

**Detoxification of Pine Terpenoids by Mountain Pine Beetle Cytochromes P450**

by

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Detoxification of Pine Terpenoids by Mountain Pine Beetle Cytochromes P450

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## Abstract

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is an irruptive bark beetle species affecting pine forests of western North America. A recent outbreak has spread over more than 25 million hectares of pine forests now affecting pine species of sensitive boreal and mountain ecosystems. Pine hosts produce a viscous oleoresin comprised of terpenoids; monoterpene, sesquiterpenes and diterpene resin acids, as a defense against insects and other herbivores. The MPB is exposed to terpenoids for most of its life cycle and these compounds act as host defenses, kairomones, and as pheromone precursors. Cytochromes P450 (P450s) have been proposed to function in MPB detoxification of host defenses, olfaction, and pheromone biosynthesis. My research addressed the role of terpenoids as toxic host defenses and pheromone precursors, and the role of MPB P450s in the modification of terpenoids in detoxification, odorant degradation, and pheromone biosynthesis.

In this thesis, I assessed the toxic effects of monoterpenes to MPB, and analyzed the terpenoid metabolic products formed by MPB and by MPB P450s from host monoterpenes and diterpene resin acids. I assessed the toxicity of ten monoterpenes to the MPB. This study helps to quantitatively define the effects of individual monoterpenes towards MPB mortality, which is critical when assessing the variable monoterpene chemical defense profiles of its host species. I identified a set of novel monoterpenyl esters in the MPB, including verbenyl ester and showed that these esters are accumulated by the female beetle early in the life cycle for future release of the MPB aggregation pheromone *trans*-verbenol. I investigated seven different MPB P450s, specifically *CYP6DE1*, *CYP6DE2*, *CYP6DJ1*, *CYP6BW1*, *CYP6BW3*, *CYP9Z18* and *CYP345E2*, for their potential roles in detoxification, odorant degradation, and pheromone biosynthesis by quantifying the transcript abundance in the antennae and alimentary canal. I have characterized the biochemical functions of four of these MPB P450s. The results of my thesis provide new insights into MPB interactions with host terpenoids defenses and the roles of P450s in these interactions.

## Lay Summary

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is a bark beetle species native to western North America. A recent outbreak of MPB has spread over more than 25 million hectares of pine forests and is now affecting sensitive boreal and mountain ecosystems. Pines produce a viscous oleoresin defense, commonly referred to as pitch. Components of this resin are toxic to insects, however the MPB also uses some of these same compounds as airborne cues to find a host tree and to produce its aggregation pheromone. My research investigates toxicity of resin terpenoid components to MPB, the genes and enzymes that it may use to detoxify these compounds, and how the MPB may use a specific resin component to produce its aggregation pheromone.

## **Preface**

**Chapter 2 Toxicity of Pine Monoterpenes to Mountain Pine Beetle.** A version of chapter 2 has been published. Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2017).

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Statistical analysis of data was conducted with input from C.I. Keeling. The field collections of MPB infested logs were conducted with assistance by S. Angus (JCH Forestry Ltd.), J. Burke (Post-doctoral fellow, UBC), S. Pokoney (Graduate, UBC) and A. Wu (Undergraduate Student, UBC). All other aspects of experimental design, methods and analysis of data were developed and implemented by the author.

**Chapter 3 Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone trans-verbenol.** A version of chapter 3 has been published. Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2018). Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone *trans-verbenol*. *Proceedings of the National Academy of Sciences*, 115(14), 3652–3657.

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The field collections of MPB-infested logs were conducted with assistance by S. Angus (JCH Forestry Ltd.), C.I. Keeling, and J. K. Booth (Graduate Student, Bohlmann Laboratory, UBC). The section “Synthesis of monoterpenyl ester standards” was conducted by C.I. Keeling. All other aspects of experimental design, methods and analysis of data were developed and implemented by the author.

**Chapter 4 Cytochromes P450 preferentially expressed in the antennae of the mountain pine beetle.** A version of chapter 4 has been published. Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2018). Cytochromes P450 preferentially expressed in the antennae of the mountain pine beetle. *Journal of Chemical Ecology*. This chapter was written by the author (C.C. Chiu). The manuscript was reviewed and revised by J. Bohlmann (supervisor). The manuscript was also reviewed by C.I. Keeling (formerly a member of the Bohlmann Laboratory; now Research Scientist, CFS). S. Angus (JCH Forestry Ltd.) and C.I. Keeling assisted with field collections of MPB-infested logs. All other aspects of experimental design, methods and analysis of data were developed and implemented by the author.

**Chapter 5 The cytochrome P450 CYP6DE1 catalyzes the conversion of  $\alpha$ -pinene into the mountain pine beetle aggregation pheromone *trans*-verbenol.** A version of chapter 5 has been submitted for publication. This chapter was written by the author (C.C. Chiu). The manuscript was reviewed and revised by J. Bohlmann (supervisor). The manuscript was also reviewed by C.I. Keeling (formerly a member of the Bohlmann Laboratory; now Research Scientist, CFS). S. Angus (JCH Forestry Ltd.) and C.I. Keeling assisted with field collections of MPB-infested logs. H. Henderson (lab technician, Bohlmann Lab) assisted with early cloning work of CYP6DE2 and did the expression of the MPB cytochrome P450 reductase. All other aspects of experimental design, methods and analysis of data were developed and implemented by the author.

**Chapter 6 Functions of mountain pine beetle cytochromes P450 CYP6DJ1, CYP6BW1 and CYP6BW3 in the oxidation of pine monoterpenes and diterpene resin acids.** This chapter was written by the author (C.C. Chiu). The manuscript was reviewed and revised by J. Bohlmann (supervisor). The manuscript was also reviewed by C.I. Keeling (formerly a member of the Bohlmann Laboratory; now Research Scientist, CFS). S. Angus (JCH Forestry Ltd.) and C.I. Keeling assisted with field collections of MPB-infested logs. H. Henderson (Technician, Bohlmann Laboratory, UBC) assisted with early cloning work of CYP6DJ1 and CYP6BW1 and did the expression of the MPB cytochrome P450 reductase. All other aspects of experimental design, methods and analysis of data were developed and implemented by the author.

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*"Pine beetles eating their way across British Columbia"*  
by Sherri Rogers

# 1. Introduction

## 1.1 Significance of mountain pine beetle

The mountain pine beetle (MBP; *Dendroctonus ponderosae ponderosae* [Hopkins, 1902]) is a native disturbance agent that periodically causes landscape-scale tree mortality within the pine forests of western North America (Safranyik & Carroll, 2006).

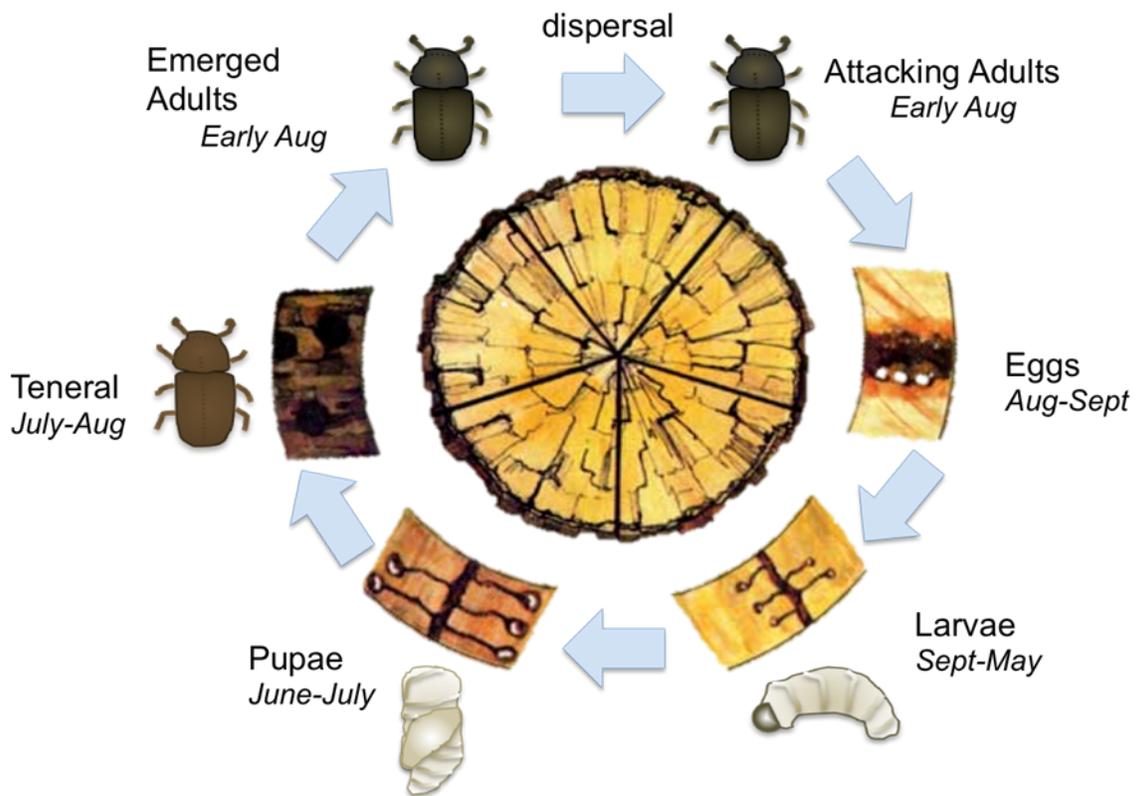
The current outbreak is the largest ever recorded, caused damage to an area of over 18 million hectares of pine forests across western Canada and the United States since the late 1990s (Aukema et al., 2006; Meddens et al., 2012) and has been exacerbated by both fire suppression leading to an overabundance of mature host trees, and climate change leading to improved beetle survival (reviewed by Raffa et al., 2008). During this outbreak, the geographic range of MPB expanded eastward across the Rocky Mountains, and ultimately invading jack pine (*Pinus banksiana*); a new host species that extends across the Canadian boreal forest (Cooke & Carroll, 2017; Cullingham et al., 2011). The current MPB epidemic has also affected pines at higher elevations than has been previously reported (Macfarlane et al., 2017). This range expansion has been attributed to the unprecedented size of the outbreak west of the Rocky Mountains, together with warming that has created climatically benign habitats within the newly invaded range (Bentz et al., 2010; Raffa et al., 2008; Safranyik et al., 2010).

MPB infests a range of different pine (*Pinus*) species, and the most common host in western Canada is lodgepole pine (*Pinus contorta* var. *latifolia*). In general, pines and other conifers, are protected against most herbivores by their production of terpenoid-rich oleoresin. However, as specialist that has co-evolved with conifer defenses, the MPB interacts with terpenoids in many different ways; e.g., terpenoids act as host

defenses that may require detoxification by MPB, they act as volatile cues for host selection, and as MPB pheromone precursors.

## **1.2 MPB Life Cycle**

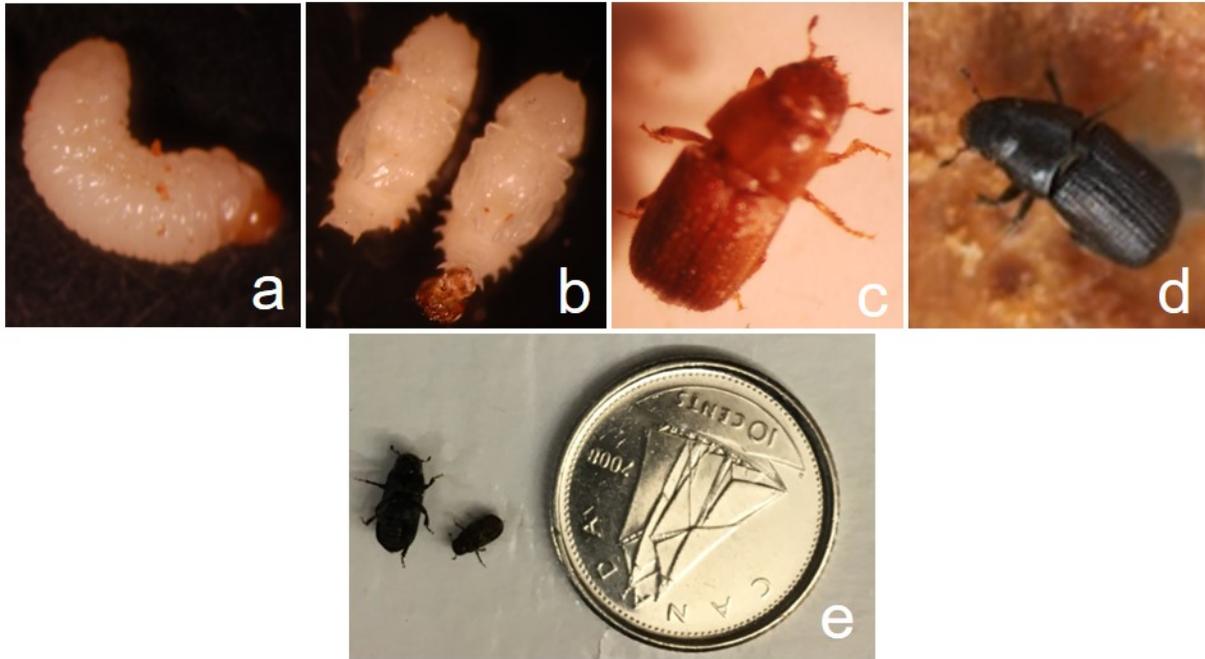
The MPB spends most of its life cycle in the cortex of its brood tree and only leaves the host for a few weeks to disperse and find a new host (**Figure 1.1**). (Bleiker & Van Hezewijk, 2016; Safranyik & Carroll, 2006). Except for the brief period of dispersal, all developmental stages from egg to adult beetles are exposed to the oleoresin terpenoids in the tree (Clark et al., 2012). In the newly colonized host, beetles mate, and females lay eggs along a vertical gallery in the phloem. Early instar larvae feed on the phloem, cambium and outermost xylem, and larvae overwinter in the 3<sup>rd</sup> or 4<sup>th</sup> instar and continue to develop during the following spring until they develop into pupae (**Figure 1.2a**) (Bentz & Mullins, 1999). The young teneral adults, which do not yet have a fully hardened exoskeleton, continue maturation feeding in the brood tree and complete sclerotization (Safranyik & Carroll, 2006) (**Figure 1.2c**). Adult beetles emerge from the brood tree in the summer to disperse and to colonize new host trees (Bleiker & Van Hezewijk, 2016; Safranyik & Wilson, 2006).



**Figure 1.1 Life cycle of the mountain pine beetle.**

Adapted from Amman & Schmitz, 1988 and Robert et al. (2013)

Diagram showing a typical one-year life cycle of MPB. After successfully colonizing the host, females lay eggs along a vertical gallery in the phloem. The early instar larvae, feed perpendicular to this gallery, overwinter in the 3<sup>rd</sup> or 4<sup>th</sup> instar and continue to develop the following spring to pupae. Newly eclosed adult beetles, called teneral adults, do not have a fully hardened exoskeleton and remain within the brood tree for maturation feeding while they complete sclerotization. Adult beetles emerge from the brood tree during a few weeks in the summer to disperse and to find new hosts to colonize.

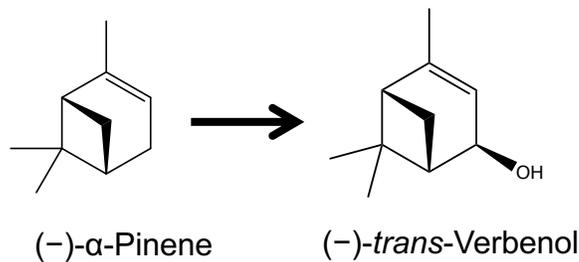


**Figure 1.2 Selected life stages of the mountain pine beetle.**

a) Larva b) Pupae c) Teneral adult d) Newly emerged adult e) An adult female MPB and adult male MPB with a Canadian dime to show the size range of the adults.

Female MPB are typically the first to locate and attack the host tree. Once a female has begun to tunnel into the bark and phloem, she releases the aggregation pheromone *trans*-verbenol that attracts males and other females (**Figure. 1.3**) (Borden et al., 1986; Progar et al., 2013; Vité & Pitman, 1968). Joining females will also release *trans*-verbenol, while joining males release the pheromone *exo*-brevicomin and smaller amounts of frontalin (Aw et al., 2010). These pheromones regulate the number of beetles that mass-attack an individual tree (Borden et al., 1986; Libbey et al., 1985). The burrowing insects damage resin ducts in the tree, which causes the release of oleoresin which is produced both constitutively and induced by the host as a chemical and physical defense at the site of insect attack (Clark et al., 2012; Raffa & Berryman, 1983). The pheromone-mediated mass attack may allow the MPB to overwhelm the

tree's oleoresin defenses. A threshold for overwhelming a tree's defenses has been estimated to be ~40 insect galleries x m<sup>2</sup> of bark area, while the optimum density for the MPB was estimated as ~60 galleries x m<sup>2</sup> (Raffa & Berryman, 1983). At the optimum density, beetles can overcome the host defenses without being affected by intraspecific competition. MPB release the anti-aggregation pheromones verbenone, which is produced by females, and frontalin, produced by males, once they reach a high density, deflecting incoming beetles to neighboring trees (Borden et al., 1986).



**Figure 1.3 *trans*-Verbenol is an oxygenated product of  $\alpha$ -pinene**

The MPB is associated with fungal and bacterial symbionts which co-inhabit the host tree. Fungal associates of MPB include *Grosmannia clavigera*, *Ophiostoma montium* and *Leptographium longiclavatum* (Khadempour et al., 2012). These fungi are transported by MPB in the maxillary mycangia and phoretically from the brood tree to the new host (Bleiker et al., 2009). The MPB-associated fungi provide ergosterol as well as nutrients for the developing larvae and teneral adults (Bentz & Six, 2006; Bleiker & Six, 2007; Goodsman et al., 2012). MPB-associated fungi and bacteria may also contribute to the metabolism of host terpenoid defenses (Adams et al., 2011; Boone et al., 2013; Wang et al., 2013, 2014). The bacterial and fungal communities change in abundance throughout the host colonization process and throughout the galleries of MPB (Adams et al., 2009; Adams & Six, 2007; Khadempour et al., 2012).

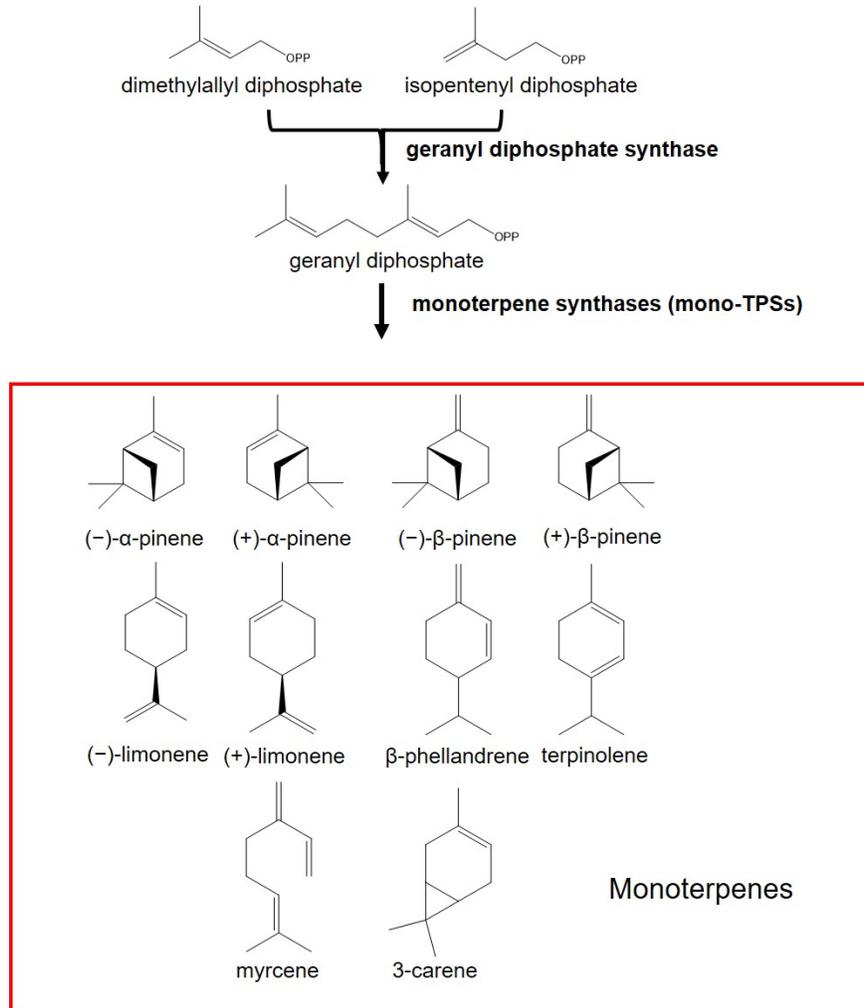
### **1.3 MPB population dynamics**

Prior to the current outbreak, major outbreaks of MPB occurred in the 1870s, 1900s, 1930s, 1940s and 1980s in British Columbia (Alfaro et al., 2010). Between these times, MPB existed on the landscape at lower population densities. MPB populations go through cycles that consist of four phases: endemic, incipient, epidemic and post epidemic (Safranyik & Carroll, 2006). At the low population density of the endemic phase, MPB beetles are unable to colonize healthy well-defended trees (Boone et al., 2011; Raffa & Berryman, 1983), which limits their reproduction to weak, poorly defended trees that are relatively rare on the landscape (Bleiker et al., 2014). In this phase, an increase in the availability of suitable hosts as well as favourable climatic conditions can lead to higher rates of MPB reproduction and increased winter survival, which results in the higher population densities of the incipient and epidemic phases (Bentz et al., 2010; Raffa et al., 2008). In the incipient and epidemic phases, MPB reach a critical threshold population density that allows them to successfully attack healthier and better defended trees which are typically also more abundant (Boone et al., 2011). This in turn favours rates of reproduction and survival, which then fuels the epidemic state (Safranyik & Carroll, 2006). It is in the epidemic state that MPB causes widespread damage to pine forests (Raffa et al., 2008).

### **1.4 Pine oleoresin defenses**

A common defense of conifers of the family Pinaceae is the production and accumulation of oleoresin, which is composed mostly of monoterpenes and diterpene

resin acids (**Figure 1.4 and 1.5**) (Bohlmann, 2012; Keeling & Bohlmann, 2006a, 2006b). In pines, oleoresin accumulates in resin ducts throughout the plant (Franceschi et al., 2005; Krokene, 2015). Oleoresin is constitutively produced and stored in the resin ducts, however oleoresin production can also be induced as a response to biotic stress (Raffa & Berryman, 1982; Raffa et al., 2017; Zulak & Bohlmann, 2010). Oleoresin serves both as a physical and chemical defense. When bark beetles damage resin ducts, the flow of resin can physically hinder the insect from progressing into the tissue (**Figure 1.6**). At the surface of the trees, the volatile monoterpenes of the oleoresin evaporate, allowing the diterpene resin acid to harden, which may trap invading insects and close the wound. In addition to acting as a physical defense, terpenoids can have toxic effects on insects (Stamopoulos et al., 2007; Tak et al., 2016).



**Figure 1.4 Monoterpene biosynthesis in pines.** Geranyl diphosphate is produced by dimethylallyl diphosphate and isopentenyl diphosphate condensed together by geranyl diphosphate synthase. Geranyl diphosphate is the substrate for monoterpene synthases which produce a diversity of monoterpenes. Monoterpenes shown here represent those most abundant in lodgepole pine.

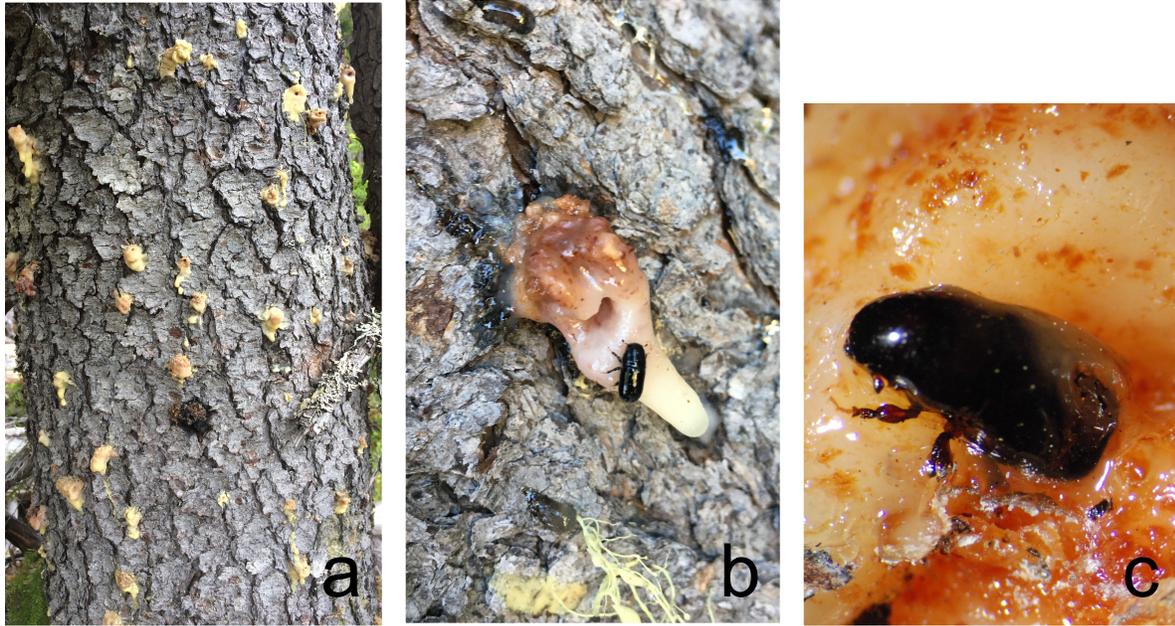


## 1.5 Biosynthesis of oleoresin terpenoids in conifers

Terpenoids are biosynthesized from the common five-carbon molecules isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which originate from the methyl-erythritol 4-phosphate pathway or the mevalonate pathway (Tholl, 2015). Isoprenyl diphosphate synthases, also referred to as prenyl transferases, use IPP and DMAPP as substrates to produce geranyl diphosphate (GPP, 10 carbon), farnesyl diphosphate (FPP, 15 carbon), and geranylgeranyl diphosphate (GGPP, 20 carbon) (Keeling & Bohlmann, 2006b). Conifer terpene synthases (TPS) then convert DMAPP, GPP, FPP and GGPP into hemiterpenes, monoterpenes, sesquiterpenes and diterpenes, respectively (Chen et al., 2011; Keeling & Bohlmann, 2006b; Keeling et al., 2011; Sharkey et al., 2013; Warren et al., 2015). Conifer diterpenes can be oxidized by cytochromes P450 (P450s) to produce diterpene alcohols, aldehydes and acids (Geisler et al., 2016; Hamberger et al., 2011; Ro et al., 2005). The oleoresin of pines consists primarily of monoterpenes and diterpene resin acids, with smaller amounts of sesquiterpenes (Keeling & Bohlmann, 2006b). Pines also emit volatile hemiterpenes, monoterpenes and sesquiterpenes from needles and damaged tissues (Lusebrink et al., 2013, 2011; Sharkey et al., 2013).

The MPB hosts lodgepole pine (*P. contorta*) and jack pine (*P. banksiana*) are well characterized for their oleoresin terpenes (Clark et al., 2012, 2014, Erbilgin et al., 2017, 2014, Hall et al., 2013a, 2013b). Several genes and enzymes of terpene biosynthesis have been characterized in these two species (Hall et al., 2013a, 2013b). Seven different prenyl transferases have been identified in lodgepole pine and nine in jack pine

(Hall et al., 2013a). In addition, eight lodgepole pine mono-TPSs have been functionally characterized and nine jack pine mono-TPSs (Hall et al., 2013a). Each of the mono-TPSs characterized in lodgepole and jack pine produce multiple monoterpenes, including (+)- $\alpha$ -pinene, (-)- $\alpha$ -pinene, (-)- $\beta$ -pinene, (-)- $\alpha$ -phellandrene, (-)- $\beta$ -phellandrene, (+)-3-carene, (-)-limonene, terpinolene, terpin-4-ol, (+)- $\alpha$ -terpineol, (+)- $\alpha$ -terpineol, (-)-camphene, (-)-sabinene and myrcene. Eleven diTPSs have also been identified in jack pine and lodgepole pine, and shown to produce the diterpenes abietadiene, pimaradiene, sandaracopimaradiene, isopimaradiene, palustradiene, levopimaradiene, dehydroabietadiene, and neoabietadiene, which are precursors for the formation of diterpene resin acids found in pine oleoresin (Hall et al., 2013b). Members of the P450 family CYP720B have been functionally characterized in lodgepole and jack pine to produce some of the diterpene resin acids found in these species (Geisler et al., 2016). The combination of multiple TPSs, many of which are multi-product enzymes, and multiple terpene-oxidizing P450s account for the large diversity of terpenes that are found in pine oleoresin and that MPB must contend with.



**Figure 1.6 Oleoresin defense of pine trees.** a) Oleoresin (yellow) that has been exuded from the resin ducts and is present on the bark of lodgepole pine after MPB attack b) Closer view of the oleoresin with attacking MPB. c) Beetle embedded in the oleoresin. Most of the monoterpenes have evaporated from the oleoresin in this picture, leaving the beetle embedded.

### 1.6 Interaction of MPB with host terpenoids

While monoterpenes can be toxic to MPB (Reid et al., 2017) and can serve as both chemical and physical defenses, MPB has co-opted some of these compounds as kairomones and pheromone precursors. Earlier studies tested the general toxicity of pine oleoresin to MPB and other bark beetles (Reid, 1960; Smith, 1961, 1963). While limonene was identified as the most toxic monoterpene to MPB (Reid & Purcell, 2011;

Reid et al., 2017), the relative toxicity of a large number of monoterpene components of the oleoresin was not known prior to the work of this thesis (Chiu et al., 2017).

Monoterpene volatiles released by pines can act as kairomones during MPB host location (Huber et al., 2000; Pureswaran et al., 2004). Female pioneer beetles are guided to a suitable host by host volatiles, avoidance of non-host volatiles, and the visual cue of a dark silhouette (Campbell & Borden, 2006a, 2006b). These cues allow MPB to distinguish a pine host from non-host reducing the energetic cost of host finding relative to randomly landing on trees (Bruce et al., 2005; Byers & Zhang, 2012). In the presence of pheromones, the monoterpene volatiles terpinolene and myrcene synergise with the aggregation pheromones and increase MPB attraction to the tree (Borden et al., 2008; Miller & Borden, 2000; Pureswaran et al., 2004). The MPB must be able to detect low concentrations of monoterpene volatiles during dispersal and host search, and must also be able to cope with the high concentrations of the same compounds in the attacked tree.

The pine monoterpene (–)- $\alpha$ -pinene is the precursor to the MPB aggregation pheromone (–)-*trans*-verbenol (Libbey et al., 1985). It is plausible that the pheromonal role of *trans*-verbenol (see **Figure 1.3**) may have evolved from it being a product of MPB detoxification of host monoterpenes. The concept that *trans*-verbenol may be derived from conversion by MPB of host  $\alpha$ -pinene (Pitman et al., 1968) was supported by work that showed that *trans*-verbenol appeared in extracts and volatile collections of female MPB when feeding on pine phloem (Libbey et al., 1985; Pureswaran et al., 2000;

Taft et al., 2015b; Vité & Pitman, 1968) or treated with  $\alpha$ -pinene (Hughes, 1973b; Hunt & Borden, 1989). However, the release of *trans*-verbenol can also be induced in female MPB without concurrent exposure to  $\alpha$ -pinene, such as by the application of juvenile hormone III (Conn et al., 1984; Keeling et al., 2016). Therefore, the biosynthetic relationship between host  $\alpha$ -pinene and production and release of *trans*-verbenol by MPB is likely to be more complicated than a simple oxidation of host  $\alpha$ -pinene and the immediate release of the resulting *trans*-verbenol.

### **1.7 Studies on host terpenoids in the current MPB epidemic**

Recent studies highlighted differences in the amounts and composition of terpenoids when comparing trees that were attacked or not attacked by MPB, as well as trees that were treated in experiments by wounding, fungal inoculation, or methyl jasmonate application (Boone et al., 2011; Burke et al., 2017; Raffa et al., 2017). In general, trees responded with increased terpenoid content in tissue or increased resin flow after a real or simulated MPB attack. Terpenoid profiles of induced resin also changed; for example, lodgepole pines treated with fungal inocula had higher proportions of  $\beta$ -pinene, 3-carene, limonene,  $\beta$ -phellandrene,  $\alpha$ -thujone, camphene and terpinolene compared to untreated trees (Boone et al., 2011). Oleoresin terpenoids have been compared between pines that are thought to have co-evolved with MPB in the historical range of the insect and pines in the area of recent or potential future MPB range expansion. Examples of historical host species would include in the southern range of British Columbia lodgepole pine (*Pinus contorta*), limber pine (*Pinus flexilis*) and ponderosa pine (*Pinus ponderosae*). Examples of novel or naïve hosts would include

lodgepole pine in the northern range of British Columbia (*Pinus contorta*), jack pine (*Pinus banksiana*), foxtail pine (*Pinus balfouriana*), whitebark pine (*Pinus albicaulis*) and bristlecone pine (*Pinus longaeva*). Generally, these studies showed that historical hosts have higher amounts of  $\beta$ -phellandrene than the naïve hosts (Bentz et al., 2015, 2017; Burke et al., 2017; Clark et al., 2010, 2014; Erbilgin et al., 2014; Raffa et al., 2017; Rosenberger et al., 2017). However, novel hosts such as jack pine, whitebark pine, bristlecone pine and foxtail pine have higher proportions of  $\alpha$ -pinene, and when specified (–)- $\alpha$ -pinene than historical hosts as part of the monoterpene profile (Bentz et al., 2015; Clark et al., 2014; Erbilgin et al., 2014; Raffa et al., 2017; Rosenberger et al., 2017; Taft et al., 2015a). Terpenoid defenses were also compared in lodgepole pines that survived MPB attack in a given area to trees that did not survive. Generally, trees that survived had higher levels of limonene compared to those that did not (Clark et al., 2012; Erbilgin et al., 2017; Raffa & Berryman, 1982). A higher abundance of resin ducts was reported in lodgepole and limber pine that appeared to be resistant compared to susceptible trees of these species (Ferrenberg et al., 2014). Together, these studies showed that oleoresin terpenoids play a variable role in defense of hosts trees against MPB in the current epidemic. This, by extension means that successful host colonization by MPB depends on the nature of the host defense and on the ability to detoxify, or in some other form, cope with terpene defenses.

### **1.8 Insect P450s in the detoxification of host defenses**

Detoxification and elimination are major mechanisms that insect herbivores use to cope with chemical defenses of their hosts. Insects generally use enzymes of three different

families for detoxification: P450s, glutathione-S-transferases (GSTs), and carboxylesterases (CCEs) (Rane et al., 2016). These enzymes are encoded by gene families, and the size of these gene families in a given species has been correlated with the species diversity of the insect's hosts and exposure to different host chemicals (Rane et al. 2016). P450s are the best studied of these detoxification enzymes and a number of P450s of insect herbivores have been shown to detoxify host defense compounds (Feyereisen, 2012). For example, members of the CYP6B family in the genus *Papilio* metabolize linear and angular furanocoumarins produced by host plants of the Apiaceae and Rutaceae families (Li et al., 2007). Gossypol is a sesquiterpene aldehyde and a major defense compound in cotton plants. The RNAi-mediated knockdown of *CYP6AE14* in the cotton bollworm (*Helicoverpa armigera*) decreased the ability of larvae to feed on a gossypol-spiked diet (Mao et al., 2007; Tao et al., 2012).

Insects may also use P450s to biosynthesize the same defense compounds as their host plant or use P450s to re-purpose host molecules. For example, the burnet moth (*Zygaena filipendulae*) can both sequester cyanogenic glucosides from its host plants and synthesize them *de novo* (Jensen et al., 2011). Two P450s, CYP405A2 and CYP332A3, are involved in the insect's *de novo* biosynthesis of the cyanogenic glucosides, linamarin and lotaustralin (Jensen et al., 2011). In the bark beetle *Ips pini*, males produce the pheromones ipsdienol and ipsenol by hydroxylation and reduction of the monoterpene myrcene (Tittiger & Blomquist, 2016). *Ips pini* can obtain myrcene from the host or synthesize it *de novo* (Gilg et al., 2009; Martin et al., 2003; Tillman et al., 1998). The insect P450 CYP9T2 hydroxylates myrcene to produce ipsdienol, which

is further modified by a dehydrogenase to produce the pheromone (Sandstrom et al., 2008, 2006). P450s have also been described as odorant- degrading enzymes in insect olfactory systems (Maïbèche-Coisne et al., 2004; Pottier et al., 2012), including the monoterpene-oxidizing P450 CYP345E2 in the MPB (Keeling et al., 2013b).



**Figure 1.7. P450 monooxygenase reaction.** The best-known mechanism of P450s is the monooxygenase reaction, in which one atom of molecular oxygen (O<sub>2</sub>) is inserted into the substrate and the other forms water.

### 1.9 P450s

P450s are a large family of heme-thiolate containing enzymes. In insects, P450s are associated with the biosynthesis of ecdysteroids (molting hormones), juvenile hormones, and pheromones, and metabolism of host defenses and pesticides (Feyereisen 2006; Li et al. 2007; Feyereisen 2011). The best-known mechanism of P450s is the monooxygenase reaction, in which one oxygen atom of molecular oxygen (O<sub>2</sub>) is inserted into the substrate and the other is used to form water. In the overall reaction (**Figure 1.7**), P450s typically oxidize the substrate resulting in the formation of a hydroxyl group or an epoxide group. Beyond monooxygenases, P450s account for over 60 different types of catalytic activities including dealkylations, dehalogenations, and carbon-carbon cleavages, and they act on a seemingly infinite number of substrates (Feyereisen, 2012). The heme group of P450s is important to the activation of the oxygen, and the most conserved regions of P450s are associated with heme

binding (Werck-Reichhart & Feyereisen, 2000a). At one point in the P450 catalytic cycle, the  $\text{Fe}^{3+}$  in the heme group is in low spin and is bound at the sixth position to  $\text{H}_2\text{O}$ . The binding of the substrate within the enzyme and in close proximity to the heme group displaces the  $\text{H}_2\text{O}$  which leads to the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and the shift of the iron from low spin to high spin. This shift can be observed by an increase in absorbance at 410 nm and decrease at 380 nm (Type I spectrum), and this shift can be used in substrate binding assays (Denisov et al., 2005). Molecular oxygen binds to the  $\text{Fe}^{2+}$ , and this complex is subsequently reduced and then protonated, forming short-lived, highly reactive transient compounds (Munro et al., 2013). At this point carbon monoxide (CO) can also competitively bind to the  $\text{Fe}^{2+}$ , forming a stable CO-P450 complex with an absorbance maximum at 450nm (Guengerich et al., 2002). The name-defining absorbance at 450 nm is used in CO-binding assays to quantify the amount of active P450 (Schenkman & Jansson, 2006). The reduction of the heme requires a supply of electrons from NADPH, which are transferred to the P450 by a redox partner. The redox partner varies between different classes of P450s. Class I P450s are reduced by adrenodoxin ( $\text{Fe}_2\text{S}_2$ ) in the mitochondrial membrane (Werck-Reichhart & Feyereisen, 2000b). Class II P450s are reduced by an FAD/FMN-containing P450 reductase and are located at the outer membrane surface of the endoplasmic reticulum (Werck-Reichhart & Feyereisen, 2000a). For convenience, the P450 nomenclature uses a hierarchical system of clades, families and sub-families based on amino acid sequence identity (Nelson & Werck-Reichhart, 2011). In this nomenclature, the prefix CYP forms the root of the name, followed by a first number representing the family (cut-off 40% identity), letters for the subfamily (cut-off 55% identity), and a second number that

identifies the specific P450 enzyme. For example, CYP6DE1 shares at least 40% identity with other members of the P450 family CYP6, and at least 55% identity with other members of the subfamily CYP6DE. Members of a given P450 subfamily may be associated with similar biochemical functions or similar biological roles in a group of more or less closely related organisms. For example, many members of the insect CYP6 subfamily are involved in detoxification.

### **1.10 MPB P450s**

P450s in MPB and other bark beetles have been studied for their role in metabolizing conifer terpenes (reviewed in Keeling, 2016). Examples of early work that implicated P450s in MPB were the findings of oxidase activity in MPB midguts (Sturgen & Robertson, 1985) and the presence of oxygenated monoterpenes in female MPB extracts (Pierce et al., 1987). In addition, female MPB treated with P450 inhibitors and then exposed to  $\alpha$ -pinene and myrcene had increased levels of monoterpenes and reduced levels of the hydroxylated products *trans*-verbenol and ipsdienol (Hunt & Smirle, 1988). These observations indicated that P450s might be important for the metabolism of monoterpenes in MPB. More recently, the MPB genome was annotated to contain 85 different P450 genes (Keeling et al., 2013c). Additional transcriptome and proteome studies identified patterns of P450 expression in MPB (Aw et al., 2010; Bonnett et al., 2012; Keeling et al., 2012, 2016; Nadeau et al., 2017; Pitt et al., 2014; Robert et al., 2016, 2013). Only a few MPB P450s have been functionally characterized prior to or in parallel with the work described in this thesis. Initially identified in a transcriptome analysis as a male pheromone biosynthesis gene, MPB CYP6DE3 was

characterized to metabolize (+)- $\alpha$ -pinene, 3-carene and (+)-limonene and may have a role in the detoxification of these compounds (Nadeau et al., 2017). MPB CYP345E3, which is most highly expressed in MPB antennae, metabolized the host volatiles (+)-3-carene, (–)-camphene and both enantiomers each of  $\alpha$ -pinene,  $\beta$ -pinene and limonene, with a possible role in odorant degradation (Keeling et al., 2013b). MPB CYP6CR1 was functionally characterized to epoxidize (Z)-6-nonen-2-one as part of the *exo*-brevicommin pheromone biosynthesis (Song et al., 2014).

### **1.11 Focus of this thesis**

This thesis focuses on the interaction between MPB and terpenes of the pine host. This includes research on the (i) toxicity of host terpenes to MPB and metabolites produced by MPB from host terpenes; (ii) biochemical functions of MPB P450s with host terpenes; and (iii) roles of MPB P450s in olfaction, detoxification and pheromone production which are essential for MPB to successfully colonize a host tree. I have tested the toxicity of pine monoterpenes to MPB (Chapter 2). I have investigated terpenoid products of MPB after treatment with terpenes or during MPB development in the terpenoid rich environment of the phloem (Chapter 3, 5, 6). And I have investigated MPB P450s that are involved in the modification of terpenoids by characterizing transcript expression

patterns and by identification of enzymatic activity of several P450s abundant in the antennae and alimentary canal (Chapter 4, 5, 6).

My thesis addresses the following questions and hypotheses:

**Question 1:**

How do MPB tolerate the high levels terpenoid host defenses during host colonization?

**Hypothesis 1:**

Terpenes are toxic to MPB in a dose-dependent manner that varies with the different terpenes.

In Chapter 2, I test toxicity of ten different monoterpenes that are abundant in pines and show the range of concentration that have toxic effect in MPB. These results informed other experiments that I did with MPB that involved the exposure of monoterpenes to MPB.

**Question 2:**

How does the metabolism of host terpenoids by the MPB relate to olfaction, detoxification and pheromone production?

**Hypothesis 2:**

Host terpenes are modified by MPB to reduce their toxicity and/or permit elimination. Some of these modifications may also be biochemically related to *trans*-verbenol pheromone biosynthesis.

In Chapter 3, I investigate the production of monoterpenols and monoterpenyl esters throughout the MPB life cycle and after treatment with monoterpenes with respect to the role of these metabolites in detoxification and pheromone production. In Chapter 5, I investigate the monoterpenols produced by beetles after treatment with both enantiomers of  $\alpha$ -pinene. In Chapter 6, I investigate the monoterpenols produced by beetles after treatment with terpinolene and both enantiomers of limonene. In Chapter 6, I also investigate the hydroxy-diterpene resin acids produced after treatment with diterpene resin acids.

**Question 3:**

What is the role of cytochromes P450 in the modification of terpenoids with regards to odorant clearance, detoxification and pheromone production in the MPB?

**Hypothesis 3:**

The cytochromes P450 identified in the antennal tissues are involved in the modification of terpenoids relevant to the mountain pine beetle host colonization processes. This degradation is important for odorant clearance in the antenna and detoxification or pheromone biosynthesis in other tissues.

In Chapter 4, I explore the role of seven cytochrome P450s in olfaction, detoxification and pheromone production by quantifying their transcript abundance in the antennae and alimentary canal of MPB. In Chapter 5, I functionally characterized CYP6DE1 enzyme as a P450 possibly involved in detoxification and pheromone production. In Chapter 6, I functionally characterize CYP6DJ1, CYP6BW1 and

CYP6BW3 as P450s involved in the detoxification of monoterpenes and diterpene resin acids.

## 2. Toxicity of Pine Monoterpenes to Mountain Pine Beetle<sup>1</sup>

### 2.1 Summary

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is an eruptive bark beetle species affecting pine forests of western North America. MPB are exposed to volatile monoterpenes, which are important host defense chemicals. I assessed the toxicity of the ten most abundant monoterpenes of lodgepole pine (*Pinus contorta*), a major host in the current MPB epidemic, against adult MPB from two locations in British Columbia, Canada. Monoterpenes were tested as individual volatiles and included (-)- $\beta$ -phellandrene, (+)-3-carene, myrcene, terpinolene, and both enantiomers of  $\alpha$ -pinene,  $\beta$ -pinene and limonene. Dose-mortality experiments identified (-)-limonene as the most toxic (LC<sub>50</sub>: 32  $\mu$ L/L), and (-)- $\alpha$ -pinene (LC<sub>50</sub>: 290  $\mu$ L/L) and terpinolene (LC<sub>50</sub>: >500  $\mu$ L/L) as the least toxic. MPB body weight had a significant positive effect on the ability to survive most monoterpene volatiles, while sex did not have a significant effect with most monoterpenes. This study helps to quantitatively define the effects of individual monoterpenes towards MPB mortality, which is critical when assessing the variable monoterpene chemical defense profiles of its host species.

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<sup>1</sup> A version of this chapter has been published. Chiu CC, Keeling CI, Bohlmann J (2017) Toxicity of pine monoterpenes to mountain pine beetle. Sci Rep 7:8858.

## 2.2 Introduction

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is an eruptive bark beetle that infests different pine (*Pinus sp.*) species in its native range of western North America. Since the late 1990's, a continuous MPB outbreak has affected over 25 million hectares of lodgepole pine (*Pinus contorta*) forests (Hart et al., 2015). The MPB has crossed the geographic barrier of the Rocky Mountains and is expanding its host range from lodgepole pine, which is dominant west of the Rocky Mountains, into jack pine (*P. banksiana*) east of the Rocky Mountains (Cullingham et al., 2011). To overcome a well-defended host tree, MPB employs aggregation pheromones such as female-released (-)-*trans*-verbenol and male-released *exo*-brevicomin to recruit a critical threshold of conspecifics. MPB also benefits from symbiotic relationships with fungal and bacterial associates (Adams et al., 2011; DiGuistini et al., 2011).

A major chemical defense system of conifers is the production of oleoresin, which consists primarily of a complex mixture of different volatile monoterpenes, non-volatile diterpenoids, and lesser abundant sesquiterpenes (Keeling & Bohlmann, 2006b; Kolosova & Bohlmann, 2012; Zulak & Bohlmann, 2010). Oleoresin terpenoids are produced constitutively and biosynthesis is induced when trees are exposed to biotic stress (Boone et al., 2011; Martin et al., 2002; Zulak & Bohlmann, 2010). The terpenoid profiles of the oleoresin vary substantially across different conifer species and between populations and individuals of the same species (Clark et al., 2010; Raffa et al., 2013; Robert et al., 2010). These variations may be explained, at least in part, by genomic

variations of terpene synthase genes (Hall et al., 2011; Roach et al., 2014). For example the monoterpene profile of lodgepole pine has higher relative amounts of  $\beta$ -phellandrene and terpinolene compared to jack pine, which has higher relative amounts of (+) and (-)- $\alpha$ -pinene, (+)-3-carene, and (-)-limonene (Clark et al., 2014; Hall et al., 2013a).

Coevolution of MPB with conifer monoterpenes resulted in complex chemo-ecological interactions (Raffa, 2014; Seybold et al., 2006). While pines produce monoterpenes as part of a chemical defense system, MPB can exploit pine monoterpenes as signals for identification of a suitable host, and also incorporates certain monoterpenes to produce pheromones for mass attack to overcome the host defense. For example, (-)- $\alpha$ -pinene serves as the precursor to (-)-*trans*-verbenol (Blomquist et al., 2010), an aggregation pheromone produced and released by females upon initial attack of a host tree. Female MPBs produce *trans*-verbenol after phloem feeding (Pierce et al., 1987; Pureswaran et al., 2000; Renwick et al., 1976b) and after exposure to  $\alpha$ -pinene vapours at concentrations of 25-250  $\mu$ L/L (Conn et al., 1984; Gries et al., 1990; Hunt & Smirle, 1988). In addition, the host monoterpenes terpinolene and myrcene are synergists of aggregation pheromones as the addition of these monoterpenes to pheromone baits increases the number of attracted beetles compared to the pheromone alone (Borden et al., 2008; Miller et al., 2005b; Pureswaran & Borden, 2005).

There is considerable evidence that monoterpenes are toxic to insects. For example, dose-response studies of monoterpenes with cockroach (*Blattella germanica*), flour

beetle (*Tribolium confusum*), cabbage looper (*Trichoplusia ni*), and housefly (*Musca domestica*), using fumigant and contact exposure, found that monoterpenes are toxic at doses comparable to those of synthetic pesticides (Phillips et al., 2010; Stamopoulos et al., 2007; Tak et al., 2016; Tarelli et al., 2009). The mode of action of monoterpenes at the molecular level appears to include components of the insect nervous system, specifically octopamine and tyramine receptors (Enan, 2001), acetylcholinesterase (López & Pascual-Villalobos, 2010, 2015) and GABA receptors (Tong & Coats, 2012). Toxicity studies of saturated pine resin vapours with *Dendroctonus* species, specifically the western pine beetle (*D. brevicomis*), Jeffery pine beetle (*D. jeffreyi*), and mountain pine beetle (*D. ponderosae*), found that non-host pine resin is more toxic than host pine resin (Smith, 1961, 1963, 1965a). This indicates that there are differences in the toxicities of the compounds that comprise the volatile pine profiles. Studies with the western pine beetle (*D. brevicomis*), the spruce beetle (*D. rufipennis*), the larch beetle (*D. simplex*), and southern pine beetle (*D. frontalis*) showed that limonene is more toxic when compared to other monoterpenes (typically  $\alpha$ -pinene,  $\beta$ -pinene, 3-carene and myrcene) (Coyne, J.F., Lott, 1976; Smith, 1965b; Werner, 1995). For MPB however, the toxicity of the individual monoterpenes that dominate the volatile profiles of its hosts is not known.

Here, I quantitatively tested the toxicity of individual monoterpene volatiles that are abundant in lodgepole pine using dose-mortality experiments with beetles sourced from two locations in British Columbia, Canada. In line with previous work and to enable comparison with the literature, I used exposure to monoterpene volatiles, as opposed to

contact exposure. Results are reported as LC<sub>50</sub>, which is the concentration at which 50% of a MPB test population is killed by a given substance after 24 h, and thereby provides a benchmark for comparison between different monoterpenes. MPB body weight varies by over three-fold, and females, the pioneering sex, are on average heavier than males. Thus, body weight and sex were monitored to assess their influence on beetle survival with different monoterpenes.

## **2.3 Materials and methods**

### **2.3.1 Insects. Beetles were obtained from two locations in British Columbia (BC), Canada.**

Cohort 1 was from a naturally infested lodgepole pine near Mt Baldy, BC (49°06'32.8"N 119°14'48.1"W), which was felled and the logs collected in May 2015. Cohort 2 was from near Whistler, BC (50° 12'33.3"N 122°53'05.2"W), where MPB pheromone baits (Contech) were attached to two lodgepole pines on June 1st 2015 and were removed after signs of attack were apparent. The attacked trees were felled in Oct 2015. Infested logs were placed in screened cages at room temperature to rear beetles to maturity. Emerged beetles were collected every 2 to 4 days and held on moist Kimwipe paper (Kimberly-Clark) at 4°C until use. Beetles were sexed based on the dimorphism of the seventh abdominal tergite (Lyon, 1958). Beetles that responded weakly to being picked up with forceps during this step were not used in the assay. Each beetle was weighed to the nearest 0.1 mg on the day of use in toxicity assays. Beetles were between 3 and 21 days post-emergence at the time of experiments.

**Table 2.1. Monoterpenes used in toxicity assays**

Compound	CAS	Purity	Supplier	Catalog #	Lot #
(1 <i>R</i> )-(+)- $\alpha$ -Pinene	7785-70-8	98%	Sigma Aldrich	P45680	08926AE
(1 <i>S</i> )-(-)- $\alpha$ -Pinene	7785-26-4	99%	Sigma Aldrich	274399	09409MS
(1 <i>S</i> )-(-)- $\beta$ -Pinene	18172-67-3	99%	Sigma Aldrich	112089	00307LG
(1 <i>R</i> )-(+)- $\beta$ -Pinene	19902-08-0	98%	Fluka	80607	BCBK2147V
(1 <i>S</i> )-(+)-3-Carene	498-15-7	99%	Sigma Aldrich	441619	0531PH
Terpinolene	586-62-9	85%	Fluka	86485	1401137
(1 <i>S</i> )-(-)-Limonene	5989-54-8	96%	Sigma Aldrich	21836-7	14105EU
(1 <i>R</i> )-(+)-Limonene	5989-27-5	~90%	Fluka	62122	BCBH9990V
Myrcene	123-35-3	99%	Sigma Aldrich	M100005	BCBD7911V
( <i>R</i> )-(-)- $\beta$ -Phellandrene	6153-17-9	84%	Synergy Semiochemicals	Custom	Custom

**2.3.2 Monoterpene toxicity assays.** Ten monoterpenes (**Table 2.1**) were used individually for toxicity assays. Monoterpenes were selected based on relative content of 2% or higher in the monoterpene profile of lodgepole pine according to two recent reports (Clark et al., 2014; Hall et al., 2013a). They also included nine of the ten most abundant monoterpenes in jack pine. A 1.5 cm x 1.5 cm piece of Whatman filter paper was placed in a 20-mL scintillation vial (VWR). Defined volumes (see below) of

undiluted monoterpenes, monoterpenes diluted into acetone, or acetone as a control were applied to the filter paper using a microdispenser (VWR) immediately before a single beetle was added to the vial and the vial was sealed. Monoterpenes were tested at five defined doses (volume monoterpene applied / volume airspace of the assay vial) of 10  $\mu\text{L/L}$ , 50  $\mu\text{L/L}$ , 100  $\mu\text{L/L}$ , 200  $\mu\text{L/L}$  and 500  $\mu\text{L/L}$ . To achieve the doses of 50  $\mu\text{L/L}$ , 100  $\mu\text{L/L}$ , 200  $\mu\text{L/L}$  and 500  $\mu\text{L/L}$  undiluted monoterpenes were applied at volumes of 1  $\mu\text{L}$ , 2  $\mu\text{L}$ , 4  $\mu\text{L}$ , and 10  $\mu\text{L}$ , respectively. For the 10  $\mu\text{L/L}$  dose, monoterpenes were diluted five-fold in acetone and 1  $\mu\text{L}$  of the diluted monoterpene applied. To control for solvent effects, and as the 0  $\mu\text{L/L}$  control, 1  $\mu\text{L}$  of acetone was applied. MPB were exposed to volatiles for 24 hours and then removed from assay vials to assess mortality. MPB were considered dead if they did not respond after being tapped with soft forceps. In total approximately 120 MPB were tested for each of the two cohorts and with each of the monoterpenes. At each concentration plus control for each monoterpene, 20 MPB with 10 females and 10 males were used. MPB were distributed so that their average body weight was the same for all concentrations in a given cohort trial. Two trials were done on each monoterpene, one with MPB from cohort 1 and the other from cohort 2, except assays with (+)- $\beta$ -pinene and (-)- $\beta$ -phellandrene could not be completed with cohort 1 due to insufficient numbers of MPB collected from this location.

**2.3.3 Statistical Analyses.** Analyses were conducted using the statistical program language R version 3.4.0 (R Core Development Team, 2017). The average beetle weights differed significantly between cohorts (two-tailed t-test,  $t(2.097) = -9.0627$ ,  $p$ -

value < 0.0001, see also **Figures 2.3** and **2.4**). I therefore analyzed each cohort separately. The `drc_3.0-1` package (Ritz et al., 2015) was used to subject mortality data to logit analysis (Finney, 1971) using the Hill three-parameter log-logistic function (`LL2.3u(upper=1)`) from which the  $LC_{50}$ , 95% fiducial limits, Hill slope ( $b \pm S.E.$ ), and  $\chi^2$  values for each compound separately were generated. Multiple comparisons between  $LC_{50}$  values were conducted via pairwise t-tests on the  $\log(LC_{50})$  values of the Hill equation, using a pooled standard error, and correcting for experiment-wise error by the Benjamini–Hochberg procedure. Logistical regression was used for each compound separately to assess the independent effects of concentration, sex and weight on survival.

## **2.4 Results**

### **2.4.1 $LC_{50}$ varies by monoterpene with (-)-limonene being the most toxic against MPB**

I measured the  $LC_{50}$  values for ten different monoterpenes at 24 h of vapour exposure with MPB from two locations, cohorts 1 and 2 (**Tables 2.2** and **2.3**). I analyzed the cohorts separately because the beetles from these two cohorts differed significantly in weight (two sample t-test,  $p < 0.0001$ ). The  $LC_{50}$  values ranged from  $\sim 30 \mu\text{L/L}$  to  $> 500 \mu\text{L/L}$ , revealing a quantitative difference in toxicity of over ten-fold between monoterpenes. Overall, the results were consistent with beetles from the two cohorts with respect to the relative toxicity of most of the monoterpenes tested. The most toxic monoterpene was (-)-limonene, which was significantly more toxic than the (+)-limonene enantiomer and any of the other monoterpenes. The next most toxic terpenes were (+)-

3-carene, myrcene, and (-)- $\beta$ -phellandrene, followed by the two enantiomers of  $\beta$ -pinene, which were close in LC<sub>50</sub> value, and (+)- $\alpha$ -pinene. Notably, (-)- $\alpha$ -pinene was considerably less toxic than the (+) enantiomer, and this difference was significant in MPB from cohort 2. The LC<sub>50</sub> value for terpinolene could not be determined definitively, because too few of the beetles died even at the highest concentrations, revealing that terpinolene was much less toxic than any of the other monoterpenes tested.

**Table 2.2. Toxicity of monoterpene volatiles against MPB from cohort 1**

Cohort 1					
Monoterpene	N	LC <sub>50</sub> [μl/L]	Hill Slope ± SE	χ <sup>2</sup> (df)	Mean Weight [mg]
(-)-Limonene	144	49 (34-71) <sup>a</sup>	-1.6 ± 0.3	5.4 (3)	9.33 ± 0.22
(+)-Limonene	120	89 (66-120) <sup>b</sup>	-2.1 ± 0.6	8.5 (1)	8.51 ± 0.19
(+)-3-Carene	120	117 (86-158) <sup>bc</sup>	-2.3 ± 0.6	8.8 (3)	8.66 ± 0.19
Myrcene	119	163 (113-234) <sup>cd</sup>	-2.2 ± 0.6	4.0 (3)	8.54 ± 0.21
(+)-α-Pinene	120	185 (151-227) <sup>cd</sup>	-4.1 ± 1.2	4.6 (3)	8.63 ± 0.19
(-)-β-Pinene	120	221 (164-296) <sup>d</sup>	-3.3 ± 1.2	2.3 (3)	8.94 ± 0.19
(-)-α-Pinene	120	277 (160-477) <sup>d</sup>	-1.4 ± 0.4	5.3 (3)	9.07 ± 0.20
Terpinolene	120	>500			8.74 ± 0.21

Dose-response models of the logit-transformed mortality data were used to determine the LC<sub>50</sub> values, 95% fiducial limits (f.l.), and Hill slope for each monoterpene tested. LC<sub>50</sub> values denoted with the same letter were not significantly different. Multiple comparisons between LC<sub>50</sub> values were conducted via pairwise t-tests on the log (LC<sub>50</sub>) values of the Hill equation, using a pooled standard error, and correcting for experiment-wise error by the Benjamini–Hochberg procedure. The goodness-of-fit test χ<sup>2</sup> (degree of freedom) was used to assess the fit of the dose-response model. Less than 50% of the beetles died at all doses of terpinolene. The mean weight of the beetles tested for each compound in cohort 1 was not significantly different and the mean weight of the beetles tested with a given monoterpene was the same at all doses (ANOVA, p-value = not significant). As sex was not a significant factor in mortality for almost all monoterpenes tested, the data for both sexes was combined.

**Table 2.3. Toxicity of monoterpene volatiles against MPB from cohort 2**

Monoterpenes	N	Cohort 2			
		LC <sub>50</sub> [μl/L]	Hill slope ± SE	χ <sup>2</sup> (df)	Mean Weight
(-)-Limonene	120	32 (22-47) <sup>a</sup>	-2.2 ± 0.5	1.4 (2)	10.03 ± 0.19
(+)-Limonene	120	60 (52-70) <sup>b</sup>	-5.9 ± 1.6	<0.1 (1)	9.68 ± 0.23
(+)-3-Carene	120	76 (63-90) <sup>bc</sup>	-4.5 ± 1.0	0.4 (1)	9.47 ± 0.19
Myrcene	119	77 (55-108) <sup>bc</sup>	-1.6 ± 0.3	1.5 (2)	9.76 ± 0.22
(-)-β-Phellandrene	120	97 (75-124) <sup>cd</sup>	-3.0 ± 0.7	9.1 (3)	9.52 ± 0.22
(-)-β-Pinene	120	130 (105-160) <sup>d</sup>	-4.9 ± 1.4	<0.1 (1)	9.52 ± 0.18
(+)-β-Pinene	120	131 (108-158) <sup>d</sup>	-6.7 ± 1.9	0.6 (3)	9.45 ± 0.20
(+)-α-Pinene	120	131 (106-161) <sup>d</sup>	-5.0 ± 1.4	3.5 (3)	9.99 ± 0.19
(-)-α-Pinene	120	248 (187-330) <sup>e</sup>	-2.2 ± 0.5	0.6 (1)	9.73 ± 0.21
Terpinolene	120	>500			9.73 ± 0.21

Dose-response models of the logit-transformed mortality data were used to determine the LC<sub>50</sub> values, 95% fiducial limits (f.l.), and Hill slope for each monoterpene tested. LC<sub>50</sub> values denoted with the same letter were not significantly different. Multiple comparisons between LC<sub>50</sub> values were conducted via pairwise t-tests on the log (LC<sub>50</sub>) values of the Hill equation, using a pooled standard error, and correcting for experiment-wise error by the Benjamini–Hochberg procedure. The goodness-of-fit test χ<sup>2</sup> (degree of freedom) was used to assess the fit of the dose-response model. Less than 50% of the beetles died at all doses of terpinolene. The mean weight of the beetles tested for each compound in cohort 1 was not significantly different and the mean weight of the beetles tested with a given monoterpene was the same at all doses (ANOVA, p-value = not significant). As sex was not a significant factor in mortality for almost all monoterpenes tested, the data for both sexes was combined.

## 2.4.2 Factors affecting mortality

Logistical regression analyses (**Tables 2.4** and **2.5**; **Figures 2.1** and **2.2**) showed that monoterpene concentration had a significant positive relationship with mortality for all monoterpenes tested. Body weight negatively influenced mortality, i.e. heavier MPB showed increased survival with most monoterpenes. MPB from the two cohorts differed significantly in average weight (two sample t-test,  $p < 0.0001$ ). The body weight (average fresh weight  $\pm$  SD) of MPB was 8.8 mg  $\pm$  2.2 mg for cohort 1 (N=983) and 9.7 mg  $\pm$  2.2 mg for cohort 2 (N=1199) (**Figures 2.3** and **2.4**). For both locations, increased body weight correlated significantly with higher survival in the treatments with (-)-limonene, myrcene, (-)- $\beta$ -pinene and terpinolene (**Tables 2.4** and **2.5**). The monoterpene (+)- $\beta$ -pinene also showed this relationship, but was only tested with beetles from cohort 2. MPB with higher body weight also survived treatment with (+)- $\alpha$ -pinene significantly more often in cohort 1, but this relationship was not found in cohort 2, and was the opposite with (-)- $\alpha$ -pinene. Body weight did not affect the survival in treatments with (+)-limonene or (-)- $\beta$ -phellandrene. Sex did not have a significant effect on mortality with most monoterpenes. However, males from cohort 1 survived better than females in treatments with (+)-3-carene and (-)- $\beta$ -pinene, but this relationship was not found with MPB of the cohort 2.

**Table 2.4. The independent effects of concentration, body weight and sex on mortality for each monoterpene tested with MPB from cohort 1.**

		Cohort 1		
Monoterpene		Concentration	Weight	Sex
(-)-Limonene	Coefficient ( $\pm$ S.E.)	0.018 $\pm$ 0.004	-0.38 $\pm$ 0.12	-0.05 $\pm$ 0.48
	<i>P</i>	8.75e-07 *	0.001*	0.915
(+) -Limonene	Coefficient ( $\pm$ S.E.)	0.018 $\pm$ 0.004	-0.23 $\pm$ 0.15	0.52 $\pm$ 0.59
	<i>P</i>	1.21e-06 *	0.115	0.379
(+) -3-Carene	Coefficient ( $\pm$ S.E.)	0.016 $\pm$ 0.003	-0.54 $\pm$ 0.17	-1.22 $\pm$ 0.59
	<i>P</i>	5.77e-06 *	0.002 *	0.039*
Myrcene	Coefficient ( $\pm$ S.E.)	0.012 $\pm$ 0.003	-0.40 $\pm$ 0.14	0.78 $\pm$ 0.54
	<i>P</i>	2.95e-06 *	0.005*	0.148
(+) - $\alpha$ -Pinene	Coefficient ( $\pm$ S.E.)	0.019 $\pm$ 0.004	-0.46 $\pm$ 0.21	0.30 $\pm$ 0.73
	<i>P</i>	5.61e-06 *	0.030*	0.680
(-)- $\beta$ -Pinene	Coefficient ( $\pm$ S.E.)	0.013 $\pm$ 0.003	-0.84 $\pm$ 0.23	-1.35 $\pm$ 0.67
	<i>P</i>	9.79e-07 *	<0.001*	0.046*
(-)- $\alpha$ -Pinene	Coefficient ( $\pm$ S.E.)	0.005 $\pm$ 0.001	-0.10 $\pm$ 0.11	0.26 $\pm$ 0.47
	<i>P</i>	5.11e-05 *	0.360	0.579
Terpinolene	Coefficient ( $\pm$ S.E.)	0.004 $\pm$ 0.001	-0.52 $\pm$ 0.17	0.26 $\pm$ 0.53
	<i>P</i>	0.00436 *	0.002*	0.625

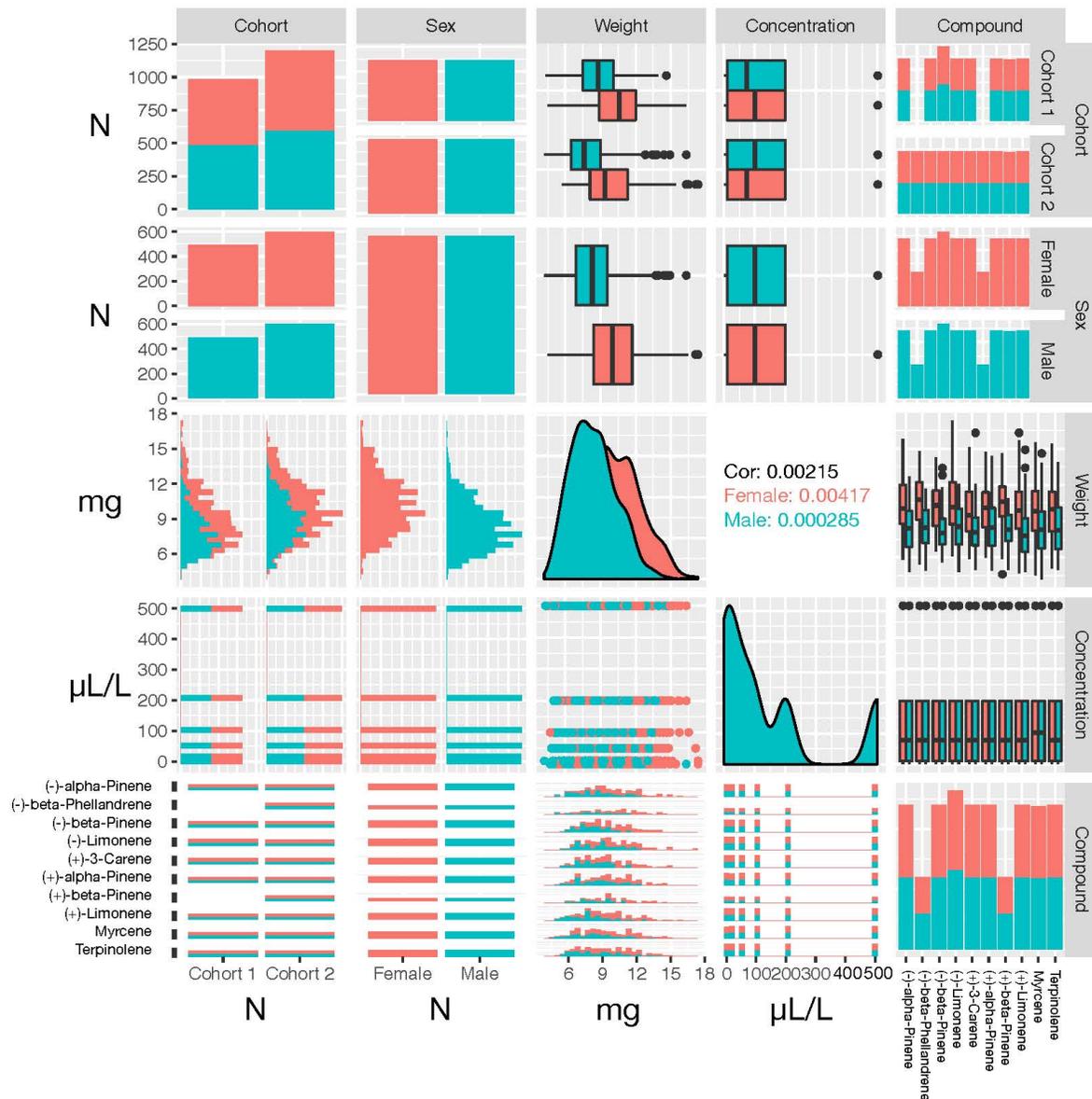
Logistic regression analysis was used to assess the effects of monoterpene concentration, body weight, and sex on mortality for each of the monoterpenes tested. The coefficient indicates the magnitude and direction of each of these effects on mortality. Concentration: A positive coefficient indicates a positive relationship between

increasing concentration and mortality. A significant positive coefficient was found for all monoterpenes tested indicating increasing toxicity at increasing concentrations. Weight: A positive coefficient indicates a positive relationship between increasing weight and mortality. Most monoterpenes had a significant negative coefficient, indicating heavier beetles survived more often. Sex: A positive coefficient indicates females survived more often than males. A negative coefficient indicates males survived more often than females. Most monoterpenes did not show a significant coefficient indicating that sex did not influence survival. \* denotes  $P$ -value  $<0.05$ .

**Table 2.5. The independent effects of concentration, body weight and sex on mortality for each monoterpene tested with MPB from cohort 2.**

Cohort 2				
Monoterpene		Concentration	Weight	Sex
(-)-Limonene	Coefficient ( $\pm$ S.E.)	0.084 $\pm$ 0.019	-0.66 $\pm$ 0.27	-1.14 $\pm$ 0.92
	<i>P</i>	8.3e-06*	0.017*	0.215
(+) -Limonene	Coefficient ( $\pm$ S.E.)	0.064 $\pm$ 0.013	-0.25 $\pm$ 0.17	-0.51 $\pm$ 0.83
	<i>P</i>	1.77e-06*	0.147	0.540
(+) -3-Carene	Coefficient ( $\pm$ S.E.)	0.051 $\pm$ 0.011	-0.42 $\pm$ 0.22	0.36 $\pm$ 0.78
	<i>P</i>	1.76e-06*	0.059	0.650
Myrcene	Coefficient ( $\pm$ S.E.)	0.030 $\pm$ 0.006	-0.60 $\pm$ 0.17	0.67 $\pm$ 0.63
	<i>P</i>	9.74e-07*	<0.001*	0.290
(-)- $\beta$ -Phellandrene	Coefficient ( $\pm$ S.E.)	0.020 $\pm$ 0.004	-0.18 $\pm$ 0.12	-0.14 $\pm$ 0.57
	<i>P</i>	9.42e-07*	0.158	0.805
(-)- $\beta$ -Pinene	Coefficient ( $\pm$ S.E.)	0.028 $\pm$ 0.005	-0.58 $\pm$ 0.21	-0.28 $\pm$ 0.70
	<i>P</i>	3.6e-07*	0.005*	0.686
(+) - $\beta$ -Pinene	Coefficient ( $\pm$ S.E.)	0.035 $\pm$ 0.007	-0.65 $\pm$ 0.23	-1.13 $\pm$ 0.86
	<i>P</i>	7.7e-07*	0.006*	0.187
(+) - $\alpha$ -Pinene	Coefficient ( $\pm$ S.E.)	0.022 $\pm$ 0.004	0.05 $\pm$ 0.15	0.70 $\pm$ 0.61
	<i>P</i>	2.15e-07*	0.733	0.254
(-)- $\alpha$ -Pinene	Coefficient ( $\pm$ S.E.)	0.012 $\pm$ 0.002	-0.40 $\pm$ 0.16	0.72 $\pm$ 0.71
	<i>P</i>	1.08e-07*	0.014*	0.313
Terpinolene	Coefficient ( $\pm$ S.E.)	0.005 $\pm$ 0.001	-0.37 $\pm$ 0.12	0.44 $\pm$ 0.50
	<i>P</i>	0.000413*	0.002*	0.375

Logistic regression analysis was used to assess the effects of monoterpene concentration, body weight, and sex on mortality for each of the monoterpenes tested. The coefficient indicates the magnitude and direction of each of these effects on mortality. Concentration: A positive coefficient indicates a positive relationship between increasing concentration and mortality. A significant positive coefficient was found for all monoterpenes tested indicating increasing toxicity at increasing concentrations. Weight: A positive coefficient indicates a positive relationship between increasing weight and mortality. Most monoterpenes had a significant negative coefficient, indicating heavier beetles survived more often. Sex: A positive coefficient indicates females survived more often than males. A negative coefficient indicates males survived more often than females. All monoterpenes did not show a significant coefficient indicating that sex did not influence survival. \* denotes  $P$ -value  $<0.05$ .



**Figure 2.1: Graphical summary of the raw data.**

Matrix of graphics representing the relationship of the factors *Cohort*, *Sex* (female, red;

male, blue), beetle *Weight*, compound *Concentration*, and *Compound* to each other. For

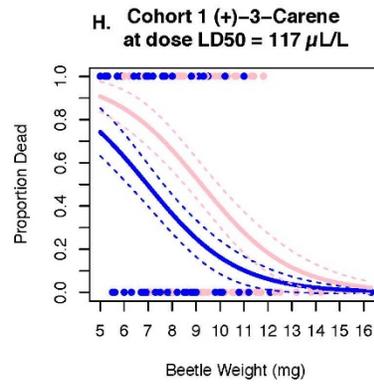
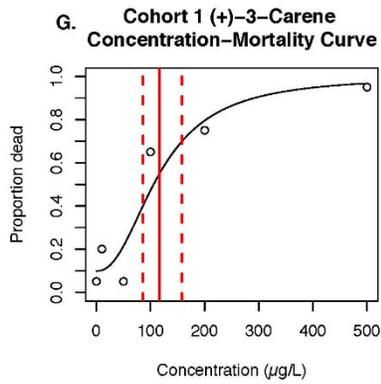
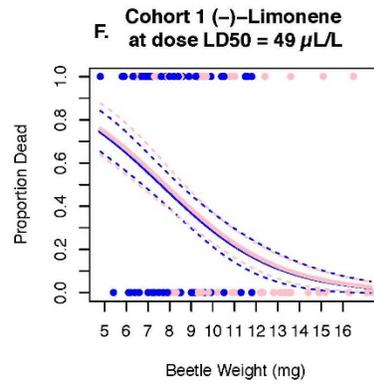
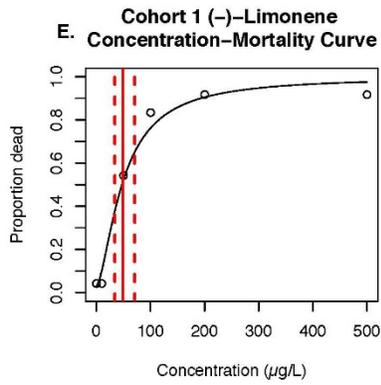
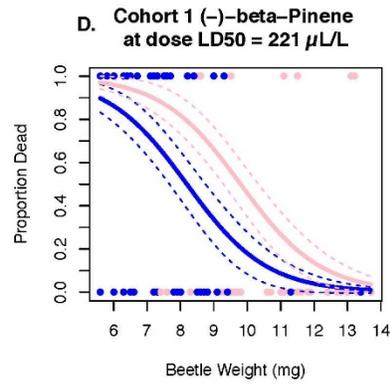
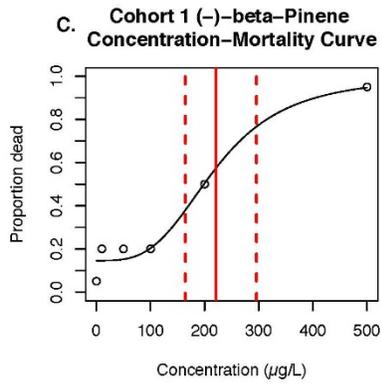
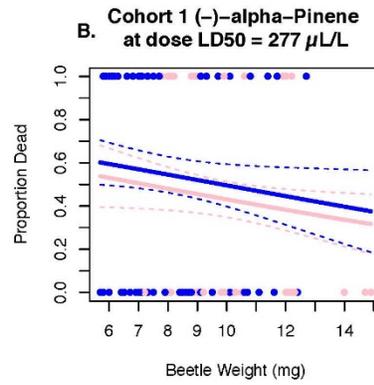
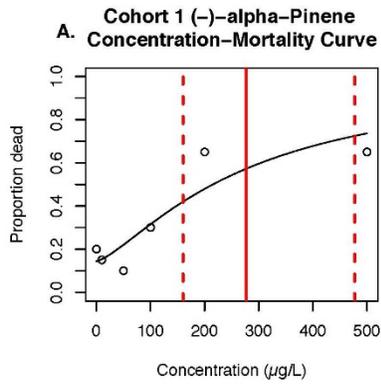
examples, (1) the *Weight* column x *Cohort* row graph shows the distribution of beetle

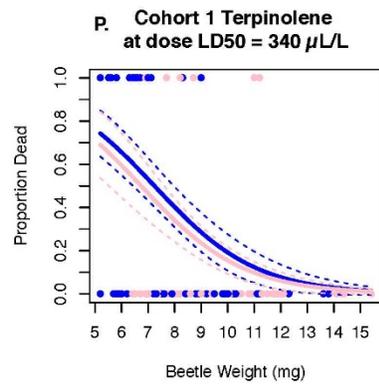
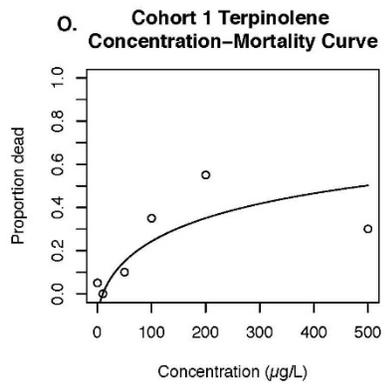
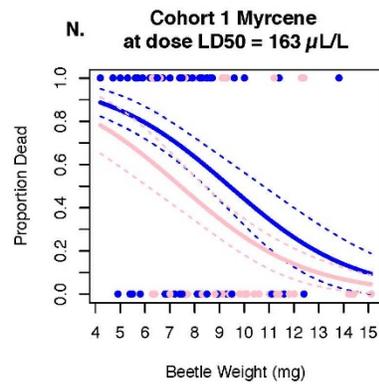
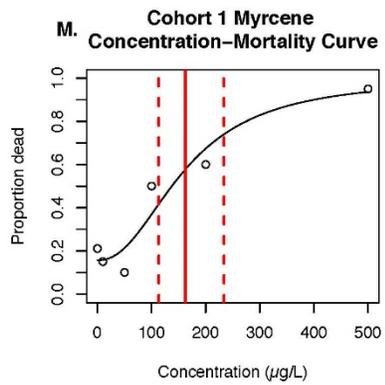
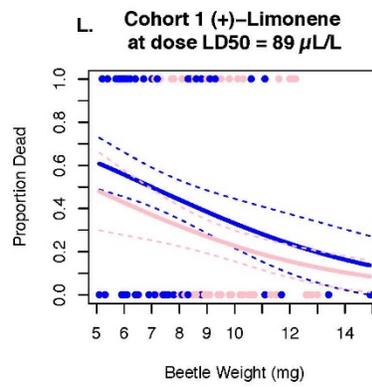
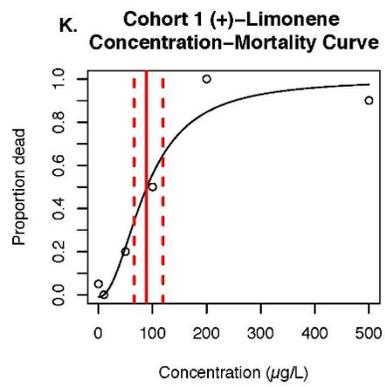
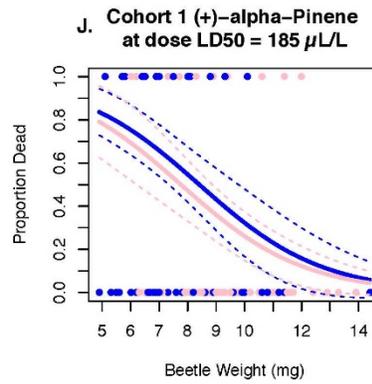
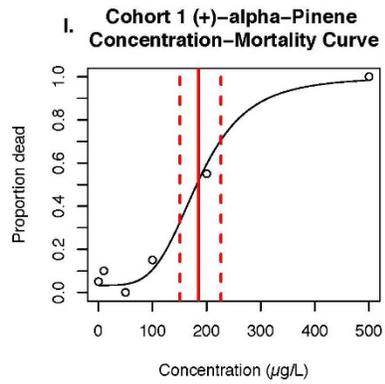
weights for each sex, for each cohort; (2) the *Cohort* column x *Compound* row graph

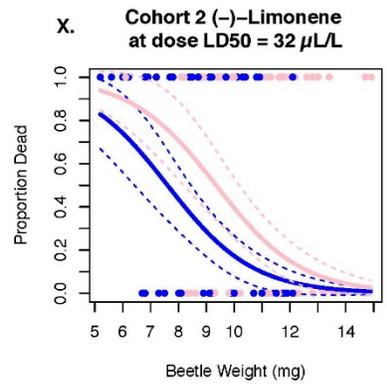
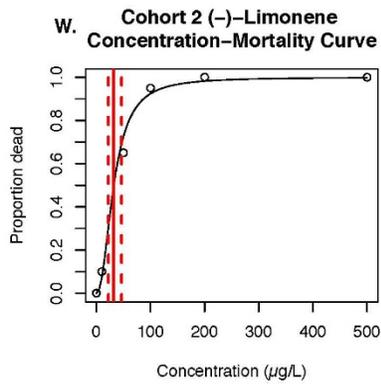
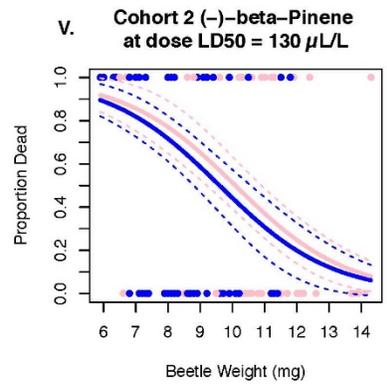
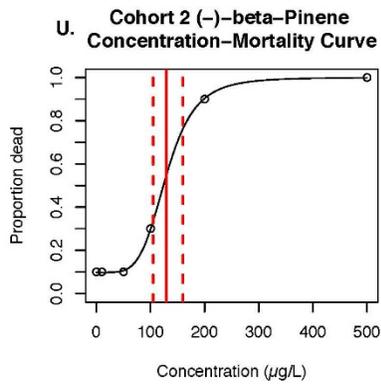
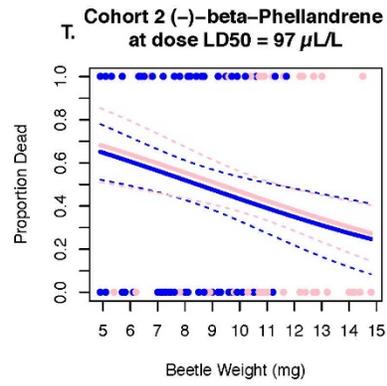
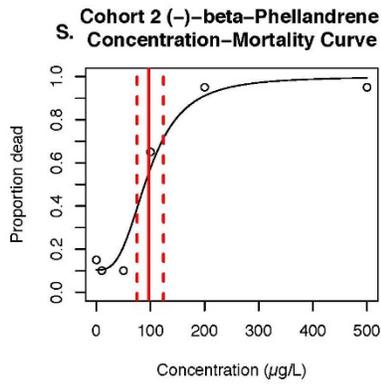
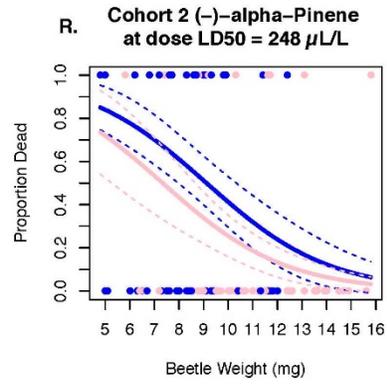
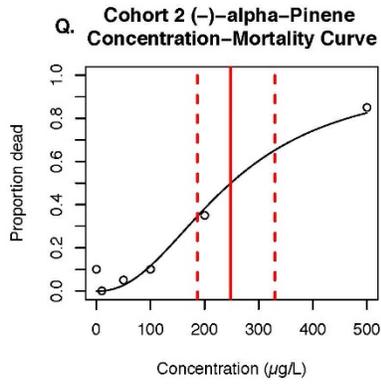
shows the number of beetles of each sex, for each cohort, and for each compound. In

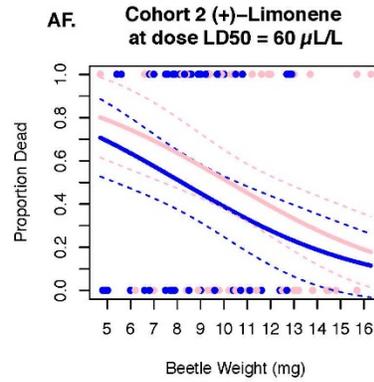
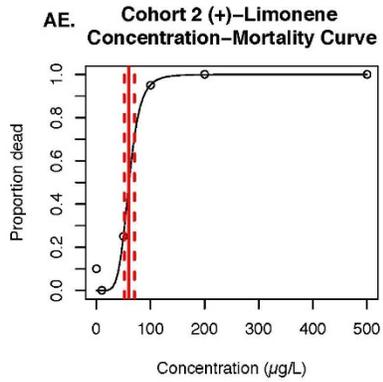
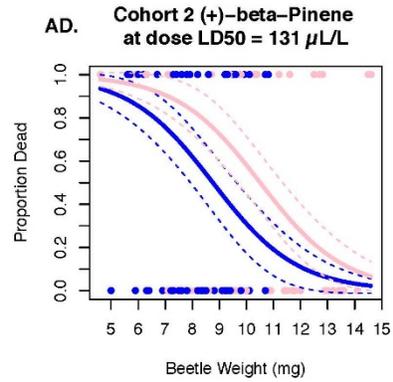
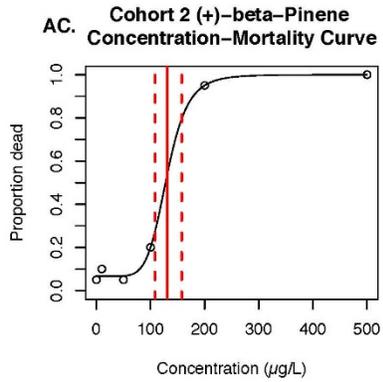
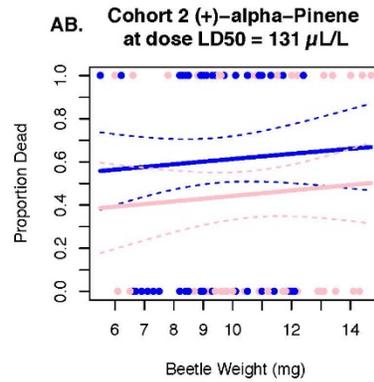
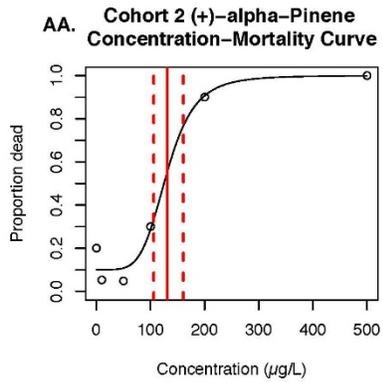
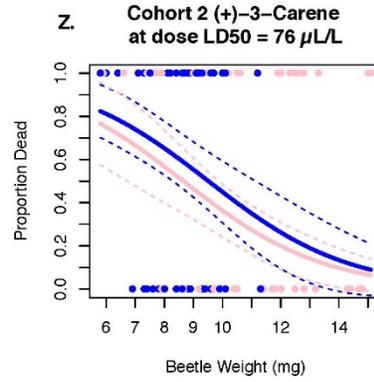
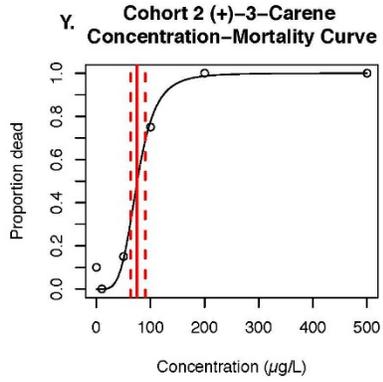
this graph one can see that equal numbers of beetles of each sex for each compound

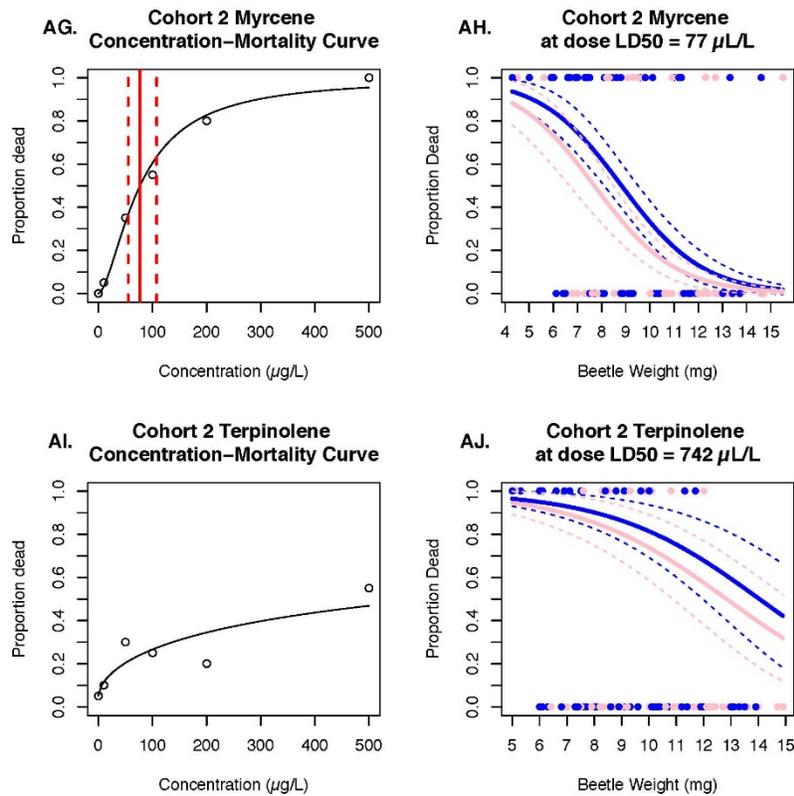
were tested, and that (-)-beta-phellandrene and (+)-beta pinene were only tested with cohort 2.







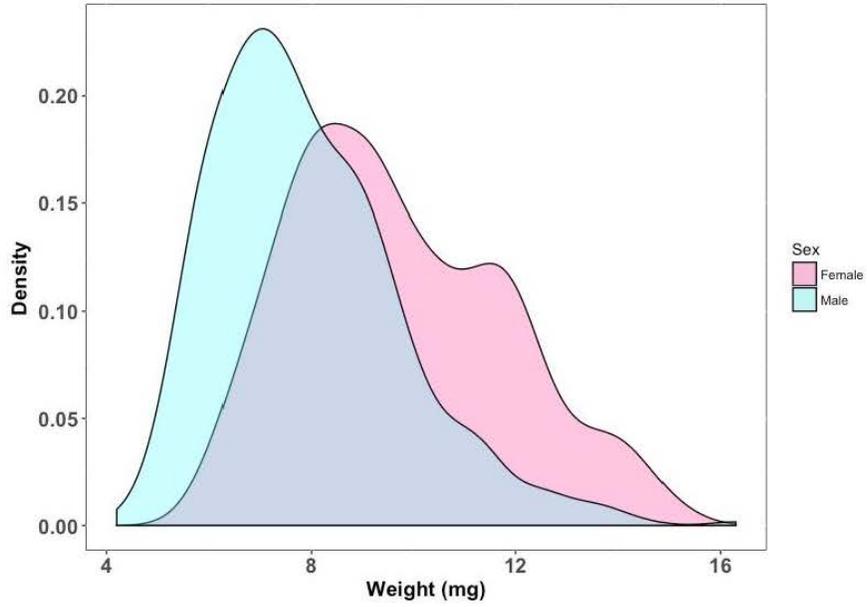




**Figure 2.2: LC<sub>50</sub> and logit curves for each compound and cohort.**

Subfigures A, C, E, G, I, K, M, O, Q, S, U, W, Y, AA, AC, AE, AG, and AI: Concentration mortality curves for each compound and cohort. Dots represent the proportion of beetles dead at each concentration tested. The solid red line indicates the LC<sub>50</sub> value. Dotted red lines indicate the fiducial limits of the LC<sub>50</sub> value.

Subfigures B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, and AJ: The predicted relationship between beetle weight and mortality for each sex at the LC<sub>50</sub> value. The solid lines represent the predicted relationship between beetle weight and mortality at the LC<sub>50</sub> based on logistic regression. Lines that slope down towards the right indicate that heavier beetles survive more often. The further apart the male (blue) and female (pink) lines are, the greater the difference in mortality between the sexes, independent of weight. Dotted lines indicate the 95% confidence intervals. The dots represent each beetle tested that either survived ( $y=0$ ) or died ( $y=1$ ) after treatment at all doses.

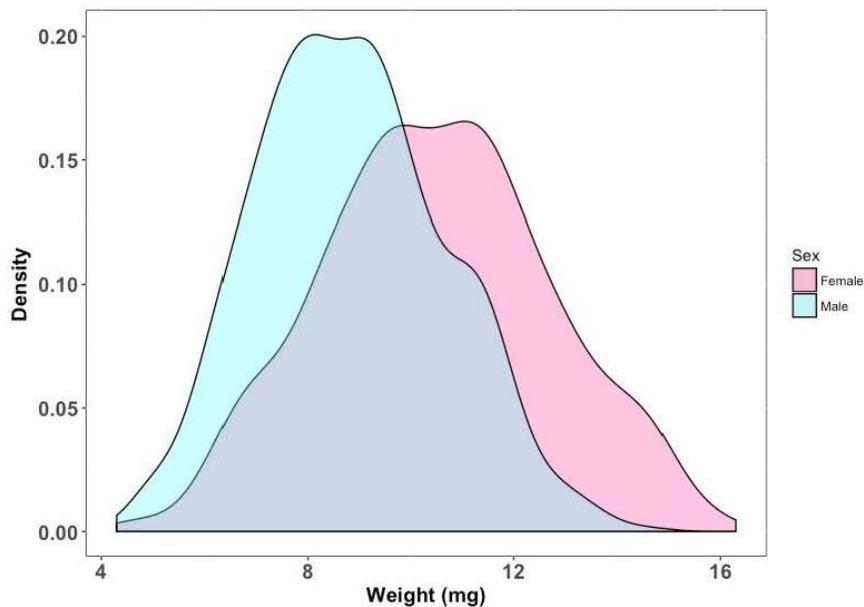


**Fig. 2.3 Weight distribution of MPB from cohort 1**

Average weight ( $\pm$ SD) =  $8.81 \pm 2.24$  mg

Female average weight ( $\pm$ SD) =  $9.73 \pm 2.20$  mg (N=492)

Male average weight ( $\pm$ SD) =  $7.90 \pm 1.87$  mg (N=491)



**Fig. 2.4 Weight distribution of MPB from cohort 2**

Average weight ( $\pm$ SD) =  $9.69 \pm 2.24$  mg

Female average weight ( $\pm$ SD) =  $10.53 \pm 2.24$  mg (N=599)

Male average weight ( $\pm$ SD) =  $8.85 \pm 1.90$  mg (N=600)

## 2.5 Discussion

MPB are exposed to monoterpenes as volatiles as well as through contact and feeding.

For the purpose of this study to assess and compare toxicity of individual

monoterpenes, I focused on the exposure to monoterpene volatiles. The effects that

monoterpenes have on MPB through physical contact or ingestion were not tested in

this study. It is also important to note that there are limitations in the comparison of the

LC<sub>50</sub> values reported here, which are in units of relative volumes, with the absolute

amounts of monoterpenes in host trees, which are typically determined after solvent

extraction and reported as  $\mu$ g of monoterpene per g of dry weight of tissue sample.

Nevertheless, it is reasonable to compare the relative ranking of toxicity of the different individual monoterpenes against MPB described here with the reported relative composition of monoterpene profiles of the host pines. The three most abundant compounds of the monoterpene profile of lodgepole pine are (-)- $\beta$ -phellandrene (>50%), (-)- $\beta$ -pinene (up to 35%), and (+)-3-carene (up to 10%) (Clark et al., 2014; Hall et al., 2013a). In another host, ponderosa pine (*Pinus ponderosa*), the three most abundant monoterpenes are (+)-3-carene,  $\beta$ -pinene, and myrcene (West et al., 2016a). In whitebark pine (*Pinus albiculus*), an occasional host of MBP, the three most abundant monoterpenes are (-)- $\beta$ -phellandrene, (-)- $\beta$ -pinene, and myrcene (Raffa et al., 2013). These monoterpenes that are relatively abundant in lodgepole pine, ponderosa pine and whitebark pine are of only mid-range toxicity to MPB compared to the other monoterpenes present (**Tables 2.2** and **2.3**). Conversely, the two most toxic monoterpenes, the two enantiomers of limonene, account for only 1% - 5% of the monoterpene profile of lodgepole pine and an even lower percentage of the whitebark pine and ponderosa pine profile (Clark et al., 2014; Hall et al., 2013a; Raffa et al., 2013; West et al., 2016b). In other bark beetle species, both enantiomers of limonene were also more toxic than other monoterpenes. For example, (+)-limonene volatiles had a shorter LT<sub>50</sub> (lethal time) compared to other monoterpenes in the spruce beetle (*D. rufipennis*) and the larch beetle (*D. simplex*), and the exposure of 50  $\mu$ L/L of limonene to western pine beetle (*D. brevicomis*) for four days caused more mortality than other monoterpenes (Smith, 1965b; Werner, 1995). (-)- $\alpha$ -Pinene, the precursor to the aggregation pheromone (-)-*trans*-verbenol (Blomquist et al., 2010), was one of the least toxic monoterpenes to MPB. (+)- $\alpha$ -Pinene, which was significantly more toxic than (-)- $\alpha$ -

pinene, is the precursor to (+)-*trans*-verbenol, a compound that is also produced by female MPB, but has not been shown to be attractive to beetles (Borden et al., 1986).

The most abundant monoterpenes in the co-evolved lodgepole pine and ponderosa pine host were not the most toxic to MPB, and the most toxic monoterpenes, i.e. the limonenes, are not the most abundant. The most abundant monoterpenes in jack pine, a host that does not share the same co-evolutionary association with MPB, are (+)- $\alpha$ -pinene (up to 40%), (+)-3-carene (up to 20%), and (-)- $\beta$ -pinene (up to 18%) (Clark et al., 2014; Hall et al., 2013a), and these were also of mid- to low-range toxicity against MPB. Like lodgepole pine, the two enantiomers of limonene account for only a small proportion (1% - 8%) of the jack pine phloem. Based on these comparisons, it is not obvious if the monoterpene defense of jack pine will be more or less effective against MPB adults than that of lodgepole pine. This is consistent with studies in other bark beetle systems, showing that minor monoterpene components were of either greater or equal toxicity than the major components. For example, the toxicity of jack pine and red pine components to *Ips pini*, loblolly and other southern pine components to *D. frontalis*, and grand fir components to *Scolytus ventralis* (Coyne, J.F., Lott, 1976; Raffa et al., 1985; Raffa & Smalley, 1995).

Boone *et al.* 2011 reported the profiles of constitutive and induced monoterpenes during the endemic, incipient and epidemic population phases of MPB in lodgepole pine. Trees that were subsequently attacked by MPB during the endemic and incipient phases had higher proportions of  $\alpha$ -pinene and  $\beta$ -pinene in the constitutive resin, while the

proportions of these compounds were lower in trees that were attacked during the epidemic phase. Similar to the induced defenses, the amount of  $\beta$ -pinene was also higher in trees that were attacked during endemic and incipient phases (Boone et al., 2011). The relative abundance of the less toxic  $\alpha$ -pinene and  $\beta$ -pinene could make trees an easier target at lower MPB population densities. Myrcene, a monoterpene of mid-range toxicity to MPB, was more highly induced in trees that were attacked in the epidemic phase (Boone et al., 2011). Limonene, which appears to be the most toxic monoterpene against MPB, was also the most strongly induced monoterpene; however, it did not explain the likelihood of a tree being attacked at any of the MPB population phases (Boone et al., 2011). In another study, lodgepole pines with higher induced limonene levels (but lower constitutive limonene) were more likely to survive MPB attack (Raffa & Berryman, 1982).

The diversity and variability of host monoterpene profiles is determined by the multi-member monoterpene synthase (mono-TPS) gene family and the expression of these genes, which differs between conifer species and between individuals of the same species (Hall et al., 2013a). Within a given tree, monoterpene profiles can change as a result of induced changes of mono-TPS gene and enzyme expression in response to insect attack (Miller et al., 2005a; Zulak & Bohlmann, 2010). Many of the conifer mono-TPSs also produce multiple monoterpenes and the specific profiles of such multi-product mono-TPSs can vary even between very closely related orthologous or paralogous mono-TPSs (Roach et al., 2014) adding to the overall complexity and variability of conifer monoterpene profiles. Several mono-TPSs of lodgepole pine and

jack pine have been functionally characterized (Hall et al., 2013a). Most of the monoterpenes in these two species are produced by more than one mono-TPS. For example, (-)- $\beta$ -phellandrene, the most abundant monoterpene in lodgepole is the major product of two mono-TPSs, PcTPS-(-)- $\beta$ -phell1 and PcTPS-(-)- $\beta$ -phell2. (-)- $\beta$ -pinene is a major product of PcTPS-(-)- $\beta$ -pin1 but a minor product of PcTPS-(-)- $\alpha$ -pin1 and PcTPS-(-)-camp/(+)- $\alpha$ -pin1. To date, no mono-TPS that produces either enantiomer of limonene has been functionally characterized from lodgepole pine. However, one mono-TPS, PbTPS-aterp from jack pine produces (-)-limonene as a minor component (5%) of the product profile of this enzyme (Hall et al., 2013a). Thus, it is currently not known which mono-TPS gene in either of these two host species of MPB is responsible for the formation of the most toxic monoterpene volatile.

Given the evolutionary and life cycle context of MPB exposure to monoterpenes, it is reasonable to hypothesize that MPB has a higher tolerance to monoterpenes than insects that are not typically experiencing high concentrations of monoterpenes in their environment. Indeed, volatile (+)-limonene has an LC<sub>50</sub> of 5  $\mu$ L/L in the red flour beetle (*Tribolium castaneum*), and a KD<sub>50</sub> (knockdown dose) of 7.5  $\mu$ L/L in the housefly (*Musca domestica*) (Stamopoulos et al., 2007; Tarelli et al., 2009). By comparison, I showed a higher tolerance of MPB to (+)-limonene with LC<sub>50</sub> of 60-89  $\mu$ L/L. The relatively high tolerance of MPB to monoterpene volatiles may be characteristic of coniferophagous bark beetles. For example, the great spruce bark beetle (*Dendroctonus micans*) and the European spruce bark beetle (*Ips typographus*)

appeared to be unaffected or had low mortality when exposed to saturated vapours of  $\alpha$ -pinene,  $\beta$ -pinene, (+)-3-carene, limonene, and myrcene (Everaerts et al., 1988).

Tolerance of insects to insecticides or plant defense compounds often increases in proportion to body weight (Robertson et al., 2007). I found a significant relationship between MPB survival and body weight for most monoterpenes. This is in agreement with findings of Reid and Purcell (Reid & Purcell, 2011), who showed that the body condition index (a metric calculated from the body weight) explained survival of MPB to high concentrations of (-)- $\alpha$ -pinene, myrcene, (+)-limonene and terpinolene vapours. After accounting for body weight, sex did not have a significant effect on the survival with most of the monoterpenes. This is also in agreement with the findings of Reid and Purcell (Reid & Purcell, 2011), despite female MPB being the first to attack a host tree. Reid *et al.* 2017 recently tested the effect of monoterpene diversity, beetle size, and beetle body condition on beetle mortality. Their results complement ours. Although they tested only four monoterpenes, they also found a significant difference in toxicity between monoterpenes.

Pierce *et al.* 1987 explored possible routes of detoxification for monoterpenes and identified oxidized metabolites of monoterpenes in extracts of female MPBs that had fed on pine phloem. We recently showed that the MPB cytochrome P450 enzyme CYP345E2 hydroxylates or epoxidizes  $\alpha$ -pinene,  $\beta$ -pinene, (+)-3-carene, limonene and terpinolene, but is found only in the antennae and likely plays an olfaction-specific role, rather than a detoxification role (Keeling et al., 2013b). Genome and transcriptome

annotation of the MPB revealed 85 different P450 genes (Keeling et al., 2013c; Li et al., 2007). The MPB P450 gene family showed blooms in the CYP6 and CYP9 clades. Members of these clades have been shown in other insects to be involved in the detoxification of plant host defense compounds and insecticides (Robert et al., 2013). Robert *et al.* 2013 found several CYP6 and CYP9 members up-regulated in MPB after feeding on host phloem. In *Ips pini*, a member of the CYP9 family, CYP9T2 hydroxylates myrcene to ipsdienol, a pheromone of this species; and in a related bark beetle, *Ips paraconfusus*, CYP9T1 is up-regulated nearly five orders of magnitude in males following feeding on monoterpene-laden pine phloem (Huber et al., 2007; Sandstrom et al., 2006).

Beyond directly affecting MPB, monoterpenes may also affect other components and interactions of the symbiotic complex of MPB and its fungal and bacterial microbiomes. For example, the MPB fungal associate *Grosmannia clavigera* utilizes (+)-limonene as a sole carbon source and uses a unique ABC transporter for controlling levels of this monoterpene (Wang et al., 2013, 2014). Thus, (+)-limonene, which is highly toxic to MPB, may be better tolerated by the fungal associate. Bacterial associates of the MPB also seem to tolerate limonene, bacterial isolates from MPB grew or were stimulated by the presence of 1-5% limonene in culture (Adams et al., 2011). Metagenomic analysis of species of the *Serratia*, *Pseudomonas*, *Burkholderia*, and *Rahnella* genera, have revealed several genes annotated in the limonene degradation pathway (Adams et al., 2013).

### 3. Monoterpenyl Esters in Juvenile Mountain Pine Beetle and Sex-Specific Release of the Aggregation Pheromone *trans*-Verbenol<sup>2</sup>

#### 3.1 Summary

A recent outbreak of mountain pine beetle (MPB) has spread over more than 25 million hectares of pine forests in western North America, affecting pine species of sensitive boreal and mountain ecosystems. During initial host colonization, female MPB produce and release the aggregation pheromone *trans*-verbenol to coordinate mass attack of individual trees. *trans*-Verbenol is formed by hydroxylation of  $\alpha$ -pinene, a monoterpene of the pine oleoresin defense. It is thought that adult females produce and immediately release *trans*-verbenol when encountering  $\alpha$ -pinene on a new host tree. Here, I showed that both sexes of MPB accumulate the monoterpenyl esters verbenyl oleate and verbenyl palmitate during their development in the brood tree. Verbenyl oleate and verbenyl palmitate were retained in adult female MPB until the time of emergence from brood trees, but were depleted in males. Adult females released *trans*-verbenol in response to treatment with juvenile hormone III (JHIII). While both sexes produced verbenyl esters when exposed to  $\alpha$ -pinene, only females responded to JHIII with release of *trans*-verbenol. Accumulation of verbenyl esters at earlier life stages may allow adult females to release the aggregation pheromone *trans*-verbenol upon landing on a new host tree, independent of access to  $\alpha$ -pinene. Formation of verbenyl esters may be part of a general detoxification system to overcome host monoterpene defenses

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<sup>2</sup> A version of this chapter has been published. Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2018). Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone *trans*-verbenol. *Proceedings of the National Academy of Sciences*, 115(14), 3652–3657.

in both sexes, from which a specialized and female-specific system of pheromone biosynthesis and release may have evolved.

### 3.2 Introduction

The current mountain pine beetle (MPB; *Dendroctonus ponderosae*) epidemic has affected over 25 million hectares of pine forests in western North America (Hart et al., 2015; Meddens et al., 2012) and is moving into the boreal forest and sensitive high elevation ecosystems (Cullingham et al., 2011; Raffa et al., 2013). Female MPB produce the aggregation pheromone *trans*-verbenol to coordinate a mass colonization behaviour (Borden et al., 1986; Raffa & Berryman, 1983) that allows MPB to overcome host defenses such as toxic monoterpenes (Chiu et al., 2017; Libbey et al., 1985; Vité & Pitman, 1968). MPB are exposed to host monoterpenes during most of their life cycle from eggs to adults in the bark of the brood tree, followed by a brief period of emergence and dispersal flight, and colonization of the bark of a new host where they mate and oviposit.

*trans*-Verbenol is thought to be produced during host colonization by female MPB through oxidation of the host monoterpene  $\alpha$ -pinene (Erbilgin et al., 2014; Hughes, 1973b; Hunt & Borden, 1989; Libbey et al., 1985; Pureswaran et al., 2000; Renwick et al., 1976a; Vité & Pitman, 1968). Female MPB also release *trans*-verbenol when treated with juvenile hormone III (JHIII) without concurrent exposure to  $\alpha$ -pinene (Conn et al., 1984; Keeling et al., 2016). In other *Dendroctonus* species, exposure of larvae or pupae to high levels of  $\alpha$ -pinene vapours caused increased levels of *trans*-verbenol in adults (Hughes, 1975). It has been hypothesized that some *Dendroctonus* species

accumulate *trans*-verbenol conjugates in pre-adult life stages or produce *trans*-verbenol *de novo* (Hughes, 1975; White et al., 1980). However, since these hypotheses were first proposed (Conn et al., 1984; Hughes, 1975), additional evidence for either conjugation or *de novo* biosynthesis of *trans*-verbenol has been lacking. Microbial biosynthesis does not appear to contribute substantially to *trans*-verbenol production (Conn et al., 1984; Gries et al., 1990; Hunt & Borden, 1989).

Here, I report the discovery of monoterpenyl esters in MPB larvae and pupae, including verbenyl oleate and verbenyl palmitate, which may be part of a monoterpene detoxification system in young male and female MPB, and may also serve as a reservoir for female-specific release of *trans*-verbenol as an aggregation pheromone.

### 3.3 Materials and Methods

**3.3.1 Chemicals.** The following chemicals were obtained from Sigma-Aldrich (Mississauga, ON, Canada): racemic juvenile hormone III (JHIII; cat. No. J2000, CAS No. 24198-95-6), *N,N'*-dicyclohexylcarbodiimide (DCC, cat. No. D8002), 4-(dimethylamino)pyridine (DMAP, cat. No. 39405), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, cat. No. 15209), (+)- $\alpha$ -pinene (CAS No. 7785-70-8), and (-)- $\alpha$ -pinene (CAS No. 7785-26-4), (-)-carveol (cat. No. 192384), (-)-myrtenol (cat. No. W343900), oleic acid (cat. No. O1008), palmitic acid (cat. No. P0500), (*S*)-(-)-perillyl alcohol (cat. No. 218391), stearic acid (cat. No. 85679), (-)-*trans*-pinocarveol (cat. No. 80613), (-)- $\beta$ -pinene (cat. No. 112089), (+)-3-carene (cat. No. 441619), (-)-limonene (cat. No. 218367), and myrcene (cat. No. M100005). *trans*-Verbenol (approx 20(+):80(-) optical purity) (lot. No. W06-00141) and *cis*-verbenol

(approx 20(+):80(-) optical purity) (lot. No. CV001129) were obtained from PheroTech (Delta, BC, Canada). (-)-*trans*-Myrtanol (cat. No. 5134 S) was obtained from Extrasynthese (Genay, France), and (-)- $\beta$ -phellandrene was obtained by purification from lodgepole pine (*Pinus contorta*) turpentine by Synergy Semiochemicals (Burnaby, Canada).

**3.3.2 MPB and phloem collection.** MPB infested lodgepole pine (*Pinus contorta*) stems were collected near Whistler, British Columbia, Canada (50°12'46.6"N, 122°53'20.8"W). In June 2016, MPB pheromone baits (Contech) were attached to trees and removed after attack four weeks later. In September 2016, infested trees were felled and the logs placed in screened cages at room temperature. Phloem samples were collected from the infested logs. Larvae, pupae and teneral adults were dissected from infested logs and stored at -80°C. Larval instar was estimated by head capsule width according to Bleiker and Régnière (Bleiker & Régnière, 2014). In addition, emerging adults were collected every 3-4 days from inside the screened cages. Adult MPB were sexed using the shape of the seventh abdominal tergite according to Lyon (1958) and larvae and pupae were sexed by PCR.

**3.3.3 MPB sexing by PCR.** Sex-specific PCR primers were designed to identify the sex of MPB larvae and pupae (**Appendix A**). Similar to a method developed to identify the sex in *Tribolium castaneum* (Lagisz et al., 2010), these primers amplify regions of the neo-X and neo-Y chromosomes in a multiplex polymerase chain reaction (PCR). PCR of males (neo-X and neo-Y) gives two major DNA products, and PCR of females (neo-X

and neo-X) gives one major DNA product as detected on an agarose gel. Primers were validated with 60 individual male and female adults that were sexed using morphological characteristics (Lyon, 1958). DNA was extracted from larval and pupal samples that had previously been extracted with pentane for *trans*-verbenyl esters analysis. A volume of about 1 mm<sup>3</sup> MPB sample was used to produce a 20 µl DNA extract using the prepGEM® insect kit (ZyGEM) according to manufacturer's instructions.

**3.3.4 MPB treatment.** Emerging adult female and male MPB were separately treated with acetone, JHIII,  $\alpha$ -pinene, and other monoterpenes to test for the production of *trans*-verbenol. For treatment with JHIII, 0.5 µl of JHIII (20 mg/ml in acetone) was topically applied to the abdomen. Topical application of 0.5 µl acetone served as a carrier control. Treated beetles were placed individually into sealed 20-ml glass vials. Both enantiomers of  $\alpha$ -pinene were used to treat beetles, as both enantiomers are present in the pine hosts of MPB. A nearly racemic mixture (44(+):56(-)) of  $\alpha$ -pinene was used in treatments, of which 2 µl was applied to a 1 cm<sup>2</sup> Whatman filter paper and placed into a sealed 20-ml glass vial with an individual beetle. Beetles were allowed to come in contact with the  $\alpha$ -pinene carrying filter paper. Other monoterpene treatments consisted of 2 µl of (-)- $\beta$ -phellandrene, 2 µl of (-)- $\beta$ -pinene, or 1 µl of (-)-limonene applied to a 1 cm<sup>2</sup> Whatman filter paper. Beetles were treated for 24 h and then removed from the vial, frozen in liquid N<sub>2</sub> and stored at -80°C until extraction.

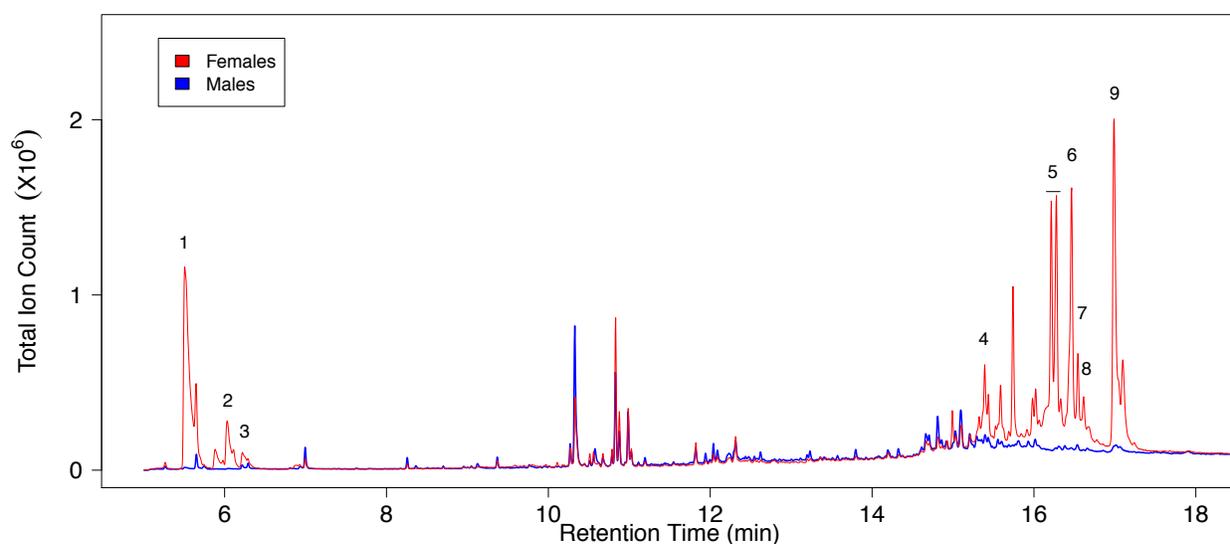
**3.3.5 MPB dissection.** Emerging females were dissected into head, thorax, and abdomen. The alimentary canal was removed from the dissected abdomen and separated into the fat body (perivisceral layer), midgut, Malpighian tubules, and hindgut. The perivisceral layer of the fat body surrounds the alimentary canal and was removed cleanly without contamination from other tissues. The rest of the fat body (parietal layer), located between the muscles in the abdomen, thorax and head could not be removed cleanly or complete and was left in place.

**3.3.6 Metabolite extraction.** Frozen beetles were crushed in a 2-ml Safe-Lock tube (Eppendorf) on dry ice using a cold glass stir rod and extracted with 0.5 ml pentane containing 1 ng/ $\mu$ l of tridecane as internal standard. Smaller specimens (1st and 2nd instar larvae, and dissected alimentary canal), were extracted with 0.1 – 0.25 ml pentane. A single larva, pupa or adult was extracted per tube. Samples were removed from dry ice, allowed to thaw for a few minutes, and centrifuged for 20 s at 2,000 x *g*. Samples were frozen again on dry ice and the pentane supernatant transferred into an amber 2-ml glass vial (Agilent). Beetles were extracted a second time with 0.5 ml pentane and the two extracts combined. To remove excess amounts of fatty acids from the sample, 400  $\mu$ l of 1 mM ammonium carbonate (pH 8) was added to the combined pentane extract and vortexed. The sample was centrifuged for 10 min at 3000 x *g* and the pentane layer removed for analysis by GC/MS. Phloem samples were extracted with 1.5 ml of *tert*-butyl methyl ether (MTBE, Sigma-Aldrich).

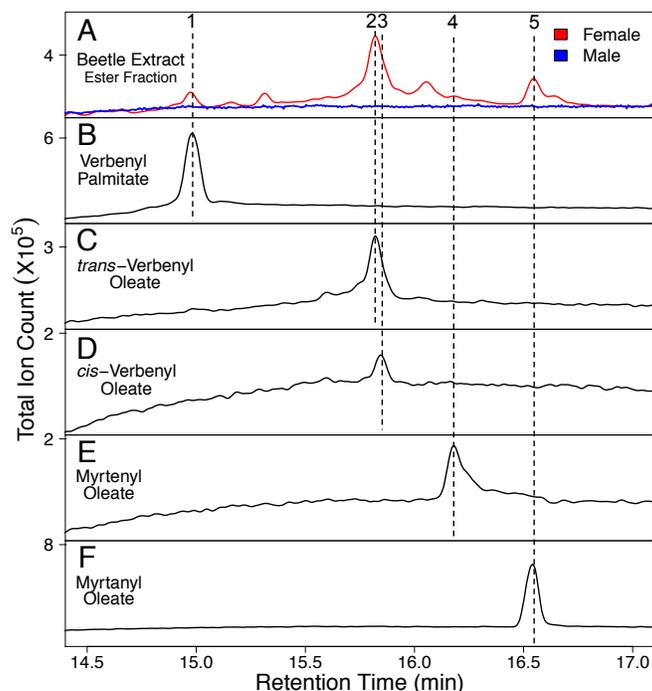
**3.3.7 Identification of monoterpenyl fatty acid esters in female MPB.** To identify female-specific compounds found as esters in emerged beetles, male and female beetle extracts were separately processed by silica chromatography and ester hydrolysis. Beetle extracts were evaporated to dryness, re-dissolved in 0.2 ml hexane, loaded onto a silica column (300 mg) (Sigma, cat. No. S2509) and washed with 4 ml hexane to remove alkanes. Esters were eluted with 6 ml of 1% (v/v) MTBE in hexane. To hydrolyse the esters, 3 ml of the ester fraction was evaporated to dryness and hydrolyzed by re-dissolving in 0.5 ml of 0.3 M methanolic KOH and incubated at 75 °C for 1 h in a sealed amber glass 2 ml vial (Agilent cat. No. 5182-0716). The liberated alcohols were then extracted twice with 0.5 ml pentane. The ester fraction and the alcohols obtained by hydrolysis were analyzed using GC/MS.

**3.3.8 Synthesis of monoterpenyl ester standards.** Monoterpenyl esters were synthesized by Steglich esterification (Neises & Steglich, 1978) for the identification of the female-specific esters. Amounts of 35 mmol of fatty acid and 105 mmol of monoterpenol were combined in 1 ml of CH<sub>2</sub>Cl<sub>2</sub>, placed on ice, and 0.2 mL of an ice-cold CH<sub>2</sub>Cl<sub>2</sub> solution containing 39 mmol of DCC and 3.5 mmol of DMAP was added. Amber GC vials (Agilent) containing the reaction mixture were briefly shaken and then held on ice for 10 min before being placed on a shaker at room temperature for 2 h. The subsequent work up involved washing with 1 M sodium acetate (pH 5.2), washing with saturated NaCl, and then drying over anhydrous MgSO<sub>4</sub>. The CH<sub>2</sub>Cl<sub>2</sub> solution was evaporated under a flow of nitrogen gas and the product re-dissolved in 0.5 ml hexane. The product was purified on a 300 mg silica column by first passing 4 ml hexane through the column and

then eluting the ester product with 6 ml of 1% (v/v) MTBE in hexane. Monoterpenyl esters were analyzed using GC/MS. However, verbenyl oleate was thermally unstable at the high temperature conditions of the GC inlet (250°C) resulting in several breakdown products detected with multiple peaks in the chromatogram of standards. In addition, the menthatrienes, cymenes and verbenes (decomposition products of verbenol) were detected in the GC/MS of beetle extracts whenever the esters were present, even when hydrocarbons had been removed by silica chromatography (**Figure 3.1**). Cool (30°C) on-column injection of ester standards and beetle ester fractions prevented the decomposition of the esters (**Figure 3.2**).



**Figure 3.1 GCMS chromatogram of the ester fraction of female (red) and male (blue) MPB extracts.** These extracts were injected onto a VF-5 column with an inlet temperature of 250°C. Peaks (1) verbene, (2) a cluster of menthatrienes and (3) cymenes were probable degradation products of verbenyl esters due to the high inlet temperature. Peaks (4) verbenyl palmitate, (5) verbenyl oleate (breaks down into multiple peaks due to the high temperatures of the inlet), (6) unidentified monoterprenyl ester 1, (7) unidentified monoterprenyl ester 2, (8) myrtenyl oleate, (9) myrtanyl oleate were monoterprenyl fatty acid esters that were present in females, but not males at the emergent stage. The mass spectra of these peaks are shown in Fig. 3.3.



**Figure 3.2 GCMS chromatogram of the ester fraction of freshly emerged adult beetles. (A)** and monoterpenyl ester standards (B-F). The standards and ester fraction were injected onto an HP-5 column at 30°C using a cool on-column injector. Verbenyl palmitate, *trans*- and *cis*-verbenyl oleate, myrtenyl oleate and myrtanyl oleate were all present in the emerging female ester fraction (red) but were not present in the emerging male ester fraction (blue). The mass spectra of peaks labeled 1-5 are shown in Fig. 3.6.

**3.3.9 GC/MS analysis.** Monoterpenyl esters were separated and analyzed using an Agilent VF-5 column (5% phenyl methyl siloxane, 27.4 m length, 250  $\mu\text{m}$  i.d., 0.25  $\mu\text{m}$  film thickness) at 0.9  $\text{ml min}^{-1}$  He on an Agilent 7890A system GC, Agilent 7683B series GC Sampler, and a 7000A GC/MS triple quad MS detector at 70eV. The GC temperature program was as follows: 40°C for 2 min, increase at 18°C  $\text{min}^{-1}$  to 300°C, hold for 7 min, using a pulsed splitless injector held at 250°C. Fatty acids were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) before analysis.

Due to their thermal instability, the monoterpenyl ester standards were also analyzed using a cool-on column injector on an Agilent HP-5 column (5% phenyl methyl

siloxane, 30 m length, 250  $\mu\text{m}$  i.d., 0.25  $\mu\text{m}$  film thickness) at 0.9  $\text{ml min}^{-1}$  He on an Agilent 6890A system GC, Agilent 7683 series GC Sampler and an Agilent 5973 Mass Selective Detector at 70eV. The GC temperature program was as follows: 30°C for 1 min, increase at 20°C  $\text{min}^{-1}$  to 300°C, hold 7 min. The temperature of the cool on-column injector increased at 20°C  $\text{min}^{-1}$ , tracking the temperature of the column.

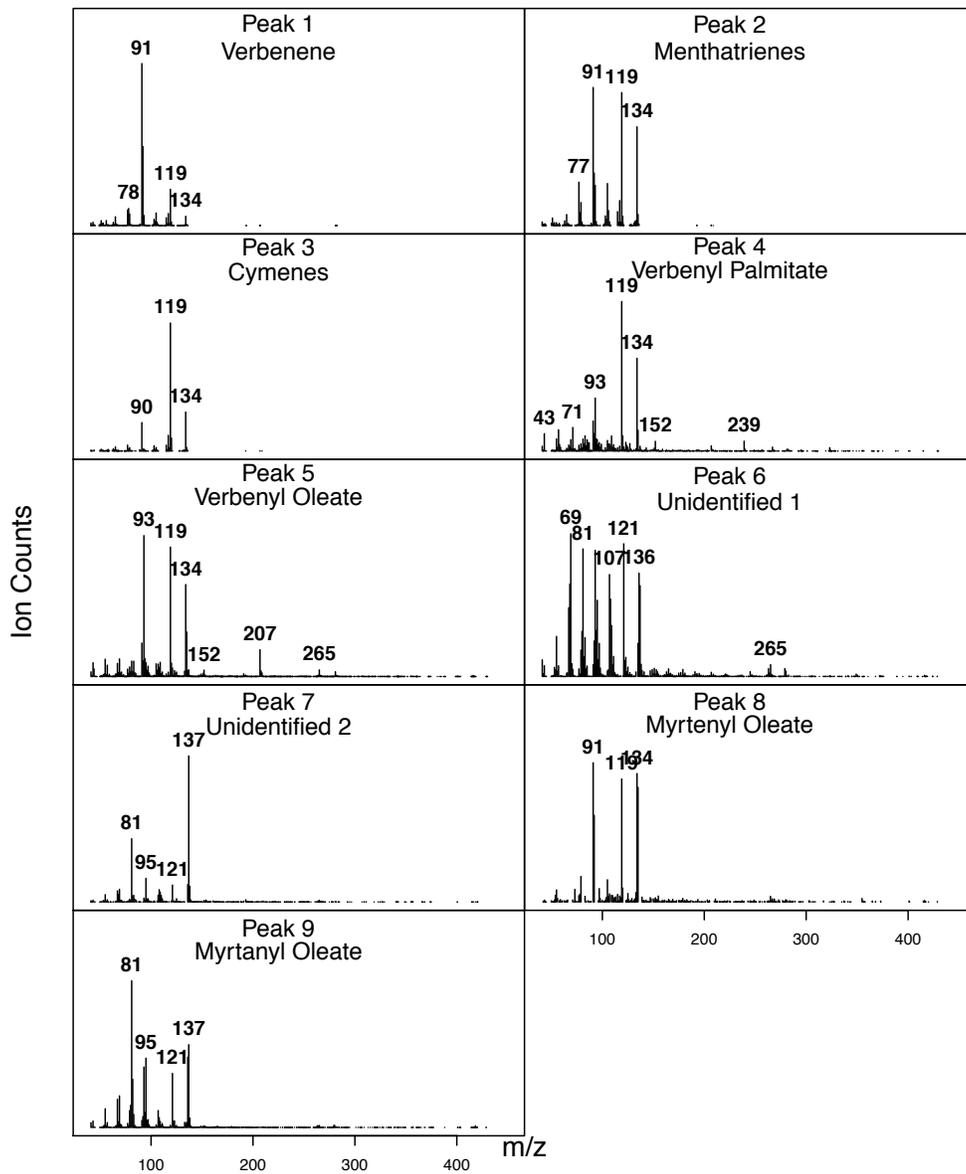
Enantiomeric purity of *trans*- and *cis*-verbenol extracted from beetles were analyzed on an Agilent CyclodexB column (10.5%  $\beta$ -cyclodextrin, 25.7 m length, 250  $\mu\text{m}$  i.d., 0.25  $\mu\text{m}$  film thickness) at 0.9  $\text{ml min}^{-1}$  He on an Agilent 7890A system GC, Agilent GC Sampler 80 and a 7000A GC/MS triple quad M5975C inert XL MSD with triple axis detector at 70eV. The GC temperature program was as follows: 40°C for 2 min, increase at 10°C  $\text{min}^{-1}$  to 100°C, 20°C  $\text{min}^{-1}$  to 230°C, hold 7 min, pulsed splitless injector held at 250°C.

**3.3.10 Statistical analysis.** Compound quantities were analyzed using non-parametric tests because they failed tests for normality and homogeneous variances (Shapiro-Wilk normality test and Barlett test of homogeneity of variances). I used the Kruskal-Wallis rank sum test followed by the Conover's test for pairwise comparisons with the p-values adjusted by the Benjamini, Hochberg, and Yekutieli correction method for multiple comparisons.

## 3.4 Results

**3.4.1 Verbenyl esters in female MPB.** Gas chromatography/mass spectrometry (GC/MS) metabolite profiling of freshly emerged male and female MPB revealed sex-specific

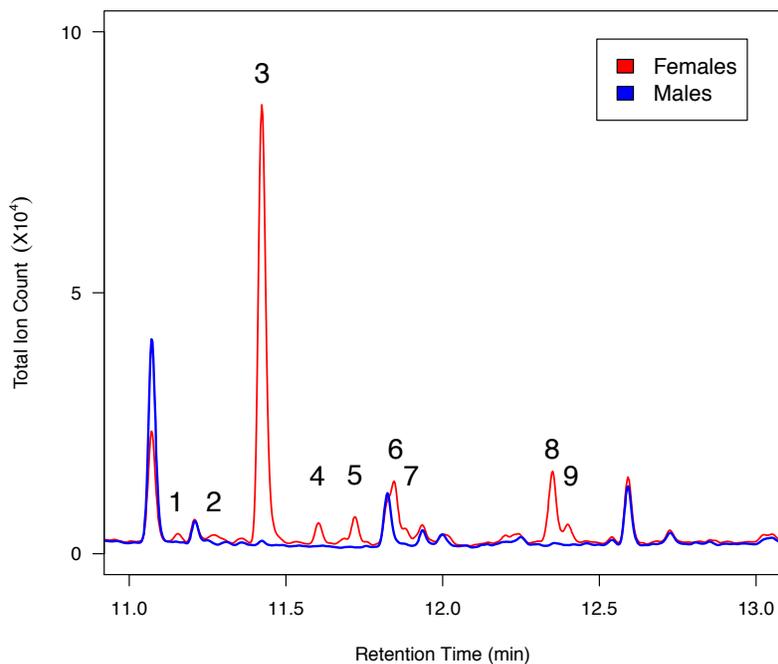
differences in a set of compounds that were present in extracts of females, but not males (**Figure 3.1, 3.2, and 3.3, Table 3.1**). Silica chromatography and ester hydrolysis, followed by GC/MS analysis revealed these compounds as fatty acid esters of mostly *trans*-verbenol along with minor amounts of fatty acid esters of *cis*-verbenol, myrtenol, myrtanol, and an unknown terpene alcohol (**Figure 3.4 and 3.5**). The female-specific esters were identified by comparison to authentic standards as verbenyl palmitate, verbenyl oleate, myrtanyl oleate, and myrtenyl oleate (**Figure 3.2 and 3.6**). *cis*-Verbenyl oleate and *trans*-verbenyl oleate did not separate under the GC conditions and could not be distinguished as these two compounds had very similar mass spectra (**Figure 3.6**). These two compounds were quantified together as “verbenyl oleate” in subsequent analyses of monoterpenol esters over the lifecycle of the beetle and in response to treatments. Monoterpenyl esters were not detected in the phloem collected from the brood trees (**Figure 3.7**).



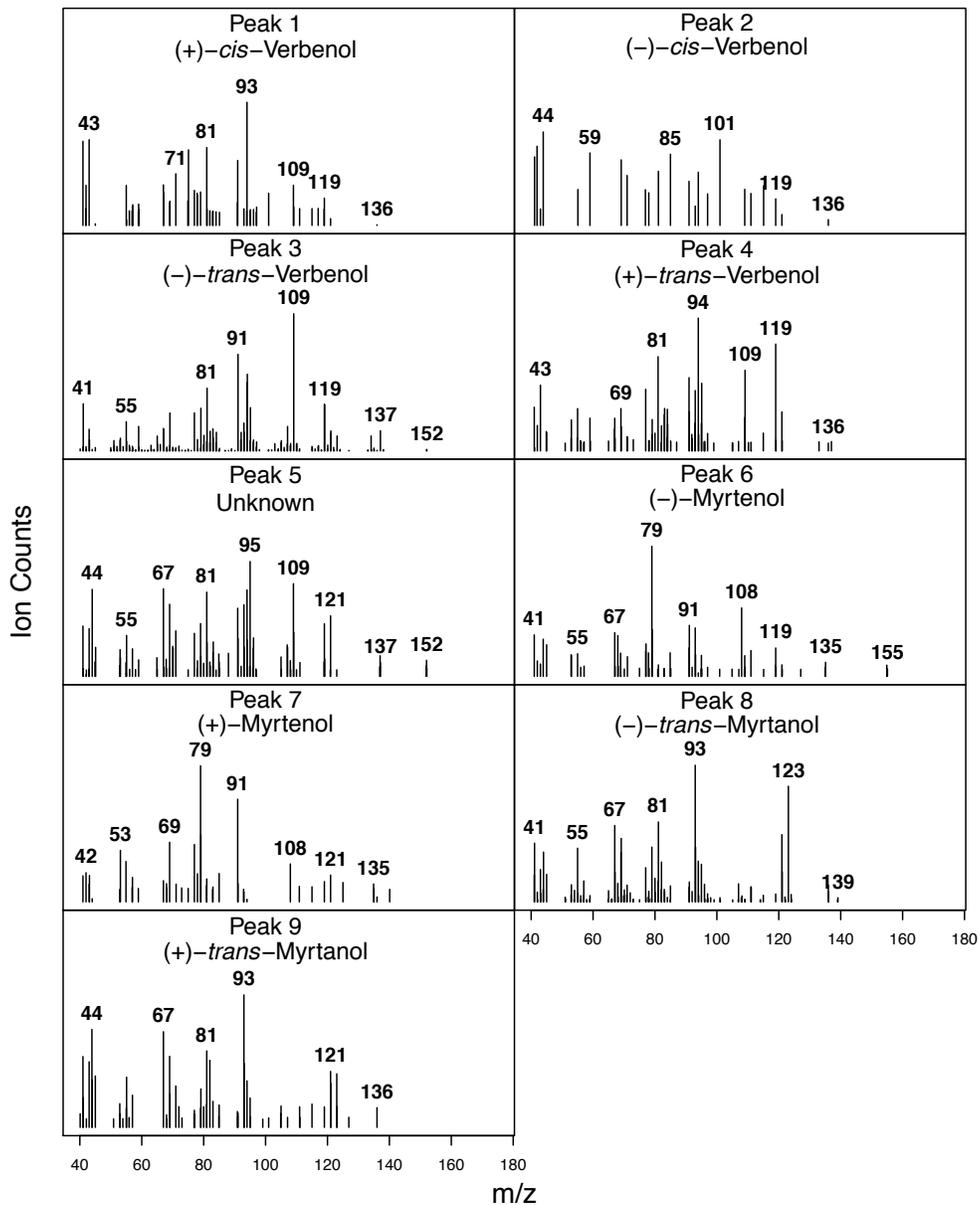
**Figure 3.3 Mass spectra of peaks 1-7 from the gas chromatogram of esters extracted from female MPB. Gas chromatograms with labeled peaks are shown in Fig 3.1.**

**Table 3.1. The retention index of all monoterpenyl esters found in extracts of MPB.** These esters were found in female emergent beetles (Fig. 3.1, 3.2, 3.3, 3.6) or in beetles treated with monoterpenes (Fig. 3.13, 3.14). All extracts and standards were injected onto a VF-5 column with an inlet temperature of 250°C. See Fig. 3.1, 3.2, 3.3, 3.6, 3.13 and 3.14 and for the gas chromatograms and mass spectra of these peaks.

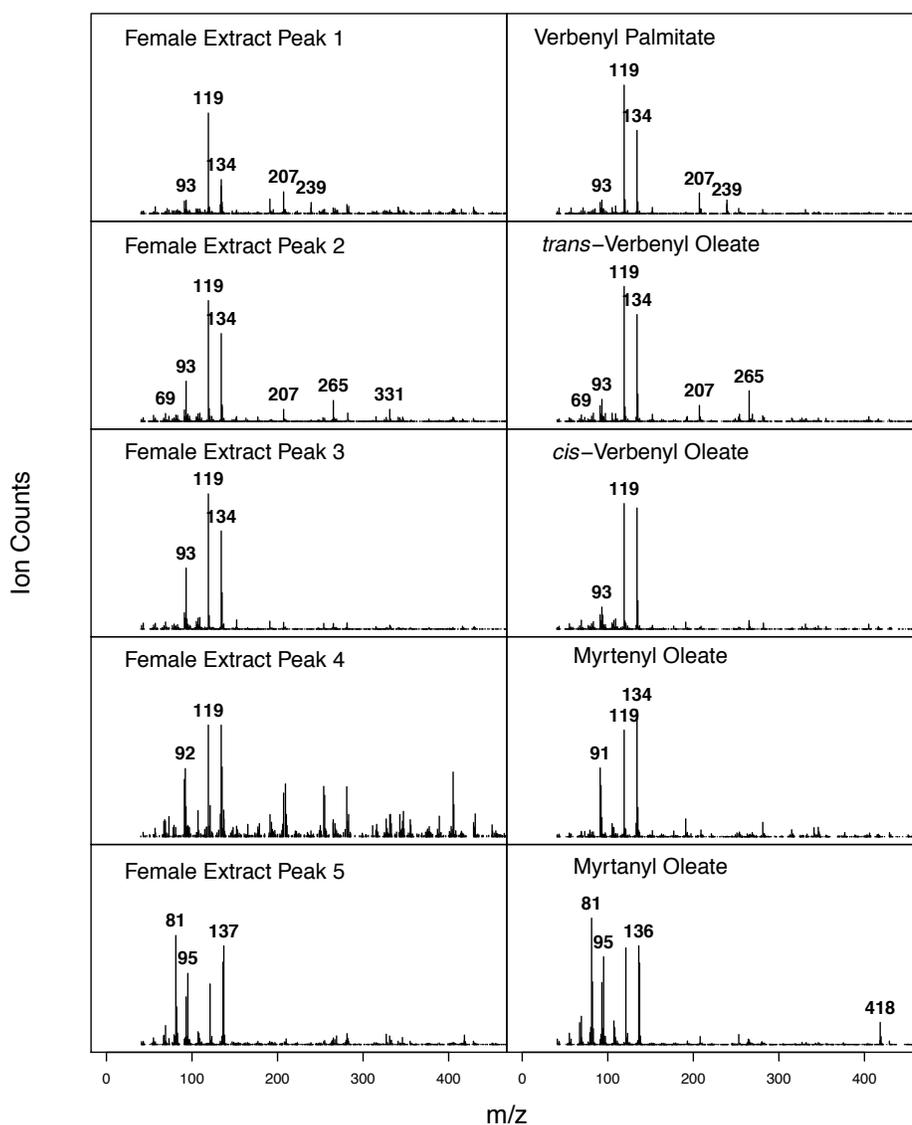
Reference	Retention Indices	Compound name
Peak 4 - Fig 3.1, 3.2	2696	verbenyl palmitate
Peak 5 - Fig 3.1, 3.2	2886	verbenyl oleate
Peak 1 - Fig 3.13 A, 3.14 A	2909	<i>cis</i> -carvyl oleate
Peak 6 - Fig 3.1, 3.2	2920	unknown monoterpenyl ester 1
Peak 4 - Fig 3.13 B, 3.14 B	2920	<i>trans</i> -pinocarvyl oleate
Peak 7 - Fig 3.1, 3.2	2942	unknown monoterpenyl ester 2
Peak 8 - Fig 3.1, 3.2	2955	myrtenyl oleate
Peak 2 - Fig 3.13 A, 3.14 A	2957	<i>trans</i> -carvyl oleate
Peak 9 - Fig 3.1, 3.2	3020	myrtanyl oleate
Peak 6 - Fig 3.13 C, 3.14 C	3023	$\beta$ -phellandrene-derived monoterpenyl ester
Peak 3 - Fig 3.13 A, 3.14 A	3048	perillyl oleate



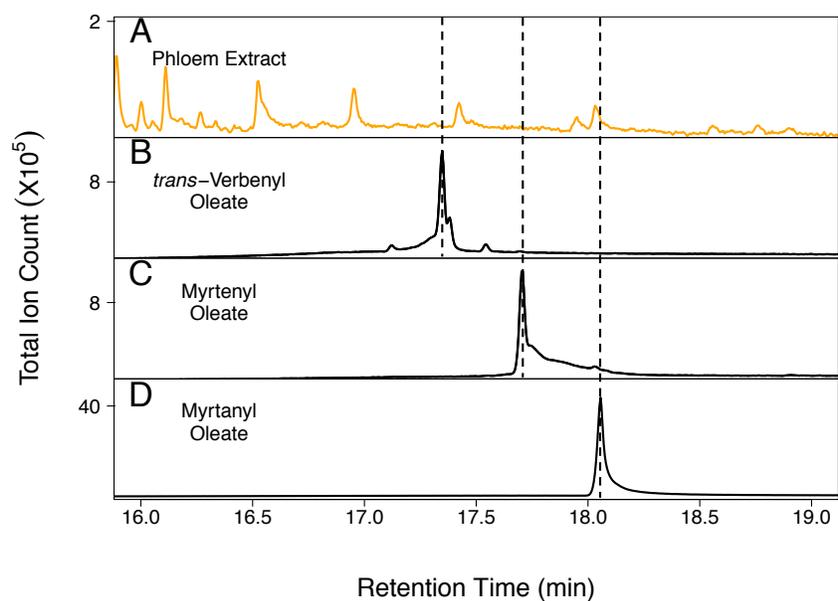
**Figure 3.4 GCMS chromatogram of the alcohol fraction of female (red) and male (blue) ester extracts after ester hydrolysis.** Peaks unique to the female hydrolyzed ester extract, (1) (+)-*cis*-verbenol, (2) (-)-*cis*-verbenol, (3) (-)-*trans*-verbenol, (4) (+)-*trans*-verbenol, (5) unknown terpene alcohol, (6) (-)-myrtenol, (7) (+)-myrtenol, (8) (-)-*trans*-myrtanol, (9) (+)-*trans*-myrtanol were alcohols released from female esters. These peaks were not present in the female extract until after saponification. The enantiomeric ratio of the *trans*-verbenol was 4%(+): 96%(-) and *cis*-verbenol was 87%(+): 13%(-). Mass spectra of labeled peaks are shown in Fig 3.5.



**Figure 3.5 Mass spectra of peaks 1-9 from the gas chromatograms of the alcohol fraction of saponified female emerged beetles.** Gas chromatograms with labeled peaks are shown in Fig 3.4. Both enantiomers of *trans*- and *cis*-verbenol (Peak 1-4), myrtenol (Peak 6-7) and myrtanol (peak 8-9) were identified as alcohols released from the female esters. Peak 5 is an unidentified terpene alcohol also released from the female esters.

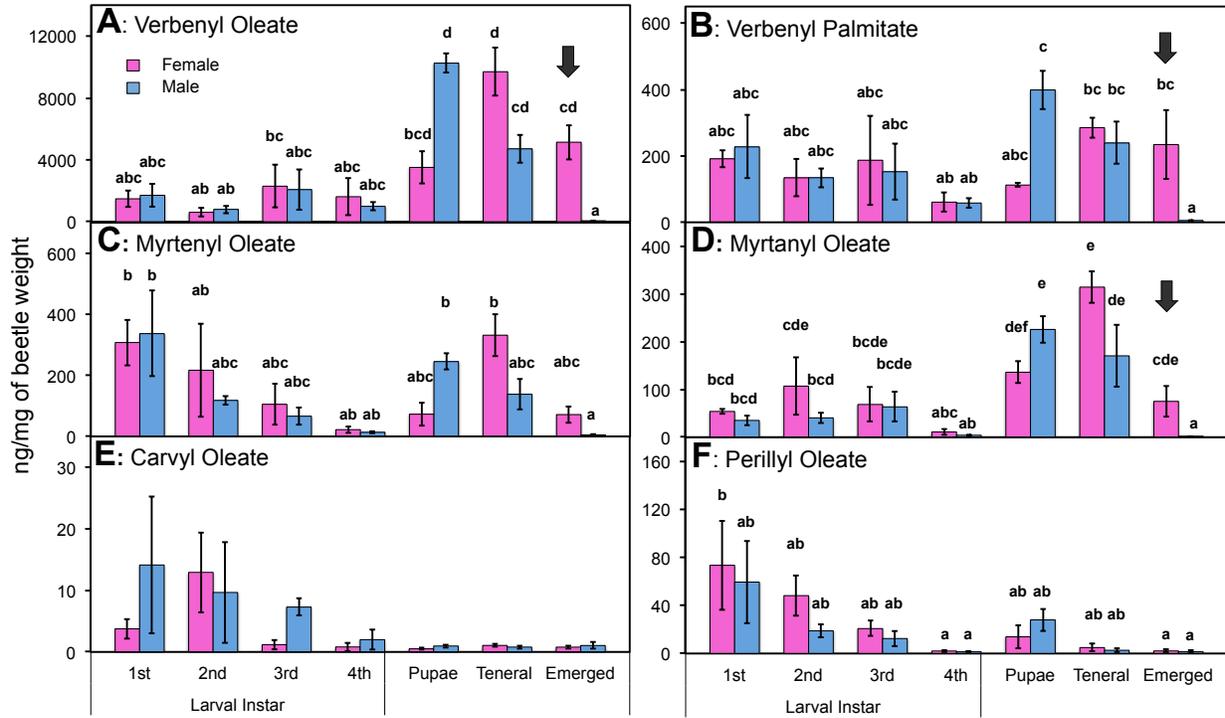


**Figure 3.6 Mass spectra of peaks 1-5 from the gas chromatogram of the female ester fraction and monoterpenyl ester standards.** Gas chromatograms with labelled peaks can be found in Fig. 3.2. Verbenyl palmitate, *trans*- and *cis*-verbenyl oleate, myrtenyl oleate and myrtanyl oleate were identified in the ester fraction of the female emergent beetle.



**Figure 3.7 GCMS chromatogram of a phloem extract (orange) from the brood tree of the MPB used in these experiments and monoterpenyl ester standards (black). The (A) phloem extract does not contain the monoterpenyl esters, (B) verbenyl oleate, (C) myrtenyl oleate and (D) myrtanyl oleate.**

**3.4.2 Sex-specific presence of verbenyl esters in freshly emerged females.** Verbenyl esters, which represent the total of oleates and palmitates of *cis*- and *trans*-verbenyl, as well as myrtenyl oleate and myrtanyl oleate, were present with similar amounts in females and males in early life stages from 1<sup>st</sup> instar larvae until pupation (**Figure 3.8 A-D**). In contrast, significant sex-specific differences appeared in the freshly emerged adult beetles that were no longer in contact with tissues of the brood tree. In the emerged adults, only females contained substantial amounts of verbenyl, myrtenyl and myrtanyl esters (**Figure 3.8 A-D**). During the early life stages until pupation, verbenyl oleate was the most abundant of the monoterpenyl esters in both sexes with 250 to 1,500 ng per mg of beetle body weight (**Figure 3.8 A**), which was at least one order of magnitude higher than any of the other monoterpenyl esters. Levels of verbenyl esters significantly increased in male pupae compared to the larval stages (**Figure 3.8 A-B**), then declined and were absent in freshly emerged adult males. In contrast, while levels of verbenyl esters in females were similar to those of the males during the four larvae stages, they were significantly increased in teneral adult females and remained high in emerged females. In contrast to the verbenyl and myrtanyl esters, the levels of other monoterpenyl esters, specifically perillyl and carvyl oleate, were independent of the sex and decreased over the life cycle from early instar larvae to the emerged adults (**Figure 3.8 E-F**).



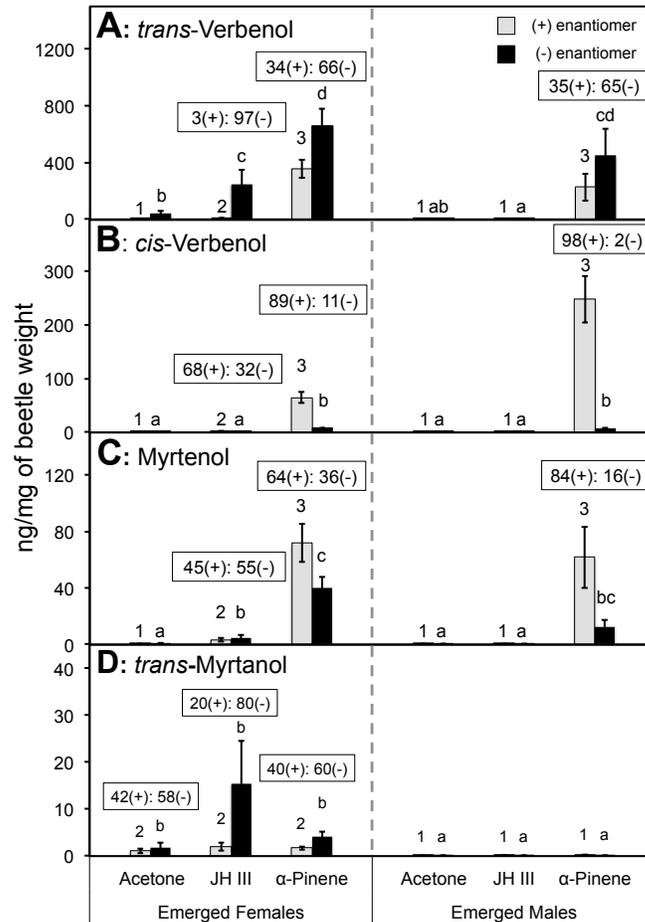
**Figure 3.8** The abundance of monoterpenyl esters in females (pink) and males (blue) over the life cycle of MPB. Columns in the same graph with the same letter were not significantly different by Conover's test ( $\alpha \geq 0.05$ ). During the larval instar to teneral adult stage, developing beetles remain in the brood tree. Tenerals are adult beetles that do not have fully sclerotized cuticle, a stage that lasts 7-14 days. Emerged beetles are fully mature adults, which leave their brood tree in search of a new host. The amount of (A) verbenyl oleate, (B) verbenyl palmitate and (D) myrtanyl oleate were significantly different between female and male emerged beetles as indicated by the arrow. No significant differences were found for carvyl oleate. (N=4)

**3.4.3 Verbenyl oleate accumulated in freshly emerged females with highest abundance in the abdomen and fat body.** Verbenyl oleate was present in freshly emerged females in the three major body parts, head, thorax and abdomen, where the alimentary canal with the fat body was removed from the abdomen (**Figure 3.9 A**). Dissection of the alimentary canal showed that verbenyl oleate was most abundant in the layer of perivisceral fat body that surrounds the alimentary canal (**Figure 3.9 B**), with much lower amounts in the midgut, Malpighian tubules and hindgut.

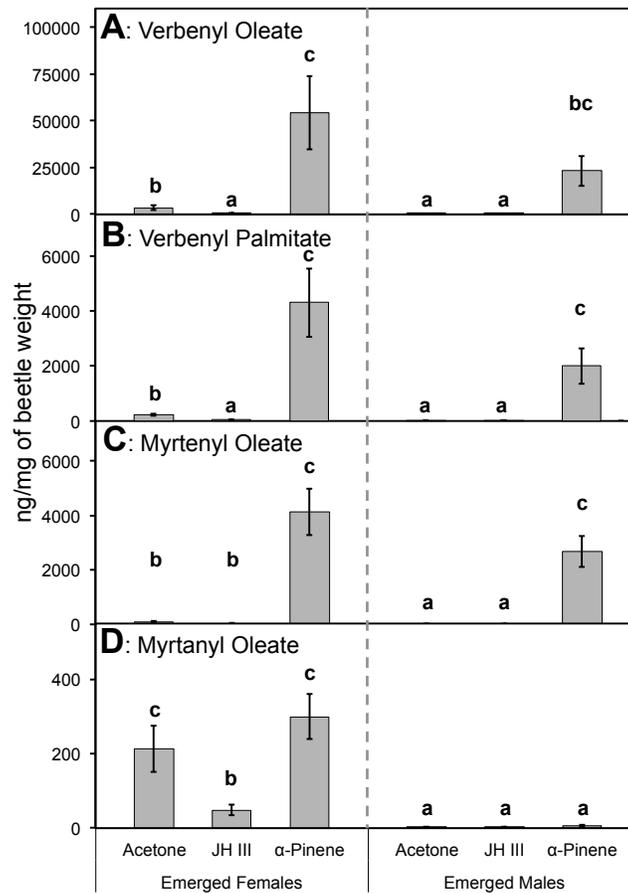


**Figure 3.9 The abundance of verbenyl oleate in dissected tissues of emerging female MPB.** The head, thorax, and abdomen were separated, then the alimentary canal with Malpighian tubules and perivisceral fat body were removed from the abdomen for further dissection into the fat body, midgut, Malpighian tubules, and hindgut. \* Denotes abdomen with the alimentary canal removed. (N=4)

**3.4.4 Levels of *trans*-verbenol increased and levels of verbenyl esters decreased in females treated with JHIII.** Following the identification of verbenyl esters in pre-adult males and females and their sex-specific abundance in freshly emerged females, I tested if their levels were affected by JHIII, which regulates pheromone release in MPB. I also tested if JHIII treatment affected levels of free verbenol and other monoterpene alcohols in female and male emerged adults. Levels of *trans*-verbenol increased in JHIII-treated females compared to acetone-treated controls (**Figure 3.10 A**), while levels of verbenyl esters decreased in JHIII-treated females compared to controls (**Figure 3.11 A**). Levels of *cis*-verbenol and myrtenol also significantly increased in females in response to JHIII (**Figure 3.10 B-C**), while the corresponding esters decreased (**Figure 3.11 B-C**). JHIII treatment did not induce elevated levels of monoterpenols in males, which lacked substantial amounts of the corresponding fatty acid esters (**Figure 3.10 D** and **3.11 D**).



**Figure 3.10** The presence of monoterpene alcohol pheromone components in MPB after treatment with acetone, juvenile hormone III (JHIII), or 44(+): 56(-)  $\alpha$ -pinene. Columns in the same graph with the same number ((+) enantiomer) or same letter ((-) enantiomer) were not significantly different by Conover's test ( $\alpha \geq 0.05$ ). Compared to the acetone control, JHIII treatment significantly increased (A) *trans*-verbenol, (B) *cis*-verbenol and (C) myrtenol production in female, but not male beetles.  $\alpha$ -Pinene treatment increased (A) *trans*-verbenol, (B) *cis*-verbenol and (C) myrtenol production in both sexes.  $\alpha$ -Pinene did not increase *trans*-myrntanol, a product of  $\beta$ -pinene metabolism. Enantiomeric ratios are shown where relevant. (N=6)



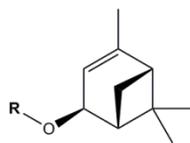
**Figure 3.11 The presence of monoterpenyl esters in MPB after treatment with acetone, JHIII, or  $\alpha$ -pinene.** Columns in the same graph with the same letter were not significantly different by Conover's test ( $\alpha \geq 0.05$ ). (A) Verbenyl oleate, (B) verbenyl palmitate and (D) myrtanyl oleate declined significantly with JHIII treatment in females. (A) Verbenyl oleate, (B) verbenyl palmitate and (C) myrtenyl oleate increased significantly with  $\alpha$ -pinene treatment in both sexes. (N=6)

### 3.4.5 Levels of trans-verbenol and verbenyl esters increased in females and males

**exposed to  $\alpha$ -pinene.** I tested the effect of  $\alpha$ -pinene vapour, which is the presumed host-derived monoterpene precursor of verbenol, on levels of monoterpenols and monoterpenyl esters in freshly emerged males and females. Exposure to a nearly racemic blend of  $\alpha$ -pinene caused increased levels of *trans*-verbenol, *cis*-verbenol, and myrtenol, as well as increased accumulation of the corresponding esters, in both sexes

(**Figure 3.10 A-C** and **3.11 A-C**). Exposure to  $\alpha$ -pinene did not induce a significant increase in the levels of myrtanol, a  $\beta$ -pinene derived monoterpene, or accumulation of myrtanyl oleate in either sex (**Figure 3.10 D** and **3.11 D**).

**3.4.6 Stereochemistry of monoterpenes induced by JHIII and  $\alpha$ -pinene treatment.** The stereochemistry of the monoterpenes detected in MPB is expected to be dependent upon the stereochemistry of the proposed precursor monoterpenes (**Figure 3.12**). I compared the enantiomeric ratio of the monoterpenes induced by treatment with JHIII or  $\alpha$ -pinene to the enantiomeric ratio of the presumed monoterpene precursors. The enantiomeric ratio of *trans*-verbenol detected in beetles differed between JHIII and  $\alpha$ -pinene treatment. The average enantiomeric ratio of *trans*-verbenol in JHIII-treated females was 3(+):97(-) for *trans*-verbenol (**Figure 3.10 A**). This ratio was similar to the ratio 4(+):96(-) of the *trans*-verbenol that comprised the verbenyl esters in freshly emergent females (**Figure 3.9**). The enantiomeric ratio of *trans*-verbenol found in freshly emerged beetles exposed to a nearly racemic  $\alpha$ -pinene vapour was 34(+):66(-) in females (**Figure 3.10 A**) and 35(+):65(-) in males (**Figure 3.11 B**), which was closer to the 44(+):56(-) ratio of  $\alpha$ -pinene used for treatment.



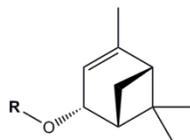
(+)-*trans*-Verbenol: **R= H**

(+)-*trans*-Verbenyl Palmitate: **R= Palmitate**

(+)-*trans*-Verbenyl Oleate: **R= Oleate**

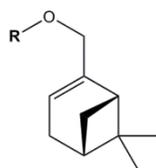


(+)- $\alpha$ -Pinene



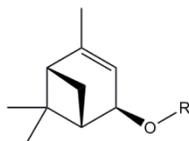
(-)-*cis*-Verbenol: **R= H**

(-)-*cis*-Verbenyl Oleate: **R= Oleate**



(+)-Myrtenol: **R= H**

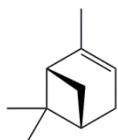
(+)-Myrtenyl Oleate: **R= Oleate**



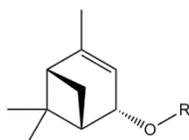
(-)-*trans*-Verbenol: **R= H**

(-)-*trans*-Verbenyl Palmitate: **R= Palmitate**

(-)-*trans*-Verbenyl Oleate: **R= Oleate**

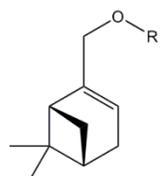


(-)- $\alpha$ -Pinene



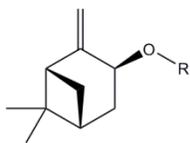
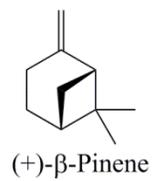
(+)-*cis*-Verbenol: **R= H**

(+)-*cis*-Verbenyl Oleate: **R= Oleate**



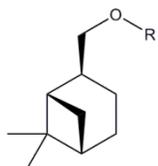
(-)-Myrtenol: **R= H**

(-)-Myrtenyl Oleate: **R= Oleate**



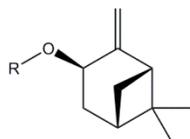
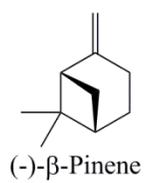
(+)-*trans*-Pinocarveol: **R**=H

(+)-*trans*-Pinocarvyl Oleate: **R**=Oleate



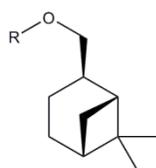
(-)-*trans*-Myrtanol: **R**=H

(-)-*trans*-Myrtanyl Oleate: **R**=Oleate



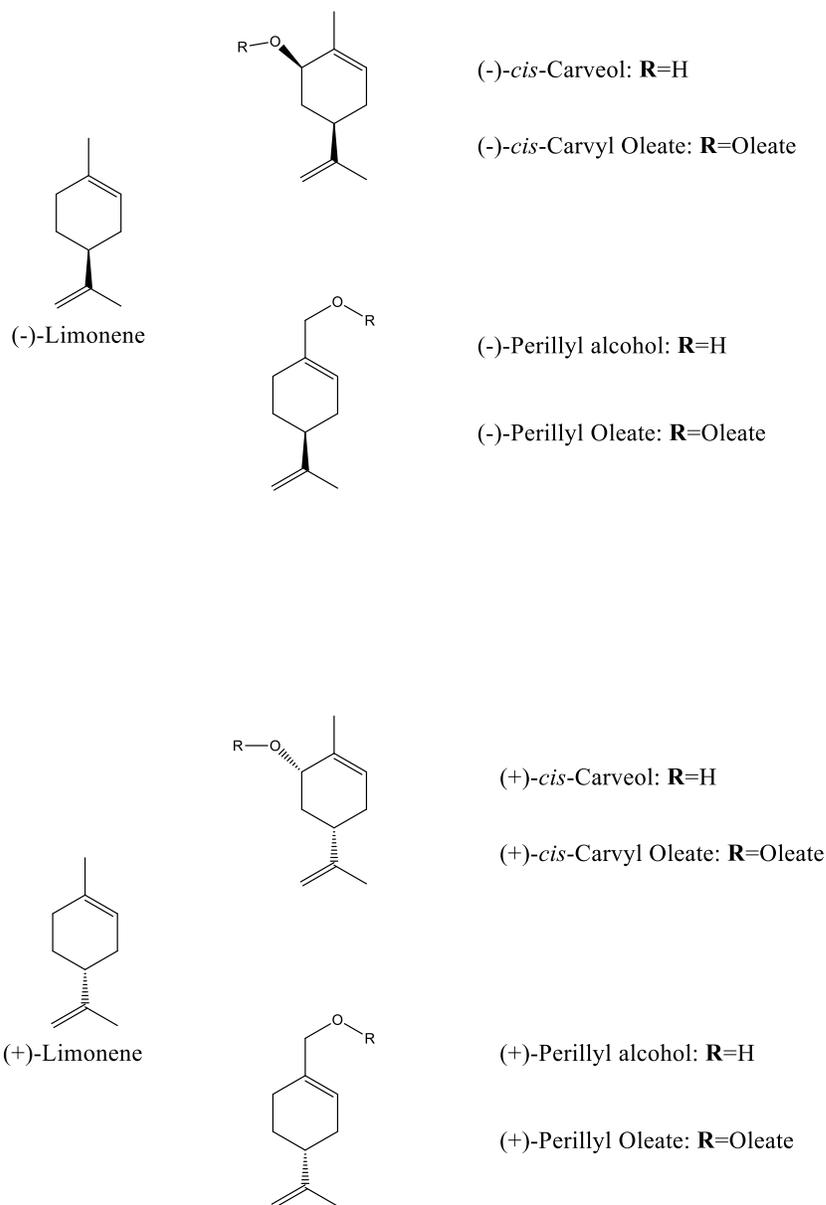
(-)-*trans*-Pinocarveol: **R**=H

(-)-*trans*-Pinocarvyl Oleate: **R**=Oleate



(+)-*trans*-Myrtanol: **R**=H

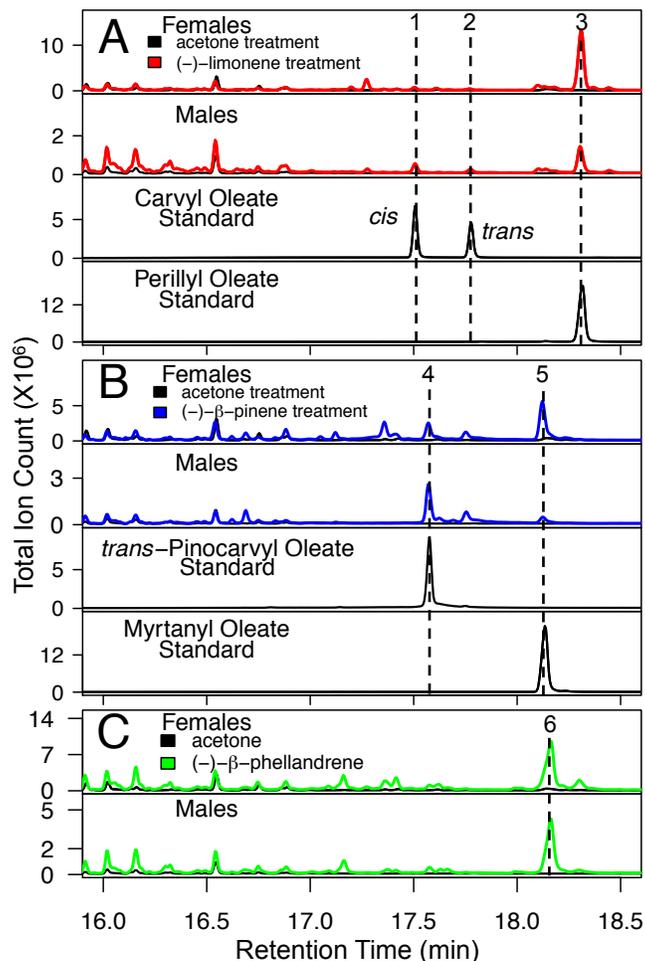
(+)-*trans*-Myrtanyl Oleate: **R**=Oleate



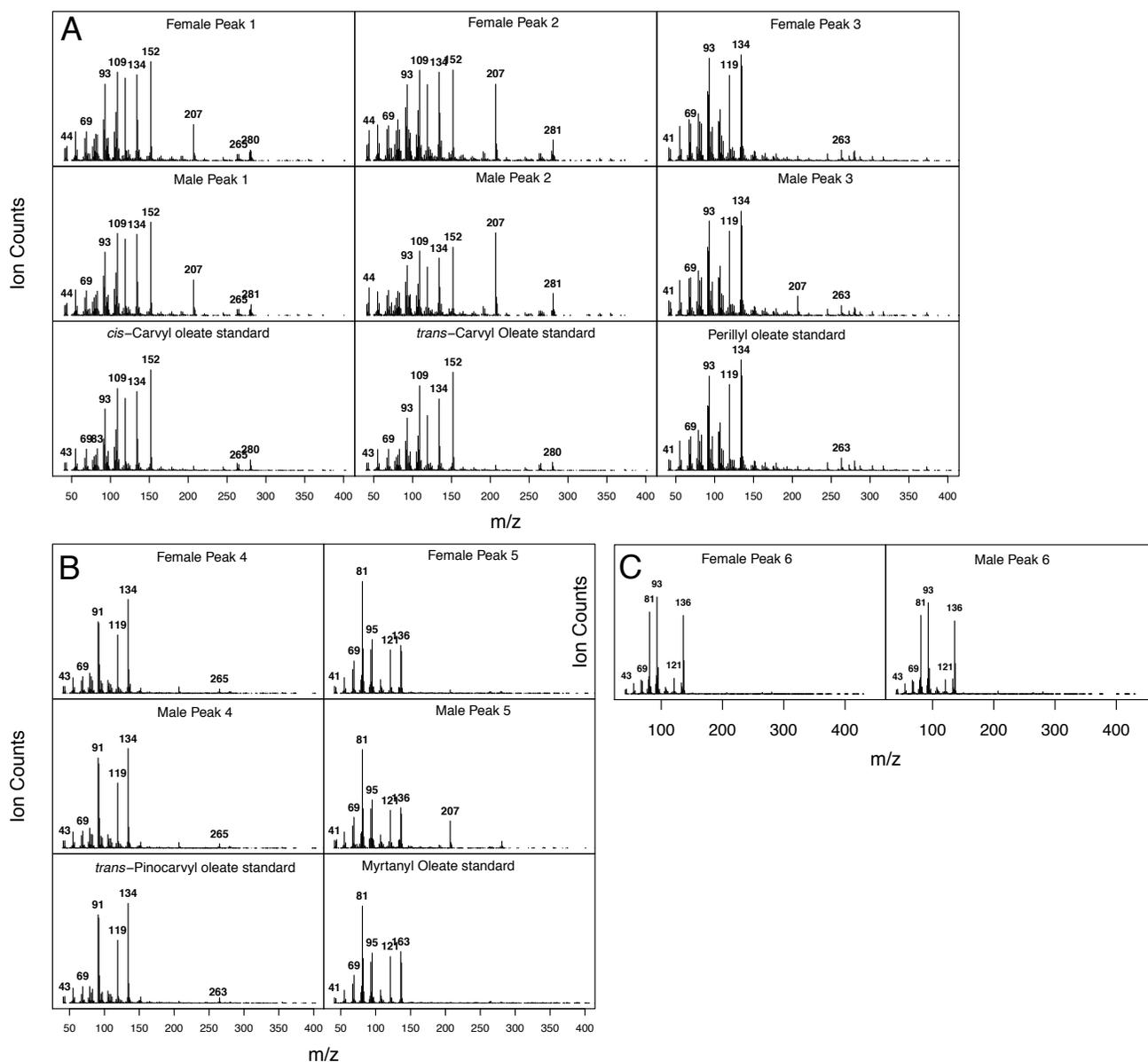
**Figure 3.12 Structures of the monoterpenes and their corresponding alcohols and esters described in the text**

**3.4.7 Monoterpenyl esters in MPB exposed to other monoterpenes.** Following the observation that exposure to  $\alpha$ -pinene vapour resulted in increased levels of verbenyl and myrtenyl esters in both females and males, I also exposed freshly emerged adult

beetles to vapours of other monoterpenes that are present in host trees to test if the occurrence of monoterpene esters was specific to  $\alpha$ -pinene or a general effect of monoterpene exposure. Females and males were individually exposed to (-)- $\beta$ -pinene, (-)-limonene, and (-)- $\beta$ -phellandrene. Exposure to all of these monoterpenes resulted in the production of monoterpene alcohols and the corresponding monoterpene esters in the beetles (**Figure 3.13**). Often, multiple products were produced from the treatment with a single monoterpene. However, the identity of only a few of the esters from (-)- $\beta$ -pinene and (-)-limonene could be verified by our synthetic standards (**Figure 3.13** and **3.14**).



**Figure 3.13 GCMS chromatograms of extracts of female and male beetles treated with acetone, (-)-limonene, (-)-β-pinene, and (-)-β-phellandrene.** The monoterpene esters, (A) carvyl oleate and perillyl oleate were identified in both sexes of limonene-treated MPB; (B) pinocarvyl oleate and myrtanyl oleate were identified in both sexes of β-pinene-treated MPB; and (C), a monoterpene ester was present in β-phellandrene-treated MPB, but could not be identified. The mass spectra of peaks labeled 1-6 are shown in Fig. 3.14.



**Figure 3.14** Mass spectra of peaks 1-6 from the gas chromatograms of extracts of female and male beetles treated with (A) (-)-limonene, (B) (-)- $\beta$ -pinene, and (C) (-)- $\beta$ -phellandrene along with monoterpenyl ester standards. Gas chromatograms with peak numbers can be found in Fig. 3.13. (A) Mass spectra of carvyl oleate and perillyl oleate were identified in limonene-treated MPB. (B) Mass spectra of *trans*-pinocarvyl oleate and myrtanyl oleate identified in  $\beta$ -pinene-treated MPB. (C) Mass spectra of an unidentified monoterpenyl ester peak found in  $\beta$ -phellandrene-treated MPB.

### 3.5 Discussion

Bark beetles (Scolytidae) commonly use monoterpenol pheromones. Their biosynthesis has been studied in MPB and *Ips* species using genomic (Keeling et al., 2013c), transcriptomic (Aw et al., 2010; Keeling et al., 2004, 2012) and proteomic (Keeling et al., 2016) approaches and functional characterization of genes (Blomquist et al., 2010; Keeling et al., 2013a; Sandstrom et al., 2006; Song et al., 2014; Tittiger & Blomquist, 2016). *I. paraconfusus* and *I. pini* produce the monoterpenol pheromones ipsenol and ipsdienol *de novo* (Gilg et al., 2005, 2009; Seybold & Tittiger, 2003) in a process regulated by JHIII (Chen et al., 1988). In general, strategies for pheromone production in bark beetles have been hypothesized to involve detoxification of host-derived monoterpenes (Hughes, 1973a; Vité et al., 1972), which may include conjugation of monoterpenes (Hughes, 1975), microbial production (Brand et al., 1975), and *de novo* biosynthesis (Seybold et al., 1995).

In the context of the present geographic and host range expansion of MPB, it is important to understand how MPB produce and release the female-specific aggregation pheromone *trans*-verbenol (Erbilgin et al., 2014; Taft et al., 2015a). This involves testing the capacity of MPB to accumulate host monoterpenes as pheromone precursors when they are most exposed to pine oleoresin during their life-cycle prior to adult emergence from brood trees.

In support of the accumulation via conjugation hypothesis of *trans*-verbenol (Conn et al., 1984; Hughes, 1975; White et al., 1980), I identified the monoterpenyl esters verbenyl oleate and verbenyl palmitate, along with myrtenyl oleate and myrtanyl oleate, in developing MPB. The effect of JHIII in females on the decrease of verbenyl oleate with

the simultaneous increase of *trans*-verbenol in the absence of direct contact with  $\alpha$ -pinene supports the conclusion that verbenyl esters can serve as a metabolite pool for the female-specific pheromone release. JHIII triggers the sex-specific up-regulation of gene expression, including the female midgut specific up-regulation of an esterase gene as observed in transcriptome data (Keeling et al., 2016).

The ability of MPB to accumulate host monoterpenes via esterification of hydroxylated intermediates may be a general mechanism of detoxification of pine monoterpenes that is not sex-specific. Such a detoxification system would be similar to those of various lepidopteran species that conjugate phytoecdysteroids with fatty acids as a detoxification mechanism (Rharrabe et al., 2007). In the MPB, the increased polarity of the monoterpenyl fatty acid ester, compared to the monoterpene hydrocarbon, along with the lipophilic fatty acid side chain, may facilitate the transport and localization of the monoterpene derivative into the abdomen and specifically the fat body as a long-term storage site.

In female MPB, the accumulation of verbenyl esters as a detoxification mechanism may have further evolved into a sex-specific pheromone systems, by which the release of *trans*-verbenol may have become independent of immediate contact with  $\alpha$ -pinene. The presence of verbenyl esters becomes a sex-specific trait at the end of MPB development in the brood tree and when beetles emerge. At this stage of their life cycle MPB cease being in direct contact with the monoterpenes of the brood trees and have not yet come in contact with monoterpenes of a new host. Females retained their verbenyl esters until the stage when they emerge and disperse in search of a new host,

while emerged males appeared to have metabolized or otherwise lost these compounds.

Monoterpenyl esters of short chain fatty acids, such as bornyl acetate have been identified in related *Dendroctonus* species (Chen et al., 2015; Madden et al., 1988).

Monoterpenyl esters of longer chain fatty acids similar to verbenyl oleate, have not been found previously in *Dendroctonus* but do exist in plants, such as chrysanthenyl hexanoate and octanoate in flowers of the flat sea holly (*Eryngium planum*) (Korbel, 2008) and geranyl, neryl and citronellyl palmitate and stearate in rose (*Rosa x hybrida*) petals (Dunphy, 2006). Interestingly, these monoterpenyl esters have also been proposed to act as a reservoir for the subsequent release of the volatile monoterpenols as part of the floral scent (Dunphy, 2006).

Similar to the present study, previous work (Conn et al., 1984; Gries et al., 1990; Hunt & Borden, 1989) also showed that male and female MPB can produce similar amounts of *trans*-verbenol upon treatment with  $\alpha$ -pinene. However, males produced very little or no *trans*-verbenol when in contact with a new host tree, although both males and females come in contact with host-derived  $\alpha$ -pinene (Libbey et al., 1985; Pureswaran et al., 2000). These observations suggest that *trans*-verbenol released by the female MPB at the new host tree may be released, at least in part, from the female-specific reservoir of verbenyl esters perhaps triggered by exposure to host cues and regulated by JHIII.

A previous study (Erbilgin et al., 2014) showed that the enantiomeric ratio of *trans*-verbenol released by attacking females does not correspond to the enantiomeric ratio of the  $\alpha$ -pinene supplied by the attacked tree. Our results show there was a small

preference towards the (-) enantiomer in the conversion of  $\alpha$ -pinene to *trans*-verbenol in adults. However, there appears to be a greater preference for the (-) enantiomer during the hydroxylation and esterification steps in earlier life stages, as evidenced by the enantiomeric excess of (-)-*trans*-verbenol in the accumulated esters and released by the female adults.

The results reported here may have broader implications for understanding pheromone production in other destructive *Dendroctonus* species that produce *trans*-verbenol as an aggregation, anti-aggregation, or synergist component in their communication systems. These include the southern pine beetle (*D. frontalis*) (Sullivan, 2016), western pine beetle (*D. brevicomis*) (Byers et al., 1984), and the red turpentine beetle (*D. valens*) (Hughes, 1973a), which cause damage across forests in North America and Asia.

## 4. Cytochromes P450 Preferentially Expressed in Antennae of Mountain Pine Beetle<sup>3</sup>

### 4.1 Summary

The mountain pine beetle (MPB, *Dendroctonus ponderosae*) is a forest insect pest endemic to western North America. During dispersal and host colonization, MPB identify suitable host trees by olfaction of monoterpene volatiles, contend with host terpene defenses, and communicate with conspecifics using terpenoid and other pheromones. Cytochromes P450 (P450s) have been proposed to function in MPB olfaction, terpene detoxification, and pheromone biosynthesis. Here, I identified P450s that were abundant in the antennae transcriptome. Analysis of transcript levels across different life stages and tissues in females and males showed additional expression of most of these P450s in the midgut or fat body. These expression profiles suggest specific or overlapping functions in olfaction, detoxification, and pheromone biosynthesis.

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<sup>3</sup> A version of this chapter has been published. Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2018). Cytochromes P450 preferentially expressed in the antennae of the mountain pine beetle. *Journal of Chemical Ecology*.

## 4.2 Introduction

Insects and plants interact through a myriad of small molecules (Hartmann 2008), including the large class of terpenes (Pichersky and Raguso 2016). A prominent example of plant insect-interactions shaped by terpene metabolites are those of conifers of the Pinaceae family and bark beetles of the Scolytinae subfamily (Franceschi et al., 2005; Raffa, 2014; Seybold et al., 2006). Coniferophagous bark beetles can tolerate high concentrations of mono- and diterpenes that are present in the oleoresin defenses of conifers (Chiu et al., 2017; Keeling & Bohlmann, 2006b). These insects also use some of the host monoterpenes as precursors for their pheromone biosynthesis (Keeling et al., 2016; Tittiger & Blomquist, 2016, 2017).

Mountain pine beetles (*Dendroctonus ponderosae*, MPB) infest pine (*Pinus* spp.) forests across western North America (Rosenberger et al., 2017). They spend most of their life cycle in the bark and phloem of pine hosts, where eggs, larvae, pupae and adults are exposed to oleoresin terpenes. The oleoresin of MPB host species such as lodgepole pine (*P. contorta*) is composed mostly of mixtures of different monoterpenes, sesquiterpenes, and diterpenes (Boone et al., 2011; Hall et al., 2013a, 2013b). While some terpenes can be toxic to MPB and the insect-associated microbiome (Chiu et al., 2017; Kopper et al., 2005), MPB and its microbial associates appear to have mechanisms to metabolize or detoxify host terpenes (Adams et al., 2013; Boone et al., 2013; Chiu et al., 2018c; Wang et al., 2013, 2014). MPB also aggregate in large numbers to overcome host defenses. They use olfaction of volatile host terpenes to cue in on suitable host trees and release pheromones to coordinate mass colonization. For example, female MPB produce the aggregation pheromone *trans*-verbenol, which is

derived from the host monoterpene  $\alpha$ -pinene (Hughes, 1973b; Tittiger & Blomquist, 2016). Other host monoterpenes, such as terpinolene and myrcene, synergize with MPB aggregation pheromones and help beetles locate suitable hosts (Borden et al., 2008). As part of olfaction, MPB antennae respond to pheromones and host volatiles (Huber et al., 2000; Pureswaran et al., 2004). During dispersal flight, MPB can detect low concentrations of pheromones and host volatiles. Upon landing on a new host tree, and during the rest of the life cycle while in contact with the tree, MPB cope with high concentration of oleoresin terpenes.

Cytochromes P450 (P450s) have been proposed as important components of the MPB biochemical systems of olfaction, pheromone biosynthesis, and host defense detoxification (Hunt & Smirle, 1988; Keeling et al., 2013b, 2013c; Robert et al., 2013; Tittiger & Blomquist, 2016). The MPB genome has been annotated to contain 86 different P450 genes (Keeling et al., 2013c). In addition, the MPB transcriptome has been sequenced from a suite of tissue-specific and developmental stage-specific cDNA libraries (Keeling et al., 2012). From these genome and transcriptome resources, only one P450 with preferential expression in antennae, *CYP345E2*, has been functionally characterized. This P450 enzyme oxidizes various pine monoterpenes, including (+)-3-carene,  $\alpha$ -pinene,  $\beta$ -pinene, limonene, and terpinolene (Keeling et al., 2013b).

As a foundation for future functional characterization of additional MPB P450s, I performed a transcriptome analysis to identify MPB P450 that are expressed in antennae. I hypothesized that preferential expression in antennae, along with

expression in midgut or fat body tissue, may identify MPB P450s with potentially overlapping functions in olfaction, pheromone biosynthesis, and detoxification.

### **4.3 Materials and Methods**

**4.3.1 Identification of Candidate Cytochrome P450s.** I identified six P450s with the highest number of expressed sequence tags (ESTs) in the antennae-specific cDNA library (Keeling et al., 2012) and one related P450 (**Table 4.1**). P450 ESTs were identified by performing a BLASTx of all the sequences in the antennae-specific cDNA library against the NCBI nr database (NCBI website). The resulting P450s were also assessed for presence of ESTs in other MPB cDNA libraries (**Table 4.1**). Each cDNA library in the MPB transcriptome contained different total numbers of ESTs and some libraries were normalized (Keeling et al., 2012). Therefore, the abundance of a particular transcript could not be directly compared between libraries. All P450s identified in the antennal cDNA have a representative full-length cDNA clone that were fully sequenced by Keeling et al. (2012) and these cDNA clone IDs are shown in **Table 4.1** P450 candidates that had more than five ESTs in the antennal cDNA library were selected for qPCR analysis (**Table 4.1**). In addition, another P450 CYP6BW1 was selected for qPCR analysis. Although there were no ESTs found for CYP6BW1 in the antenna library, its high sequence similarity (94.3% nucleotide identity and 96.4% amino acid identity) suggested that it may have the same or very similar function to CYP6BW3 in other tissues. Thus, to identify CYP6BW1 expression in these tissues, and to confirm that the primers I designed to quantify CYP6BW3 were not amplifying CYP6BW1 transcripts as well, I also designed primers for, and quantified, CYP6BW1 expression

levels. I performed qPCR as described below to analyze transcript abundances across developmental stages and tissue types.

**Table 4.1 Cytochromes P450 identified in cDNA libraries made from mountain pine beetle antennae and other tissues associated with olfaction, detoxification or pheromone biosynthesis**

P450 Candidate	FLcDNA Clone ID	NCBI Accession #	# of Expressed Sequence Tags (ESTs)						
			Tissue cDNA Libraries			Development Stage cDNA Libraries			
			Antennae  DPO04	Head  DPO13	Midgut and Fat Bodies  DPO09 DPO10 DPO11 DPO12	Larvae  DPO01 DPO14	Pupae  DPO02 DPO03	Teneral Adult  DPO05	Emerged Adult  DPO06 DPO07 DPO08
<b>CYP345E2</b>	DPO043_F01	JQ855645	464	8	2	4	0	0	3
<b>CYP6DJ1</b>	DPO0411_I13	JQ855677	65	0	20	2	0	0	0
<b>CYP6DE1</b>	DPO0814_E19	JQ855668	33	3	11	8	2	0	12
<b>CYP6DE2</b>	DPO047_M21	JQ855669	21	0	11	3	0	4	0
<b>CYP9Z18</b>	DPO1028_G02	JQ855645	11	0	17	0	0	0	5
<b>CYP6BW3</b>	DPO049_N22	JQ855663	10	2	1	0	0	0	0
CYP393A1	DPO047_M16	JQ855649	2	0	0	0	0	0	2
CYP6BW2	DPO1127_B15	JQ855662	2	0	7	2	0	1	3
CYP9AN1	DPO0410_B16	JQ855680	2	4	2	0	0	0	0
CYP9AP1	DPO1319_O05	JQ855681	2	3	8	0	0	0	1
CYP9Z20	DPO042_F03	JQ855684	2	3	9	4	0	0	3
CYP9Z22	DPO0111_H17	JQ855686	2	4	2	10	0	6	0
<b>CYP6BW1</b>	DPO079_G21	JQ855661	0	0	34	8	0	0	4
<b>ESTs Total</b>			7680	11520	36864	16128	9216	4608	13824

All P450s identified in the antennal cDNA library (DPO04) are shown in this table. P450 candidates selected for qPCR analysis in this study (**shown in boldface**) had more than five ESTs identified in the antennae cDNA library, with the exception of CYP6BW1 which was selected for study based on high sequence similarity with CYP6BW3. EST abundances in other tissue- and life stage-specific cDNA libraries are shown for comparison along with the total number of ESTs for each cDNA library. cDNA libraries were described and identified by the DPO number according to Keeling et al. (2012).

**4.3.2 Insect Collection.** MPB-infested and non-infested lodgepole pine bolts were collected from two separate trees, one infested and one non-infested, near Whistler, British Columbia, Canada (50°10'17.6"N, 122°52'35.4"W) on April 24<sup>th</sup> 2014. All insects used in this study were collected from bolts of the infested tree. Infested bolts were placed in screened cages and stored at room temperature. Fourth instar larvae, pupae, and teneral adults were collected by removing the outer layer of bark of infested logs so that specimens could be collected from the exposed phloem layer. Larvae were collected and dissected between May 2<sup>nd</sup> and 7<sup>th</sup> 2014. Larval instar was determined by head capsule width according to Bleiker & Régnière (2014). Transcript assays were performed with 4<sup>th</sup> instar larvae, which was the dominant developmental stage at the time of sampling. Pupae were collected between May 4<sup>th</sup> and 7<sup>th</sup> 2014. Teneral adults were collected between May 20<sup>th</sup> and 24<sup>th</sup> 2014. Newly emerged adults were collected between June 3<sup>rd</sup> and 11<sup>th</sup> 2014. To obtain adult MPB that simulated the early host colonization stage (colonizing adults), I used a method previously described by Pureswaran et al. (2000). In brief, newly emerged female MPB were individually placed into drill-holes in the bark of a non-infested lodgepole pine bolt. After 24 h, an individual male beetle was paired with each female. Pairs were left to feed on the host phloem for an additional 24 h and then collected. Adults that had fed on the host were collected between June 5<sup>th</sup> and 15<sup>th</sup> 2014. All samples were flash frozen in liquid N<sub>2</sub> and stored at -80°C immediately after collection.

**4.3.3 Insect Dissections.** MPB of different life stages were dissected into antennae, heads without antennae, midguts, and fat bodies. Dissections were done in sterile

saline solution (150 mM NaCl, 3 mM KCl, 1.8 mM CaCl<sub>2</sub>, and 22 mM glucose, pH 7.2) under a stereomicroscope. The fat body connected to the alimentary canal was defined as the fat body sample. The midgut was detached from the rest of the alimentary canal. Heads and antennae were collected from adult MPB. Antennae included the club and pedicle. Remaining carcasses were used for sex identification. Dissected samples were flash frozen in liquid N<sub>2</sub> and stored at -80°C.

**4.3.4 Sex Identification.** Teneral adults, freshly emerged adults, and colonizing adults were sexed using the shape of the seventh abdominal tergite (Lyon, 1958). Larvae and pupae were sexed using multiplex PCR assays with a set of primers that amplified a 600 bp region of the neo-X chromosome or a 500 bp region of the male-specific neo-Y chromosome as described in Appendix A. DNA was extracted in one of two different ways: (1) In experiments with intact insects, both DNA and RNA were isolated from the same insects using the RNeasy® Plant Mini Kit (Qiagen) and a modified protocol (Martins, 2009). In brief, after elution of RNA, 50 µl of 8 mM NaOH was added to the RNeasy spin column, followed by incubation at 55°C for 5 min and centrifugation of the column at 2000 x *g* for 3 min. The pH of the recovered DNA sample was adjusted to 7.5 using 8 µl of HEPES. (2) For experiments with dissected tissues, DNA was isolated from a sample volume of approximately 1 mm<sup>3</sup> of the carcass that remained after insect dissection using the prepGEM® insect kit (ZyGEM).

**4.3.5 RNA Preparations and cDNA Synthesis for qPCR.** RNA was extracted using the RNeasy® Plant Mini Kit according to the manufacturer's instructions; except, for

dissected tissue samples, 50 to 100  $\mu$ l (instead of 750  $\mu$ l) of the RLT buffer was used for extractions. For all samples, 30  $\mu$ l of RNase-free H<sub>2</sub>O was used to elute RNA from RNeasy spin columns. RNA quality and concentration were determined using the Agilent RNA 6000 Nano or Agilent RNA 6000 Pico Kit (Agilent). RNA with a RIN > 7 was used for cDNA synthesis with the Maxima First Stand cDNA Synthesis Kit with dsDNase (ThermoScientific). The cDNA was diluted to 1.8 ng/ $\mu$ l for qPCR analysis of the different life stages and 0.2 ng/ $\mu$ l for qPCR analysis of dissected tissues.

**4.3.6 Selection of Reference Genes for qPCR.** The reference genes used for normalization in the qPCR analysis were *ubiquitin conjugating E2* and *tubulin* for qPCR of different developmental stages and *cyclin-dependent kinase 10* and *NADH dehydrogenase flavoprotein 1* for qPCR of dissected tissues. They were selected and validated as follows: A set of genes that showed stable expression across different conditions were selected from published MPB RNA-Seq transcriptome data representing 64 samples of larvae and adults (Keeling et al., 2016; Robert et al., 2016, 2013). The RNA-Seq reads were filtered and trimmed with Trimmomatic version 0.30 (Lohse et al., 2012), bases with quality < 20 were trimmed from the 3' end of each read, and trimmed sequences < 30 bp in length were removed. RNA-Seq reads were then counted using Salmon version 0.6.3 as part of Sailfish (Patro et al., 2014) against gene models identified in the MPB genome APGK01 (Keeling et al., 2013c), and normalized between samples in R version 3.1.2 (R Core Development Team, 2017) using edgeR\_3.8.5 (Robinson et al., 2009). A simple ANOVA was performed, and genes that had no significant difference in transcript levels across the 64 different conditions were

selected. Genes were annotated by BLASTx search against the NCBI nr database and four genes were selected based on predicted functions in essential processes and for being conserved across different species: *Ubiquitin conjugating E2* (GenBank YQE\_02422), *NADH dehydrogenase flavoprotein 1* (YQE\_11061), *acyl-CoA-binding domain-containing protein* (YQE\_10263), and *cyclin-dependent kinase 10* (YQE\_12354). A geNorme analysis (Vandesompele et al., 2002) was performed with these four genes along with *tubulin* (EZ115624) and *ubiquitin* (EZ115790), which were used previously as reference genes for qPCR in MPB (Aw et al., 2010).

**4.3.7 Primer Design for qPCR.** To identify sequence regions of low identity to other MPB transcripts, the open reading frame of each target gene was searched by BLASTn against the MPB transcriptome (Keeling et al., 2012) and genome (Keeling et al., 2013c). Each primer pair was designed to amplify 100-150 bp and one primer of each pair spanned an intron. The melting temperature of each primer was between 60 and 64°C and differed by no more than 1°C from its primer pair. Each primer was analyzed for self-complementation and complementation with its primer pair. Primers were obtained from Integrated DNA Technologies. All PCR primers used in this study are shown in **Table 4.2**.

**Table 4.2 Olfaction and detoxification candidate cytochromes P450 - number of ESTs found in each stage specific and tissue specific cDNA libraries from Keeling et al. 2012.**

Gene Name	Genbank	Primer Sequence (5'-3')	T <sub>m</sub> (°C)	Spans Intron	Amplicon Size (bp)	Primer Efficiency
<i>Ubiquitin conjugating E2</i>	YQE_02422	GGAAGAGATCCTCCAGCACAATGTTTCAGC	62	Yes	100	1.85
		CCTGGTATGGACTGTCAGGTGGTCC	62	Yes		
<i>acyl-CoA-binding domain-containing protein</i>	YQE_10263	CAAGCTGGGTGGCCGTTTCAG	61	Yes	113	1.87
		GTGCTTCAGGTAGGCTGCTACTTGG	61	No		
<i>NADH dehydrogenase flavoprotein 1</i>	YQE_11061	CAGTATCACCGTCAATCTGCCGAAGG	61	Yes	117	1.88
		CTGTGCAGGGCCTGTTTCACG	61	No		
<i>cyclin-dependent kinase 10</i>	YQE_12354	GGCTGTGTGAAGATTGCCGACTTTGG	62	Yes	119	1.79
		GGAGCTTGCAGTAGCAGTTCTGGTG	62	No		
<i>tubulin</i>	EZ115624	CCAGATTGGAGCTAAGTTTTGG	60	Yes	127	1.91
		ACCGATGCTTCGTTGTAAT	60	No		
<i>ubiquitin</i>	EZ115790	AAGTTGCAGGATGCAGATCTTC	60	Yes	120	1.99
		GGGGATTCTTCTTTGTCCT	59	No		
<i>CYP6BW1</i>	JQ855661	GCATTGGCTTGAGACTTGGC	63	Yes	118	1.89
		CTCGTTTCTCCATCTGAATTGGA	63	No		
<i>CYP6BW3</i>	JQ855663	GCATTGGCTTGAGACTTGGGA	61	Yes	117	1.82
		CTCGTTTCTCCATCTGAATAGGG	61	No		
<i>CYP6DE1</i>	JQ855668	ATTGACAAAGGAACCCGACTG	61	Yes	102	1.84
		ACTGAATCTTTCAGGGTCAAATCTT	61	No		
<i>CYP6DE2</i>	JQ855669	AACATGTTTCGATCAATTGGAAGG	63	Yes	99	1.82
		TCCAATATTGCCAAGAATTGGCTAG	63	No		
<i>CYP6DJ1</i>	JQ855677	TCCTCTTGGAACAATGATTGC	60	Yes	152	1.81
		AGTCTGGATCCACCACAAGC	61	No		
<i>CYP9Z18</i>	JQ855682	TGGTGTTCAGAGGCACTTAGG	63	Yes	123	1.82
		CTCCAGGCTTCAGGTGAACC	63	No		
<i>CYP345E2</i>	JQ855645	CCAACCTCAGAAATACGATCCAC	62	No	114	1.99
		AACCTATTGCCAATACATATTCTAGGC	61	Yes		

Olfaction P450s were selected as P450s with the highest number of ESTs in the antennae cDNA library from Keeling *et al.* 2012. Other stage specific and tissue specific cDNA libraries from Keeling *et al.* 2012 are showed for comparison along with the total number of Sanger ESTs from the cDNA libraries. ESTs of all P450s also appear in the midgut and fatbody specific cDNA libraries indicating that they might have a wider role in general detoxification apart from degrading odorants. Each cDNA library is specified by DPO number labelled in Keeling *et al.* 2012. Publications which these P450s or close homologs are mentioned: <sup>1</sup>Keeling *et al.* 2016, <sup>2</sup>López *et al.* 2013, <sup>3</sup>Bonnett *et al.* 2012, <sup>4</sup>Pitt *et al.* 2014, <sup>5</sup>Robert *et al.* 2013, <sup>6</sup>Cano-Ramírez *et al.* 2013, <sup>7</sup>Keeling *et al.* 2013

**4.3.8 Test of Primer Efficiency.** Primer concentrations were adjusted to 3.6  $\mu\text{M}$  and forward and reverse primers for each primer pair combined. Equal amounts of cDNA of each developmental stage were combined into a pooled cDNA template with a concentration of 2.5 ng/ $\mu\text{l}$ , and the same was done for cDNAs of the different tissues (0.5 ng/ $\mu\text{l}$ ). A series of six four-fold dilutions were made from each pooled cDNA sample. qPCR was performed for each primer pair on these cDNA serial dilutions. Ct values were plotted against the log values of each dilution, except the first dilution, and a slope of this relationship was calculated. The primer efficiency was calculated using the formula  $\text{efficiency} = 10^{(-1/\text{SLOPE})}$ , where 100% efficiency equals 2 (**Table 4.2**).

**4.3.9 qPCR Analyses.** qPCR was performed separately for each gene in 96 well plates (Bio-Rad) and separately for developmental stages and tissue types. qPCR assays contained 6  $\mu\text{l}$  of SsoFast EvaGreen Supermix (Bio-Rad), 1  $\mu\text{l}$  of primer pair (3.6  $\mu\text{M}$ ), 5  $\mu\text{l}$  of cDNA (1.8 ng/ $\mu\text{l}$  for developmental stages; 0.2 ng/ $\mu\text{l}$  for dissected tissues) in a total volume of 12  $\mu\text{l}$ . A negative control that contained product from a cDNA synthesis reaction without RNA was included for each plate. Assays were conducted using a C1000 Thermal Cycler with CFX96 Real Time PCR Detection System (Bio-Rad). The PCR conditions were: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 5 s, extension at 65°C for 5 s. Melting curve analysis was performed with reaction products from 65-95°C in increments of 0.5°C. qBase software (Biogazelle) was used to normalize the Ct values

to the two reference genes and to calculate the normalized relative expression level using the primer efficiency.

**4.3.10 Statistical Analysis.** Statistics were performed separately for each target P450 using the normalized Ct values. The normalized Ct values were subjected to and rejected the Shapiro-Wilk test for normality and the Bartlett test for homogeneity of variances (Bartlett, 1937; Royston, 1982). Therefore, transcript abundance was analyzed using non-parametric tests. The Kruskal-Wallis rank sum test was used followed by the Conover's test for pairwise comparisons with the  $p$ -values ( $p < 0.05$ ) adjusted by the Benjamini, Hochberg, and Yekutieli correction method for multiple comparisons (Benjamini & Yekutieli, 2001; Iman & Conover, 1979; Kruskal & Wallis, 1952).

## 4.4 Results

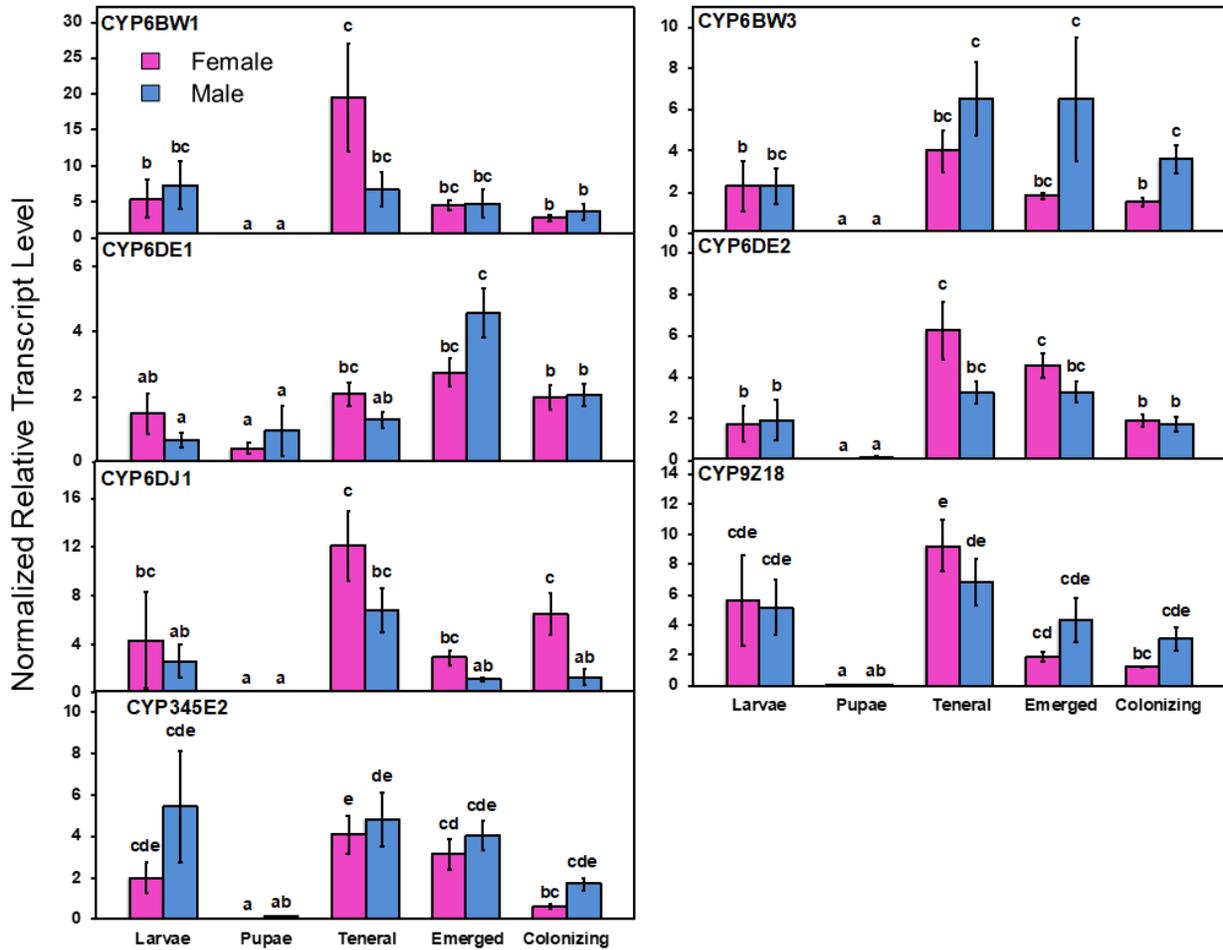
**4.4.1 Identification of P450s That May Function in Olfaction, Pheromone Biosynthesis, or Detoxification.** I identified twelve different P450s in the antennal cDNA library (**Table 4.1**). Of these, I selected six different P450s, *CYP345E2*, *CYP6DE1*, *CYP6DE2*, *CYP6BW3*, *CYP6DJ1* and *CYP9Z18* that were the most abundant P450 transcripts in the antennal cDNA library and also present in the midgut and fat body cDNA libraries (**Table 4.1**) for qPCR analysis. In addition, since *CYP6BW3* has over 96% amino acid identity with *CYP6BW1*, the latter P450 was also selected for qPCR analysis as it allowed us to assess divergence of closely related P450 genes by patterns of

expression. These seven P450s were included in the gene expression analysis across the different developmental stages of the MPB life cycle and different tissues.

**4.4.2 P450 Expression over the MPB Life Cycle.** The first objective of the qPCR analysis was to obtain quantitative information on the transcript abundance of the seven P450s over the MPB life cycle, during which beetles may use P450s for olfaction, pheromone biosynthesis or detoxification. The qPCR analysis of the life cycle covered five stages, specifically 4<sup>th</sup> instar larvae, pupae, teneral adults, freshly emerged adults, and a life stage that mimics the attack of a new host tree (colonizing adults). Analyses were performed across these life stages separately for females and males. Each replicate consisted of one individual, with eight replicates for each developmental stage and sex.

Transcript abundance varied by developmental stage, with the most pronounced difference being the relatively low levels of the P450 transcripts in the pupae (**Figure 4.1**). Specifically, for all of the P450 genes except *CYP6DE1* transcript abundance was significantly lower in the pupae compared to other life stages. For *CYP6DE1*, the transcript abundance was similar at the larval, pupal and teneral adult stages; however, transcript abundance was significantly lower in pupae compared to emerged and colonizing adults. For *CYP6BW1*, *CYP6DE1*, *CYP6DE2*, *CYP9Z18*, and *CYP345E2*, no significant differences were found between sexes at the same life stage. For *CYP6BW3*, transcript abundance was higher in male colonizing adults, compared to female

colonizing adults. And for *CYP6DJ1*, transcript abundance was significantly higher in female colonizing adults, compared to male colonizing adults.



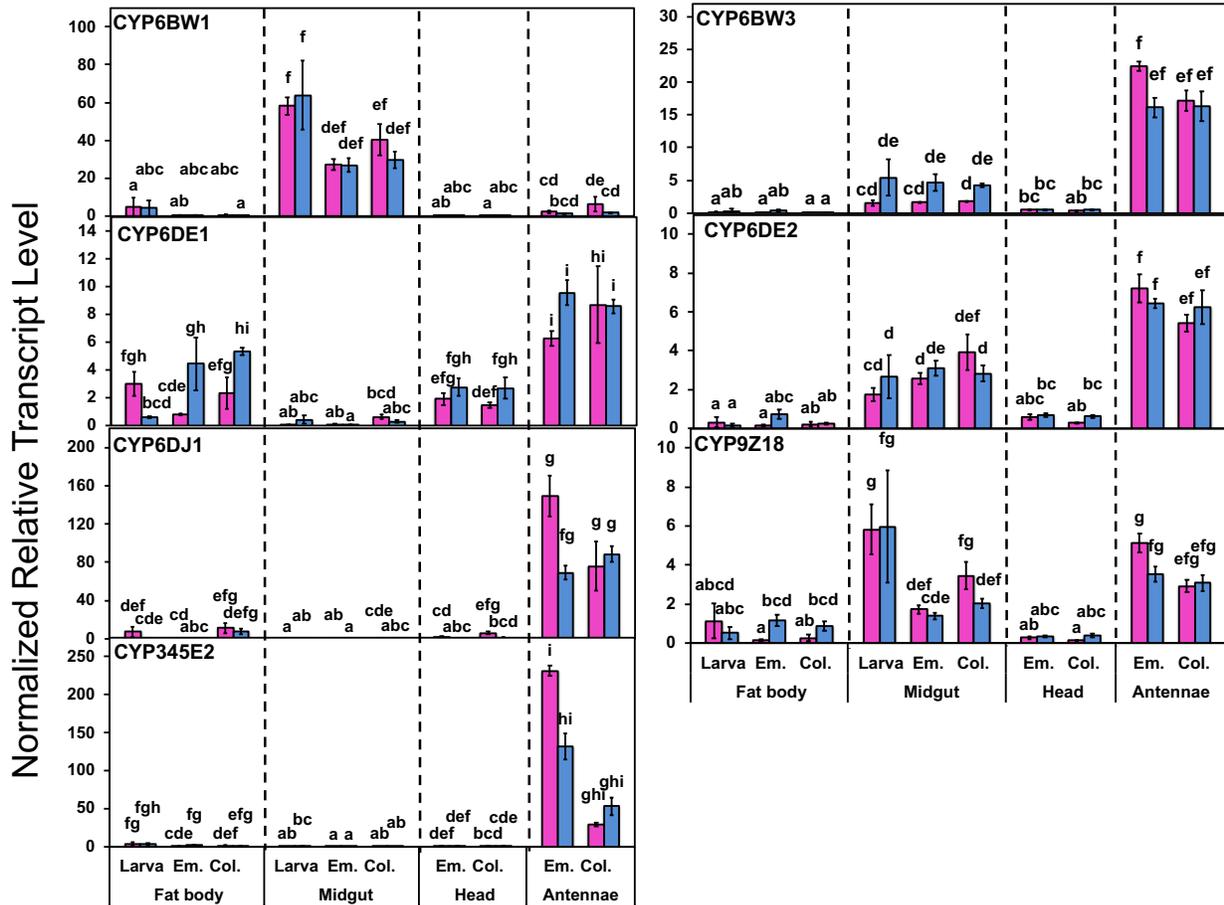
**Figure 4.1** The relative abundance of cytochrome P450 transcripts in female (pink) and male (blue) individuals over the life cycle of MPB. Each replicate consisted of a single insect, with n=8 replicates per life stage and sex. Columns in the same graph with different letters were significantly different by Conover's test ( $\alpha \leq 0.05$ ). Error bars represent standard error. During the larval to teneral adult stage, developing beetles remain in the brood tree. Emerged beetles are fully mature adults, which leave their brood tree in search of a new host. Colonizing beetles are insects that have been introduced to a new log, simulating the conditions of beetles attacking a new host

**4.4.3 P450 Expression across Different Tissue Types.** The second objective was to compare the P450 transcript abundance in different body parts, antennae, head, midgut, and fat body tissues. I performed qPCR analysis that sampled these body parts at three different stages of the life cycle, specifically 4<sup>th</sup> instar larvae, emerged adult, and colonizing adults. Analyses were performed across the different body parts for the three life stages separately for females and males. Antennae and head were not present for larvae. Each replicate consisted of dissected tissue from five (head, fat body, midgut) or ten (antennae) individuals, with four replicates for each sex, tissue and developmental stage (**Figure 4.2**).

*CYP6BW1* transcript abundance was 5 to 60 times higher in the midgut compared to the fat body, head or antennae. *CYP6BW3* transcripts were 5 to 20 times more abundant in the antennae than the head, midgut, or fat body. *CYP6DE2* transcripts were 5 to 7 times more abundant in the antennae compared to the head and fat body, and 2 to 4 times more abundant in the midgut compared to the head and fat body. *CYP6DJ1* transcripts were 10 to 150 times more abundant in the antennae than the head, midgut or fat body. Transcripts for *CYP9Z18* were 2 to 5 times more abundant in the antennae and midgut compared to the head and fat body. *CYP345E2* transcripts were 50 to 200 times more abundant in the antennae than the head, midgut or fat body. For these six P450s, no significant differences in transcript abundance were observed between sexes for the same tissue type and life stage.

*CYP6DE1* transcripts were 6 to 10 times more abundant in the antennae compared to the midgut tissues, and transcripts were also significantly more abundant in the fat body and head compared to the midgut tissue. *CYP6DE1* had significant sex-

specific differences in transcript abundance in the fat body tissue. In larval fat bodies, transcript abundance was higher in females. In contrast, in emergent and colonizing adult beetle fat bodies, the transcript abundance was higher in males.



**Figure 4.2** The relative abundance of cytochrome P450 transcripts in fat body, midgut, head, and antennae tissues in female (pink) and male (blue) MPB larvae, emerged beetles (Em.) and colonizing (Col.) beetles. Each replicate consisted of pooled tissue from five (n=5; head, fat body, midgut) or ten (n=10; antennae) dissected individuals. Each column represents four replicates (n=4). Columns in the same graph with different letters were significantly different by Conover's test ( $\alpha \leq 0.05$ ). Error bars represent standard error.

## 4.5 Discussion

The MPB genome contains 86 different P450 genes (Keeling et al., 2013c). Of these, only a few have been functionally characterized. Specifically, MPB *CYP345E2* is thought to be involved in olfaction, and the encoded enzyme oxidizes several different pine monoterpenes (Keeling et al., 2013b); *CYP6CR1* is involved in the biosynthesis of the male pheromone *exo-brevicommin* (Song et al., 2014); and the functional characterization of *CYP6DE3* suggests a role in the oxidation of various monoterpenes as part of detoxification (Nadeau et al., 2017). In general, MPB P450s have been proposed as important for processes such as olfaction, pheromone biosynthesis, and detoxification of host defenses. The present study was designed to identify and analyze transcript expression profiles of new P450 candidates potentially involved in those processes.

Our analysis showed that MPB *CYP6BW1* was highly expressed in the midgut while *CYP6BW3* was highly expressed in the antennae, which is in agreement with previous transcriptome and proteome analyses that showed that *CYP6BW1* transcripts are significantly higher in the midgut compared to the fat body (Keeling et al., 2016). The homologues of these P450s in other *Dendroctonus* species, *CYP6BW5v1* from *D. rhizophagus* and *CYP6BW5v3* from *D. valens*, which share over 90% amino acid identity to *CYP6BW1* and *CYP6BW3*, are expressed in both the antennae and alimentary canal (foregut, midgut and hindgut) (Cano-Ramírez et al., 2013; López et al., 2013). This may suggest that functions of *CYP6BW1* and *CYP6BW3* have diverged from a single P450 that had roles in both the antennae and midgut. The midgut specific

expression of *CYP6BW1* indicates a possible role in detoxification, while the antennae specific expression of *CYP6BW3* indicates a role in olfaction, such as odorant clearance.

Based on transcript expression, *CYP6DE1* may be the only one of the seven P450s analyzed here that has a function that includes the pupal stage. Its differential expression in the fat body during MPB development, including differential expression in females and males, may suggest a role in pheromone metabolism, with an additional role in olfaction as indicated by its transcript abundance in the antennae.

Previous transcriptome and proteome analyses showed that *CYP6DJ1* transcript and protein were significantly higher in females than males, and were induced by feeding on host tissue (Robert et al., 2013) and juvenile hormone III treatment (Keeling et al., 2016). The qPCR analysis presented here also showed that *CYP6DJ1* was significantly more highly expressed in the colonizing females compared to colonizing males. Homologs of MPB *CYP6DJ1*, specifically *CYP6DJ1v1* in *D. rhizophagus* and *CYP6DJ1v3* in *D. valens*, which share 91% amino acid identity, were also more highly expressed in females compared to males (Cano-Ramírez et al., 2013; López et al., 2013). These results, which are consistent across different *Dendroctonus* species investigated with different methods, showing higher abundance of *CYP6DJ1* in females compared to males at the adult stage, may suggest a role in female pheromone formation, possibly formation of *trans*-verbenol. The abundance of *CYP6DJ1* transcripts in the antennae indicates a dual role in olfaction.

Transcripts of *CYP6DE2* and *CYP9Z18* were abundant in both the midgut and antennae, indicating a role in both general detoxification and olfaction. Similar to the

results of our analysis in MPB, homologs of *CYP9Z18* transcripts in *D. armandi* are present in antennae, gut, and reproductive organs (Dai et al., 2015). In *D. valens*, *CYP9Z18* is down-regulated in female antennae and gut regions, but up-regulated in male antennae and gut after monoterpene exposure (López et al., 2013).

I found *CYP345E2* to be the most abundant P450 EST in the antennal cDNA library. The highly antenna-specific expression of *CYP345E2* transcript strongly suggests a role in olfaction. Previous work identified host odorants:  $\alpha$ -pinene,  $\beta$ -pinene, limonene, (+)-3-carene, (-)-camphene and (+)-sabinene as substrates for *CYP345E2*. (Keeling et al., 2013b).

In summary, differential transcript abundance in different tissues suggests specific or overlapping roles of P450s in olfaction for *CYP6BW3*, *CYP6DE1*, *CYP6DE2*, *CYP6DJ1*, *CYP9Z18*, and *CYP345E2*, in detoxification for *CYP6BW1*, *CYP6DE2*, and *CYP9Z18*, and in pheromone biosynthesis for *CYP6DE1* and *CYP6DJ1*. These results will inform the future functional characterization of these P450s to determine their specific substrates, products, and roles in MPB biology.

## 5. The cytochrome P450 CYP6DE1 catalyzes the conversion of $\alpha$ -pinene into the mountain pine beetle aggregation pheromone *trans*-verbenol<sup>4</sup>

### 5.1 Summary

A recent outbreak of the mountain pine beetle (*Dendroctonus ponderosae*; MPB) has affected over 20 M hectares of pine forests in western North America. During the colonization of host trees female MPB release the aggregation pheromone (–)-*trans*-verbenol. (–)-*trans*-Verbenol is thought to be produced from the pine defense compound (–)- $\alpha$ -pinene by cytochrome P450 (P450) dependent hydroxylation. Here I describe the functional characterization of MPB CYP6DE1 as a *trans*-verbenol synthase. CYP6DE1, but not the closely related CYP6DE2, converted the bicyclic monoterpenes (–)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (–)- $\beta$ -pinene, (+)- $\beta$ -pinene and (+)-3-carene. CYP6DE1 was not active with other monoterpenes or diterpene resin acids that were tested as substrates. CYP6DE1 produced *trans*-verbenol from blends of (–)- $\alpha$ -pinene and (+)- $\alpha$ -pinene with an enantiomeric product profile that was similar to that produced by female MPB exposed to the  $\alpha$ -pinene enantiomers.

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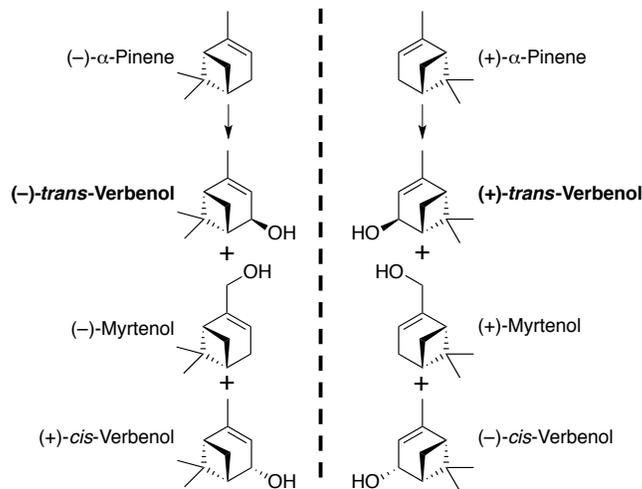
<sup>4</sup> A version of this chapter has been accepted to Scientific Reports

## 5.2 Introduction

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is a pest of several pine (*Pinus*) species in western North America (Safranyik & Carroll, 2006). While endemic MPB populations are mostly confined to weakened host trees, during epidemic outbreak phases MPB successfully colonize healthy mature trees (Boone et al., 2011; Burke & Carroll, 2017). Pines and other conifers are generally well defended against most herbivores by their production, accumulation and secretion of oleoresin terpenes (Bohlmann, 2012; Keeling & Bohlmann, 2006a; Raffa, 2014). However, conifer-feeding bark beetles and their associated microbiomes have evolved mechanisms to detoxify host terpenes (Boone et al., 2013; Wang et al., 2013, 2014). Bark beetles may also coopt metabolized host terpenes as signal molecules for their intraspecific communications (Seybold et al., 2006; Tittiger & Blomquist, 2016).

MPB can tolerate high concentrations of oleoresin monoterpenes (Chiu et al., 2017), which they experience throughout the life cycle from egg to adult and during different phases of activity from host colonization to dispersal. MPB also use volatile host monoterpenes as cues during the dispersal flight to locate new hosts (Borden et al., 2008). A fascinating chemo-ecological feature of MPB is their release of the aggregation pheromone *trans*-verbenol during host colonization (Borden et al., 1986; Pitman et al., 1968). (–)-*trans*-Verbenol [(1*S*,2*R*,5*S*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol] is a hydroxylated derivative of (–)- $\alpha$ -pinene, and is the active enantiomer, while (+)-*trans*-verbenol [(1*R*,2*S*,5*R*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol], which is inactive as a pheromone in MPB, is derived from (+)- $\alpha$ -pinene (**Figure 5.1**). Both (–)- $\alpha$ -

pinene and (+)- $\alpha$ -pinene are common monoterpenes of the oleoresin and volatile emissions of pines (Clark et al., 2010; Hall et al., 2013a).



**Figure 5.1** The two enantiomers of  $\alpha$ -pinene and their hydroxylated products *trans*-verbenol, myrtenol and *cis*-verbenol. (-)- $\alpha$ -Pinene is the precursor of (-)-*trans*-verbenol, (-)-myrtenol and (+)-*cis*-verbenol. (+)- $\alpha$ -Pinene is the precursor of (+)-*trans*-verbenol, (+)-myrtenol and (-)-*cis*-verbenol.

It was previously thought that only female MPB produce, and immediately release, *trans*-verbenol by oxidation of  $\alpha$ -pinene upon landing on a new host tree (Hughes, 1973a; Tittiger & Blomquist, 2016). However, I recently showed that both male and female MPB accumulate verbenol as fatty acid esters during their juvenile life stages, suggesting that formation of verbenol and verbenyl esters occurs in both sexes possibly as part of monoterpene detoxification in the feeding larvae (Chiu et al., 2018c). While both sexes accumulate verbenyl esters as larvae, only females retain these metabolites when they emerge as adults from the brood tree and may use them as a source for sex-specific pheromone release (Chiu et al., 2018c).

The enzymes and biochemical processes involved in MPB monoterpene detoxification and terpenoid pheromone formation may be, at least in part, identical (Chiu et al., 2018a; Nadeau et al., 2017). It is conceivable that MPB first evolved genes and enzymes for monoterpene detoxification as an essential process to survive host defenses, which then resulted in the formation of MPB-specific monoterpene derivatives that were coopted as pheromones. A prominent gene family in the MPB genome that may serve functions in terpene detoxification and pheromone biosynthesis as well as in olfaction of monoterpenes are the cytochrome P450s (P450s) (Keeling et al., 2013c). Of the 86 P450s identified in the MPB genome (Keeling et al., 2013c), only three have been reported as functionally characterized for their substrates and products. Specifically, CYP345E2, an antennae-specific P450 epoxidizes or hydroxylates (+)-3-carene, (–)-camphene and both enantiomers of  $\alpha$ -pinene,  $\beta$ -pinene and limonene (Keeling et al., 2013b). CYP6CR1 epoxidizes (Z)-6-nonen-2-one to 6,7-epoxynonan-2-one, a precursor of the male MPB pheromone *exo*-brevicommin (Song et al., 2014). CYP6DE3 converts (+)- $\alpha$ -pinene, 3-carene and (+)-limonene and produces (+)-*trans*-verbenol from (+)- $\alpha$ -pinene (Nadeau et al., 2017).

In a recent transcriptome screen of the MPB P450 gene family I identified P450s that are expressed in different body parts where monoterpene oxidation may occur, specifically in antennae for olfaction, as well as in the alimentary canal and fat body for detoxification and pheromone formation (Chiu et al., 2018a). Of the seven different MPB P450s identified in this screen, transcripts of *CYP6DE1* were highly abundant in antennae and fat body. The closest related gene family member *CYP6DE2* (83% amino acid identity) was most highly expressed in antennae and midgut tissues. *CYP6DE3*,

which is 72% identical to CYPDE1, was found in another study to be more highly expressed in unfed males compared to fed males, unfed and fed females (Nadeau et al., 2017).

Here I describe the heterologous expression of CYP6DE1 and CYP6DE2 proteins and their reconstitution with MPB cytochrome P450 reductase (CPR) for functional characterization. CYP6DE1 converted (–)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (–)- $\beta$ -pinene, (+)- $\beta$ -pinene and (+)-3-carene, and it produced *trans*-verbenol from blends of (–)- $\alpha$ -pinene and (+)- $\alpha$ -pinene with an enantiomeric product profile that closely resembled that of female MPB exposed to the two  $\alpha$ -pinene enantiomers.

## **5.3 Materials and Methods**

### **5.3.1 Insects**

MPB infested lodgepole pine (*P. contorta*) trees were felled near Whistler, BC, Canada (50°12'33.3"N 122°53'05.2"W) in October 2015 and (50°12'46.6"N 122°53'20.8"W) in September 2016. Stems were cut into bolts, which were placed in screened cages and stored indoors at the University of British Columbia at room temperature. Emerged beetles were collected every three to four days and segregated by sex based on abdominal tergite shape (Lyon, 1958).

### **5.3.2 Chemicals**

Chemicals obtained from Sigma-Aldrich (Mississauga, ON, Canada) were: *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, cat. No. 15209), methyl *tert*-butyl ether

(MTBE, cat. No. 650560), pentane (cat. No. 34956), (+)- $\alpha$ -pinene (cat. No. P45680), and (-)- $\alpha$ -pinene (cat. No. 274399), (+)- $\beta$ -pinene (cat. No. 80607), (-)- $\beta$ -pinene (cat. No. 112089), (+)-3-carene (cat. No. 441619), (-)-limonene (cat. No. 218367), (+)-limonene (cat. No. 62122), myrcene (cat. No. M100005), terpinolene (cat. No. 86485), (-)-*trans*-myrtanol (cat. No. W343900), (-)-*trans*-pinocarveol (cat. No. 80613). Chemicals obtained from Helix Biotech (Richmond, BC, Canada) were: abietic acid (cat. No. R002), dehydroabietic acid (cat. No. R001), neoabietic acid (cat. No. R003), levopimaric acid (cat. No. R005), pimaric acid (cat. No. R011). Chemicals obtained from PheroTech (Delta, BC, Canada) were: *trans*-verbenol (approx. 20(+):80(-) enantiomeric purity) (lot. No. W06-00141) and *cis*-verbenol (approx. 20(+):80(-) enantiomeric purity) (lot. No. CV001129). The (-)-*trans*-myrtanol (cat. No. 5134 S) was from Extrasynthese (Genay, France). The (-)- $\beta$ -phellandrene was obtained by purification from lodgepole pine turpentine by Synergy Semiochemicals (Burnaby, Canada).

### 5.3.3 Heterologous expression of CYP6DE1, CYP6DE2, and CPR

The full-length open reading frames of *CYP6DE1* (JQ855668, DPO0814\_E19) and *CYP6DE2* (JQ855669, DPO047\_M21) (Keeling et al., 2012) were cloned into the pFastBac vector (Invitrogen) and transformed into MAX Efficiency DH10Bac Competent cells (Invitrogen, cat. # 10361-012) to generate recombinant bacmids. Empty pFastBac vector was used to generate a recombinant bacmid for negative controls. Bacmids were used to transfect *Spodoptera frugiperda* Sf9 cells (Invitrogen, cat. # 1265-017) for production of baculovirus to a titer of  $2-5 \times 10^7$  IFU  $\times$  mL<sup>-1</sup>. The resulting baculovirus

was used to infect 250 mL of Sf9 cell culture (cell density  $7.5 \times 10^6$  cells  $\times$  mL<sup>-1</sup>) at a multiplicity of infection of one. The pelleted seed culture was incubated with the baculovirus culture for 1 h at 27°C and then resuspended in 250 mL of Sf-900 II serum-free media (Invitrogen, cat # 10902-088) with 10% fetal bovine serum. Hemin HCl (Sigma cat #51280) was added to a concentration of 2  $\mu$ g  $\times$  mL<sup>-1</sup> 24 h after the infection. Cells were harvested 72 h after infection. Cells were pelleted and washed three times with 50 mM potassium phosphate buffer (KPB), pH7.4. Cells were resuspended in P450 buffer (50 mM of KPB pH 7.4, 20% glycerol, 1 mM EDTA and 0.1 mM DTT), disrupted by sonification, and centrifuged for 1 h at 100,000  $\times$  g to collect the microsomes. Microsomes were suspended in 5 mL of P450 buffer and aliquots were frozen at -80°C until use. To test for the presence of the P450 proteins, 5  $\mu$ L of denatured microsomes were analyzed on a 12% SDS-PAGE gel, and P450 activity was checked by carbon monoxide (CO)-difference spectrum analysis (Guengerich et al., 2009; Omura & Sato, 1964). MPB cytochrome P450 reductase (CPR) (JQ855645) was expressed *E. coli* in as previously described (Keeling et al., 2013b).

### 5.3.4 Enzyme assays

*In vitro* assays with CYP6DE1 or CYP6DE2 were performed individually with ten different monoterpenes [(+)- $\alpha$ -pinene, (-)- $\alpha$ -pinene, (+)- $\beta$ -pinene, (-)- $\beta$ -pinene, (+)-limonene, (-)-limonene, (+)-3-carene, myrcene,  $\beta$ -phellandrene, and terpinolene] and five different diterpene resin acids (DRAs; abietic acid, dehydroabietic acid, neoabietic acid, levopimaric acid and pimaric acid). Assays with microsomes from empty vector expression, as well as assays with CYP6DE1 or CYP6DE2 microsomes without

NADPH, were used as negative controls. Microsomes from P450 or empty vector expression were combined with CPR microsomes and kept on ice for 1 h before being used in assays. Assays were prepared as follows: 25  $\mu\text{L}$  of P450 microsome (0.5-2.0  $\mu\text{M}$ ) and 2  $\mu\text{L}$  of CPR microsome (1U/mL) were added to 2-mL amber glass vials (Agilent, cat# 5182-00716), and KPB (pH 7.4) and NADPH were added for a final concentration of 50 mM KPB and 1 mM NADPH. To start the assays, 3  $\mu\text{L}$  of individual monoterpenes (10 mM in pentane) or DRAs (1 mM in MTBE) was added and the vial was immediately capped. The total assay volume was 300  $\mu\text{L}$ . Assays were incubated for 1 h at 30°C and then extracted three times with pentane (for assays with monoterpenes) or MTBE (for assays with DRAs). Extracts were concentrated under a  $\text{N}_2$  stream to 300  $\mu\text{L}$ . Extracts from assays with DRAs were derivatized by adding 5  $\mu\text{L}$  of BSTFA to 50  $\mu\text{L}$  of assay extract and letting the sample incubate overnight. Assay products were analyzed by gas chromatography coupled mass spectroscopy (GC-MS).

Assays to determine enzyme kinetics of CYP6DE1 were performed in triplicate with (+)- $\alpha$ -pinene and (-)- $\alpha$ -pinene at concentrations of 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 200  $\mu\text{M}$ , 400  $\mu\text{M}$ , 600  $\mu\text{M}$ , 800  $\mu\text{M}$ , and 1200  $\mu\text{M}$ . Assays were incubated for 1 h, then immediately frozen in liquid  $\text{N}_2$ , and kept frozen until extraction with pentane. Under these conditions, product formation maintained linearity for at least 100 min. Kinetic parameters were determined by nonlinear regression with the Michaelis-Menten model using ANEMONA (Hernández & Ruiz, 1998).

### 5.3.5 Treatment of MPB with $\alpha$ -pinene, $\beta$ -pinene and 3-carene

Female MPB used in this experiment were from lodgepole pine bolts collected in October 2015 and were 1-3 weeks past emergence at the time of treatment. Emergent beetles were exposed to vapours of (+)- $\alpha$ -pinene, (-)- $\alpha$ -pinene, (+)- $\beta$ -pinene, (-)- $\beta$ -pinene, or (+)-3-carene corresponding to 0.1  $\mu$ L volume of monoterpene per mL of airspace as previously described (Chiu et al., 2017). A 1.5 cm x 1.5 cm Whatman filter paper was placed into a 20 mL scintillation vial (VWR) and 2  $\mu$ L of the individual monoterpene was added to the filter paper. For controls, 1  $\mu$ L of acetone was used instead of monoterpene. Beetles were placed into the vials with one beetle per vial, and vials were capped. After 24 h living beetles were collected, frozen with liquid N<sub>2</sub> and kept at 80°C until extraction. Each beetle was extracted individually and monoterpene and monoterpenols analyzed by GC-MS as previously described (Chiu et al., 2018c). Treatment experiments were performed with four replicates for each monoterpene, each replicate consisting of a single female beetle.

#### *Tests for the stereoselective use of $\alpha$ -pinene by MPB and comparison with CYP6DE1*

Female MPB used in this experiment were from lodgepole pine bolts collected in September 2016 and were 9 days past emergence a time of treatment. Treatments of beetles with monoterpenes were performed as described above, except instead of individual monoterpenes, mixtures of (+)- $\alpha$ -pinene and (-)- $\alpha$ -pinene were used at different ratios with 2  $\mu$ L of blend placed on a 1.5 cm x 1.5 cm Whatman filter paper in a 20 mL glass vial. The ratios of (+)- $\alpha$ -pinene : (-)- $\alpha$ -pinene were 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10. Three replicates were performed for each ratio, each

replicate consisting of a single female beetle. For comparison, enzyme assays with CYP6DE1 were performed as described above with mixtures of (+)- $\alpha$ -pinene and (-)- $\alpha$ -pinene at the same ratios, with 3  $\mu$ L of 1 mM substrate in pentane added to the 300  $\mu$ L total assay volume. Enzyme assays were performed with three replicates. Data from tests of the stereoselective use of  $\alpha$ -pinene by MPB and CYP6DE1 were analyzed for differences in the proportion of (-)-*trans*-verbenol produced compared to the proportion (-)- $\alpha$ -pinene given as a substrate tested using a t-test ( $p < 0.05$ ).

### 5.3.6 GC-MS analysis

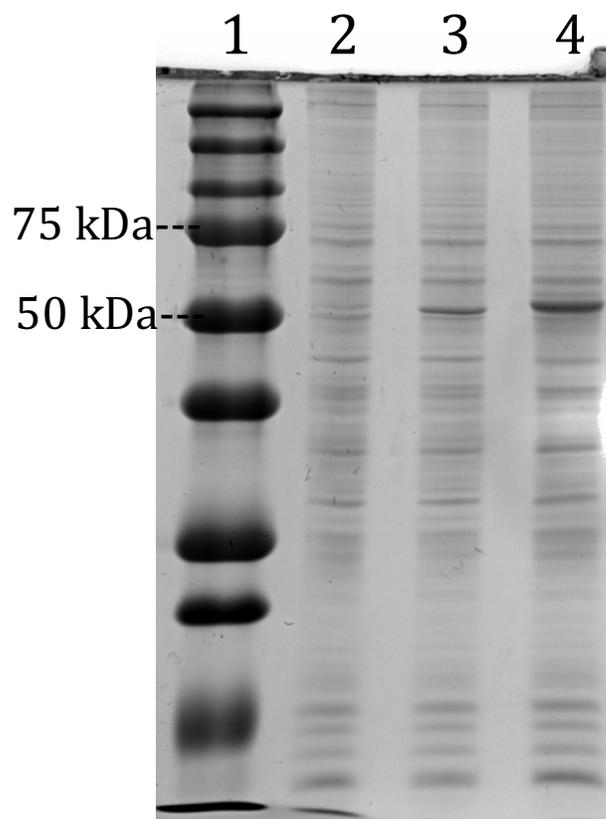
GC-MS analyses were performed on an Agilent 7890A system GC, Agilent GC Sampler 80, and a 7000A GC/MS triple quad M5975C inert XL MSD with triple axis detector at 70 eV. Monoterpenes and monoterpenols from the enzyme assays and treatment of MPB were analyzed by injecting sample volumes of 1  $\mu$ L onto a DB-WAX column (J&W, polyethylene glycol, 30 m, 250  $\mu$ m i.d., 0.25  $\mu$ m film thickness). Oven temperature for analysis of products from enzyme assays with monoterpenes was 40°C for 2 min, 8°C min<sup>-1</sup> to 100°C, 20°C min<sup>-1</sup> to 230°C and then held for 5 min. Oven temperature for analysis of beetle extracts was 40°C for 2 min, 8°C min<sup>-1</sup> to 100°C, 20°C min<sup>-1</sup> to 250°C and then held for 10 min. Products from enzyme assays with DRAs were analyzed by injecting a sample volume of 1  $\mu$ L onto a HP-5 column (J&W, 5% phenyl methyl siloxane, 27.4 m length, 250  $\mu$ m i.d., 0.25  $\mu$ m film thickness). Oven temperature for analysis of products from enzyme assays with DRAs was 40°C for 1 min, 20°C min<sup>-1</sup> to 300°C and then held for 8 min. Stereochemistry of  $\alpha$ -pinene and *trans*-verbenol from the enzyme assays and MPB treatment with mixtures of (+)- $\alpha$ -pinene and (-)- $\alpha$ -pinene were

analyzed by injecting sample volumes of 1  $\mu\text{L}$  onto a CyclodexB column (10.5%  $\beta$ -cyclodextrin, 25.7m length, 250  $\mu\text{m}$  i.d., 0.25- $\mu\text{m}$  film thickness). Oven temperature for analysis of products from enzyme assays and MPB extracts was 40°C for 2 min, increase at 10  $^{\circ}\text{C min}^{-1}$  to 100  $^{\circ}\text{C}$ , 20  $^{\circ}\text{C min}^{-1}$  to 230  $^{\circ}\text{C}$ , hold for 7 min with pulsed splitless injector held at 250  $^{\circ}\text{C}$ .

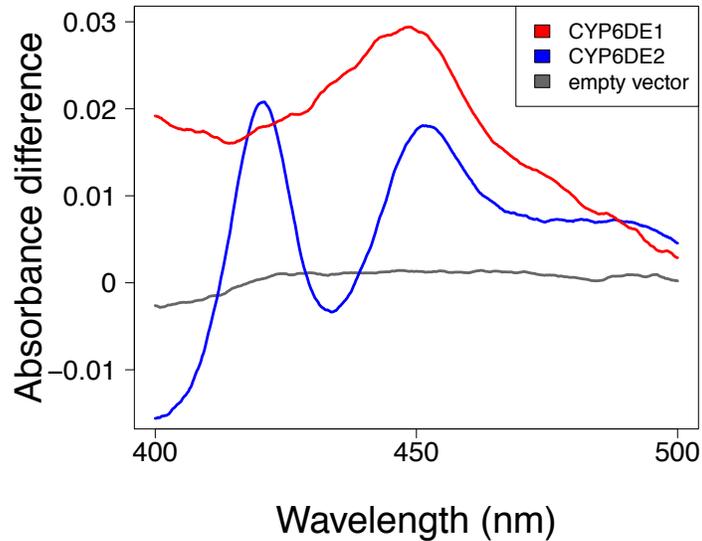
## 5.4 Results

### 5.4.1 CYP6DE1 converts some, but not all, host monoterpenes and is not active with diterpene resin acids

MPB CYP6DE1 and CYP6DE2 were produced in *S. frugiperda* cells, isolated as microsomal membrane-bound proteins (**Figure 5.2**), and identified as functional P450s based on CO-spectra (**Figure 5.3**). Both P450s were reconstituted with MPB CPR and tested in *in vitro* enzyme assays with ten different monoterpenes and five different DRAs. The substrates that were tested represent typical monoterpenes and DRAs found in the phloem of the MPB host lodgepole pine (Clark et al., 2014; Hall et al., 2013a, 2013b). While I did not detect activity of CYP6DE2 with any of the substrates tested (**Table 5.1**), CYP6DE1 oxidized both enantiomers of the bicyclic monoterpenes  $\alpha$ -pinene and  $\beta$ -pinene as well as (+)-3-carene (**Figure 5.4, Table 5.1**).



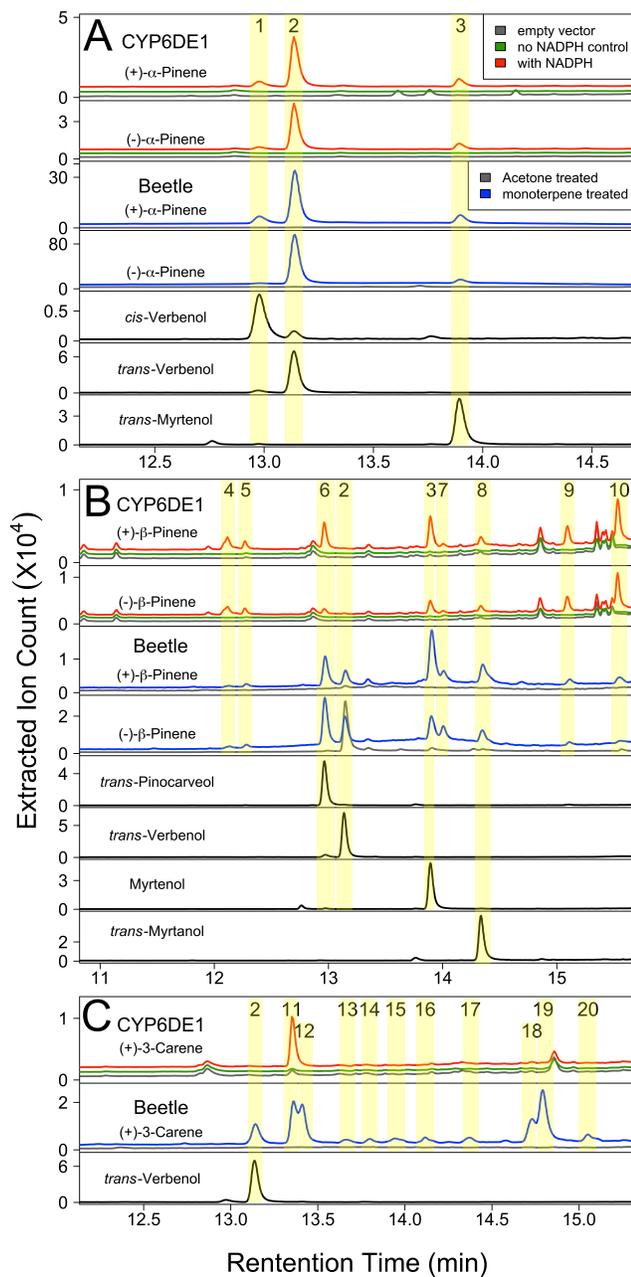
**Figure 5.2. Denatured CYP6DE1, CYP6DE2 and empty vector control microsomes on a 12% SDS-Page gel.** P450s were heterologously expressed in Sf9 cells. Lane 1: Precision Plus Protein ladder (Bio-rad). Lane 2: empty vector microsomes. Lane 3: CYP6DE1 microsomes, protein band is visible between 75 kDa and 50 kDa. Lane 4: CYP6DE2 microsomes, protein band is visible between 75 kDa and 50 kDa.



**Figure 5.3. CO Spectra of CYP6DE1, CYP6DE2 and empty vector control microsomes.** P450 microsomes that were harvested from Sf9 cells were tested for P450 activity according to Omura and Sato (1964). Absorbance difference is the difference in absorbance between reduced P450 microsomes and CO-bound P450 microsomes. CYP6DE1 and CYP6DE2 both produce a peak at 450 nm, indicating active P450 enzymes. CYP6DE2 also produces a peak at 420 nm, indicating inactive P450 enzymes. The empty vector control does not produce a peak at 420 or 450 nm, indicating the lack of abundant P450 enzymes in these microsomes.

**Table 5.1. Substrates tested in *in vitro* assays for activity with CYP6DE1 and CYP6DE2.**

Substrate	Activity Assay ( <i>in vitro</i> )	
	CYP6DE1	CYP6DE2
<b>Monoterpenes</b>		
(+)- $\alpha$ -pinene	✓	✗
(-)- $\alpha$ -pinene	✓	✗
(+)- $\beta$ -pinene	✓	✗
(-)- $\beta$ -pinene	✓	✗
(+)-limonene	✗	✗
(-)-limonene	✗	✗
(+)-3-carene	✓	✗
$\beta$ -phellandrene	✗	✗
myrcene	✗	✗
terpinolene	✗	✗
<b>Diterpene Resin Acids</b>		
abietic acid	✗	✗
neoabietic acid	✗	✗
dehydroabietic acid	✗	✗
palustric acid	✗	✗
isopimaric acid	✗	✗

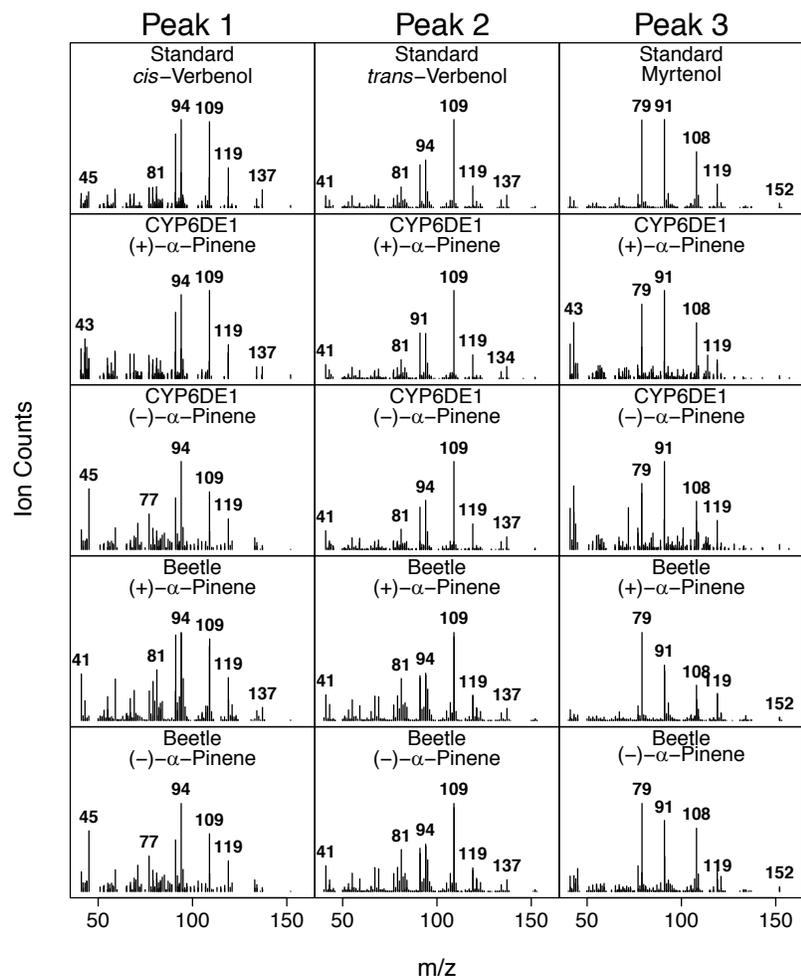


**Figure 5.4 GC-MS traces of products formed by CYP6DE1 in vitro enzyme assays with different monoterpenes, and GC-MS traces of products extracted from female MPB exposed to different monoterpenes. (A) In assays with (+)- $\alpha$ -pinene or (–)- $\alpha$ -pinene, both CYP6DE1 and beetles produced *trans*-verbenol (peak 2), with minor amounts of *cis*-verbenol (peak 1) and myrtenol (peak 3). (B) With (+)- $\beta$ -pinene or (–)- $\beta$ -pinene, both CYP6DE1 and beetles produced myrtenol (peak 3), *trans*-pinocarveol (peak 6), and *trans*-myrtanol (peak 8). Five other unidentified products were found in the products of CYP6DE1 and beetles treated with  $\beta$ -pinene (peaks 4, 5, 7, 9, 10). (C) With (+)-3-carene, both CYP6DE1 and beetle produced an unidentified product (peak 11). Nine other unidentified products were seen in (+)-3-carene treated beetles (peaks 12–21). (B) and (C) Extracts of beetles treated with acetone,  $\beta$ -pinene or (+)-3-carene also contained *trans*-verbenol (peak 2). Representative GC-MS traces are shown with the total of the extracted ions 91, 94, 108, 109, 119, 121, 137, 152. Retention indices and mass spectra are shown in **Table 5.2** and **Figures 5.5–5.6**.**

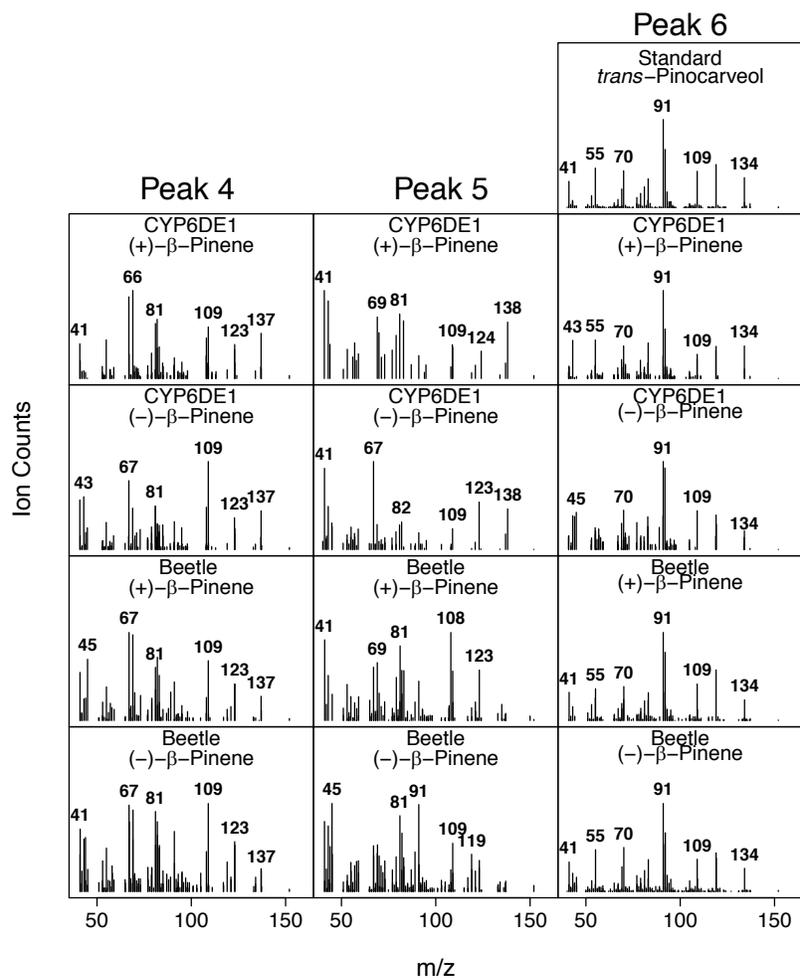
Assay products with CYP6DE1 were identified by comparison of retention times and mass-spectra with those of authentic standards (**Figure 5.5-5.9, Table 5.2**). In assays with (+)- $\alpha$ -pinene or (-)- $\alpha$ -pinene, CYP6DE1 produced *cis*-verbenol (peak 1), *trans*-verbenol (peak 2) and myrtenol (peak 3) (**Figure 5.4 A, Figure 5.5**). *cis*-Verbenol (peak 1), *trans*-verbenol (peak 2) and myrtenol (peak 3) were not detected in assays with the empty vector control, or with CYP6DE1 assays that did not contain the NADPH co-factor (**Figure 5.4 A, Figure 5.5**).

**Table 5.2. The retention index of all  $\alpha$ -pinene,  $\beta$ -pinene and 3-carene products of CYP6DE1 and from extracts of MPB after treatment.** All samples were injected onto a DB-Wax column. See Figure 5.4 to 5.9 for the gas chromatograms and mass spectra of these peaks

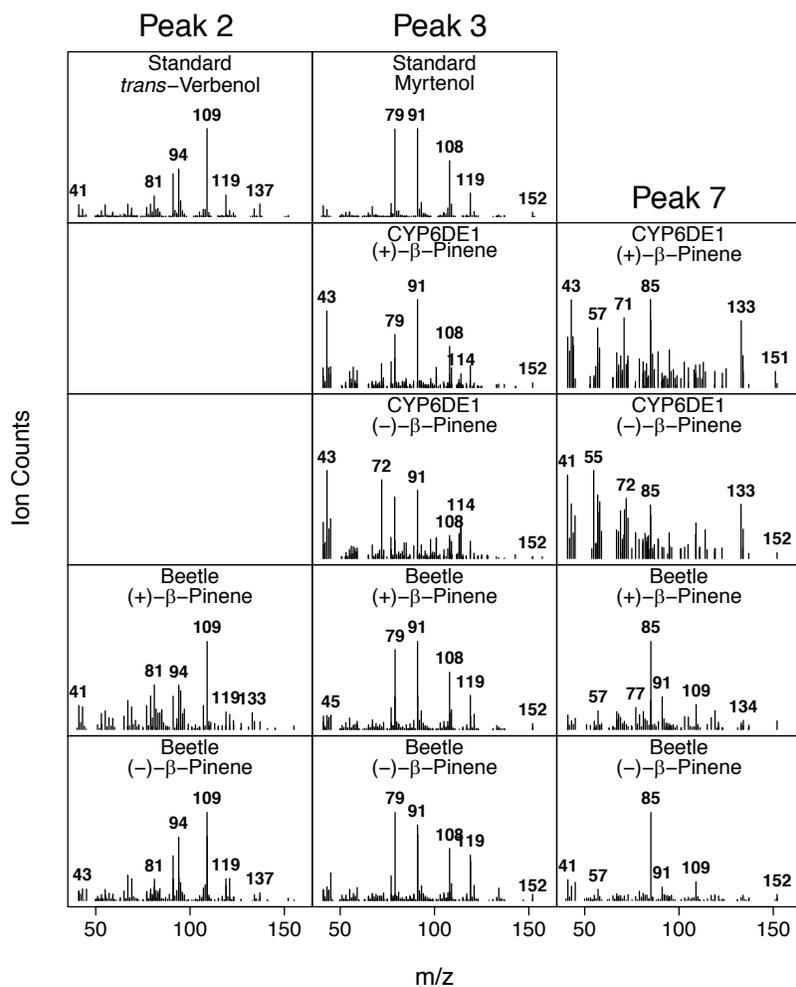
Reference	Retention Indices	Compound name
Peak 4	1553	$\beta$ -pinene product
Peak 5	1569	$\beta$ -pinene product
Peak 13	1637	3-carene product
Peak 8	1658	<i>trans</i> pinocarveol
Peak 1	1660	<i>cis</i> verbenol
Peak 2	1682	<i>trans</i> verbenol
Peak 11	1715	3-carene product
Peak 12	1723	3-carene product
Peak 14	1783	3-carene product
Peak 3	1799	myrtenol
Peak 15	1807	3-carene product
Peak 7	1817	$\beta$ -pinene product
Peak 16	1835	3-carene product
Peak 6	1873	<i>trans</i> myrtanol
Peak 17	1880	3-carene product
Peak 18	1943	3-carene product
Peak 19	1955	3-carene product
Peak 9	2007	3-carene product
Peak 20	2075	$\beta$ -pinene product
Peak 10	2093	$\beta$ -pinene product



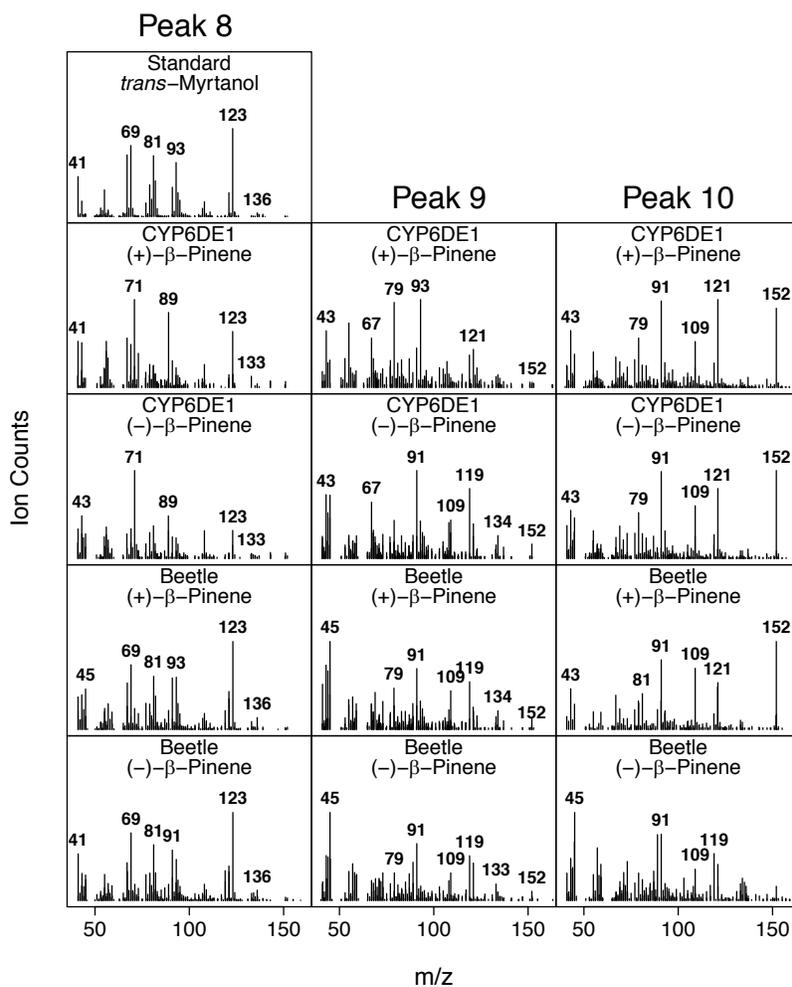
**Figure 5.5** Mass spectra of peaks 1-3 from the gas chromatograms of extracts of CYP6DE1 or female beetles treated with (+) and (-)- $\alpha$ -pinene along with the *cis* and *trans*-verbenol and myrtenol standards. Gas chromatograms with peak numbers can be found in Figure 5.4.



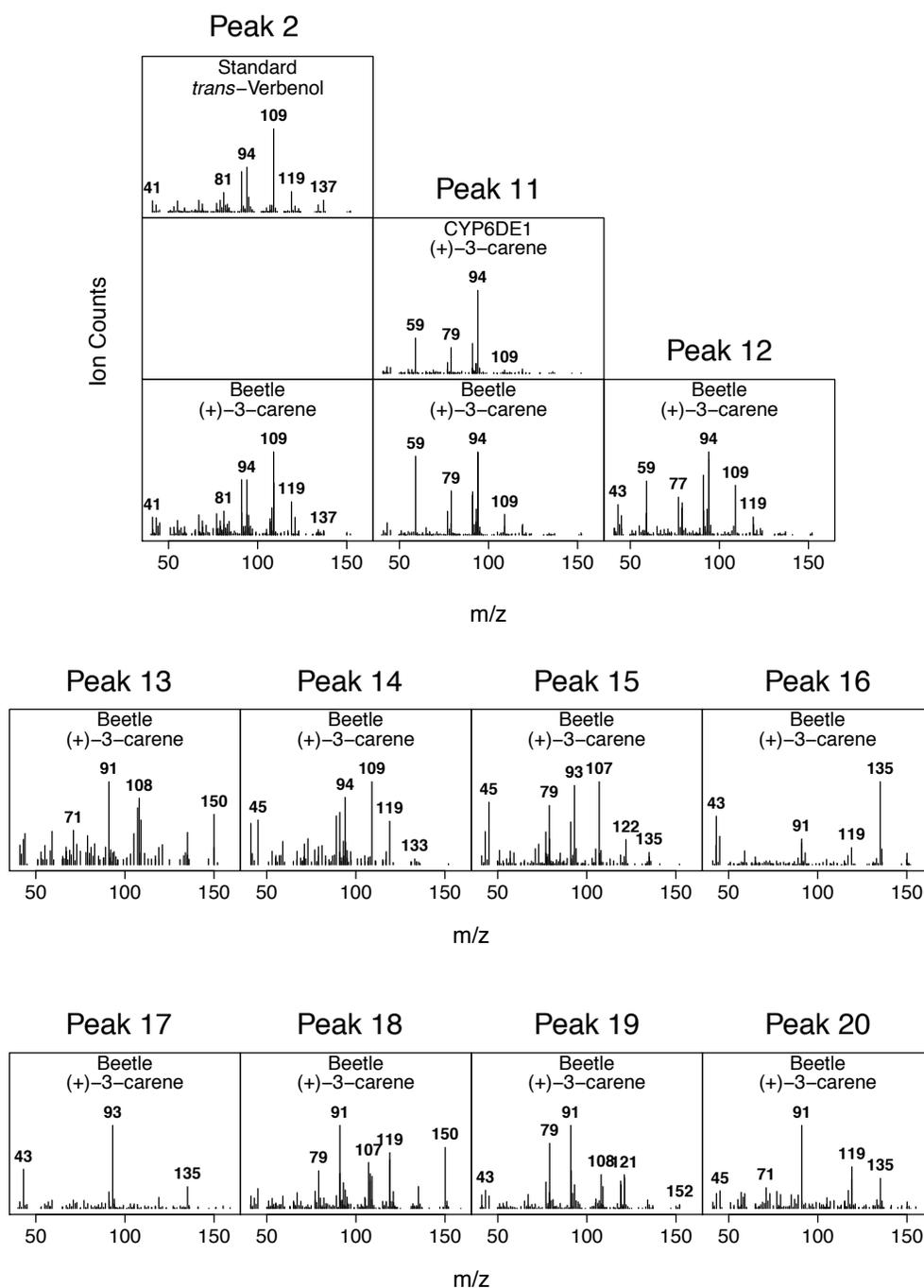
**Figure 5.6** Mass spectra of peaks 4-6 from the gas chromatograms of extracts of CYP6DE1 or female beetles treated with (+) and (-)- $\beta$ -pinene along with the *trans*-pinocarveol standard. Gas chromatograms with peak numbers can be found in Figure 5.4 B.



**Figure 5.7** Mass spectra of peaks 2,3,7 from the gas chromatograms of extracts of CYP6DE1 or female beetles treated with (+) and (-)-β-pinene along with the *trans*-verbenol and myrtenol standards. Gas chromatograms with peak numbers can be found in Figure 5.4 B.



**Figure 5.8** Mass spectra of peaks 8-10 from the gas chromatograms of extracts of CYP6DE1 or female beetles treated with (+) and (-)-β-pinene along with *trans*-myrtanol standards. Gas chromatograms with peak numbers can be found in Figure 5.4 B.



**Figure 5.9** Mass spectra of peaks 2, 11-20 from the gas chromatograms of extracts of CYP6DE1 or female beetles treated with (+)-3-carene along with monoterpenol standards. Gas chromatograms with peak numbers can be found in Figure 5.4 C.

Products from CYP6DE1 assays with (+)- $\beta$ -pinene or (-)- $\beta$ -pinene were myrtenol (peak 3), *trans*-pinocarveol (peak 6), and *trans*-myrtenol (peak 8), along with five other unidentified peaks (peaks 4, 5, 7, 9 and 10) (**Figure 5.4 B, 5.6-5.8**). Myrtenol (peak 3), *trans*-pinocarveol (peak 6), *trans*-myrtenol (peak 8), and the five unidentified peaks (peaks 4, 5, 7, 9 and 10) were not detected in assays with the empty vector control, or with CYP6DE1 assays that did not contain NADPH (**Figure 5.4 B, Figures 5.6-5.8**).

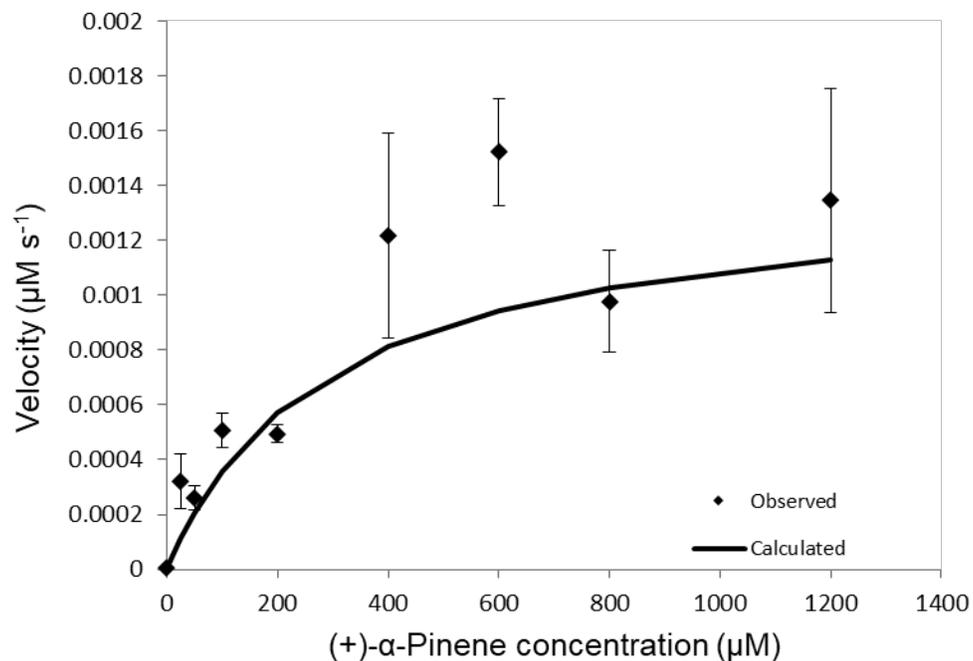
The product from CYP6DE1 assays with (+)-3-carene was a single unidentified peak (peak 11) (**Figure 5.4 C, Figure 5.9**). Peak 11 was not detected in assays with the empty vector control, or with CYP6DE1 assays that did not contain NADPH (**Figure 5.4 C, Figure 5.9**). CYP6DE1 was not active with the other five monoterpenes tested and was also not active with any of the diterpenes tested (**Table 5.1**).

#### 5.4.2 CYP6DE1 kinetics with $\alpha$ -pinene

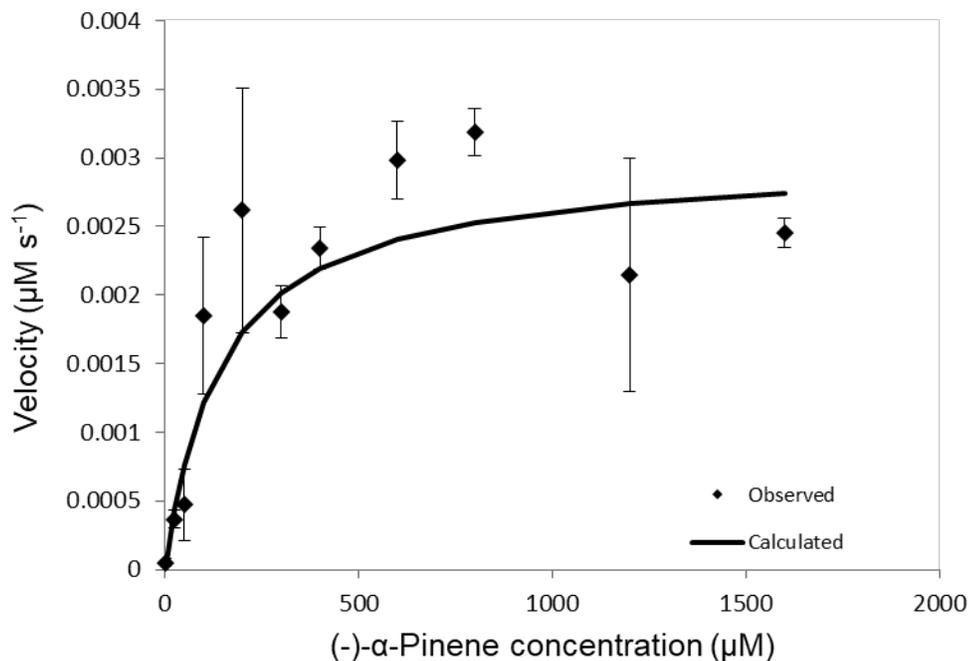
Kinetic parameters  $K_m$ ,  $k_{cat}$  and  $V_{max}$  of CYP6DE1 were in the same order of magnitude with (+)- $\alpha$ -pinene and (-)- $\alpha$ -pinene as substrates (**Table 5.3, Figure 5.10-5.11**). The enzyme appeared to be slightly more efficient *in vitro* with (-)- $\alpha$ -pinene with a catalytic efficiency ( $k_{cat}/K_m$ ) of  $2.3 \pm 0.9 \times 10^3 \text{ s}^{-1} \times \text{M}^{-1}$ , compared to  $k_{cat}/K_m$  of  $5.8 \pm 2.6 \times 10^2 \text{ s}^{-1} \times \text{M}^{-1}$  with (+)- $\alpha$ -pinene.

**Table 5.3 Kinetic properties of CYP6DE1**

	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ ( $\mu\text{M s}^{-1}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}} / K_m$ ( $\text{s}^{-1} \text{M}^{-1}$ )
(+)- $\alpha$ -Pinene	$292 \pm 1$	$0.007 \pm 0.003$	$0.17 \pm 0.08$	$5.8 \pm 2.6 \times 10^2$
(-)- $\alpha$ -Pinene	$160 \pm 1$	$0.016 \pm 0.006$	$0.37 \pm 0.15$	$2.3 \pm 0.9 \times 10^3$



**Figure 5.10 Michaelis-Menten saturation curve of CYP6DE1 with (+)-α-pinene as a substrate.** Calculated  $V_{max}$ ,  $K_m$  and  $k_{cat}$  values are shown in Table 5.3. Kinetic parameters were determined by nonlinear regression with the Michaelis-Menten model using ANEMONA (Hernández & Ruiz, 1998).



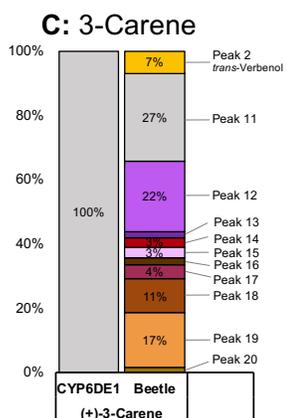
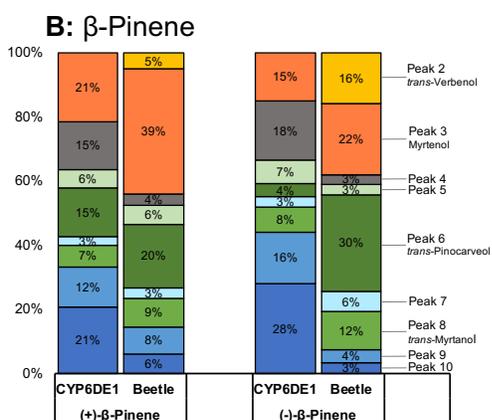
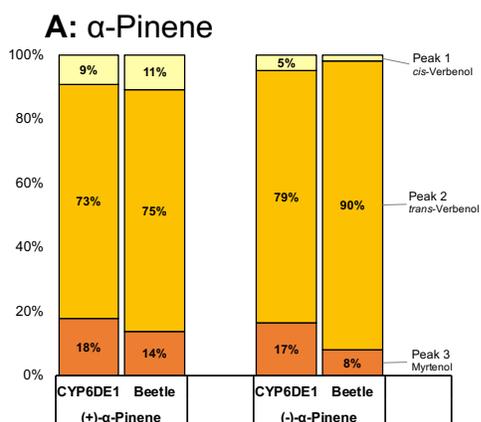
**Figure 5.11 Michaelis-Menten saturation curve of CYP6DE1 with (-)-α-pinene as a substrate.** Calculated  $V_{max}$ ,  $K_m$  and  $k_{cat}$  values are shown in Table 5.3. Kinetic parameters were determined by nonlinear regression with the Michaelis-Menten model using ANEMONA (Hernández & Ruiz, 1998).

#### 5.4.3 Product profiles of CYP6DE1 match products of female MPB exposed to monoterpenes

Since the activity of CYP6DE1 with (+)-α-pinene, (-)-α-pinene, (+)-β-pinene, (-)-β-pinene, and (+)-3-carene resulted in multiple products for each substrate, I measured the relative amounts of each product (**Figure 5.12**). I then compared the product

composition formed by CYP6DE1 *in vitro* with products extracted from female MPB that were exposed to the same five different monoterpenes.

Overall, the proportion of products formed by CYP6DE1 with (+)- $\alpha$ -pinene or (-)- $\alpha$ -pinene closely resembled the monoterpenols found in MPB exposed to the same monoterpenes (**Figure 5.4 A** and **Figure 5.12 A**). Extracts of MPB exposed to (+)- $\alpha$ -pinene contained *cis*-verbenol (peak 1), *trans*-verbenol (peak 2) and myrtenol (peak 3) (**Figure 5.4 A**). These metabolites were not identified in extracts from females exposed to acetone serving as a negative control (**Figure 5.4 A**). The relative composition of the product profile of (+)- $\alpha$ -pinene-treated MPB was 11% *cis*-verbenol, 75% *trans*-verbenol and 14% myrtenol (**Figure 5.12 A**). For comparison, the product profile of CYP6DE1 assays with (+)- $\alpha$ -pinene consisted of 9% *cis*-verbenol, 73% *trans*-verbenol and 18% myrtenol (**Figure 5.12 A**).



**Figure 5.12. Relative quantitative composition of products formed by CYP6DE1 in in vitro enzyme assays with different monoterpenes, and GC-MS traces of products extracted from female MPB exposed to different monoterpenes. (A) The percentage profile was calculated from total nanograms based upon response factors of authentic standards. (B) and (C) The percentage profile was calculated by peak area of the extracted ion chromatogram. Retention indices and mass spectra are shown in Table 5.2 and Figure 5.4-5.9. CYP6DE1 assays were performed with N=6 replicates; MPB treatment assay were performed with N=4 replicates.**

Beetles that were exposed to (–)- $\alpha$ -pinene also contained *cis*-verbenol (peak 1), *trans*-verbenol (peak 2) and myrtenol (peak 3) (**Figure 5.4 A**). The product profile of (–)- $\alpha$ -pinene-treated MPB consisted of 2% *cis*-verbenol, 90% *trans*-verbenol and 8% myrtenol (peak 3) (**Figure 5.12 A**). The product profile of CYP6DE1 assays with (–)- $\alpha$ -pinene contained 5% *cis*-verbenol, 79% *trans*-verbenol (peak 2) and 17% myrtenol (**Figure 5.12 A**).

MPB exposed to (+)- $\beta$ -pinene contained *trans*-verbenol (peak 2), myrtenol (peak 3), *trans*-pinocarveol (peak 6), *trans*-myrtanol (peak 8), and five unidentified peaks (peaks 4, 5, 7, 9 and 10) (**Figure 5.4 B**). Of these peaks, only peak 2 (*trans*-verbenol) was recorded in controls in which females had been exposed to acetone, presumably due to prior formation of this metabolite (Chiu et al., 2018c) (**Figure 5.4 B**). The terpenol profile of (+)- $\beta$ -pinene-treated MPB consisted of 5% *trans*-verbenol, 39% myrtenol, 4% peak 4, 6% peak 5, 20% *trans*-pinocarveol, 3% peak 7, 9% *trans*-myrtanol, 8% peak 9, and 6% peak 10. (**Figure 5.12 B**). For comparison, the product profile of CYP6DE1 assays with (+)- $\beta$ -pinene consisted of 21% myrtenol, 15% peak 4, 6% peak 5, 15% *trans*-pinocarveol, 3% peak 7, 7% *trans*-myrtanol, 12% peak 9, and 21% peak 10 (**Figure 5.12 B**).

MPB exposed to (–)- $\beta$ -pinene contained *trans*-verbenol (peak 2), myrtenol (peak 3), *trans*-pinocarveol (peak 6), *trans*-myrtanol (peak 8), and five unidentified peaks (peaks 4, 5, 7, 9 and 10) (**Figure 5.4 B**). The product profile of (–)- $\beta$ -pinene-treated MPB consisted of 16% *trans*-verbenol, 22% myrtenol, 3% peak 4, 3% peak 5, 30% *trans*-pinocarveol, 6% peak 7, 12% *trans*-myrtanol, 4% peak 9, and 3% peak 10 (**Figure**

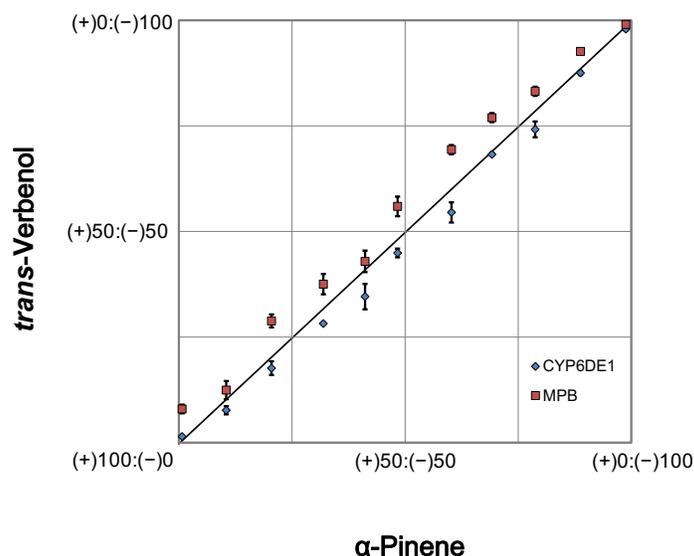
**5.12 B**). For comparison, the product profile of CYP6DE1 assays with (–)-β-pinene consisted of 15% myrtenol, 18% peak 4, 7% peak 5, 4% *trans*-pinocarveol, 3% peak 7, 8% *trans*-myrtenol, 16% peak 9, and 28% peak 10 (**Figure 5.12 B**).

MPB exposed to (+)-3-carene contained *trans*-verbenol (peak 2) and nine other unidentified peaks (peaks 11-20) (**Figure 5.4 C**). Again, only peak 2 (*trans*-verbenol) was recorded in controls with acetone treated females (**Figure 5.4 C**). The product profile of (+)-3-carene-treated beetle extracts consisted of 7% *trans*-verbenol, 27% peak 11, 22% peak 12, 2% peak 13, 3% peak 14, 3% peak 15, 2% peak 16, 4% peak 17, 11% peak 18, 17% peak 19, and 2% peak 20 (**Figure 5.12 C**). The product profile of CYP6DE1 assays with (+)-3-carene consisted of 100% peak 11 (**Figure 5.12 C**).

#### **5.4.4 In vitro activity of CYP6DE1 and female MPB show minor enantiomeric preferences in their utilization of α-pinene**

The conversion of both (+)-α-pinene and (–)-α-pinene by CYP6DE1 was of particular interest for two reasons: first, the product profiles of CYP6DE1 with these substrates closely matched the products observed in female MPB exposed to the same compounds (**Figure 5.12 A**); and second, only the (–) enantiomer of α-pinene can yield the pheromone-active (–)-enantiomer of *trans*-verbenol (**Figure 5.1**). In a host tree such as lodgepole pine, MPB are exposed to both enantiomers of α-pinene and their ratios may vary by pine species and genotype (Hall et al., 2013a; Taft et al., 2015b). I therefore tested the *in vitro* activity of CYP6DE1 with different mixtures of (+)-α-pinene and (–)-α-pinene, and I compared the enantiomeric composition of (+)-*trans*-verbenol

and (–)-*trans*-verbenol products with those detected in female MPB exposed to the same ratios of (+)- $\alpha$ -pinene and (–)- $\alpha$ -pinene (**Figure 5.13**).



**Figure 5.13.** The proportion of (–)-*trans*-verbenol and (+)-*trans*-verbenol produced by CYP6DE1 or female MPB in assays or treatments, respectively, with different ratios of (–)- $\alpha$ -pinene and (+)- $\alpha$ -pinene. A series of enantiomeric ratios of (–)- $\alpha$ -pinene and (+)- $\alpha$ -pinene were used as either a substrate for CYP6DE1 enzyme assays or in treatment assays with female MPB. The enantiomeric ratios of the corresponding *trans*-verbenol products were measured. CYP6DE1 assays were performed with N=3 replicates; MPB treatment assay were performed with N=3 replicates.

At all enantiomeric ratios of (+)- $\alpha$ -pinene : (–)- $\alpha$ -pinene exposure tested, female MPB produced a enantiomeric ratio of (+)-*trans*-verbenol : (–)-*trans*-verbenol that was slightly shifted towards a higher proportion of the (–)-enantiomer in the product compared to the substrate (**Figure 5.13**). For example, beetles that were treated with a (+)52%:(–)48% blend of  $\alpha$ -pinene produced a (+)44%:(–)56% ratio of *trans*-verbenol,

indicative of a minor preference (ratio shift by 8%) of utilizing the (–)- $\alpha$ -pinene. On average, for all ratios tested except for the 100% and 0% ratios, beetles had a 5% preference in utilizing the (–)- $\alpha$ -pinene over the (+)- $\alpha$ -pinene based on the observed product ratios of (–)-*trans*-verbenol : (+)-*trans*-verbenol. Differences in the enantiomeric ratio of (–)-*trans*-verbenol produced compared to the enantiomeric ratio of (–)- $\alpha$ -pinene used to treat beetles was tested using a t-test ( $p < 0.05$ ) and found to be significant ( $t = 8.0726$ ,  $df = 32$ ,  $p\text{-value} < 0.001$ ).

Using the set of different mixtures of (+)- $\alpha$ -pinene : (–)- $\alpha$ -pinene as substrate in *in vitro* assays with CYP6DE1 I observed a slight enantiomeric preference towards formation of (+)-*trans*-verbenol relative (–)-*trans*-verbenol. On average CYP6DE1 had an apparent 3% preference in utilizing the (+)- $\alpha$ -pinene over the (–)- $\alpha$ -pinene based on the observed product ratios of (–)-*trans*-verbenol : (+)-*trans*-verbenol. Differences in the proportion of (–)-*trans*-verbenol produced compared to the proportion (–)- $\alpha$ -pinene given as a substrate were tested using a t-test ( $p < 0.05$ ) and found to be significant ( $t = -5.3198$ ,  $df = 30$ ,  $p\text{-value} < 0.001$ ).

## 5.5 Discussion

I showed that CYP6DE1 has a biochemical function in the oxidation of the bicyclic monoterpenes (–)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (–)- $\beta$ -pinene, (+)- $\beta$ -pinene and (+)-3-carene. To our knowledge, these results are the first to show that a bark beetle P450 produces (–)-*trans*-verbenol, a monoterpene that serves as a pheromone in the aggregation behaviour of the MPB. The MPB P450 CYP6DE3 was previously reported to convert (+)- $\alpha$ -pinene into (+)-*trans*-verbenol (Nadeau et al., 2017), but assays with (–)- $\alpha$ -pinene were not reported. It is therefore not known whether CYP6DE1 and

CYP6DE3 have overlapping or distinct biochemical functions in their activity with the two enantiomers of  $\alpha$ -pinene. However, unlike with CYP6DE3, I did not find  $\alpha$ -pinene oxide to be a product of CYP6DE1.  $\alpha$ -Pinene oxide is not released by attacking female MPB.

In contrast to CYP6DE1 and CYP6DE3, no enzyme activity has been reported for CYP6DE2. The use of several, but not all, monoterpenes as substrates suggest that CYP6DE1 may serve a general role in monoterpene detoxification and monoterpene removal, while it may also contribute to pheromone biosynthesis. Testing the possible biological roles of CYP6DE1 further *in vivo* will require gene editing or RNAi knock down experiments. I have successfully used RNAi with a few other MPB genes (Keeling et al., 2013a), but I have not been able to use RNAi to significantly repress the *CYP6DE1* transcript in beetles.

The monoterpenol product profiles of CYP6DE1 with (+)- $\alpha$ -pinene or (-)- $\alpha$ -pinene were the same as those found in female MPB exposed to these monoterpenes, both in terms of qualitative and quantitative composition. CYP6DE1 produced mostly *trans*-verbenol with minor amounts of *cis*-verbenol and myrtenol, similar to the monoterpenol composition released by female beetles as an aggregation pheromone when colonizing a new host tree (Taft et al., 2015a). By comparison, the product profiles of CYP6DE1 with (+)- $\beta$ -pinene, (-)- $\beta$ -pinene or (+)-3-carene were substantially different in their quantitative or qualitative composition from the monoterpenols extracted from beetles treated with the same compounds. These results suggest that additional P450s are active in MPB that substantially contribute to the conversion of (+)- $\beta$ -pinene, (-)- $\beta$ -

pinene and (+)-3-carene, while CYP6DE1 may be a product-defining enzyme in the conversion of (+)- $\alpha$ -pinene or (-)- $\alpha$ -pinene in MPB.

In MPB exposed to  $\beta$ -pinene, (+)-3-carene or acetone (control), I also observed *trans*-verbenol, which can be explained by the prior formation of this compound during the early life stages prior to the experiments (Chiu et al., 2018c). I did not detect *trans*-verbenol in assays of CYP6DE1 with  $\beta$ -pinene or (+)-3-carene.

The aggregation pheromone produced by female MPB consists of 87% - 97% (-)-*trans*-verbenol compared to (+)-*trans*-verbenol (Chiu et al., 2018c; Erbilgin et al., 2014; Keeling et al., 2016). I tested if female MPB in *in vivo* assays, or CYP6DE1 in *in vitro* assays, would be similarly selective for utilizing (-)- $\alpha$ -pinene over (+)- $\alpha$ -pinene in the formation of *trans*-verbenol. Interestingly, female MPB only had a minor preference for utilizing (-)- $\alpha$ -pinene over (+)- $\alpha$ -pinene, while CYP6DE1 appeared to have a minor preference for converting (+)- $\alpha$ -pinene when both substrates tested simultaneously. The CYP6DE1 *in vitro* kinetic parameters were similar for (-)- $\alpha$ -pinene and (+)- $\alpha$ -pinene, and CYP6DE1 appeared to be only slightly more catalytically efficient with (-)- $\alpha$ -pinene. Thus the availability of (-)- $\alpha$ -pinene relative to (+)- $\alpha$ -pinene in the host oleoresin may be a critical factor in controlling the formation (-)-*trans*-verbenol compared to (+)-*trans*-verbenol, while female MPB and CYP6DE1 do not appear to be substantially selective for one substrate enantiomer or the other.

*CYP6DE1* transcripts are expressed in female and male MPB at different stages of the life cycle, including larvae, pupae, teneral adults, freshly emerged adults and host colonizing adults (Chiu et al., 2018a). In both sexes of emerged and colonizing MPB, *CYP6DE1* transcripts are expressed most abundantly in antennae and fat body, and at

relative lower levels in the midgut (Chiu et al., 2018a). Female and male MPB accumulate verbenyl esters during the juvenile life stages from larvae to teneral adults, and in female emerging adults verbenyl esters are most abundant in the abdomen and specifically in the fat body of the alimentary canal (Chiu et al., 2018c). The combined knowledge on transcript profiles and biochemical functions of CYP6DE1, together with the recent discovery of accumulation of verbenyl esters in the early life stages of female and male MPB (Chiu et al., 2018c), suggest that CYP6DE1 may be involved in the formation of verbenyl esters via hydroxylation of  $\alpha$ -pinene.

The biochemical functional characterization of an MPB P450 that catalyzes the formation of (–)-*trans*-verbenol from (–)- $\alpha$ -pinene, along with the oxidation of other host monoterpenes, may provide either a biochemical tool to produce pheromones for pest control agents against MPB.

## **6. Functions of mountain pine beetle cytochromes P450 CYP6DJ1, CYP6BW1 and CYP6BW3 in the oxidation of pine monoterpenes and diterpene resin acids**

### **6.1 Summary**

The mountain pine beetle (MPB; *Dendroctonus ponderosae*) is a bark beetle that attacks various pine species in western North America. Species of pine (*Pinus*) produce oleoresin, comprised mainly of monoterpenes and diterpene resin acids, as a defense against herbivores and pathogens. I have functionally characterized three MPB cytochromes P450, CYP6DJ1, CYP6BW1 and CYP6BW3 that have been identified in previous transcriptome analyses and show that these P450s oxidize various terpenoids present in pine resin. CYP6DJ1 oxidizes the monoterpenes limonene and terpinolene and produces *cis*-limonene-8,9-epoxide, *trans*-limonene-8,9-epoxide, perilla alcohol and several unidentified oxidized compounds. These products of CYP6DJ1 were also identified in extracts of limonene-treated MPB. CYP6BW1 and CYP6BW3 both oxidized the diterpene resin acids abietic acid, dehydroabietic acid, neoabietic acid, levopimaric acid, palustric acid, and isopimaric acid, and produced hydroxylated and epoxidized products. I propose that CYP6DJ1, CYP6BW1 and CYP6BW3 contribute to the metabolism of terpenoids in the MPB, potentially enabling the beetle to cope with these hosts defenses.

## 6.2 Introduction

The mountain pine beetle (MPB; *Dendroctonus ponderosae*) is a native eruptive forest insect, that occasionally causes the mortality of mature pine trees across forested landscapes of western North America (Safranyik & Wilson, 2006). As part of its lifecycle, which is spent mostly beneath the bark of its host trees, the MPB is exposed to oleoresin defenses (Clark et al., 2012). Oleoresin is mostly comprised of monoterpenes and diterpene resin acids (DRAs) (Zulak & Bohlmann, 2010). The relationship between MPB and terpenoids have been studied extensively as these compounds have a role as defensive chemicals, kairomones, and pheromone precursors in the complex chemical ecology of the MPB-pine host-associate system (Raffa, 2014). Terpenoids in high concentration are toxic to MPB (Chiu et al., 2017); however, the MPB and its microbial associates can also detoxify and in some case repurpose some of these compounds (Chiu et al., 2018c; Pierce et al., 1987; Wang et al., 2013, 2014). For example, female MPB produce an aggregation pheromone *trans*-verbenol from  $\alpha$ -pinene (Pitman et al., 1968; Seybold et al., 2006), a monoterpene abundant in the pine hosts.

Cytochromes P450 (P450s) are enzymes that have been proposed to be involved in detoxification and pheromone biosynthesis in the MPB (Hunt & Smirle, 1988; Keeling et al., 2013b, 2013c; Robert et al., 2013; Tittiger & Blomquist, 2016). In a recent study (Chapter 4), I analyzed the transcript abundance of seven MPB P450 genes in different tissues where monoterpene and DRA oxidation may occur, specifically in antennae for olfaction, as well as in the alimentary canal and fat body for detoxification and pheromone formation (Chiu et al., 2018a). Of the seven different MPB P450s identified in this screen, transcripts of *CYP6DJ1* and *CYP6BW1* were highly abundant in

antennae, while *CYP6BW3* was highly abundant in the midgut. *CYP6BW1* and *CYP6BW3* share 96% amino acid identity and their expression in different tissues may reflect divergent roles in olfaction versus detoxification. Analysis of transcript abundance in male and female MPB at different life stages, specifically 3<sup>rd</sup> instar larvae, pupae, teneral, emerging and colonizing adults revealed sex-specific differences in the expression of these P450s (Chiu et al., 2018a). Transcript abundance of *CYP6DJ1* was significantly higher in colonizing females compared to colonizing males, while transcript abundance of *CYP6BW3* was higher in colonizing males. These results may suggest different roles for *CYP6DJ1* and *CYP6BW3* in female and male pheromone formation. Here, I investigate the role of *CYP6DJ1*, *CYP6BW1* and *CYP6BW3* in monoterpene and diterpene oxidation by testing the heterologously expressed P450s *in vitro* against ten monoterpenes and six DRAs as substrates.

## **6.3 Materials and Methods**

### **6.3.1 Insects**

MPB infested lodgepole pine (*P. contorta*) trees were felled near Whistler, BC, Canada (50°12'33.3"N 122°53'05.2"W) in October 2015. Stems were cut into 50 cm bolts, which were placed in screened cages and stored indoors at the University of British Columbia at room temperature. Emerged beetles were collected every three to four days and segregated by sex based on abdominal tergite shape (Lyon, 1958).

### 6.3.2 Chemicals

Chemicals obtained from Sigma-Aldrich (Mississauga, ON, Canada) were: *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, cat. No. 15209), methyl *tert*-butyl ether (MTBE, cat. No. 650560), pentane (cat. No. 34956), (+)- $\alpha$ -pinene (cat. No. P45680), and (–)- $\alpha$ -pinene (cat. No. 274399), (+)- $\beta$ -pinene (cat. No. 80607), (–)- $\beta$ -pinene (cat. No. 112089), (+)-3-carene (cat. No. 441619), (–)-limonene (cat. No. 218367), (+)-limonene (cat. No. 62122), myrcene (cat. No. M100005), terpinolene (cat. No. 86485), limonene-1,2-epoxide (cat. No. 218332), limonene 1,2-diol (W440900), perilla alcohol (cat. No. 218391), carveol (cat. No. 192384), and  $\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH, cat. No. N7505). Chemicals obtained from Helix Biotech (Richmond, BC, Canada) were: abietic acid (cat. No. R002), dehydroabietic acid (cat. No. R001), neoabietic acid (cat. No. R003), levopimaric acid (cat. No. R005), and isopimaric acid (cat. No. R011). *trans*-Verbenol (approx 20(+):80(–) optical purity) (lot. No. W06-00141) was obtained from PheroTech (Delta, BC, Canada). Limonene-8,9-epoxide (cat. No. ZEP0020) was obtained from Endeavour Speciality Chemicals (Daventry, UK). (–)- $\beta$ -Phellandrene was obtained by purification from lodgepole pine turpentine by Synergy Semiochemicals (Burnaby, Canada).

### 6.3.3 Identification and heterologous expression of CYP6DJ1, CYP6BW1 and CYP6BW3

*CYP6DJ1* (JQ855677), *CYP6BW1* (JQ855661) and *CYP6BW3* (JQ855663) were identified in the MPB transcriptome (Keeling et al., 2012) as described in (Chiu et al.,

2018a). The full open reading frames of the pDNR-LIB EST clones *CYP6DJ1* (DPO0411\_I13), *CYP6BW1* (DPO079\_G21), *CYP6BW3* (DPO049\_N22) were sub-cloned into the pFastBac vector (Invitrogen) and transformed into MAX Efficiency DH10Bac Competent cells (Invitrogen, cat. # 10361-012) to generate recombinant bacmids. Empty pFastBac vector was used to generate a recombinant bacmid for negative controls. Bacmids were used to transfect *Spodoptera frugiperda* Sf9 cells (Invitrogen, cat. # 1265-017) for production of baculovirus to a titer of  $3-4 \times 10^7$  IFU mL<sup>-1</sup>. The resulting baculovirus was used to infect 250 mL of Sf9 cell culture (cell density  $1.5 \times 10^6$  cells mL<sup>-1</sup>) at a multiplicity of infection of one. The pelleted seed culture was incubated with the baculovirus culture for 1 h at 27°C and then resuspended in 250 mL of Sf-900 II serum-free media (Invitrogen, cat # 10902-088) with 10% fetal bovine serum. Hemin HCl (Sigma cat #51280) was added to a concentration of 2 µg mL<sup>-1</sup> 24 h after the infection. Cells were harvested 72 h after infection. Cells were pelleted and washed three times with a 50 mM potassium phosphate buffer solution (KPB), pH7.4. Cells were resuspended in P450 buffer (50 mM of KPB pH 7.4, 20% glycerol, 1 mM EDTA and 0.1 mM DTT), disrupted by sonification, and centrifuged for 1 h at 100,000 x g to collect the microsomes. Microsomes were suspended in 5 mL of P450 buffer and aliquots were frozen at -80°C until use. To test for the presence of the P450 proteins, 5 µL of denatured microsomes were analyzed on a 12% SDS-PAGE gel, and P450 activity was checked by carbon monoxide (CO)-difference spectrum analysis (Guengerich et al., 2009; Omura & Sato, 1964). MPB CPR (JQ855645) was expressed in *E. coli* as previously described (Keeling et al., 2013b).

### 6.3.4 Enzyme assays

*In vitro* assays with CYP6DJ1, CYP6BW1 and CYP6BW1 were performed individually with each P450 and substrate using ten different monoterpenes [(+)- $\alpha$ -pinene, (-)- $\alpha$ -pinene, (+)- $\beta$ -pinene, (-)- $\beta$ -pinene, (+)-limonene, (-)-limonene, (+)-3-carene, myrcene,  $\beta$ -phellandrene, and terpinolene] and five different DRAs (abietic acid, dehydroabietic acid, neoabietic acid, levopimaric acid, and isopimaric acid). The substrates that were tested represent typical monoterpenes and DRAs found in the phloem of the MPB host lodgepole pine (Clark et al., 2014; Hall et al., 2013a, 2013b). Assays with microsomes from empty vector expression, as well as assays with P450 microsomes without NADPH, were used as negative controls. Microsomes from P450 or empty vector expression were combined with CPR microsomes and kept on ice for 1 h before being used in assays. Assays were prepared as follows: 25  $\mu$ L of P450 microsome (0.5-2.0  $\mu$ M) and 2  $\mu$ L of CPR microsome (1U mL<sup>-1</sup>) were added to 2 mL amber glass vials (Agilent, cat# 5182-00716), and KPB (pH 7.4) and NADPH were added for a final concentration of 50 mM KPB and 1 mM NADPH. To start the assays, 3  $\mu$ L of individual monoterpenes (10 mM in pentane) or DRAs (1 mM in MTBE) were added and the vial was immediately capped. The total assay volume was 300  $\mu$ L. Assays were incubated for 1 h at 30°C and then extracted three times with pentane (for assays with monoterpenes) or MTBE (for assays with DRAs). Extracts were concentrated under a N<sub>2</sub> stream to 300  $\mu$ L. Extracts from assays with DRAs were derivatized by adding 5  $\mu$ L of BSTFA to 50  $\mu$ L of assay extract and letting the sample incubate overnight. Assay products were analyzed by gas chromatography coupled mass spectroscopy (GC-MS).

### 6.3.5 Beetle Treatment with limonene and terpinolene

Female emergent beetles were exposed to vapours of (+)-limonene, (–)-limonene or terpinolene corresponding to 0.05  $\mu\text{L}$  ((+) and (–)-limonene) or 0.1  $\mu\text{L}$  (terpinolene) volume of monoterpene per mL of airspace as described in (Chiu et al., 2017). Beetles were 6-13 days past emergence at time of treatment. A 1.5 cm x 1.5 cm Whatman filter paper was placed into a 20 mL scintillation vial (VWR) and 1  $\mu\text{L}$  of (+) or (–)-limonene or 2  $\mu\text{L}$  of terpinolene was added to the filter paper. For controls, 1  $\mu\text{L}$  of acetone was used instead of monoterpene. Females were placed into the vials with one beetle per vial, and vials were capped. After 24 h living females were collected, frozen with liquid  $\text{N}_2$  and kept at  $80^\circ\text{C}$  until extraction. Each beetle was extracted individually with MTBE and the products analyzed by GC-MS. Each replicate consisted of a single extracted female beetle and four replicates were performed per monoterpene treatment. Frozen beetles were crushed individually in a 2.0 ml Safe-Lock tube (Eppendorf) over dry ice, using a cold glass stir rod, in 0.5 ml of MTBE containing 1  $\text{ng } \mu\text{L}^{-1}$  of tridecane added as an internal standard. The crushed beetle was removed from dry ice, allowed to thaw for a few minutes and centrifuged for 20s at  $2000 \times g$ . The crushed beetle was then frozen again on dry ice before the MTBE was transferred to an amber 2 mL glass vial (Agilent). This process was repeated with another 0.5 ml of MTBE. Extracts of beetles were treated to remove excess fatty acids from the sample; 400  $\mu\text{L}$  of 1 mM ammonium carbonate (pH 8) was added to the combined MTBE extract and vortexed. The sample was centrifuged for 10 min at  $3000 \times g$  and the MTBE layer removed for analysis by GC/MS.

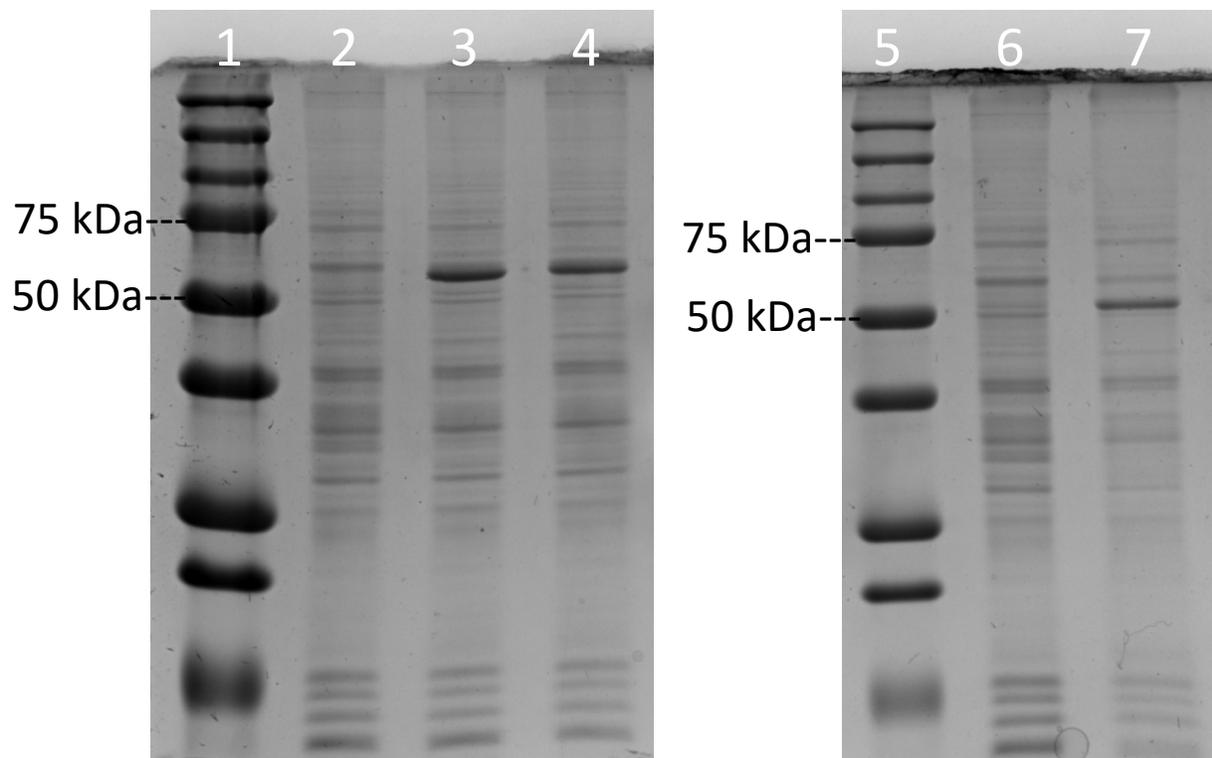
### 6.3.6 GC-MS analysis

GC-MS analyses were performed on an Agilent 7890A system GC, Agilent GC Sampler 80, and a 7000A GC/MS triple quad M5975C inert XL MSD with triple axis detector at 70 eV. Monoterpenols were analyzed by injecting 1  $\mu\text{L}$  of sample onto a DB-WAX column (J&W, polyethylene glycol, 30 m, 250  $\mu\text{m}$  i.d., 0.25  $\mu\text{m}$  fil). Oven temperature for analysis of products from enzyme assays with monoterpenes was 40°C for 2 min, 8°C  $\text{min}^{-1}$  to 100°C, 20°C  $\text{min}^{-1}$  to 230°C and then held for 5 min. Oven temperature analysis of beetle extracts treated with monoterpenes was 40°C for 2 min, 8°C  $\text{min}^{-1}$  to 100°C, 20°C  $\text{min}^{-1}$  to 250°C and then held for 10 min. DRA enzyme assay products were analyzed by injecting 1  $\mu\text{L}$  of sample onto an HP-5 column (J&W, 5% phenyl methyl siloxane, 27.4 m length, 250  $\mu\text{m}$  i.d., 0.25- $\mu\text{m}$  film thickness). Oven temperature was 40°C for 1 min, 20°C  $\text{min}^{-1}$  to 300°C and then held for 8 min.

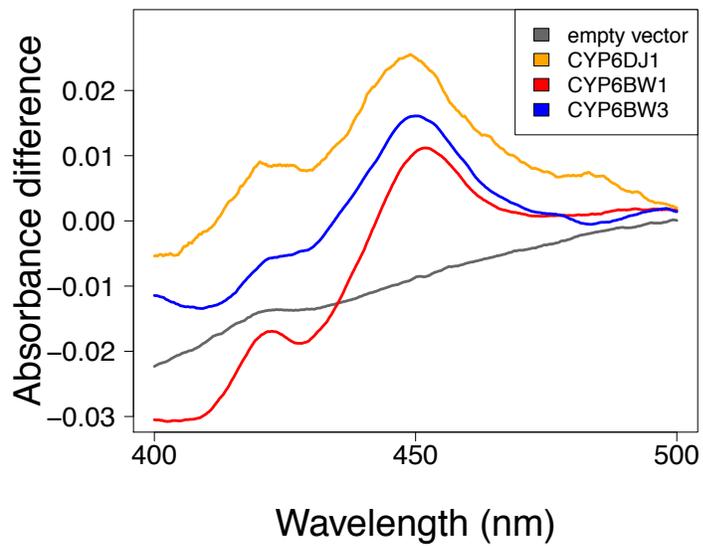
## 6.4 Results

MPB CYP6DJ1 was produced in Sf9 insect cells, isolated as microsomal membrane-bound proteins (**Figure 6.1**), and identified as a functional P450 based on its CO-spectrum (**Figure 6.2**). CYP6DJ1 was reconstituted with MPB CPR and tested in *in vitro* enzyme assays with ten different monoterpenes and six different DRAs (**Table 6.1**). CYP6DJ1 oxidized terpinolene as well as both enantiomers of limonene (**Figure 3A**, **Figure 6.3C**, **Figure 6.3E**, **Table 6.1**). The products were identified by comparison of retention times (**Figure 6.3A**, **Figure 6.3C**, **Figure 6.3E**, **Table 6.2**) and mass-spectra (**Figures 6.4 – 6.7**) with those of authentic standards. The products from CYP6DJ1 assays with (+)-limonene were *cis*-limonene-8,9-epoxide (peak 3), *trans*-limonene-8,9-

epoxide (peak 4) and perilla alcohol (peak 9) (**Figure 6.3A, Figure 6.3C, Figure 6.4, and Figure 6.6**). The products from CYP6DJ1 assays with (–)-limonene were *cis*-limonene-8,9-epoxide (peak 3), *trans*-limonene-8,9-epoxide (peak 4) and perilla alcohol (peak 9) (**Figure 6.3A, Figure 6.3C, Figure 6.4, and Figure 6.6**). *cis*-Limonene-8,9-epoxide (peak 3), *trans*-limonene-8,9-epoxide (peak 4) and perilla alcohol (peak 9) were not detected in assays with the empty vector control, or with CYP6DJ1 assays that did not contain NADPH, a co-factor for P450 activity (**Figure 6.3A, Figure 6.3C**). The products from CYP6DJ1 assays with terpinolene was a single unidentified peak (peak 11) (**Figures 6.3E and Figure 6.7**). Peak 11 was not detected in assays with the empty vector control, or with CYP6DJ1 assays that did not contain NADPH. CYP6DJ1 was not active with the other seven monoterpenes tested and was also not active with any of the DRAs tested (**Table 6.1**).



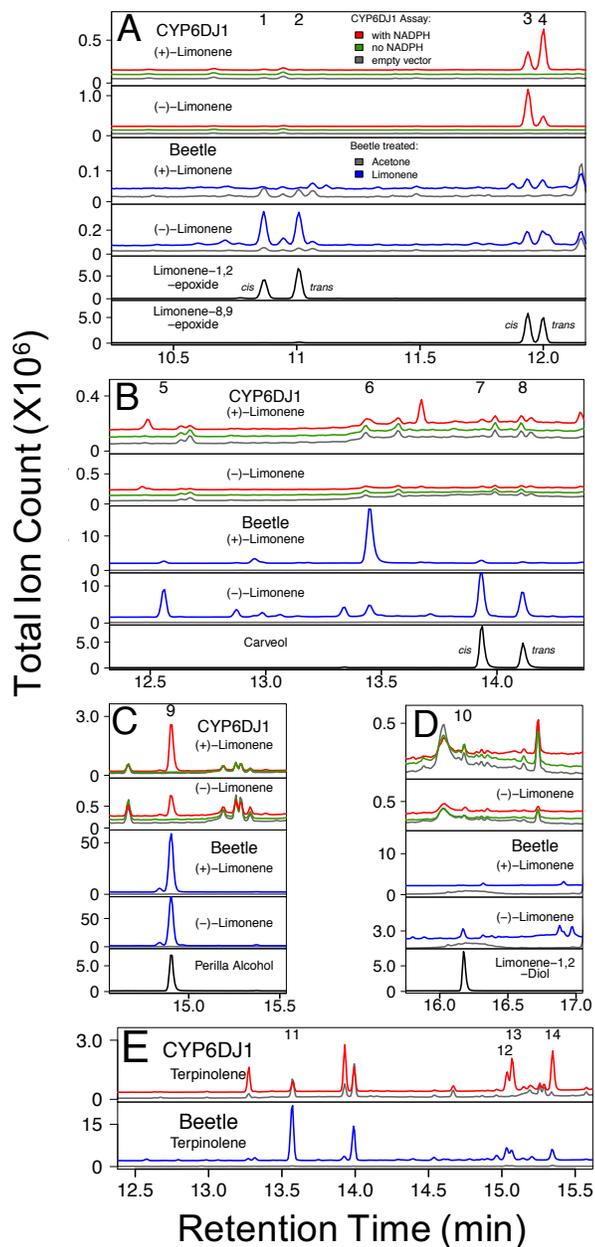
**Figure 6.1. Denatured CYP6DJ1, CYP6BW1, CYP6BW3 and empty vector control microsomes on a 12% SDS-PAGE gel.** Lane 1: Precision Plus Protein ladder (Bio-rad). Lane 2: empty vector microsomes. Lane 3: CYP6BW1 microsomes, protein band is visible between 75 kDa and 50 kDa. Lane 4: CYP6BW3 microsomes, protein band is visible between 75 kDa and 50 kDa. Lane 5: Precision Plus Protein ladder (Bio-rad). Lane 6: empty vector microsomes. Lane 7: CYP6DJ1 microsomes, protein band is visible between 75 kDa and 50 kDa.



**Figure 6.2. CO Spectra of CYP6DJ1, CYP6BW1, CYP6BW3 and empty vector control microsomes.**

**Table 6.1. Activity assay of CYP6DJ1, CYP6BW1 and CYP6BW3 with ten selected monoterpenes and six diterpene resin acid substrates.** Red crosses indicate that no product was detected in GC chromatograms of the assay, green checkmarks indicate that one or more products were detected in the assay.

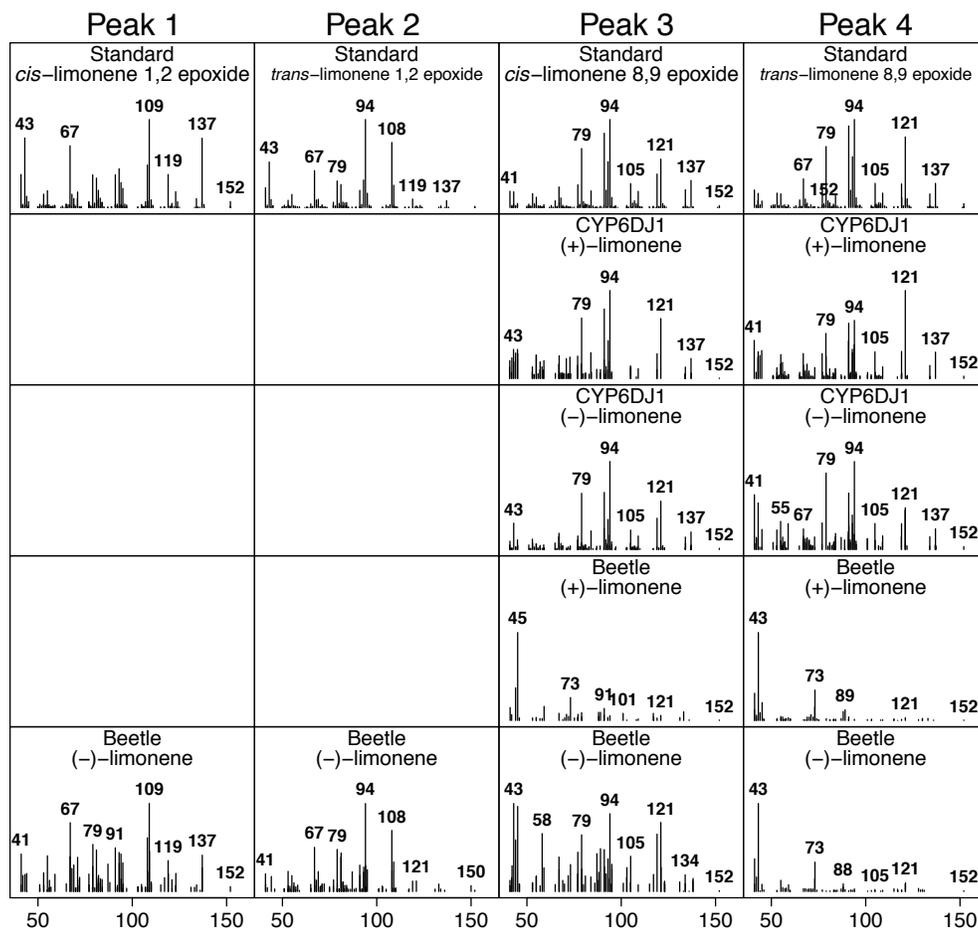
Substrate	Activity Assay ( <i>in vitro</i> )		
	CYP6DJ1	CYP6BW1	CYP6BW3
<b>Monoterpenes</b>			
(+)- $\alpha$ -pinene	✗	✗	✗
(-)- $\alpha$ -pinene	✗	✗	✗
(+)- $\beta$ -pinene	✗	✗	✗
(-)- $\beta$ -pinene	✗	✗	✗
(+)-limonene	✓	✗	✗
(-)-limonene	✓	✗	✗
(+)-3-carene	✗	✗	✗
$\beta$ -phellandrene	✗	✗	✗
myrcene	✗	✗	✗
terpinolene	✓	✗	✗
<b>Diterpene Resin Acids</b>			
abietic acid	✗	✓	✓
dehydroabietic acid	✗	✓	✓
neoabietic acid	✗	✓	✓
levopimaric acid	✗	✓	✓
palustric acid	✗	✓	✓
isopimaric acid	✗	✓	✓



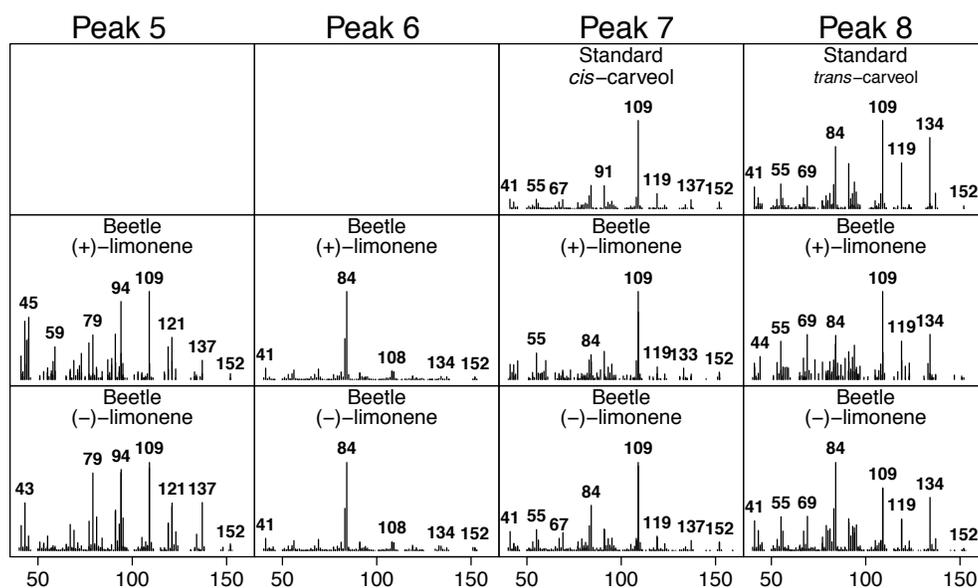
**Figure 6.3. Products of recombinant CYP6DJ1 and female beetle extracts after treatment with (+) and (-)-limonene.** Subfigures A-D highlight different regions of the same chromatograms of CYP6DJ1 assays and beetles treated with (+) and (-)-limonene. (A) *cis* and *trans* limonene-1,2-epoxide (peaks 1-2) are present in the beetles treated with (-)-limonene. *cis* and *trans* limonene-8,9-epoxide (peaks 3-4) are present in the (+) and (-)-limonene CYP6DJ1 assays and beetles treated with (+) and (-)-limonene. (B) Two unidentified limonene metabolites (peaks 5-6) are present in the beetles treated with (+) and (-)-limonene. *cis* and *trans* carveol (peaks 7-8) are present in the beetles treated with (+) and (-)-limonene. (C) Perilla alcohol (peak 9) is present in the (+) and (-)-limonene CYP6DJ1 assays and beetles treated with (+) and (-)-limonene. (D) Limonene-1,2-diol (peak 10) is present in beetles treated with (-)-limonene. (E) Chromatograms of CYP6DJ1 assays with terpinolene and terpinolene treated beetles. Four unidentified terpinolene metabolites are present in terpinolene treated beetle extracts (peaks 11-14), three of which, (peaks 12-14) are found in CYP6DJ1 assays with terpinolene. Chromatograms are shown with the total of the extracted ions 91, 94, 108, 109, 119, 121, 137, 152 m/z. Retention indices and mass spectra of peaks 1-10 are shown in Table 6.2 and Fig. 6.4-6.7.

**Table 6.2 The retention index of all limonene and terpinolene products of CYP6DJ1 and from extracts of MPB after treatment. All samples were injected onto a DB-Wax column. See Fig. 6.3 and 6.4-6.7 for the gas chromatograms and mass spectra of these peaks. Gas chromatograms with these peak numbers can be found in Fig. 6.3**

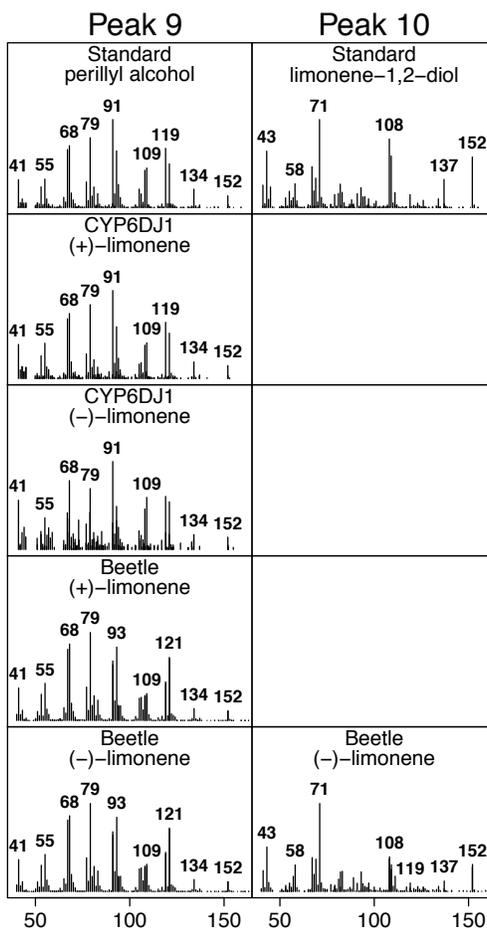
<b>Reference</b>	<b>Retention Index</b>	<b>Compound name</b>
Peak 1	1443	Limonene 1,2 epoxide
Peak 2	1456	Limonene 1,2 epoxide
Peak 3	1551	Limonene 8,9 epoxide
Peak 4	1556	Limonene 8,9 epoxide
Peak 5	1662	Unknown limonene product
Peak 6	1754	Unknown limonene product
Peak 7	1832	<i>cis</i> -Carveol
Peak 8	1863	<i>trans</i> -Carveol
Peak 9	2008	Perilla alcohol
Peak 10	2165	Limonene 1,2 diol
Peak 11	1773	Unknown terpinolene product
Peak 12	2032	Unknown terpinolene product
Peak 13	2040	Unknown terpinolene product
Peak 14	2094	Unknown terpinolene product



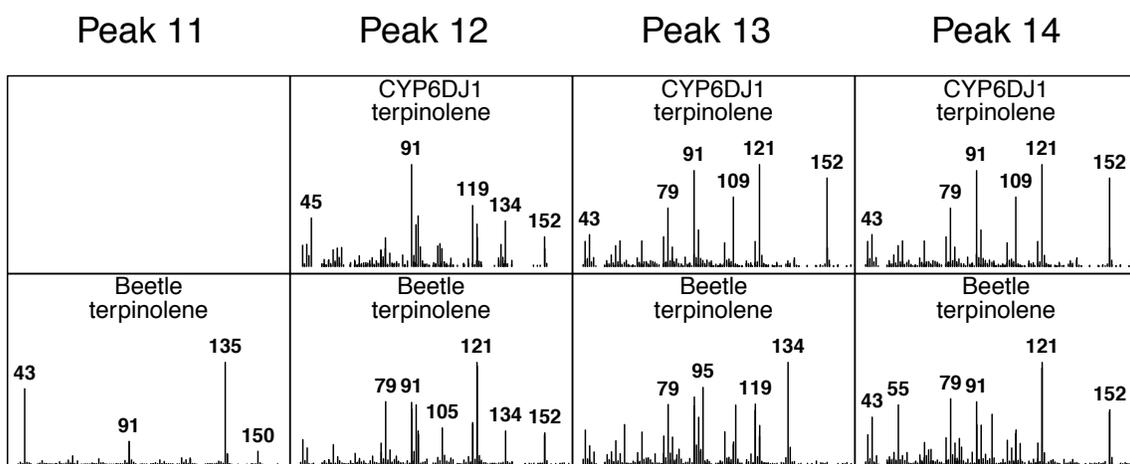
**Figure 6.4** Mass spectra of peaks 1-4 from the gas chromatograms of extracts of CYP6DJ1 or female beetles treated with (+) and (-)-limonene along with the *cis*- and *trans*-limonene-1,2-epoxide and *cis*- and *trans*-limonene-8,9-epoxide standards. Gas chromatograms with peak numbers can be found in Fig. 6.3. These compounds are not TMS derivatives.



**Figure 6.5** Mass spectra of peaks 5-8 from the gas chromatograms of extracts of CYP6DJ1 or female beetles treated with (+) and (-)-limonene along with the *cis*- and *trans*-carveol standards. Gas chromatograms with peak numbers can be found in Fig. 6.3. These compounds are not TMS derivatives.



**Figure 6.6. Mass spectra of peaks 9 and 10 from the gas chromatograms of extracts of CYP6DJ1 or female beetles treated with (+) and (-)-limonene along with the perilla alcohol and limonene-1,2-diol standards. Gas chromatograms with these peak numbers can be found in Fig. 6.3. These compounds are not TMS derivatives.**



**Figure 6.7. Mass spectra of peaks 11-14 from the gas chromatograms of extracts of CYP6DJ1 or female beetles treated with terpinolene. These compounds are not TMS derivatives.**

#### 6.4.2 Products of monoterpenes in MPB exposed to limonene and terpinolene

Female beetles were exposed to monoterpenes (+)-limonene, (–)-limonene and terpinolene, and products extracted from beetles were compared with those found in the CYP6DJ1 assays with these monoterpenes. Extracts of beetles exposed to (+)-limonene contained *cis*-limonene-8,9-epoxide (peak 3), *trans*-limonene-8,9-epoxide (peak 4), two unidentified products (peak 5 and 6), *cis*-carveol (peak 7) and *trans*-carveol (peak 8) and perilla alcohol (peak 9) (**Figure 6.3A, Figure 6.3B, Figure 6.3C, Figure 6.4, Figure 6.5, Figure 6.6**). These products were not identified in females that had been exposed to acetone (**Figure 6.3A, Figure 6.3B, Figure 6.3C**). Extracts of beetles exposed to (–)-limonene contained *cis*-limonene-1,2-epoxide (peak 1), *trans*-limonene-1,2-epoxide (peak 4), *cis*-limonene-8,9-epoxide (peak 3), *trans*-limonene-8,9-epoxide (peak 4), unidentified product peaks 5 and peak 6, *cis*-carveol (peak 7), *trans*-carveol (peak 8), perilla alcohol (peak 9) and limonene-1,2-diol (**Figure 6.3A, Figure 6.3B, Figure 6.3C, Figure 6.3D, Figure 6.4, Figure 6.5, Figure 6.6**). These products were not identified in females that had been exposed to acetone (**Figure 6.3A, Figure 6.3B, Figure 6.3C, Figure 6.3D**). Extracts of female beetles that had been exposed to terpinolene contained four unidentified products, peak 11, peak 12, peak 13 and peak 14 (**Figure 6.3E and Figure 6.7**). These products were not identified in females that had been exposed to acetone (**Figure 6.3E**).

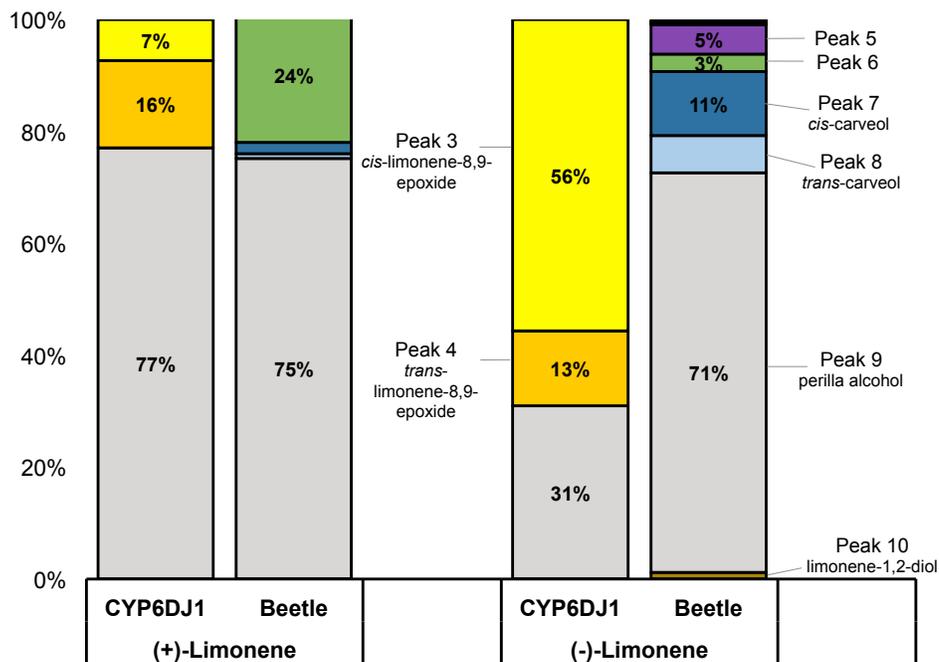
### 6.4.3 Comparison of product profiles of CYP6DJ1 *in vitro* activity with limonene and terpinolene activity and MPB exposed to the same monoterpenes

The activity of CYP6DJ1 with (+)-limonene, (–)-limonene, and terpinolene resulted in multiple products for each substrate. I measured the relative amounts of each product formed in *in vitro* assays by CYP6DJ1 and compared them with those detected in extracts of beetles exposed to (+)-limonene, (–)-limonene, and terpinolene (**Figure 6.8**).

The product profile of CYP6DJ1 with (+)-limonene as substrate consisted of 7% *cis*-limonene-8,9-epoxide (peak 3), 16% *trans*-limonene-8,9-epoxide (peak 4), and 77% perilla alcohol (peak 9) (**Figure 6.8**). The product profile of (+)-limonene-treated beetle consisted of <1% *cis*-limonene-8,9-epoxide (peak 3), <1% *trans*-limonene-8,9-epoxide (peak 4), 1% peak 5, 24% peak 6, 2% *cis*-carveol (peak 7), 1% *trans*-carveol (peak 8) and 75% perilla alcohol (peak 9) (**Figure 6.8**).

The product profile of CYP6DJ1 with (–)-limonene consisted of 56% *cis*-limonene-8,9-epoxide (peak 3), 13% *trans*-limonene-8,9-epoxide (peak 4) and 31% perilla alcohol (peak 9) (**Figure 6.8**). The product profile of (–)-limonene-treated beetle consisted of 5% peak 5, 3% peak 6, 11% *cis*-carveol (peak 7), 7% *trans*-carveol (peak 8), 71% perilla alcohol (peak 9), and limonene-1,2-diol (peak 10) (**Figure 6.8**).

The peak area of *cis*-limonene-1,2-epoxide (peak 1), *trans*-limonene-8,9-epoxide (peak 2), *cis*-limonene-8,9-epoxide (peak 3), and *trans*-limonene-8,9-epoxide (peak 4) together accounted for less than 2% of the product profile detected in the beetle extracts (**Figure 6.8**).



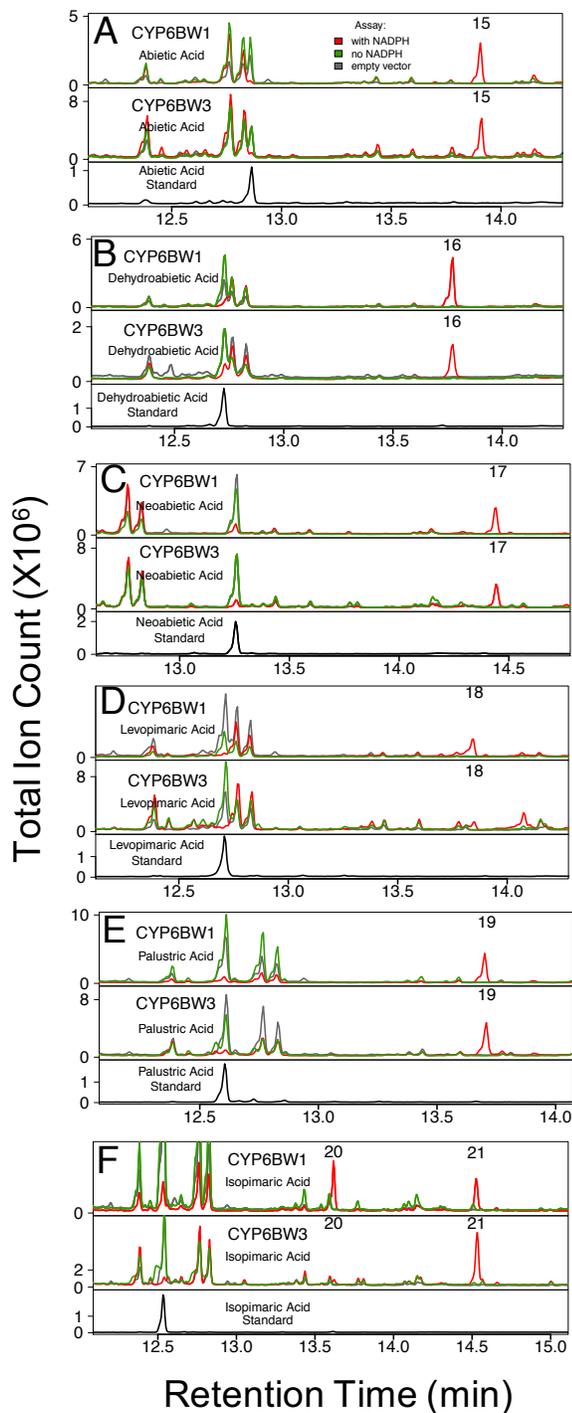
**Figure 6.8. Product profiles of recombinant CYP6DJ1 and female beetle extracts after treatment with (+)-limonene and (-)-limonene.** The percentage profile was calculated by peak area of the extracted ion chromatogram. Retention indices and mass spectra are shown in Table 6.2 and Fig. 6.4-6.6. CYP6DE1 profile N=3, Beetle profile N=3.

#### 6.4.4 CYP6BW1 and CYP6BW3 used diterpene resin acids as substrates

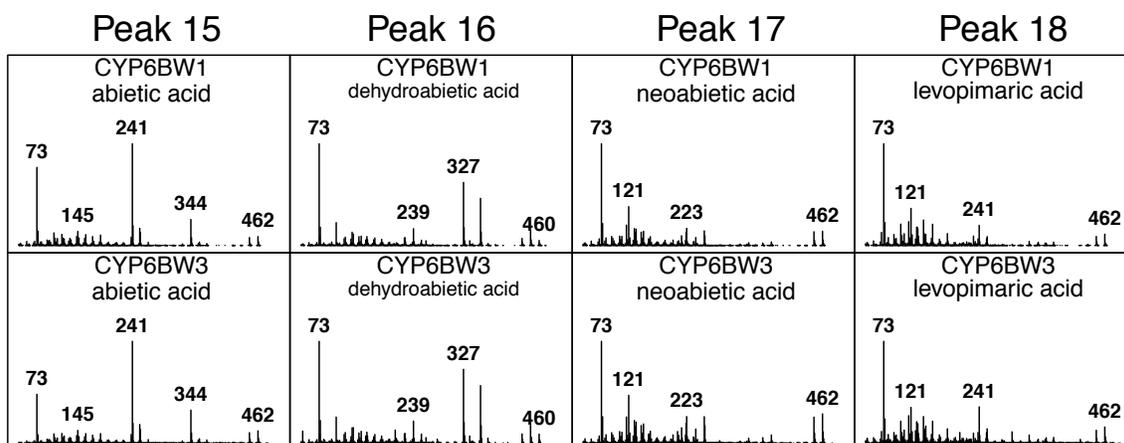
MPB CYP6BW1 and CYP6BW3 were produced in Sf9 insect cells, isolated as microsomal membrane-bound proteins (**Figure 6.1**), and identified as functional P450s based on CO-spectra (**Figure 6.2**). CYP6BW1 and CYP6BW3 were reconstituted with MPB CPR and tested in *in vitro* enzyme assays with ten different monoterpenes and six different DRAs. Both CYP6BW1 and CYP6BW3 oxidized all six diterpene resin acids, and overall CYP6BW1 and CYP6BW3 gave similar product profiles (peaks 15 – 21) with these DRAs (**Figure 6.9, Table 6.1**). Although authentic standards could not be obtained for compounds corresponding to peaks 15 - 21, mass spectra were used to deduce the likely identity of products to support future work on product identification.

A single product (peak 15) was detected in CYP6BW1 and CYP6BW3 assays with abietic acid (**Figures 6.9A and 6.10**). Peak 15 had a mass spectrum with a molecular ion of 462 m/z. Since BSTFA was used to derivatize the products of the *in vitro* assays before analysis by GC/MS, resulting in a trimethylsilyl group covalently bound to each free hydroxyl group of the enzyme-formed product where each trimethylsilyl group adds an additional 72 m/z to the mass. M/z 462 is consistent with a singly hydroxylated abietic acid. CYP6BW1 and CYP6BW3 also had a single product (peak 16) in assays with dehydroabietic acid (**Figures 6.9B and 6.10**). Peak 16 had a mass spectrum with a molecular ion of 460 m/z. This is consistent with a singly hydroxylated dehydroabietic acid. The single product (peak 17) detected in assays of CYP6BW1 and CYP6BW3 assays with neoabietic acid (**Figures 6.9C and 6.10**) had a mass spectrum with a molecular ion of 462 m/z, consistent with a singly hydroxylated neoabietic acid. In

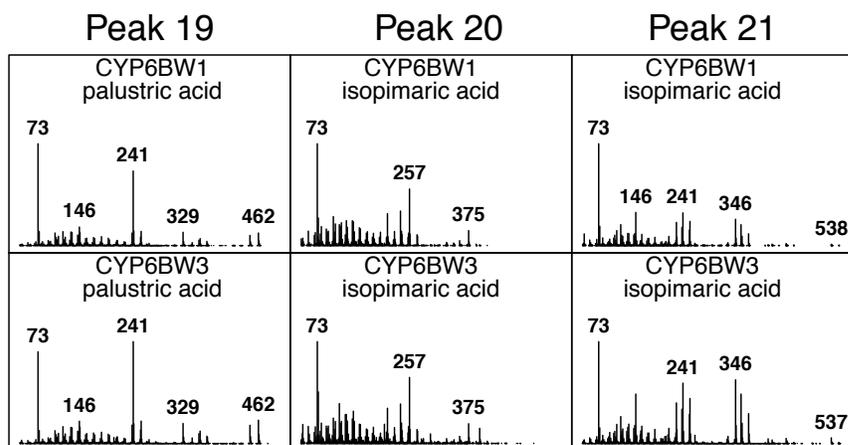
assays with levopimaric acid CYP6BW1 and CYP6BW3 also produced a single product (peak 18) (**Figures 6.9D and 6.10**) with a mass spectrum with a molecular ion of 462 m/z, consistent with a singly hydroxylated levopimaric acid. The single product (peak 19) in CYP6BW1 and CYP6BW3 assays with palustric acid (**Figures 6.9E and 6.11**) had a mass spectrum with a molecular ion of 462 m/z consistent with a singly hydroxylated palustric acid. I found two product peaks (peak 20 and 21) in assays of CYP6BW1 and CYP6BW3 assays with isopimaric acid, although peak 20 in the CYP6BW3 assay was a very minor peak (**Figure 6.9F, 6.11**). Peak 20 had a mass spectrum with a molecular ion of 390 m/z. This is consistent with a singly epoxidized isopimaric acid. Peak 21 had a mass spectrum with a molecular ion of 537 m/z. This is consistent with an  $[M-15]^+$  of a TMS-derivatized vicinal diol due to the hydrolysis of the epoxidized isopimaric acid. Hydroxylated and epoxidized products of the DRA were not detected in assays with the empty vector control, or with CYP6BW1 and CYP6BW3 assays that did not contain NADPH, an important co-factor for P450 activity (**Figure 6.9**). CYP6BW1 and CYP6BW3 were not active with the ten monoterpenes tested (**Table 6.1**).



**Figure 6.9. Products of recombinant CYP6BW1 and CYP6BW3 with diterpene resin acids.** CYP6BW1 and CYP6BW3 assays with (A) abietic acid (B) dehydroabietic acid, (C) neoabietic acid, (D) levopimaric acid, (E) palustric acid, (F) isopimaric acid, as substrates. Mass spectra of peaks 15-21 are shown in Fig. 6.4-6.7.



**Figure 6.10. Mass spectra of peaks 15-18 from the gas chromatograms of extracts of CYP6BW1 or CYP6BW3 with abietic, dehydroabietic, neoabietic and levopimaric acid as substrates. Gas chromatograms with peak numbers can be found in Fig. 6.9A-D.**



**Figure 6.11. Mass spectra of peaks 19-21 from the gas chromatograms of extracts of CYP6BW1 or CYP6BW3 with palustric or isopimaric acid as substrates. Gas chromatograms with peak numbers can be found in Fig. 6.9E-F.**

## 6.5 Discussion

CYP6DJ1 oxidized (+)-limonene, (–)-limonene, and terpinolene. When either enantiomer of limonene was presented as a substrate, CYP6DJ1 produced *cis*- and *trans*-limonene-8,9-epoxide and perilla alcohol. These products were also present in limonene-treated females beetles, indicating that CYP6DJ1 may contribute to the metabolism of limonene in the beetle. However, the presence of limonene metabolites in beetles, that were not CYP6DJ1 products also indicates the contribution of other enzymes to limonene metabolism. Several studies have shown that limonene has particularly high toxicity to MPB compared to other monoterpenes, with (–)-limonene being significantly more toxic than (+)-limonene (Chiu et al., 2017; Reid et al., 2017). In addition, the presence of (+)-limonene in host volatiles has been implicated in the disruption of host selection by female MPB (Gray et al., 2015). However, despite having a potentially higher defensive potential against MPB attack, limonene accounts for only a minor percentage of the monoterpene profile of pine host species for MPB (Clark et al., 2014; Hall et al., 2013a; Taft et al., 2015b).

Previous transcriptome and proteome analyses indicated that the *CYP6DJ1* transcript and CYP6DJ1 protein is upregulated in female MPB, compared to males, during the host colonization process, specifically after feeding (Chiu et al., 2018a; Nadeau et al., 2017; Robert et al., 2013) or treatment with juvenile hormone III (Keeling et al., 2016). The upregulation of CYP6DJ1 in colonizing females suggest that it could be involved in the oxidation of  $\alpha$ -pinene and the biosynthesis of *trans*-verbenol, a female produced pheromone. However, CYP6DJ1 did not show activity with  $\alpha$ -pinene. Furthermore, I

recently showed that *trans*-verbenol released from colonizing females is derived from verbenyl esters accumulated by females earlier in the life cycle (Chiu et al., 2018c). The production of the pheromone *trans*-verbenol via  $\alpha$ -pinene hydroxylation may not be occurring in colonizing females, as previously thought. Therefore, a P450 involved in *trans*-verbenol biosynthesis specifically for pheromone production may not be expected to be upregulated in colonizing females. Instead, the role of CYP6DJ1 in detoxifying limonene and terpinolene, may be more important to colonizing females than males since females are the pioneer sex and are the first to contend with host defenses.

I showed that both CYP6BW1 and CYP6BW3 oxidize the same set of different DRAs and produce mostly the same hydroxylated and epoxidized products. Future research will need to identify these products and their presence in MPB exposed to DRAs to assess their biological role in MPB. Since CYP6BW1 and CYP6BW3 share 96% amino acid identity, it is not surprising that these two enzymes were active with the same DRAs. Although these P450s both metabolized DRAs, they may be playing different roles in different tissues, as CYP6BW1 is highly expressed in the midgut and CYP6BW3 is highly expressed in the antennae (Chiu et al., 2018a; Keeling et al., 2012). CYP6BW1 may be metabolizing DRAs as part of general detoxification in the midgut, while CYP6BW3 may have a more specialized role in the protection of the olfactory system in the antennae. Putative orthologues of MPB CYP6BW1 and CYP6BW3 have been identified in other *Dendroctonus* species, sharing over 90% amino acid identity to CYP6BW1 and CYP6BW3. *CYP6BW5v1* from *D. rhizophagus* and *CYP6BW5v3* from *D. valens* appear to be single copy genes that are expressed in both the antennae and

midgut (Cano-Ramírez et al., 2013; López et al., 2013). CYP6BW1 and CYP6BW3 may have evolved by gene duplication from a single copy gene that was expressed in both the antennae and midgut and may have subsequently diverged with different patterns of expression indicative of sub-functionalization. Previous transcriptome analyses also identified *CYP6BW3* transcripts as upregulated in colonizing males, compared to colonizing females, which may have suggested a role in the biosynthesis of the male produced pheromone frontalin (Chiu et al., 2018a; Nadeau et al., 2017). Our results do not support such a function.

The activity of CYP6DJ1 with limonene and terpinolene as substrates, and the activity of CYP6BW1 and CYP6BW3 with DRAs as substrates, may protect the beetle against the pine chemical defenses and contributes to the survival of MPB in the terpenoid-saturated environment of the phloem.

## 7. Conclusions

### 7.1 MPB P450s and terpenes of the host defense system

The interactions of MPB with monoterpenes and diterpenes of the host defense system are complex, where terpenes can be both harmful and beneficial to the beetle's success at colonizing a host tree and completing its life cycle. Oleoresin terpenes are produced by pines and other conifers as a major chemical and physical defense system (Bohlmann, 2012; Boone et al., 2013; Raffa, 2014). However, presumably as a result of a history of co-evolution between conifers and bark beetles, the MPB has evolved not only to overcome some of the terpene defenses but also co-opted some of the host monoterpenes as kairomones for host search or as precursors for pheromone biosynthesis (Hughes, 1973b; Pureswaran & Borden, 2005). Whether a given terpene compound acts as a toxic defense against the beetle or serves as a kairomone or pheromone precursors depends on (i) the specific structure of the terpene molecule, (ii) the concentration at which MPB is experiencing the compound, and (iii) to what extent the beetle can metabolize a given terpene into potentially less harmful or even beneficial metabolites.

My thesis explored the toxicity of different pine terpenes to MPB. I then explored members of the MPB cytochrome P450 (P450) gene family for functions in oxidizing pine terpenes, in parallel with investigating the metabolites that may be produced in beetles that were exposed to individual terpenes. In the course of this thesis, I established reference MPB LC<sub>50</sub> data for a set of pine terpenes; I discovered P450s that are differentially expressed in MPB organs and tissues that may be involved in

metabolism of host terpenes; I identified the biochemical functions of a set of P450s with terpene substrates; and I discovered a new group of monoterpenol fatty acid esters in the MPB that may serve as accumulated intermediates in the formation and release of the female sex pheromone *trans-verbenol*.

During host colonization, adult MPB, eggs, larvae, pupae and teneral adults are exposed to terpenes as airborne volatiles, through contact with oleoresin or by feeding on phloem containing oleoresin (Clark et al., 2012). Despite the MPB's co-evolution with pines as their required hosts, the oleoresin terpenes of pines can be toxic towards MPB in high concentrations (Chiu et al., 2017; Reid et al., 2017). In my thesis, I quantified the lethal concentration<sub>50</sub> (LC<sub>50</sub>) of individual monoterpenes that are abundant in MPB hosts, as such reference data surprisingly appeared to be missing in the literature prior to my work (Chiu et al., 2017) and the parallel work by Reid et al., (2017). The fact that MPB can tolerate terpenes at relatively high levels, but is affected by high concentration of some terpenes, suggested that MPB has efficient biochemical or other mechanisms to cope with these molecules. Since not all terpenes had the same LC<sub>50</sub>, these results also suggested that different host terpenes may be converted by one or more enzymes of a detoxification system that differ with regard to their substrate specificities or catalytic efficiencies with different terpenes. As insect P450s are known to contribute to the detoxification of host defense chemicals, I investigated members of the MPB P450 gene family (Keeling et al., 2013c). I identified candidate P450s, by analysis of transcripts that were differentially expressed in different body parts of MPB, indicative of roles in detoxification, olfaction, or pheromone biosynthesis (Chiu et al., 2018a).

While dispersing from a brood tree and searching for a new host, MPB are exposed to monoterpene volatiles released from host and non-host species, and some of these monoterpenes may act as kairomones (Pureswaran & Borden, 2005). These monoterpenes are detected by receptors in the antennae and subsequently need to be removed by odorant-degrading enzymes (Pottier et al., 2012). I identified seven different MPB P450 genes (CYP6DE1, CYP6DE2, CYP6DJ1, CYP6BW1, CYP6BW3, CYP9Z18) that displayed expression patterns indicative of candidates as monoterpene degrading enzymes in the antennae. Some of these P450s were also expressed in other tissues suggesting broader functions in monoterpene detoxifications. From this set of seven differentially expressed P450s that showed expression in antennae, I functionally characterized the P450s CYP6DE1, CYP6DJ1, CYP6BW3 and CYP6BW1 (Chiu et al., 2018a, 2018d, 2018b), which revealed different substrate specificities and product profiles when tested against a panel of different pine monoterpenes and diterpene resin acids. These results suggested some level of functional specialization, while the CYP6BW1 and CYP6BW3 enzymes also retain some level of functional overlap, within a group of terpene-oxidizing MPB P450s. In parallel to the *in vitro* biochemical characterization of MPB P450s for their activities in oxidizing various terpene substrates, I tested the same terpene substrates *in vivo* with adult MPB. These experiments showed that the cloned P450s and MPB produce similar monoterpenols and hydroxy-diterpene resin acids, which validated the relevance of the results from the biochemical characterization of cloned MPB P450s for drawing conclusions on their possible roles in MPB terpene metabolism. Ultimately, such conclusions will have to be

substantiated with MPB in which the expression or function of MPB genes has been altered, for example by RNAi or CRISPR gene editing.

In the course of this work that compared P450 *in vitro* activities and *in vivo* terpene-derived metabolites in MPB, I made the unexpected discovery of a group of monoterpenyl fatty acid esters that accumulate in pre-adult MPB (Chiu et al., 2018c). These esters may originate from the P450-dependent detoxification of host monoterpenes followed by conjugation to a fatty acid for sequestration of a potentially less toxic derivative of the host monoterpene. Patterns of accumulation of verbenyl oleate in developing MPB and sex-specific release of *trans*-verbenol from emerging females, suggested that this particular monoterpenol ester may have been co-opted for the accumulation of pheromone precursors by female MPB.

During the attack of a new host tree, female MPB release the monoterpenol aggregation pheromone *trans*-verbenol (Pureswaran et al., 2000). (–)-*trans*-Verbenol is thought to be produced in adult females by P450-dependent oxidation of (–)- $\alpha$ -pinene, which is abundant in the host oleoresin and air-borne volatiles. This notion was supported by production of *trans*-verbenol in beetles that were exposed to  $\alpha$ -pinene or were feeding on host tissue, (Conn et al., 1984; Hunt & Smirle, 1988). My discovery that earlier life stages of the MPB, ranging from larvae to teneral adults, accumulate verbenyl oleate and other monoterpenyl esters during their development in the brood tree, adds a new dimension to this picture of host-derived *trans*-verbenol formation and its release (Chiu et al., 2018c). These esters appear to be produced from  $\alpha$ -pinene in the phloem-feeding

larvae, are being stored, and appear to serve as a reservoir for the sex-specific release of *trans*-verbenol by adult female MPB during dispersal and host search (Chiu et al., 2018c). The formation of monoterpenyl esters may be part of, or derived from, the overall monoterpene detoxification systems in MPB, whereby MPB first oxidize  $\alpha$ -pinene to *trans*-verbenol and then produce verbenyl fatty acid esters, which may be stored without being toxic to the insect. As the first gene and enzyme involved in MPB formation of *trans*-verbenol, I identified and functionally characterized the P450 CYP6DE1, which produces *trans*-verbenol from  $\alpha$ -pinene. This P450 may be involved in pheromone production as well as monoterpene detoxification.

Overall, the characterization of CYP6DE1, CYP6DJ1, CYP6BW1 and CYP6BW3 for their activities with various different monoterpenes or diterpene resin acids that are abundant in MPB hosts, and the corresponding transcript expression analyses and metabolite analyses in the MPB, revealed both overlapping and distinct functions of individual MPB P450s that may contribute to processes of detoxification, olfaction, and pheromone biosynthesis as the MBP copes with the terpene-rich environment of the pine defense system.

## **7.2. Future work**

In the following sections of the conclusions, I highlight individual conclusions from the different results chapters of my thesis as the foundation for proposing potential future work, that builds upon the findings of my thesis.

### 7.2.1 Chapter 2: Toxicity of pine monoterpenes to MPB

This research was designed to obtain reference MPB LC<sub>50</sub> data for different monoterpenes present in the host pine oleoresin and its volatile emissions, specifically (-)- $\beta$ -phellandrene, (+)-3-carene, myrcene, terpinolene, and both enantiomers of  $\alpha$ -pinene,  $\beta$ -pinene and limonene. The LC<sub>50</sub> value is a standard metric in toxicity studies and can be used to compare the toxicity of different monoterpenes relative to each other in one system, or to compare the toxicity of the same monoterpene in different species.

The comparison of MPB LC<sub>50</sub>s for the different monoterpenes and the abundance of these monoterpenes in the host tree revealed that the most toxic monoterpenes to MPB, (-)-limonene and (+)-limonene, are not the most abundant in the MPB pine hosts. Conversely,  $\beta$ -phellandrene which is the most abundant monoterpene of lodgepole pine (>50% of the total oleoresin monoterpenes) (Hall et al., 2013a), only displayed moderate toxicity towards MPB. These results suggest that the MPB has successfully evolved to cope with the major monoterpenes in its abundant host, lodgepole pine, but would be affected by the higher toxicity of other monoterpenes, if expanding into potential new hosts with a high abundance of (-)-limonene or (+)-limonene. Pines that abundantly accumulate limonene in their oleoresin include whitebark pine (*Pinus albicaulis*), a keystone species within high elevation ecosystems that are currently being invaded by MPB due to a warming environment (Bentz et al., 2015, 2010; Raffa et al., 2017).

Comparisons of the MPB monoterpene LD<sub>50</sub> values to such LD<sub>50</sub> values of other insects showed that MPB tolerance of monoterpenes is more than ten-fold greater than insects that do not inhabit terpene-rich environments (Stamopoulos et al., 2007). This comparison highlights the fact that MPB must have evolved mechanisms to reduce the effect of conifer monoterpenes, while many other herbivorous insects would be excluded from conifers as hosts.

In future work, an extended analysis of toxicity of monoterpenes in MPB needs to include different methods of exposure, specifically contact and feeding and it should include all stages of the MPB life cycle from egg to adult. Different combinations of monoterpenes sesquiterpenes and diterpenes, representative of different host species under threat from MPB invasion need to be tested for possible synergic or neutralizing effects. Another major aspect of monoterpene toxicity that is unknown is their mode of action at the level of the complete MPB organism, MPB physiology and cell biology. A detailed understanding of the toxicity of individual monoterpenes and mixtures thereof, along with knowledge of modes of MPB tolerance or detoxification, would enable greater predictive power as to which conifer species may or may not likely be suitable hosts of the MPB as it expands its geographic range and may involve interactions with conifers that have not previously acted as MPB hosts.

### **7.2.2 Chapter 3: Monoterpenyl esters and sex-specific release of trans-verbenol**

The most surprising discovery of my thesis was the finding of various monoterpenyl esters in developing MPB and the potential of verbenyl oleate to serve as a reservoir for

the release of the aggregation pheromone *trans*-verbenol by female MPB. Verbenyl oleate accumulates in both sexes during their development from larvae to teneral adult in the brood tree, but only females had retained this compound when they emerged from the brood trees. This discovery added new information towards answering long-standing questions about the metabolic origin of the female-specific aggregation pheromone *trans*-verbenol (Conn et al., 1984; Hughes, 1973a). The accumulation of the monoterpenyl esters in MPB may be a metabolic link between monoterpene detoxification by feeding larvae and evolution of a MPB aggregation pheromone.

Future studies need to identify the complete set of genes and enzymes of MPB larvae involved in the conversion of  $\alpha$ -pinene to verbenyl fatty acid esters, the mechanisms and tissues that facilitate the accumulation of these molecules, and ultimately the molecular and biochemical mechanisms that control the sex-specific release of *trans*-verbenol and its temporal and spatial regulation. New work should explore the presence of verbenyl fatty acid esters in other *Dendroctonus* species that release *trans*-verbenol, including *D. frontalis*, *D. brevicornis* and *D. terebrans* (Byers et al., 1984; Sullivan, 2016). A study across different *Dendroctonus* species and other more-or-less closely related species, may resolve patterns of the evolution of pheromone formation relative to monoterpene detoxification. This improved knowledge of the mountain pine beetle pheromone system can inform prediction of bark beetle outbreaks and invasion of new habitats.

### **7.2.3 Chapter 4: P450s with preferential expression in MPB antennae**

I identified seven P450 genes, CYP6BW1, CYP6BW3, CYP6DE1, CYP6DE2, CYP6DJ1, CYP9Z18, and CYP345E2, that had relatively high transcript abundance in MPB antennae. Based on the patterns of transcript abundance, I proposed roles in olfaction for CYP6BW3, CYP6DE1, CYP6DE2, CYP6DJ1, CYP9Z18, and CYP345E2, in detoxification for CYP6BW1, CYP6DE2, and CYP9Z18, and in pheromone biosynthesis for CYP6DE1 and CYP6DJ1. Some of these P450s were functionally characterized in my subsequent work. In future work, a comprehensive transcriptome analysis of all 85 P450s identified in the MPB genome (Keeling et al., 2013c) across different tissues and life stages needs to be completed as a foundation for understanding the function and evolution of this large gene family in MPB, and how it may have contributed to the MPB's ability to colonize well-defended pine hosts.

#### **7.2.4 Chapter 5: CYP6DE1 converts $\alpha$ -pinene into *trans*-verbenol**

I showed that CYP6DE1, but not the closely related CYP6DE2, used the bicyclic monoterpenes (–)- $\alpha$ -pinene, (+)- $\alpha$ -pinene, (–)- $\beta$ -pinene, (+)- $\beta$ -pinene and (+)-3-carene as substrates. CYP6DE1 converted (–)- $\alpha$ -pinene and (+)- $\alpha$ -pinene into *trans*-verbenol, with minor amounts of *cis*-verbenol and myrtenol, a product profile that closely matched extracts of female beetles treated with the same compounds. I concluded that CYP6DE1 may contribute to formation of *trans*-verbenol in MPB, while also contributing to the metabolism of  $\beta$ -pinene and 3-carene indicative of an original role in monoterpene detoxification. A function for CYP6DE2 remained elusive.

Future *in vivo* work needs to validate a role of CYP6DE1 in *trans*-verbenol production. This could entail RNAi or CRISPR approaches. Experiments need to be designed to delineate, if possible, the proposed role of CYP6DE1 in the formation of *trans*-verbenol as a biologically important, female-specific aggregation pheromone from other, general roles of CYP6DE1 in monoterpene detoxification.

### **7.2.5 Chapter 6: CYP6DJ1, CYP6BW1 and CYP6BW3 oxidize pine terpenes**

I showed that the MPB P450 CYPDJ1 metabolizes the monoterpenes limonene and terpinolene and that the oxidized monoterpene enzymatic products were also found in MPB extracts treated with the same compounds. Limonene is the most toxic monoterpene in lodgepole pine (Chiu et al., 2017). CYP6DJ1 has higher expression in female compared to male MPB (Chiu et al., 2018a; Keeling et al., 2016; Nadeau et al., 2017; Robert et al., 2013), however females did not survive limonene vapours significantly better than males (Chiu et al., 2017). The transcript abundance of CYP6DJ1 in males and females may be below a threshold that would impact the toxicity of limonene.

One underlying assumption in much of my thesis has been that the MPB P450 oxidation products of pine monoterpenes will be less toxic than the monoterpenes themselves. However, without future experimental work it cannot be excluded that the oxidized monoterpene products remain toxic or could potentially be even more toxic than the monoterpene olefins of the pine oleoresin. Having identified the products of various MPB P450 activities, it is now possible to test in future work both toxicity of the pine

monoterpenes (see conclusions from chapter 2) and the toxicity of P450-generated oxidized monoterpene derivatives. Evidence of herbivore metabolism producing potentially more toxic compounds from plant defense metabolites exists, as for example with pyrrolizidine alkaloids (Hartmann, 1999; Heckel, 2014).

I also showed that MPB CYP6BW1 and CYP6BW3 oxidize diterpene resin acids. CYPBW1 transcripts are abundant in the midgut, while CYP6BW3 is highly expressed in the antennae (Chiu et al., 2018a, 2018b). Putative orthologues of *CYP6BW1/3* are found in other *Dendroctonus* species, *D. rhizophagus* and *D. valens*, as single copy genes expressed in both the antennae and the midgut in those species (Cano-Ramírez et al., 2013; López et al., 2013). The pair of MPB CYP6BW1 and CYP6BW3 may have originated from a common ancestral gene via gene duplication followed by divergence (i.e., possibly sub-functionalization) of their spatial expression. Future work should investigate the promoters and transcription factors of these two genes for regulation of their expression in antennae and midgut, respectively. As with the aforementioned MPB P450, other future work needs to test *in vivo* functions also for CYP6DJ1, CYP6BW1 and CYP6BW3. *In vivo* analysis of CYP6BW1 and CYP6BW3 may help us understand their specific roles in the midgut and antennae and the possible sub-functionalization of CYP6BW1 and CYP6BW3 in MPB.

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## **Appendix A. The Development of PCR-based markers for Sex Identification in the Mountain Pine Beetle<sup>5</sup>**

### Background

In the mountain pine beetle, each sex has a distinct role in host colonization. Females are the pioneer beetles and males follow the females to the host. Each sex produces a different set of pheromones. It is therefore important to distinguish the sex of each individual when assessing traits associated with the host colonization process such as pheromone production. The sex can be distinguished in MPB adult and pupae using morphological characteristics (Lyon, 1958; Schofer & Lanier, 1970). However, the sex of larvae can only be distinguished using molecular genetic techniques (James et al., 2016). I have developed a simple PCR-based assay to determine the sex of an individual beetle using reagents common to most molecular biology labs.

### Identification of neo-X and neo-Y regions of the genome

The MPB has eleven pairs of autosomes and two sex chromosomes neo-X and neo-Y (11AA + neoXY) (Lanier & Wood, 1968). Females have two neo-X chromosomes and males have a neo-X and a neo-Y chromosome. This karyotype has probably diverged from an ancestral state of 14AA + Xyp (Zuñiga et al., 2002). An autosome is thought to have fused with the original X chromosome to form the neo-X chromosome. The original yp chromosome was lost and the other autosome became the neo-Y. Therefore, the ancestral autosomal regions of the neo-X and neo-Y share sequence similarity although

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<sup>5</sup> A published version of this appendix can be found in SI Appendix: Chiu, C. C., Keeling, C. I., & Bohlmann, J. (2018). Monoterpenyl esters in juvenile mountain pine beetle and sex-specific release of the aggregation pheromone trans-verbenol. *Proceedings of the National Academy of Sciences*, 115(14), 3652–3657.

they have diverged since they became sex chromosomes. Keeling et al. (2013) identified a horizontally transferred gene in scaffold KB632308 of the female assembly and KB741144 and KB737807 of the assembly of the MPB genome (**Figure A1 A**). The differences in length of these scaffolds between male and female assemblies are consistent with this locus being on the ancestral autosomal portion of the neo-X and neo-Y chromosomes.

### Primer Design

I explored these scaffolds for regions of perfect sequence identity adjacent to regions of high diversity in which to design one common primer and two unique primers for the neo-X and neo-Y chromosomes (**Figure A1 B**). The neo-X primer was designed to be specific to the neo-X chromosome and with the common primer amplify a 587 bp region of the neo-X (**Figure A1 B, Table A1**). The neo-Y primer was designed to be specific to the neo-Y chromosome and with the common primer amplify a 494 bp region of the neo-Y (**Figure A1 B, Table A1**). I performed a Blastn of these primers to the MPB genome to check that no amplicons from other sites in the genome would be expected. With these three primers in a multiplex PCR reaction, males will have two products corresponding to the neo-X and neo-Y amplicon, and females will have one product corresponding to the neo-X amplicon.

### DNA Extraction

**Pre-sexed adult beetles:** Forty adult and twenty teneral, male and female MPB were used to test the primer design. These samples were sexed using the shape of the

seventh tergite (Lyon, 1958). The RNA of these samples had already been extracted from each individual using the RNeasy Plant Mini Kit (Qiagen). Therefore, after the RNeasy Plant Mini protocol had been executed, the DNA was eluted from the mini spin columns in the following manner (Martins, 2009). 50 µl of NaOH (8 mM) was pre-heated to 55°C and applied to the column. After 5 min, the column was centrifuged at 5000 RPM for 3 min to collect the DNA extract. The pH of the DNA extract was then adjusted by adding 8 µl of 0.1M HEPES buffer. **Larval and pupal samples:** The MPB larval and pupal samples shown in **Figure A2** were first extracted with pentane to analyze the monoterpenyl esters, then DNA was extracted using the prepGEM® insect kit (ZyGEM). A volume of about 1 mm<sup>3</sup> MPB sample was used to produce a 20 µl DNA extract according to the manufacturer's instructions. 10 first instar, 14 second instar, 13 third instar 12 fourth instar and 12 pupal samples were extracted using the PrepGEM kit method.

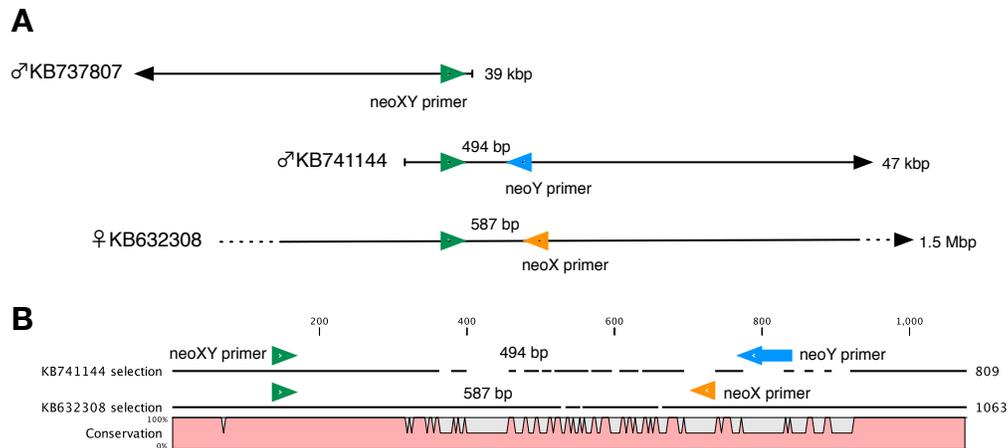
### Polymerase Chain Reaction

Each 10 µl PCR reaction mix contained 1.0 µl of 1:5 dilution of beetle DNA extract (either from PrepGEM or Qiagen spin columns), 5.0 pmol of each neo-X, neo-Y and XY primer, 0.4 mg/ml of bovine serum albumin, 2.1 µl of ddH<sub>2</sub>O, and 5 µl of Mango Mix (Bioline). The PCR reaction conditions were as follows: 96°C for 2 min for initial denaturation, followed by 35 cycles of 96°C for 15s, 66°C for 20s, 72°C for 45s with a final extension step of 72°C for 4 min. 5 µl of the PCR reaction was loaded onto a 2% agarose gel and electrophoresed to differentiate the band size.

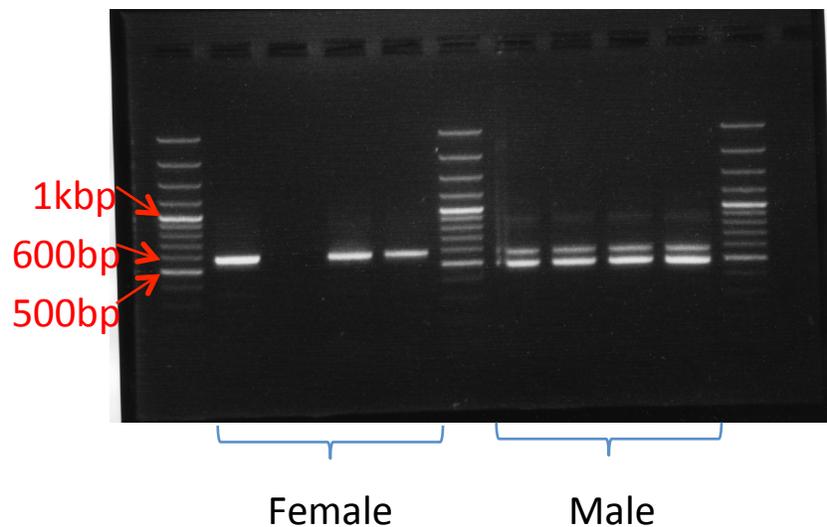
## Results

PCR of the pre-sexed adult samples produced amplicon sizes corresponding to the correct sex in 20 of 20 male adults, 10 of 10 male teneral, 20 of 20 female adult and 10 of 10 female teneral samples. Typical amplicon sizes are shown in **Figure A2**. If the PCR failed to produce an amplicon, redilution of the beetle DNA extract to 1:10 or 1:20 usually corrected this problem. PCR of the larval and pupal samples produced amplicon sizes corresponding to either male or female in 9 of 10 first instar, 12 of 14 second instar, 10 of 13 third instar, 11 of 12 fourth instar and 9 of 12 pupal samples. Approximate 16% of the PCRs with the larval and pupal samples did not produce any amplicon.

This method is an effective method of determining the sex of the beetle life stages when distinguishing morphological features are absent using basic molecular biology laboratory equipment and without sequencing.



**Figure A1. (A) Schematic of the male and female scaffolds the primers were designed on.** Female scaffold with NCBI accession KB632308 = female Seq\_985433, male scaffold KB741144 = male Seq\_1102890, and male KB737807 = male Seq\_1099472 as described in Keeling et al. (2013). These scaffolds do not contain any recognizable genic regions within 5 kbp of the primer binding sites. The HGT locus is over 30 kbp away from the primer binding sites. (B) Nucleotide alignment of local regions of putative neo-X scaffold from the female assembly with the putative neo-Y scaffold from the male assembly showing sequence diversity and location of primers.



**Figure A2. Amplicons from the multiplex PCR reaction using the neo-Y, neo-X and XY primers and DNA extracted from four female and four male adult MPB that were sexed using morphological features.** These PCR products were separated on a 2% agarose gel. A single amplicon of ~600 bp can be seen in samples amplified from female DNA extracts. Two amplicons of ~600 bp and ~500 bp can be seen in samples amplified from female DNA extracts. Note that one female sample failed to amplify, this can typically be resolved by adjusting the dilution of the DNA extract.

**Table A1: List of primers designed for sex identification in the MPB**

<b>Primer Name</b>	<b>Primer Sequence 5'-3'</b>	<b>Product with XY Primer</b>	<b>Tm (°C)</b>
XY primer:	5'-GTTGCCCGTAACTTCGTAAATGTCCAG-3'	-	60
neo-X:	5'-CACCTAGAGGGTATCCTGCGACAC-3'	X - specific	61
neo-Y:	5'-GCTAAGAGCGATGAAGGAGAGGC-3	Y - specific	60