend (Zaman et al., 2023a). The combined negative impacts of bark beetles and their symbiotic fungi on host tree defenses have been examined in prior research (Agbulu et al., 2022; Hammerbacher et al., 2013; Kandasamy et al., 2023). Similarly, studies reported differential induction of defense metabolites by various species of phytopathogens in different study systems (Burns et al., 2023; Dudney et al., 2020; Holtz and Schoettle, 2018).

Prior-pathogen infection alters both the chemical and anatomical defenses of pines, which could impact their suitability to subsequent biotic disturbances (Klutsch and Erbilgin, 2018). For example, infections by dwarf mistletoe (*Arceuthobium americanum*) on jack pines (*P. banksiana*) impaired the trees' ability to produce defensive compounds to the subsequent infection by *G. clavigera*. Additionally, defensive traits such as phloem thickness and moisture content were also

reduced as a result of the dwarf mistletoe infection. Similar outcomes were observed in this study, where *C. harknessii* reduced the defense metabolites of lodgepole pines in a manner akin to fungal symbionts of MPB. In other host species of MPB, similar results were reported. For instance, MPB preferred attacking limber pines (*Pinus flexilis*) weakened by the prior *Cronartium ribicola* infection (Holtz and Schoettle, 2018). I found that elevated CO_2 levels enhanced the virulence of both *G. clavigera* and *L. longiclavatum* on lodgepole pine phloem, resulting in significantly larger lesions than ambient conditions (Appendix B: **Figure B4**). These results further complicate the mutualistic interactions between MPB and fungal species.

6.3 Ozone impairs MPB's reproduction but enhances broods' plasticity

My study revealed the harmful impacts of O_3 exposure to MPB by potentially degrading pheromone components, disrupting their communication and ultimately reducing mating success (Chapter 4). In addition, O_3 also influenced the MPB to exhibit altered physiological responses such as changes in respiration, locomotory and olfactory behaviours. Changes in physiological parameters may ultimately affect MPB's attack strategy. Prior studies also reported that forest insects are experiencing changes in behaviour, life cycles, and distribution patterns due to climate change (Halsch et al., 2021). Degradation of pheromone components has previously been reported in several insect species due to O_3 exposure, which caused impaired communications (Jiang et al., 2023; Masui et al., 2020; Saunier et al., 2023). Besides, I found that O_3 exposure caused enhanced survival in both MPB parents and their broods. This can be attributable to the modified survival strategy due to abiotic factors. For instance, oxidative stress caused by O_3 exposure may influence the beetle to re-calculate energy consumption and resource allocation such as changes in respiration phases, and metabolism (Contreras and Bradley, 2009; Hoffmann, 2017; Pimentel, 1994). Furthermore, I found that broods that emerged from O_3-exposed parents exhibited improved mating success, gallery construction, and pheromone production. Prior studies similarly reported that epigenetic modulation of gene expression in insects experiencing environmental stress led to phenotypic plastic changes in their broods (Bueno et al., 2023; Dukes et al., 2009; Garnas, 2018).

6.4 Mountain pine beetle dynamics under predicted climate change

My study showed the intricate relationship between climatic factors studied and MPB behaviour, reproduction, and overall impact on their fungal symbionts. Elevated CO_2 levels accelerated development and an earlier emergence in MPB, likely influencing their life history traits and population dynamics (Zaman et al., 2024). Previous reviews have addressed these issues and anticipated similar concerns in other biological systems (Boullis et al., 2015; Kaunisto et al., 2016; Roggatz et al., 2022). Furthermore, I showed that elevated O_3 levels reduced MPB oviposition behaviour, altered reproductive patterns, and disruptions in pheromone communication (Chapter 4). However, their broods showed enhanced plasticity. In contrast, reduced humidity, showed improved mating success and brood fitness (Zaman et al., 2024). On the other hand, fungal symbionts of MPB specifically *G. clavigera* and *L. longiclavatum* were not much affected by elevated CO_2 when grown on artificial media (Zaman et al., 2024) but their virulence was enhanced when grown on lodgepole pine phloem (Appendix B: **Figure B4**). Similar results were reported in the Norway spruce (*Picea abies*)- *Endoconidiophora polonica* pathosystem, where infection of Norway spruce seedlings by *E. polonica* under elevated CO_2 enhanced disease severity in terms of both mortality and virulence (Linnakoski et al., 2017). However, elevated O_3 restricted the fungal growth on both artificial media and phloem (Appendix B: **Figure B4**). Despite the negative impacts of O_3 on both MPB and their symbiotic fungi, subsequent generations of MPB can develop enhanced resilience (Zaman et al., 2024). Considering the negation of harmful impacts of O_3 by elevated CO_2 on forest ecosystems (Xia et al., 2021) under predicted climate change including warmer temperatures and reduced humidity, MPB may develop plasticity (Soderberg et al., 2021) and further range expansion may be possible due to climate adaptability. Collectively, the findings of my studies illustrate how biodiversity and forest ecosystems are particularly susceptible to climate change, underscoring the necessity for a comprehensive understanding of the interrelated effects of rising CO_2 and O_3 levels on bark beetles, their cohabiting partners, and adversaries.

6.5 Limitations of the study system and future directions

Several limitations should be considered when evaluating the conclusions from my experimental approach. Firstly, I used logs to rear MPB under various environmental conditions in Chapters 4 and 5. While this approach provides insights into insect biology in controlled settings, it does not

fully replicate natural conditions that beetles expose during host colonization. For instance, naturally infested trees offer different microclimates for bark beetles, potentially involving interactions with various insect species and pathogens beyond non-fungal phoretic symbionts, leading to positive and negative feedback. To achieve a more holistic approach, larger setups such as Free Air Carbon Dioxide Enrichment (FACE) facilities would be ideal. Additionally, artificially infesting live trees with MPB in the forest poses risks is challenging, due to restrictions imposed by the provincial government regulatory agencies. Consequently, my study provides a more accessible and affordable laboratory-based setup that can be easily reproduced.

Secondly, after inoculating trees with fungi in Chapters 2 and 3, I measured lesion length as a proxy to assess pine resistance or susceptibility. This approach is commonly used in this and other systems (Klutsch and Erbilgin, 2018; Rice et al., 2007; Zhao et al., 2019). This approach has an assumption that the pathogen will grow better in less defended trees, hence the lesion length is expected to be longer. However, it is difficult to verify the assumption because there are so many factors that could potentially affect fungal growth including its virulence, inherent resistance of trees inoculated, and environmental factors. Perhaps at least one aspect of this assumption, the virulence of the pathogen, can be tested by inoculating trees with known highly virulent and less virulent isolates of the same pathogen species. Additional analyses, including fungal penetration into the xylem, tree mortality, fungal dissemination within the phloem, and physiological responses, can also be evaluated whether shorter lesion lengths correspond to a more resistant response.

Thirdly, while inoculating beetles into cut logs provides valuable insights into bark beetle biology, it may not fully replicate their reactions with living trees. For instance, cut logs may not have continuous chemical defenses as live trees do. Likewise, beetles enter a host tree after assessing its suitability using their chemoreceptors. In our study, they were directly introduced into the tree. Despite these limitations, studying insect-host plant interactions in logs can be useful to determine, for instance, how host quality affect insect reproduction, as acknowledged in earlier literature (Erbilgin et al., 2020; Goodsman et al., 2012; Kandasamy et al., 2023).

Lastly, we assessed the transgenerational impact of CO_2 and O_3 on the first and second generations of beetle and found that subsequent generations developed plasticity. These results suggest that conducting experiments extending multiple generations of beetles may be critical to

understanding the transgenerational plastic effects in insects and could provide valuable insights into their evolution under climate change. Similarly, assessing the effects of other environmental factors on multiple generations of symbiotic fungi would enhance our understanding of the directionality of the MPB-fungi symbiosis under the predicted future climate change scenarios. These considerations underscore the complexity of studying ecological dynamics and the importance of ongoing research to elucidate these intricate relationships.

6.6 Concluding remarks

In light of projected climate change scenarios, studying the factors driving the invasion dynamics and biological interactions of MPB and their associated fungi has become crucial. Through my research, I have developed comprehensive insights into how environmental variables influence both MPBs and their fungal partners. This contributes significantly to our comprehension of evolutionary biology and invasion processes by (1) identifying the key detoxification mechanisms responsible for the production of volatile organic and oxygenated monoterpene compounds by ophiostomatoid fungi, laying the groundwork for field tests to develop MPB monitoring tools; (2) unraveling the involvement of phytopathogens in assessing the susceptibility of host trees, thus guiding mitigation strategies to mitigate invasion risks; (3) studying the effects of environmental factors on the MPB-fungal symbionts can shed light on the crucial facets of their life history, evolutionary co-adaptation, and symbiotic strategies in the face of anticipated climate change. This knowledge stands to aid in forecasting MPB dynamics and outbreaks under future climate scenarios and devising effective management approaches.

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Appendix A: Chapter 2 supplementary and additional information

In this appendix, I included the supplementary information that was published as part of the manuscript "**Bark Beetles Utilize Ophiostomatoid Fungi to Circumvent Host Tree Defenses**" in the journal of *Metabolites*. A version of this publication is presented in chapter 2. I presented here some additional experimental data that support the main hypotheses of chapter 2. Figure A2 shows the comparative mycelial growth of 3 fungal symbionts of mountain pine beetles: *G. clavigera*, *O. montium*, and *L. longiclavatum* supplemented with 6 monoterpenes such as alpha-pinene, beta-pinene, limonene, myrcene, 3-carene, and bornyl acetate. Fungal dry mycelial weights were measured as proxy of total fungal biomass 45 days post monoterpene treatments. Figure A3-A8 shows different monoterpene conversion proportions in percentage by the 3 fungal symbionts 10 days post treatments. *G. clavigera* had the lowest growth in limonene supplementation while both *L. longiclavatum* and *O. montium* had the highest growth. *L. longiclavatum* had the lowest growth in 3-carene while *O. montium* had the lowest growth in in bornyl acetate and alpha-pinene supplementation. All the 3 symbionts converted alpha-pinene to camphene in high proportions while beta-pinene was converted mostly to alpha-pinene. Limonene was transformed into alpha-pinene mostly by *G. clavigera* while *L. longiclavatum* and *O. montium* transformed it to *p*-cymene in high percentage. 3-Carene was transformed into 47% of alpha-pinene by *O. montium*, 65% into beta-pinene by *G. clavigera*, and 80% into limonene by *L. longiclavatum*. Bornyl acetate was converted into alpha and beta-pinene in similar proportions while myrene was converted into beta-pinene and 3-carene in similar proportions by all the fungi.

Table A1: Emission of volatile organic compounds identified from the headspace collection of fresh pine bark, fourteen days after inoculation with different fungi. Analyses were conducted using GC-MS.

	RT[#]	Concentrations of compounds (ng per mg dry weight of bark)							
		Uninfected		*G. clavigera*		*L. longiclavatum*		*O. montium*	
Monoterpenes		Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
Tricyclene	8.8	ND	0.51 ± 0.51	ND	2.14 ± 1.15	ND	3.69 ± 2.54	ND	2.11 ± 1.12
α-Pinene	9.24	225.1 ± 79.3	284.40 ± 60.98	173.90 ± 40.85	370 ± 55.29	224.20 ±68.63	784.20 ± 247.10	149.80 ± 31.81	393.70 ± 94.95
Camphene	9.91	36.59 ± 13.61	40.09 ± 8.93	25.71 ± 4.55	49.62 ± 7.90	28.95 ±6.67	95.28 ± 27.86	21.13 ± 3.44	47.92 ± 9.36
Sabinene	10.96	ND	7.76 ± 2.43	ND	8.38 ± 2.92	ND	9.25 ± 2.67	ND	9.24 ± 3.08
ß-Pinene	11.09	131.60 ± 38.24	221.50 ± 31.54	113.70 ± 20.44	297.70 ± 27.74	145.70 ±34.82	440.10 ± 110.60	97.39 ± 15.04	325.40 ± 58.52
Myrcene	11.64	48.52 ± 7.34	110.90 ± 26.76	60.46 ± 10.65	163.40 ± 49.49	69.36 ±12.52	182.90 ± 35.19	47.77 ± 6.84	138.20 ± 38.24
3-Carene	12.36	108.4 ± 32.34	610.80 ± 217.10	190.40 ± 65.65	888.40 ± 338.20	206.70 ±71.57	590.20 ± 214.60	154.60 ± 44.12	684.40 ± 266.80
α-Terpinene	12.71	8.53 ± 1.05	15.21 ± 3.94	21.85 ± 3.89	23.19 ± 7.14	17.28 ±3.69	21.63 ± 4.69	11.91 ± 2.18	19.67 ± 6.72
α-Phellandrene	12.29	37.46 ± 4.72	99.29 ± 38.85	78.18 ± 14.76	106.10 ± 38.65	69.87 ±14.56	103.60 ± 20.63	46.05 ± 7.86	64.78 ± 21.35
Cymene	13.05	2.40 ± 1.47	14.36 ± 3.74	1.748 ± 0.58	21.19 ± 5.60	1.96 ±0.47	19.52 ± 3.54	0.97 ± 0.41	17.45 ± 4.63
Limonene	13.19	131 ± 36.29	230.70 ± 33.71	121 ± 12.20	320.40 ± 58.26	146.20 ±24.67	510.20 ± 115.20	102.50 ± 12.58	291.10 ± 48.00
ß-Phellandrene	13.25	657.80 ± 72.69	1621 ± 416.00	834.90 ± 155.80	2292 ± 695.00	972.50 ±177.60	2530 ± 496.50	686.30 ± 104.40	1933 ± 546.40
γ-Terpinene	14.32	4.21 ± 0.60	11.64 ± 3.28	6.82 ± 1.51	18.52 ± 6.15	6.93 ±1.63	19.60 ± 3.43	5.09 ± 0.98	16.14 ± 5.31
Terpinolene	15.30	13.71 ± 3.19	45.00 ± 15.31	23.11 ± 6.98	71.61 ± 27.59	24.80 ±7.86	69.44 ± 16.83	18.21 ± 4.44	60.66 ± 23.94
Phenylproprenes									
Allylanisole-4-ol	19.12	11.86 ± 1.73	17.44 ± 6.54	8.31 ± 1.09	17.28 ± 4.12	10.39 ±1.96	28.50 ± 5.36	8.96 ± 0.72	20.02 ± 5.33
Methyl eugenol	24.46	ND	1.59 ± 0.78	ND	2.19 ± 0.85	ND	7.67 ± 4.53	ND	2.89 ± 1.50
Oxygenated monoterpenes									
Camphor	11.92	ND	1.87 ± 1.05	ND	3.73 ± 1.42	ND	7.67 ± 2.43	ND	4.96 ± 1.32
Linalool	15.88	1.83 ± 0.15	2.37 ± 0.47	2.49 ± 0.67	4.13 ± 1.45	3.05 ±0.86	6.21 ± 2.37	2.48 ± 0.36	4.72 ± 1.99
Borneol	18.30	ND	10.55 ± 4.79	ND	65.39 ± 16.58	ND	60.55 ± 7.93	ND	45.30 ± 10.66
Terpinen-4-ol	18.6	ND	2.38 ± 1.03	ND	13.78 ± 5.47	ND	29.29 ± 5.89	ND	7.07 ± 3.35
α-Terpineol	19.03	0	1.38 ± 0.71	0.83 ± 0.57	6.56 ± 2.40	1.14 ±0.76	12.88 ± 1.76	0.75 ± 0.52	5.64 ± 2.73
Verbenone	19.49	ND	0.44 ± 0.13	ND	0.51 ± 0.18	ND	0.17 ± 0.09	ND	0.42 ± 0.24

Bornyl acetate	21.71	23.23 ± 8.46	ND	42.52 ± 16.08	ND	47.41 ±18.36	ND	35.05 ± 10.53	ND
Citronellyl acetate	23.40	ND	0.01 ± 0.01	ND	0.01 ± 0.01	ND	0.05 ± 0.02	ND	0.01 ± 0.00
Sesquiterpenes									
ß-Elemene	24.31	ND	5.30 ± 2.57	ND	8.10 ± 3.09	ND	7.93 ± 3.88	ND	7.77 ± 3.38
ß-Caryophyllene	24.85	ND	3.44 ± 1.60	ND	6.07 ± 2.45	ND	8.85 ± 2.68	ND	5.31 ± 2.13
α-Bergamotene	25.08	ND	8.60 ± 3.29	ND	12.47 ± 3.97	ND	15.32 ± 4.26	ND	12.44 ± 4.20
Aromadendrene	25.96	6.61 ± 0.72	ND	14.72 ± 3.36	ND	18.32 ±4.43	ND	12.68 ± 2.01	ND
Germacrene-4-ol	26.91	49.11 ± 10.03	ND	26.87 ± 9.04	ND	23.96 ±6.54	ND	35.46 ± 7.89	ND
γ-Cadinene	26.24	35.87 ± 6.60	ND	41.10 ± 13.80	ND	50.26 ±10.61	ND	34.53 ± 4.64	ND
δ-Cadinol	27.24	22.13 ± 3.78	ND	18.89 ± 4.84	ND	31.81 ±6.45	ND	19.32 ± 3.08	ND
Guaia-6,9-diene	25.21	16.70 ± 6.67	32.72 ± 9.17	10.76 ± 3.69	35.30 ± 8.95	14.56 ±5.58	82.02 ± 25.35	10.46 ± 3.09	38.84 ± 9.32
δ-Cadinene	26.30	69.49 ± 12.90	ND	76.71 ± 7.97	ND	93.30 ±20.00	ND	71.48 ± 12.05	ND
α-Muurolene	26.05	19.78 ± 3.67	ND	26.01 ± 4.52	ND	28.48 ±6.18	ND	19.86 ± 2.77	ND
Aliphatics/ others									
Isobutanol	2.32	ND	0.00 ± 0	ND	1.92 ± 0.11	ND	2.65 ± 0.17	ND	1.93 ± 0.11
3-Methyl-2-butanone	2.55	ND	0.50 ± 0.08	ND	0.57 ± 0.02	ND	0.60 ± 0.03	ND	0.55 ± 0.02
1-Butanol	2.593	ND	0.53 ± 0.13	ND	0.96 ± 0.08	ND	1.12 ± 0.38	ND	0.79 ± 0.34
2-Methyl-2-butanol	2.9	ND	11.90 ± 1.57	ND	14.93 ± 0.73	ND	15.19 ± 0.62	ND	15.94 ± 1.07
Acetoin	3.13	ND	2.91 ± 1.25	ND	3.75 ± 0.84	ND	4.71 ± 0.39	ND	4.97 ± 0.86
3-Methyl-1-butanol	3.54	ND	0.50 ± 0.12	ND	1.07 ± 0.32	ND	1.35 ± 0.32	ND	0.96 ± 0.18
2-Methyl-butanol	3.65	ND	0.27 ± 0.07	ND	0.71 ± 0.13	ND	1.01 ± 0.19	ND	0.41 ± 0.09
4-Methylheptane	4.087	ND	3.44 ± 0.42	ND	4.23 ± 0.28	ND	4.31 ± 0.25	ND	4.33 ± 0.23
2,4-Dimethyl-1-heptene	5.911	ND	6.02 ± 0.57	ND	6.46 ± 0.43	ND	6.83 ± 0.49	ND	6.67 ± 0.29
4-Methyloctane	6.68	ND	0.36 ± 0.03	ND	0.37 ± 0.03	ND	0.39 ± 0.03	ND	0.39 ± 0.02
2-Ethyl-1-butanol	9.802	ND	0.73 ± 0.06	ND	0.67 ± 0.01	ND	0.68 ± 0.02	ND	0.68 ± 0.02
Phenethyl alcohol	16.37	ND	0.10 ± 0.02	ND	0.23 ± 0.07	ND	0.68 ± 0.19	ND	0.27 ± 0.05
Grandisol	19.6	ND	0.28 ± 0.09	ND	0.19 ± 0.08	ND	0.04 ± 0.03	ND	0.63 ± 0.22
3,4-Dimethoxyphenol	24.841	ND	0.08 ± 0.07	ND	0.19 ± 0.11	ND	0.45 ± 0.18	ND	0.11 ± 0.07

#RT: Retention time according to GC-MS analysis; ND: Not detected.

Table A2: Relative amounts (mean ± SE, n=10) of volatiles from uninfected bark detected after various time periods (0, 14, 28 and 42 days) from the beginning of an experiment. Data from the control uninfected treatment are presented here.

Uninfected (Control)		Concentrations of compounds (ng per mg dry weight of bark)			
Monoterpenes	RT[#]	Week 0	Week 2	Week 4	Week 6
α-Pinene	9.240	1.722 ± 0.798	25.258 ± 8.169	19.375 ± 7.447	4.332 ± 1.724
Camphene	9.910	0.169 ± 0.086	0.401 ± 0.135	0.285 ± 0.083	0.133 ± 0.018
ß-Pinene	11.090	1.532 ± 0.524	5.604 ± 1.938	4.497 ± 0.920	2.535 ± 0.457
ß-Myrcene	11.640	0.339 ± 0.081	0.976 ± 0.207	0.719 ± 0.089	0.401 ± 0.039
3-Carene	12.360	3.690 ± 1.892	14.862 ± 5.296	9.408 ± 2.880	5.273 ± 1.551
p-Cymene	13.050	0.041 ± 0.017	0.196 ± 0.016	0.141 ± 0.022	0.074 ± 0.014
Limonene	13.190	1.397 ± 0.813	2.224 ± 0.750	2.348 ± 0.909	1.222 ± 0.407
ß-Phellandrene	13.250	11.539 ± 2.971	28.471 ± 5.539	24.734 ± 2.477	17.572 ± 2.137
γ-Terpinene	14.320	0.049 ± 0.015	0.155 ± 0.045	0.124 ± 0.028	0.071 ± 0.016
Terpinolene	15.300	0.321 ± 0.108	1.126 ± 0.382	0.831 ± 0.219	0.390 ± 0.094
Oxygenated monoterpenes					
Geranyl acetate	11.62	1.149 ± 0.774	1.087 ± 0.732	0.957 ± 0.524	0.734 ± 0.440
Camphor	11.92	0.005 ± 0.003	0.016 ± 0.006	0.007 ± 0.002	0.001 ± 0.000
ß-ocimene	13.9	0.004 ± 0.003	0.006 ± 0.004	0.005 ± 0.002	0.005 ± 0.002
Borneol	18.3	0.021 ± 0.009	0.034 ± 0.012	0.034 ± 0.012	0.011 ± 0.003
Terpinen-4-ol	18.6	0.015 ± 0.006	0.018 ± 0.007	0.028 ± 0.009	0.017 ± 0.004
α-Terpineol	19.03	0.015 ± 0.004	0.049 ± 0.012	0.040 ± 0.006	0.019 ±0.005
Bornyl acetate	21.71	0.458 ± 0.376	0.599 ± 0.491	0.455 ± 0.316	0.115 ± 0.060
Sesquiterpenes					
ß-Caryophyllene	24.85	0.011 ± 0.005	0.015 ± 0.007	0.011 ± 0.006	0.008 ± 0.004
Germacrene-4-ol	26.91	3.705 ± 2.387	4.388 ± 2.570	2.979 ± 1.686	2.039 ± 1.260
Diterpenes					
Epi-13-manool	23.4	4.478 ± 1.533	3.398 ± 2.071	3.598 ± 1.292	2.255 ± 0.852

Table A3: Relative amounts (mean ± SE, n=10) of volatiles from uninfected bark detected after various time periods (0, 14, 28 and 42 days) from the beginning of an experiment. Data from the control *G. clavigera* treatment are presented here.

G. clavigera		Concentrations of compounds (ng per mg dry weight of bark)			
Monoterpenes	RT[#]	Week 0	Week 2	Week 4	Week 6
α-Pinene	9.240	0.823 ± 0.368	27.440 ± 7.648	11.421 ± 3.579	6.477 ± 1.800
Camphene	9.910	0.119 ± 0.064	2.380 ± 0.294	1.164 ± 0.196	0.320 ± 0.095
ß-Pinene	11.090	0.665 ± 0.096	49.835 ± 12.396	23.910 ± 5.040	5.301 ± 1.742
ß-Myrcene	11.640	0.216 ± 0.025	6.220 ± 0.881	3.157 ± 0.539	0.838 ± 0.220
3-Carene	12.360	2.244 ± 0.812	75.335 ± 14.993	40.151 ± 8.915	10.229 ± 2.528
p-Cymene	13.050	0.025 ± 0.009	0.163 ± 0.028	0.150 ± 0.026	0.067 ± 0.014
Limonene	13.190	0.758 ± 0.304	14.796 ± 5.271	8.222 ± 2.998	2.353 ± 1.136
ß-Phellandrene	13.250	8.074 ± 0.989	86.073 ± 18.769	91.077 ± 15.564	30.374 ± 7.018
γ-Terpinene	14.320	0.033 ± 0.008	0.769 ± 0.141	0.480 ± 0.095	0.127 ± 0.025
Terpinolene	15.300	0.234 ± 0.073	5.985 ± 1.246	3.683 ± 0.833	0.786 ± 0.176
Oxygenated monoterpenes					
Geranyl acetate	11.62	0.825 ± 0.417	0.924 ± 0.464	0.660 ± 0.270	0.761 ± 0.405
Camphor	11.92	0.001 ± 0.001	0.015 ± 0.004	0.010 ± 0.004	0.004 ± 0.001
ß-ocimene	13.9	0.003 ± 0.002	0.005 ± 0.005	0.005 ± 0.004	0.007 ± 0.003
Borneol	18.3	0.012 ± 0.004	0.080 ± 0.016	0.049 ± 0.009	0.012 ± 0.005
Terpinen-4-ol	18.6	0.008 ±0.002	0.086 ± 0.023	0.096 ± 0.017	0.032 ± 0.006
α-Terpineol	19.03	0.014 ± 0.003	0.210 ± 0.081	0.183 ± 0.053	0.033 ± 0.005
Bornyl acetate	21.71	0.431 ± 0.365	1.238 ± 0.818	0.751 ± 0.415	0.342 ± 0.258
Sesquiterpenes					
ß-Caryophyllene	24.85	0.005 ± 0.003	0.002 ± 0.002	0.008 ± 0.006	0.004 ± 0.004
Germacrene-4-ol	26.91	2.751 ± 1.307	3.545 ± 1.782	2.123 ± 0.852	2.411 ± 1.194
Diterpenes					
Epi-13-manool	23.4	2.160 ± 1.358	5.922 ± 3.384	1.952 ± 1.168	3.890 ± 1.593

Table A4: Relative amounts (mean ± SE, n=10) of volatiles from uninfected bark detected after various time periods (0, 14, 28 and 42 days) from the beginning of an experiment. Data from the control *L. longiclavatum* treatment are presented here.

L. longiclavatum		Concentrations of compounds (ng per mg dry weight of bark)			
Monoterpenes	RT[#]	Week 0	Week 2	Week 4	Week 6
α-Pinene	9.240	0.979 ± 0.362	19.792 ± 9.739	14.361 ± 6.510	11.136 ± 5.035
Camphene	9.910	0.106 ± 0.028	2.172 ± 0.406	1.185 ± 0.266	0.350 ± 0.073
ß-Pinene	11.090	0.831 ± 0.195	47.351 ± 10.580	24.518 ± 5.711	8.724 ± 2.667
ß-Myrcene	11.640	0.260 ± 0.054	6.003 ± 1.021	3.188 ± 0.678	0.990 ± 0.226
3-Carene	12.360	4.255 ± 1.953	74.198 ± 15.568	43.261 ± 12.191	14.523 ± 4.648
p-Cymene	13.050	0.024 ± 0.006	0.225 ± 0.025	0.206 ± 0.035	0.125 ± 0.034
Limonene	13.190	0.713 ± 0.284	13.665 ± 4.926	7.464 ± 2.933	2.350 ± 0.676
ß-Phellandrene	13.250	10.270 ± 1.978	97.382 ± 19.394	72.063 ± 16.036	36.702 ± 7.663
γ-Terpinene	14.320	0.054 ± 0.019	0.739 ± 0.151	0.524 ± 0.147	0.182 ± 0.053
Terpinolene	15.300	0.403 ± 0.166	5.850 ± 1.295	4.071 ± 1.239	1.146 ± 0.369
Oxygenated monoterpenes					
Geranyl acetate	11.62	0.755 ± 0.430	0.667 ± 0.333	0.464 ± 0.204	0.372 ± 0.177
Camphor	11.92	0.001 ± 0.000	0.023 ± 0.011	0.011 ± 0.004	0.002 ± 0.001
ß-ocimene	13.9	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	0.005 ± 0.001
Borneol	18.3	0.013 ± 0.003	0.098 ± 0.028	0.046 ± 0.010	0.013 ± 0.003
Terpinen-4-ol	18.6	0.010 ± 0.005	0.093 ± 0.020	0.088 ± 0.023	0.040 ± 0.010
α-Terpineol	19.03	0.016 ± 0.005	0.182 ± 0.058	0.175 ± 0.072	0.056 ± 0.018
Bornyl acetate	21.71	0.288 ± 0.174	0.721 ± 0.476	0.779 ± 0.501	0.325 ± 0.178
Sesquiterpenes					
ß-Caryophyllene	24.85	0.007 ± 0.004	0.000 ± 0.000	0.006 ± 0.004	0.001 ± 0.001
Germacrene-4-ol	26.91	2.574 ± 1.382	3.005 ± 1.668	1.405 ± 0.491	1.372 ± 0.572
Diterpenes					
Epi-13-manool	23.4	7.779 ± 3.364	10.623 ± 3.266	3.709 ± 1.642	4.952 ± 1.761

Table A5: Relative amounts (mean ± SE, n=10) of volatiles from uninfected bark detected after various time periods (0, 14, 28 and 42 days) from the beginning of an experiment. Data from the control *O. montium* treatment are presented here.

O. montium		Concentrations of compounds (ng per mg dry weight of bark)			
Monoterpenes	RT[#]	Week 0	Week 2	Week 4	Week 6
α-Pinene	9.240	1.060 ± 0.442	12.922 ± 2.750	7.571 ± 1.868	10.412 ± 6.355
Camphene	9.910	0.085 ± 0.027	1.926 ± 0.330	0.875 ± 0.298	0.249 ± 0.093
ß-Pinene	11.090	0.773 ± 0.161	43.294 ± 11.255	18.546 ± 7.326	4.355 ± 1.701
ß-Myrcene	11.640	0.230 ± 0.047	5.400 ± 0.909	2.371 ± 0.745	0.664 ± 0.190
3-Carene	12.360	2.522 ± 1.127	63.853 ± 14.157	25.926 ± 7.179	7.758 ± 2.243
p-Cymene	13.050	0.023 ± 0.007	0.167 ± 0.033	0.150 ± 0.033	0.054 ± 0.011
Limonene	13.190	0.757 ± 0.313	13.448 ± 4.894	7.066 ± 3.418	1.923 ± 0.984
ß-Phellandrene	13.250	8.648 ± 1.732	97.719 ± 22.419	48.542 ± 8.344	25.756 ± 7.314
γ-Terpinene	14.320	0.036 ± 0.011	0.639 ± 0.125	0.311 ± 0.082	0.101 ± 0.027
Terpinolene	15.300	0.263 ± 0.106	4.968 ± 1.102	2.360 ± 0.691	0.609 ± 0.169
Oxygenated monoterpenes					
Geranyl acetate	11.62	1.036 ± 0.782	0.982 ± 0.648	0.938 ± 0.540	0.627 ± 0.298
Camphor	11.92	0.002 ± 0.001	0.023 ± 0.012	0.007 ± 0.003	0.002 ± 0.001
ß-ocimene	13.9	0.004 ± 0.004	0.002 ± 0.002	0.001 ± 0.001	0.005 ± 0.001
Borneol	18.3	0.011 ± 0.003	0.071 ± 0.022	0.032 ± 0.012	0.009 ± 0.003
Terpinen-4-ol	18.6	0.008 ± 0.002	0.071 ± 0.019	0.063 ± 0.013	0.028 ± 0.007
α-Terpineol	19.03	0.020 ± 0.008	0.167 ± 0.050	0.119 ± 0.043	0.044 ± 0.016
Bornyl acetate	21.71	0.167 ± 0.108	0.455 ± 0.306	0.241 ± 0.104	0.144 ± 0.073
Sesquiterpenes					
ß-Caryophyllene	24.85	0.006 ± 0.005	0.000 ±0.000	0.004 ± 0.004	0.004 ± 0.003
Germacrene-4-ol	26.91	3.271 ± 2.105	2.973 ± 1.877	2.511 ± 1.436	1.790 ± 0.857
Diterpenes					
Epi-13-manool	23.4	6.207 ± 2.451	7.840 ± 4.578	3.608 ± 1.369	3.055 ± 1.847

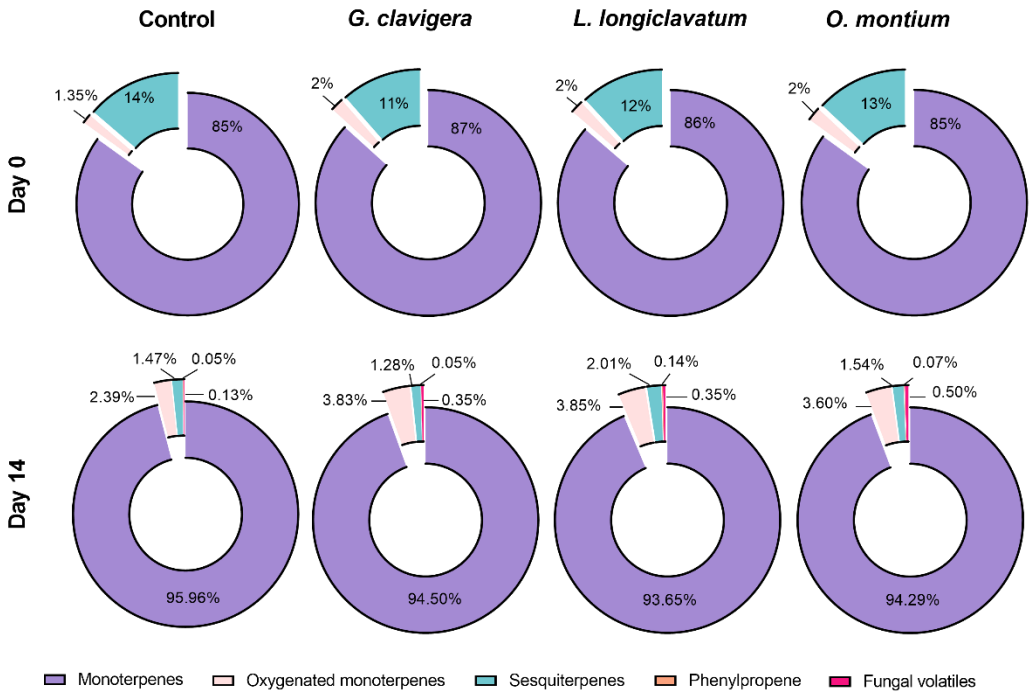

Figure A1: Pie chart representing the chemical composition of *Pinus contorta* var. *latifolia* phloem (cut logs) before and after 14 days of fungal inoculation.

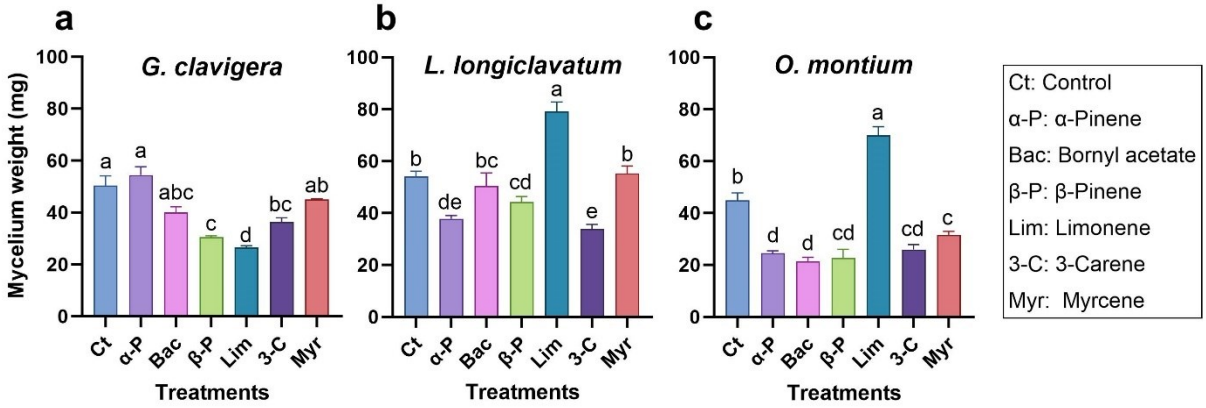

Figure A2: Growth comparison of ophiostomatoid fungal symbionts treated with individual monoterpenes. Three different fungi (a. *Grosmannia clavigera*, b. *Leptographium longiclavatum*, and c. *Ophiostoma montium*) were grown on potato dextrose broth media with 6 monoterpenes individually for 45 days. Fungi grown without monoterpene treatment served as control. Fungal mycelia were collected by buchner funnel filtration method, freeze dried and measured dry mycelium weight. One-way ANOVA was employed followed by Fisher's LSD test for multiple comparisons. Different small letters on top of the bars denote significant differences at $p < 0.05$.

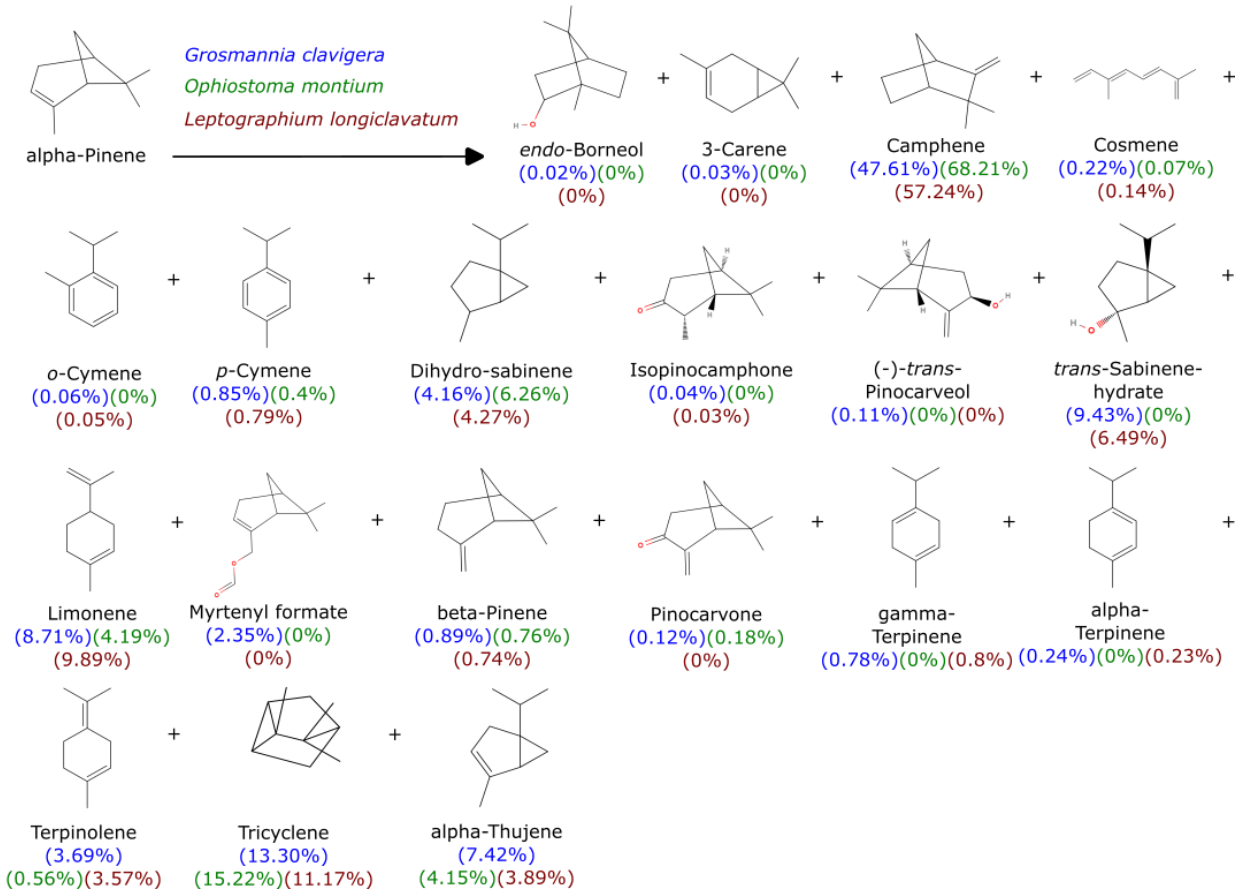

Figure A3: Percentage of alpha-pinene conversion to volatile organic compounds symbiotic fungi of mountain pine beetles after 10 days. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green - *Ophiostoma montium*, and red - *Leptographium longiclavatum*.

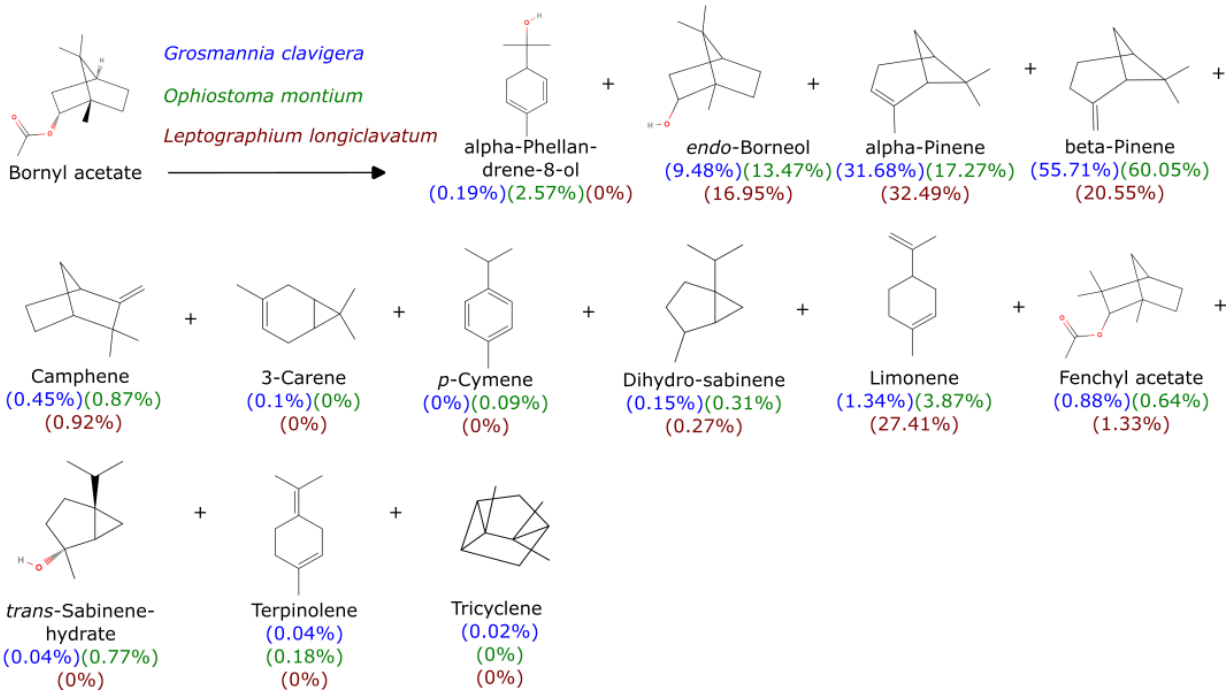

Figure A4: Percentage of bornyl acetate conversion to volatile organic compounds by symbiotic fungi of mountain pine beetles after 10 days of inoculation. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green - *Ophiostoma montium*, and red - *Leptographium longiclavatum*.

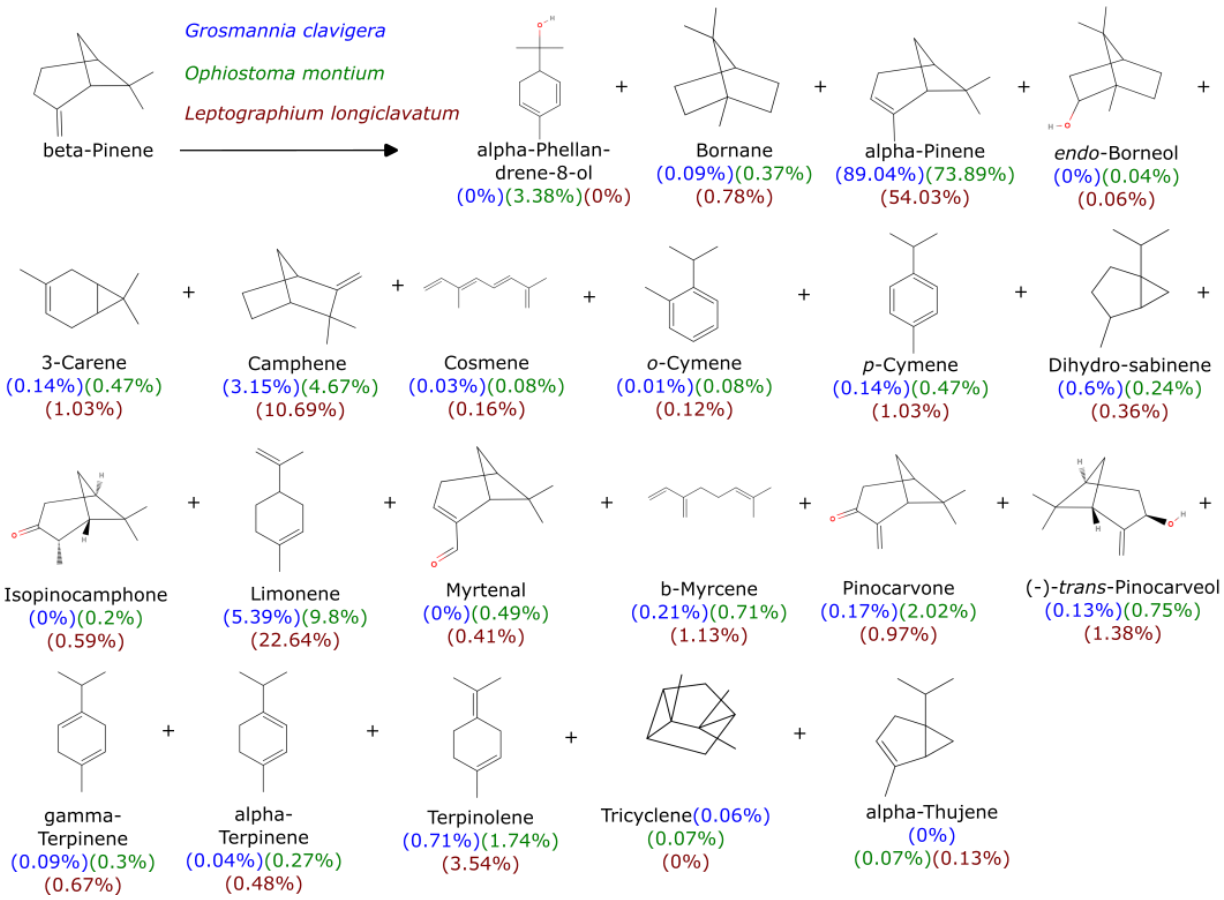

Figure A5: Percentage of beta-pinene conversion to volatile organic compounds by symbiotic fungi of mountain pine beetles after 10 days of inoculation. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green - *Ophiostoma montium*, and red - *Leptographium longiclavatum*.

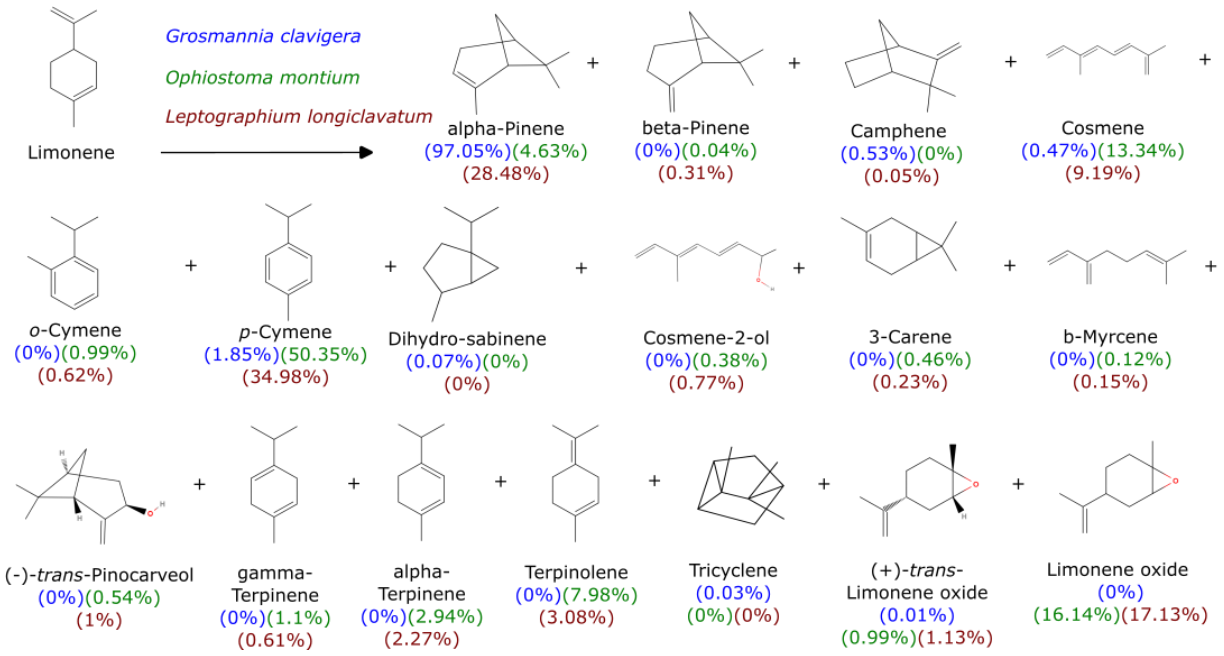

Figure A6: Percentage of limonene conversion to volatile organic compounds by symbiotic fungi of mountain pine beetles after 10 days of inoculation. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green - *Ophiostoma montium*, and red - *Leptographium longiclavatum*.

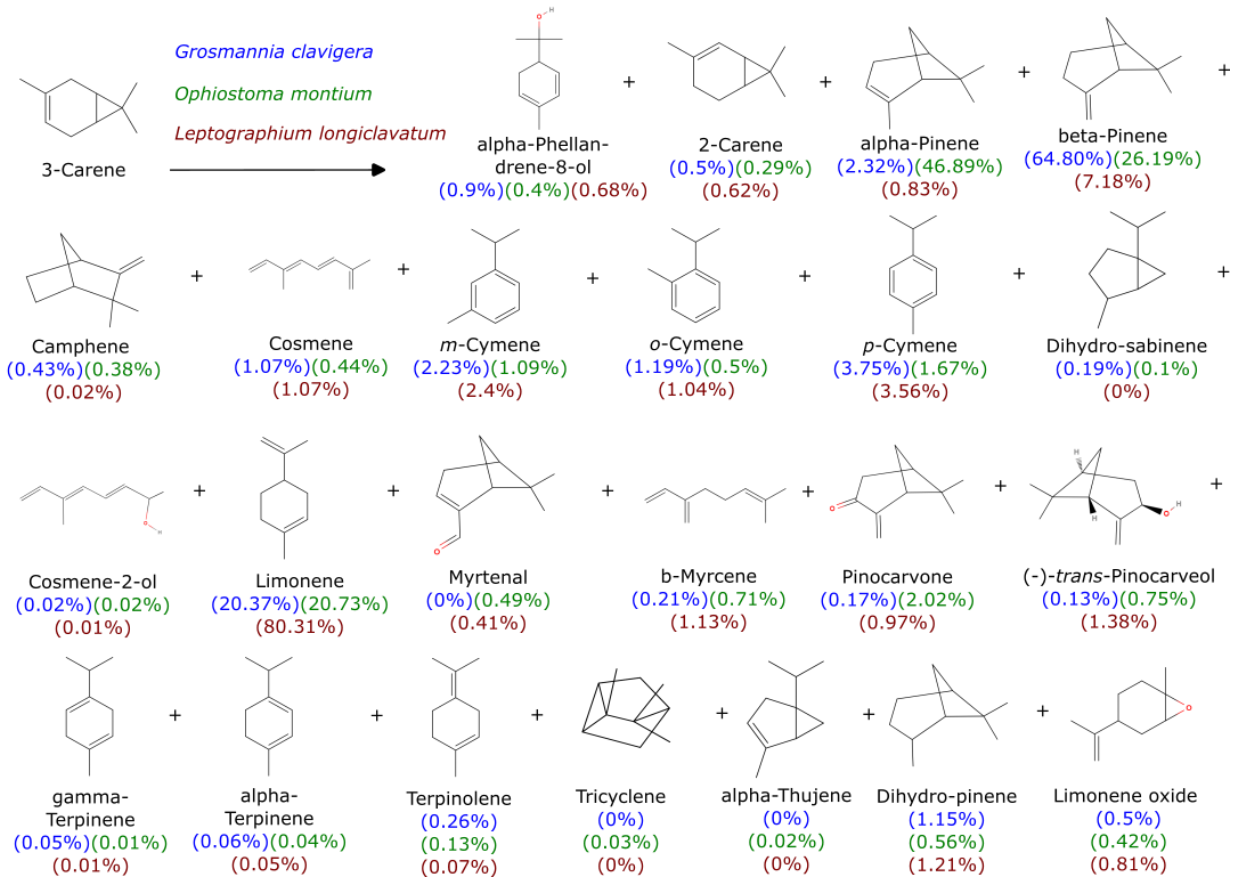

Figure A7: : Percentage of 3-carene conversion to volatile organic compounds by symbiotic fungi of mountain pine beetles after 10 days of inoculation. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green- *Ophiostoma montium*, and red- *Leptographium longiclavatum*.

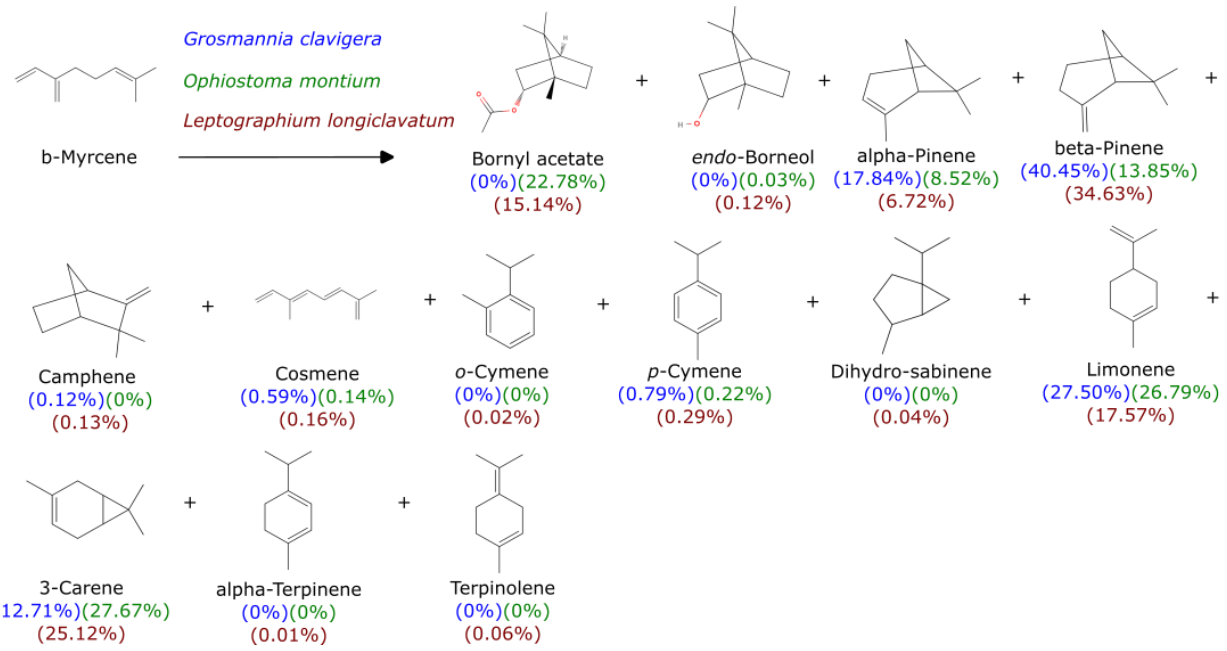

Figure A8: Percentage of myrcene conversion to volatile organic compounds by symbiotic fungi of mountain pine beetles after 10 days of inoculation. Percentages of each compound produced by each fungus are shown in different colour as follows: blue- *Grosmannia clavigera*, green- *Ophiostoma montium*, and red- *Leptographium longiclavatum*.

Appendix B: Chapter 3 supplementary and additional information

In this appendix, I included the supplementary information that was published as part of the manuscript "**A Pine in Distress: How Infection by Different Pathogenic Fungi Affect Lodgepole Pine Chemical Defenses**" in the journal of *Microbial ecology*. A version of this publication is presented in chapter 3. I presented here some additional experimental data that support the main hypotheses of chapter 3. Figure B4 was generated from additional experiments where I tested the virulence of the phytopathogens under ambient, elevated CO_2 and O_3 conditions under darkness. Fungi (*Grosmannia clavigera*, *Ophiostoma montium*, *Leptographium longiclavatum*, *Aspergillus* sp., *Trichoderma* sp., and *Atropellis piniphila*) were grown initially for 10 days on potato dextrose agar media and a 5mm plug from the actively growing margin of each fungus was transferred onto the phloem of lodgepole pine logs (on the same circumference). All the logs were then placed inside separate climate chambers for 14 days. After 14 days, barks were removed, and fungal lesion area were photographed and then estimated using ImageJ software. Both G. clavigera and L. longiclavatum significantly created larger lesion under elevated CO_2 condition than under ambient condition. Elevated O_3 condition significantly restricted the growth of all the fungi compared to the ambient condition.

Table B1: Presence/absence of metabolites in samples with and without infection of five native pathogens of lodgepole pine (*Pinus contorta* var. *latifolia*). The presence and absence of all chemical compounds were detected from the extraction and identifications by gas chromatograph-mas spectrometry in the majority of the 302 samples. The compounds present in the treatments are denoted by "x" and the absent by "–".

Chemical compounds	***Atropellis piniphila***	***Endocronartium harknessii***	***Grosmannia clavigera***	***Leptographium longiclavatum***	***Ophiostoma montium***	***P. contorta* control**
Monoterpenes						
β-Phellandrene	x	x	x	x	x	x
α-Pinene	x	x	x	x	x	x
Camphene	x	x	x	x	x	x
β-Pinene	x	x	x	x	x	x
Myrcene	x	x	x	x	x	x
3-Carene	x	x	x	x	x	x
Limonene	x	x	x	x	x	x
Thujene	x	–	–	–	–	–
β-Ocimene	x	–	x	x	x	–
Tricyclene	x	–	–	–	–	–
α-Terpinene	x	x	–	–	–	x
p-Cymene	x	x	x	x	x	x
γ-Terpinene	x	x	x	x	x	x
Terpinolene	x	x	x	x	x	x
Sabinene	x	–	–	–	–	–
Oxygenated monoterpenes						
Geranyl acetate	x	–	x	x	x	–

α-Terpineol	x	x	x	x	x	x
p-Cymen-8-ol	x	–	–	–	–	–
Terpinen-4-ol	x	–	x	x	x	–
Ascaridole	x	–	–	–	–	–
Bornyl acetate	x	x	x	x	x	x
Camphor	–	–	x	x	x	–
Borneol	x	x	x	x	x	x
Linalool	x	–	–	–	–	–
Cuminal	x	–	–	–	–	–
Endo-Borneol	x	–	–	–	–	–
Car-3-en-5-one	x	–	–	–	–	–
Pinocarvone	x	–	–	–	–	–
Citronellyl acetate	x	–	–	–	–	–
Sesquiterpenes						
α-Farnesene	x	–	–	–	–	–
Germacrene D	x	–	–	–	–	–
cis-Muurola- 3.5-diene	x	–	–	–	–	–
α-Muurolene	x	–	–	–	–	–
β-Curcumene	x	–	–	–	–	–
Aromadendrene	x	–	–	–	–	–
β-Elemene	x	–	–	–	–	–
γ-Cadinene	x	–	–	–	–	–

Cubenene	x	–	–	–	–	–
β-Farnesene	x	–	–	–	–	–
β-trans-Farnesene	x	–	–	–	–	–
α-Bisabolol	x	–	–	–	–	–
Nerolidol	x	–	–	–	–	–
tau-Cadinol	x	–	–	–	–	–
tau-Muurolol	x	–	–	–	–	–
α-Cadinol	x	–	–	–	–	–
Germacrene D4-ol	x	–	x	x	x	–
Farnesol	x	–	–	–	–	–
β-Caryophyllene	–	–	x	x	x	–
γ-Elemene	x	–	–	–	–	–
Diterpenes						
13-Epi-manool	x	–	x	x	x	x
Agathadiol	x	–	–	–	–	–
Cembrene	x	–	–	–	–	–
Levopimaral	x	–	–	–	–	–
Abietal	x	–	–	–	–	–
Sandaracopimaral	x	–	–	–	–	–
Kaurenal	x	–	–	–	–	–
Isopimaral	x	–	–	–	–	–
Isopimarol	x	–	–	–	–	–
Manoyl oxide	x	–	–	–	–	–

Torulosol	x	–	–	–	–	–
Others						
Pinosylvin dimethyl ether	x	–	–	–	–	–
Linalyl cinnamate	x	–	–	–	–	–
4-Allylanisole	x	x	x	–	–	x
β-Sitosterol	x	–	–	–	–	–
4-Ethoxy ethylbenzoate	x	–	–	–	–	–

Table B2: Comparison between healthy and infected lodgepole pine (*Pinus contorta* var. *latifolia*) trees. Results reported from One-way ANOVA; Fisher's LSD post hoc multiple comparisons were conducted between healthy and infected trees. Comparisons were based on concentrations of individual compounds.

Chemicals	**F-value**	***p*-value**	**False Discovery Rate**
β-Phellandrene	130.01	<0.0001	<0.0001
Camphene	97.02	<0.0001	<0.0001
β-Pinene	82.78	<0.0001	<0.0001
Terpinolene	63.11	<0.0001	<0.0001
α-Terpinene	38.94	<0.0001	<0.0001
γ-Terpinene	35.74	<0.0001	<0.0001
Cymene	27.11	<0.0001	<0.0001
Myrcene	22.29	<0.0001	<0.0001
Limonene	16.34	<0.0001	<0.0001
α-Terpineol	8.88	<0.0001	<0.0001
Bornyl acetate	8.47	<0.0001	<0.0001
3-Carene	8.10	<0.0001	<0.0001
Borneol	4.38	0.0007	0.0008
4-Allylanisole	3.97	0.0017	0.0018
α-Pinene	1.67	0.1418	-

Table B3: Coefficient scores of metabolites from infected by various biotic stress agents and non-infected lodgepole pine (*Pinus contorta* var. *latifolia*) phloem analyzed by Partial Least Squares Discriminant Analysis (PLS-DA).

Compounds	coef.mean	AC	Control	GC	LL	OM	WGR
Camphene	29.65	7.71	96.53	8.99	8.84	9.28	46.58
β-Phellandrene	27.96	8.02	100	7.24	6.62	6.92	38.96
α-Terpinene	27.25	0.59	15.28	18.26	22.02	23.33	84.02
β-Pinene	26.40	6.69	84.71	8.15	8.08	8.49	42.29
Terpinolene	22.28	6.45	81.76	5.44	4.84	5.06	30.12
γ-Terpinene	21.72	5.89	75.40	5.89	5.54	5.81	31.83
Myrcene	19.92	4.94	64.40	6.01	5.95	6.26	31.90
Bornyl acetate	19.87	2.58	37.51	9.65	11.01	11.65	46.82
Cymene	18.74	5.23	67.66	4.68	4.26	4.46	26.15
Limonene	17.27	3.18	44.27	6.77	7.34	7.75	34.33
α-Terpineol	16.08	3.22	44.61	5.81	6.17	6.52	30.13
3-Carene	13.98	0.90	18.05	7.85	9.27	9.83	37.96
4-Allylanisole	10.85	0.44	12.58	6.23	7.42	7.88	30.53
Borneol	9.49	1.18	20.95	4.07	4.59	4.88	21.31
α-Pinene	7.87	0	7.45	4.68	5.65	6.02	23.42

Here, coef.mean= mean of coefficient scores of all treatments, WGR = *Cronartium harknessii*, AC = *Atropellis piniphila*, GC= *Grosmannia clavigera*, LL= *Leptographium longiclavatum*, OM = *Ophiostoma montium*.

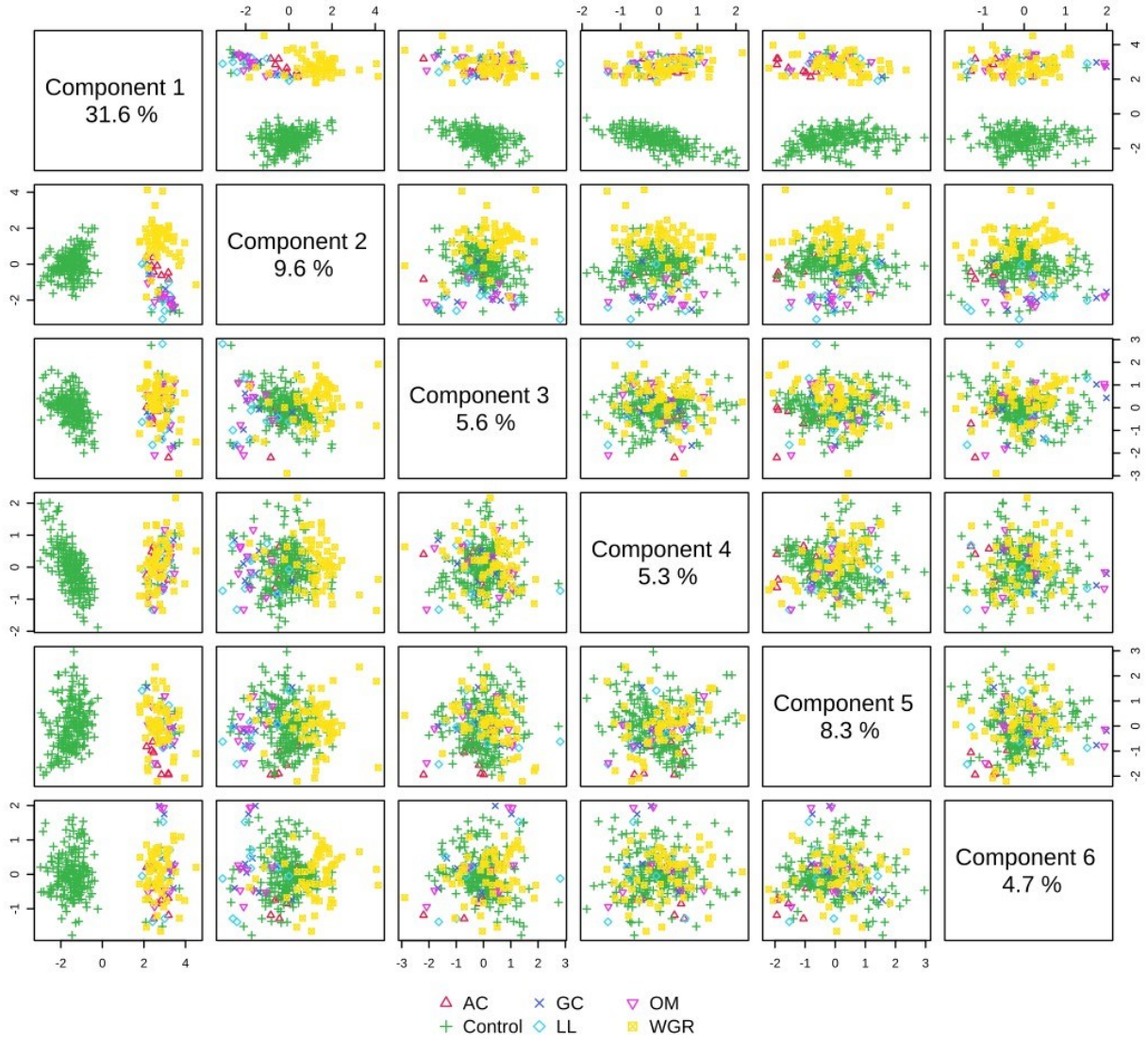

Figure B1: Partial Least Squares Discriminant Analysis (PLS-DA) representation of metabolites of lodgepole pine (Pinus contorta var. latifolia) infected with different pathogenic fungal species. WGR = C*ronartium harknessii,* AC = *Atropellis piniphila,* GC= *Grosmannia clavigera,* LL= *Leptographium longiclavatum,* OM = *Ophiostoma montium.* This model was cross validated by leave-one-out cross-validation method in Figure B2.

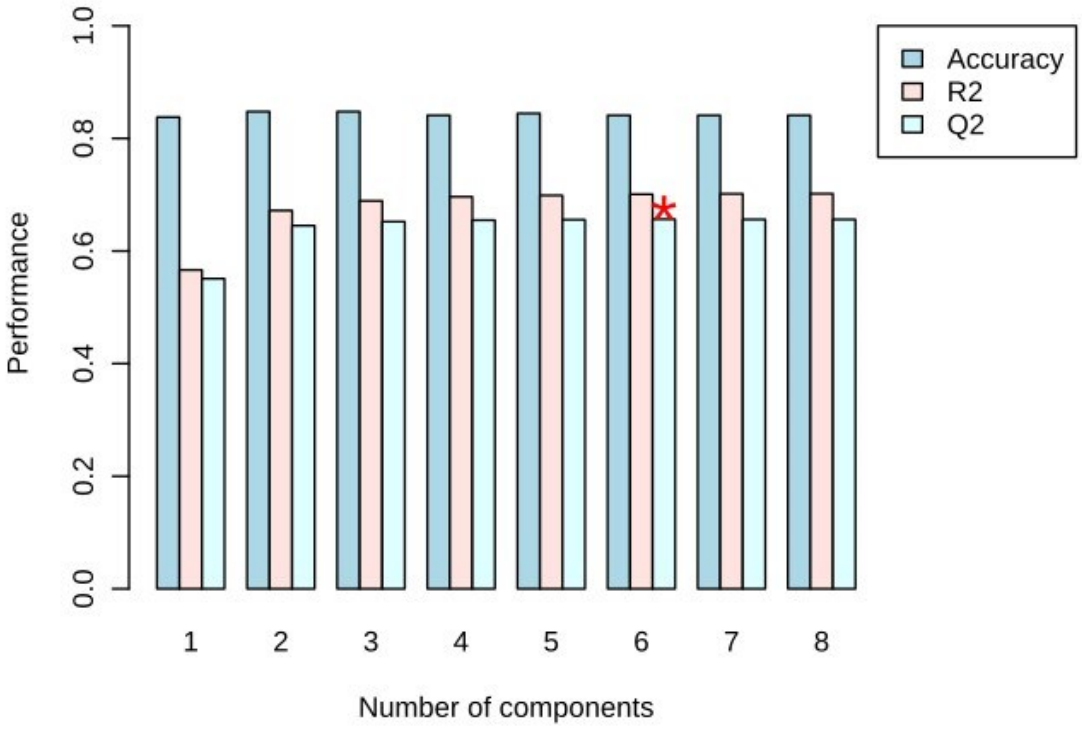

Figure B2: Cross-validation of Partial Least Squares Discriminant Analysis (PLS-DA) model by the Leave-one-out cross-validation method. Here, Q^2 is an estimate of the predictive ability of the model. The highest accuracy was observed with six components. The red star highlights the best classifier or the highest predictive ability of the model in the PLS-DA model using different numbers of components.

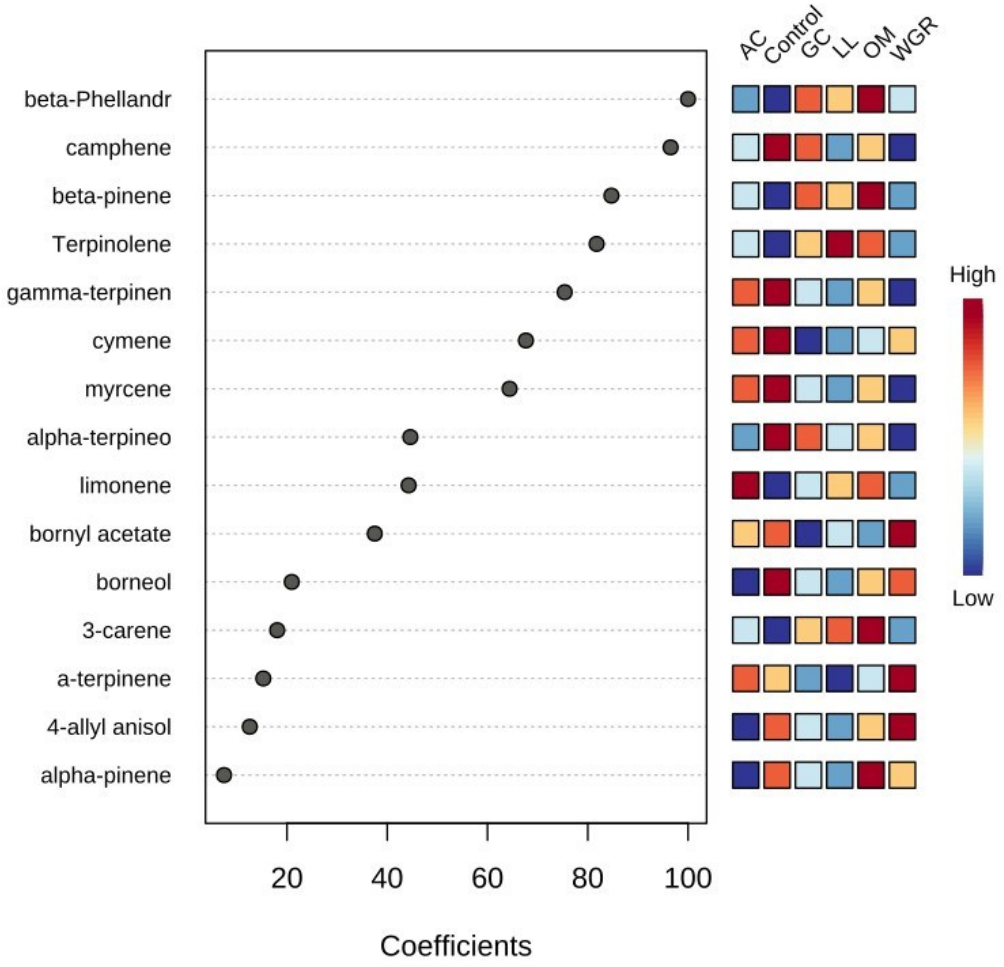

Figure B3: Important features identified by Partial Least Squares Discriminant Analysis (PLS-DA) based on coefficient scores of non-infected control treatment. Here, colored boxes show the relative concentrations of the corresponding metabolite under various conditions denoted by the scale on the right hand (darkest red= highest concentration and darkest blue= lowest concentration). Here, WGR = *Cronartium harknessii*, AC = *Atropellis piniphila*, GC= *Grosmannia clavigera*, LL= *Leptographium longiclavatum*, OM = *Ophiostoma montium*.

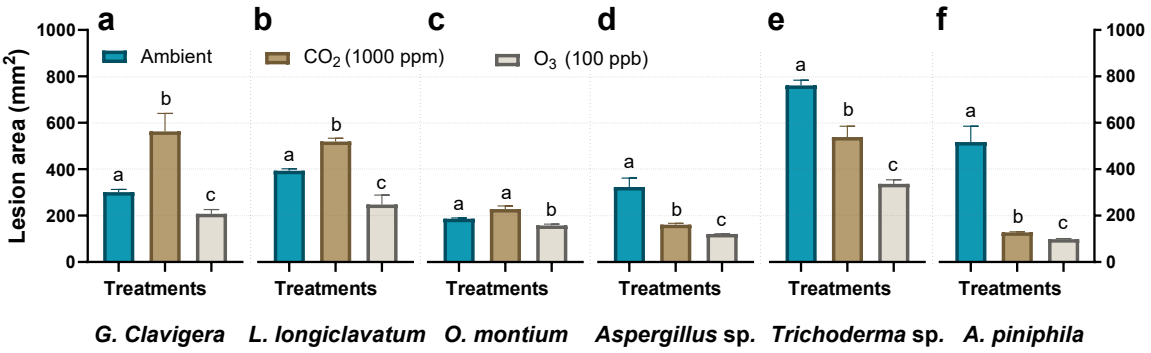

Figure B4: Effect of ambient and elevated conditions of greenhouse gases (CO_2 and O_3) on the virulence of six phytopathogens infected on lodgepole pine (LP) phloem.

(a-f) Phytopathogens used in this experiment were three fungal symbionts of mountain pine beetle such as *Grosmannia clavigera* (EL035), *Leptographium longiclavatum* (EL038), *Ophiostoma montium* (EL030), two saprophytes such as *Aspergillus* sp. (NOF560), *Trichoderma* sp. (NOF1416), and the atropellis canker pathogen *Atropellis piniphila* (NOF3174), respectively. All the fungi were allowed to grow for 14 days on LP logs inside three separate climate chambers with different conditions in each (ambient, elevated CO_2, and O_3 with temperature at 23 °C and darkness in all chambers). For each fungus, lesion area created under different environmental conditions were analyzed by one-way ANOVA followed by Fisher's LSD multiple comparison test. In each sub-figure (a-f) different small letters on top of the bars denote significant difference at $p < 0.05$.

Appendix C: Chapter 5 supplementary and additional information

Table C1: Source information of the fungal isolates used in this study.

Accession number	Location collection	Date collected	Species notes	Isolated from	Identified by
EL004 **EL035** **EL034**	Banff, Alberta, Canada	May 2016	*Grosmannia clavigera*	MPB gallery in *Pinus contorta*	Nadir Erbilgin
EL002 **EL037** **EL038**	Graham fire base, Alberta, Canada	May 2016	*Leptographium longiclavatum*	MPB gallery in *P. banksiana* × *P. contorta* hybrid	Nadir Erbilgin
EL030 **EL031** **EL032**	Westcastle, Alberta, Canada	January 2015	*Ophiostoma montium*	MPB gallery in *Pinus contorta*	Nadir Erbilgin
EL050 **EL051**	Banff, Alberta, Canada	May 2016	*Aspergillus* sp. *Trichoderma* sp.	MPB gallery in *Pinus contorta*	Nadir Erbilgin
NOF 3221	Glacier National Park, British Columbia, Canada	November 2019	*Beauveria bassiana*	*Picea glauca* infested with *Dendroctonus rufipennis*	Colin Myrholm

Table C2: Total amount of pheromones produced by F1 offspring from parents reared under various climatic factors.

Pheromone	Ambient (ng/mg)	eCO_2 (ng/mg)	eO_3 (ng/mg)	Low RH (ng/mg)	Ambient RH (ng/mg)
***cis*/*trans*-Verbenol**	6.12	6.65	6.25	1.20	1.29
***exo*-Brevicomin**	0.31	0.53	0.26	0.30	0.60
Verbenone	8.98	16.76	10.36	0.99	0.95
Frontalin	1.02	1.57	0.99	2.73	2.09

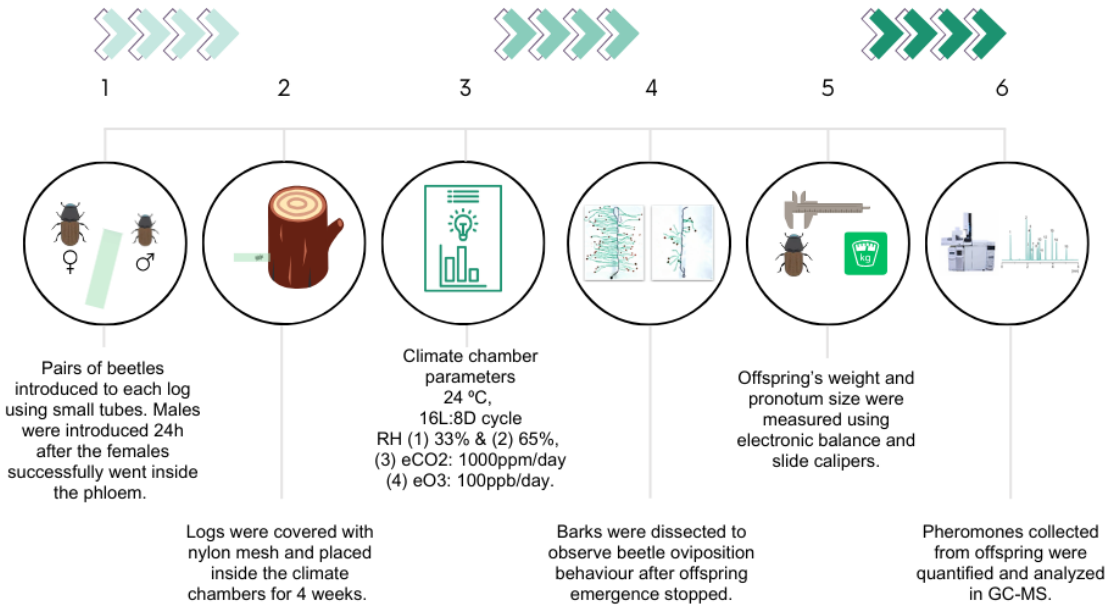

Figure C1: Experimental workflow of different climatic variables exposure and characterization of behavioural, physiological, and morphological parameters in mountain pine beetles.

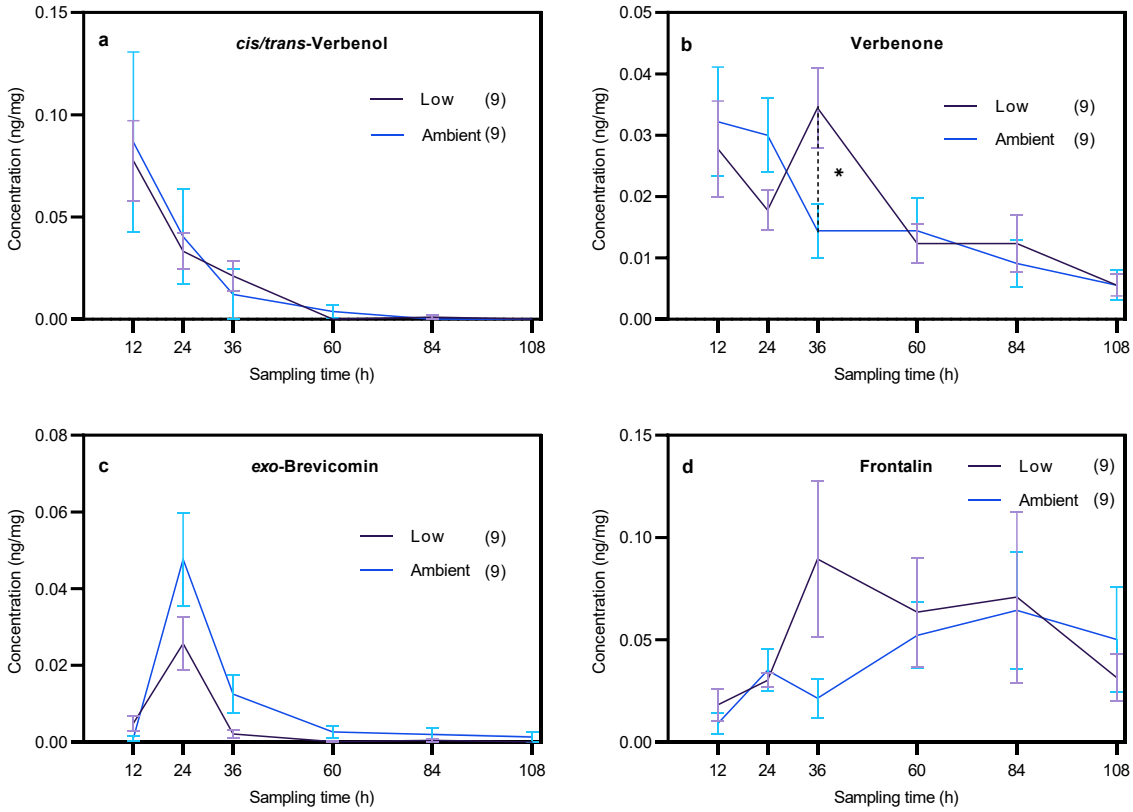

Figure C2: Effect of low and ambient relative humidity treatments on the F1 offspring's pheromone production in mountain pine beetles.

Four pheromones (a) *cis*/*trans*-verbenol, (b) verbenone, (c) *exo*-brevicomin, and (d) frontalin produced by the F_1 generation were collected at different time points, and concentrations were quantified as ng mg^{-1}. Repeated measures mixed effects model was used to analyze the data, Tukey test was used for multiple comparisons. Here, sample sizes are in the brackets, and significantly differing groups are reported as (*) $p<0.05$.

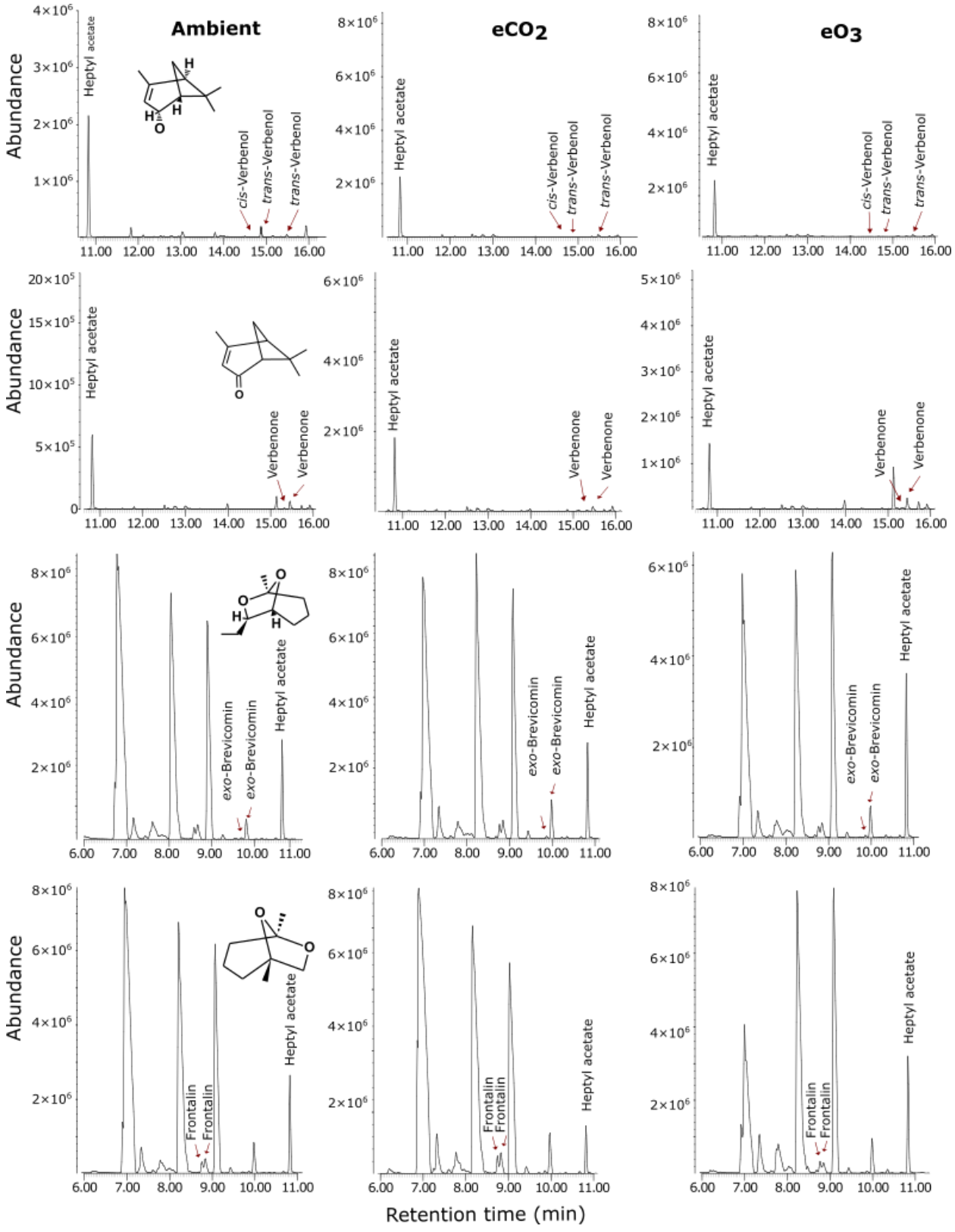

Figure C3: Pheromone detection using gas chromatography-mass spectrometry.