INVESTIGATING RNA INTERFERENCE AS A BIOPESTICIDE FOR *DENDROCTONUS PONDEROSAE*

by

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Abstract

In western North America, the mountain pine beetle (*Dendroctonus ponderosae*, MPB) is the most destructive pest of pine trees, consequently causing ecological, economic, and socio-ecological impacts. Previous and current management techniques are ecologically and economically costly, creating a need for a low-cost, ecologically safe method for MPB population management. A molecular approach, RNA interference (RNAi), is being increasingly applied to pest management strategies due to target specificity and low production costs. The RNAi pathway is a naturally occurring anti-viral pathway that degrades invading genetic material. When co-opted, the RNAi pathway can be used to silence targeted genes in insects by disrupting cellular function. Developing RNAi to use in ecologically safe trap trees can create a novel tool for managing MPB populations. Existing and new gene targets were tested using microinjection (injection of dsRNA) and oral delivery methods for causing species-specific MPB mortality. Oral delivery involved the use of both *in vitro* synthesized dsRNA and heat-inactivated, hpRNA-expressing yeast delivered through direct feeding and association with pine phloem. Success of RNAi initiation was determined using mortality observations alongside gene expression analysis.

This study has identified promising gene targets and laboratory-based dsRNA delivery methods for RNAi initiation in emerged adult MPB. This study has also provided new insights into previously established dsRNA delivery methods. High mortality in all injected MPB suggests that dsRNA microinjection is too invasive for emerged adult MPB, despite being a successful delivery method in congeneric species. A previously successful method, submerging MPB in dsRNA for twelve hours, elicited an RNAi response in four of the six gene targets. However, the results were not replicated and showed variation between bioassays. To advance

the laboratory-based delivery method and provide support for RNAi use in lethal trap trees, a phloem-based dsRNA delivery method was also tested. Successful ingestion, high MPB survival in controls, and some RNAi-induced mortality supports the use of phloem-based methods for future RNAi assays in MPB. Lastly, an investigative transcriptome analysis confirmed the presence of critical RNAi components and provided insights into the MPB response to the different delivery methods. The submerged MPB mainly responded metabolically by breaking down energy stores, and the phloem-fed MPB showed the opposite metabolic response by creating energy stores. This analysis further supports the phloem-based dsRNA delivery for future RNAi studies on MPB. Future RNAi studies in MPB should consider emergence timing and its impact on MPB health to reduce the confounding factors affecting MPB mortality.

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Chapter 1 - Introduction

At endemic levels, bark beetles are important disturbance agents in forests, increasing diversity and productivity as a part of the natural forest development cycle (Dhar et al. 2016a; Pettit et al. 2020). In British Columbia, bark beetles (Coleoptera: Curculionidae, Scolytinae) are important drivers of mature tree mortality. Four notable bark beetle species are the: mountain pine beetle (*Dendroctonus ponderosae*, MPB), spruce beetle (*Dendroctonus rufipennis*, SB), Douglas-fir beetle (*Dendroctonus pseudotsugae*, DFB) and western balsam bark beetle (*Dryocetes confuses*, WBBB) (BCMFLNRO n.d.). The MPB is arguably the most significant insect affecting forests in western North America. Outbreaks negatively impact the local and national economy and have complex impacts on the ecosystem including altered forest compositions and forest fire probabilities (McCambridge et al. 1982; Simard et al. 2011; Morris et al. 2015; Corbett et al. 2016; Dhar et al. 2016a; Audley et al. 2020). Currently, the main constraint on MPB population range and numbers are cold temperatures in the winter (Safranyik 1998). With increasing temperatures, MPB populations exhibited unprecedented outbreak magnitudes, establishing further north and threatening the naïve boreal forest of Alberta (Cullingham et al. 2011; Janes et al. 2014). Advancing research towards new tools for MPB population reduction is critical for future forest management and health. Molecular advancements offer promise for a species-specific, non-toxic insecticide for pest management.

Developing a non-toxic, species-specific insecticide for MPB requires several considerations. The biology of the species and the current management methods are important to understand. This information, including life cycle, range expansion, and anticipated application of the insecticide, impacts the MPB life stage that is targeted. Next, more complex associations should be considered, including MPB population genetics and the taxonomic history of the

species. The pathway that is co-opted for this molecular-based insecticide, RNA interference (RNAi), is a naturally occurring, anti-viral pathway found in eukaryotes, including fungi, plants, and animals (Zhu and Palli 2020). While promising for pest management tools, there are limitations to the method. This introductory chapter will cover the background of MPB, including general biology, management, population genetics, and taxonomic history, as well as the RNAi pathway and science behind the development of a non-toxic, species-specific insecticide.

1.1 Mountain pine beetle biology and management

1.1.1 Mountain pine beetle life cycle and host attack

The mountain pine beetle progresses through five main developmental stages: eggs, four larval instars, pupae, teneral adults, and finally into adults. This entire life cycle occurs under the bark of the host trees (Howe et al. 2021; Chiu and Bohlmann 2022). The time required for the MPB life cycle is usually one year, but in some instances, it occurs over two years (Cullingham et al. 2019; Thompson et al. 2020; Chiu and Bohlmann 2022). This variation in timing can be local or annual and is influenced by weather and climate, specifically the daily temperatures (Bentz and Powell 2014; Thompson et al. 2020). Most MPB adults perish after mating and laying eggs, but some adults are able to survive mild winters (Thompson et al. 2020; Koch et al. 2021). The life cycle progression is usually aligned seasonally so that the larvae overwinter in hosts (Dhar et al. 2016a; Thompson et al. 2020; Chiu and Bohlmann 2022). The timing of the life cycle is not only important for overwintering success, but also for attack success on host trees by the new generation (Bentz and Hansen 2018; Wangen et al. 2024).

The preferred host genus for MPB is *Pinus*, with MPB able to colonize 14 known *Pinus spp.*, such as lodgepole pine (P*inus contorta*) (Dhar et al. 2016a; Audley et al. 2020). Female

MPB are the first to attack a host, and release aggregation pheromones to recruit other male and female MPB (Cullingham et al. 2019; Chiu and Bohlmann 2022). At endemic levels, MPB typically select large and older or weakened trees to infest, as the tree defences are easier to overcome (Janes et al. 2014; Dhar et al. 2016b; Wittische et al. 2019; Audley et al. 2020). Overcoming tree defenses is assisted by microbes associated with MPB, including fungi (Paine et al. 1997; Koch et al. 2021; Chiu and Bohlmann 2022). Tree mortality is caused by a combination of the MPB gallery formation and phloem ingestion as well as nutrient blockages caused by MPB-associated fungi (Koch et al. 2021). Particularly, blue stain fungi are thought to contribute to tree mortality through invasion of the sapwood (Paine et al. 1997). Fungal associates may also provide nourishment for MPB larvae (Paine et al. 1997; Morris et al. 2017; Cullingham et al. 2019). At epidemic levels, MPB can infest younger, healthier trees as there are enough beetles to overcome the stronger defences (Paine et al. 1997; Dhar et al. 2016b; Chiu and Bohlmann 2022).

1.1.2 Mountain pine beetle outbreak dynamics

The MPB is a native, irruptive insect of western North America (Cullingham et al. 2019; Howe et al. 2021; Koch et al. 2021; Wangen et al. 2024). Often, MPB outbreaks are periodic, occurring every 25-40 years and lasting for approximately 10 years (Janes et al. 2014; Dhar et al. 2016a; Bleiker 2019). These outbreaks are often regulated by positive and negative feedback loops (Raffa et al. 2008). Local beetle pressure and the presence of an endemic MPB population are major drivers of epidemic-level MPB outbreaks (Howe et al. 2021; Koch et al. 2021). Sporadic outbreaks can also occur if environmental conditions, such as drought, increase the number of weakened pines in an area close to an endemic MPB population (Koch et al. 2021). Recently, outbreaks promoted by MPB population pressure, forest management, and climate

changes have resulted in MPB range expansion and attacks on naïve hosts like Whitebark (*Pinus albicaulis* Engelman) and Jack pine (*Pinus banksiana* Lamb.) (Cullingham et al. 2019; Wittische et al. 2019; Howe et al. 2021).

1.1.3 Mountain pine beetle range expansion

Historically, the range of MPB in Canada was limited to British Columbia; Banff, the southern Rockies, Kananaskis region, and Cypress Hills in Alberta; and a small population in the Cypress Hills Interprovincial Park (CHIPP) in Saskatchewan (Bleiker 2019). In the early 2010s, the range of MPB in British Columbia spread from southern areas into northeastern areas as well as across most of Alberta (Janes et al. 2014; Morris et al. 2017). Molecular data also shows that genomic differentiation is allowing for the beetles to disperse over longer distances and provides adaptations to colder climates (Janes et al. 2014). More recently, the range has expanded into the boreal forest and northwards, placing southeastern Yukon at future risk (Bleiker 2019). The MPB range has also expanded into higher elevations, reaching new susceptible stands (Howe et al. 2021; Chiu and Bohlmann 2022). Range expansion is attributed to compounding factors, including climate change and forest management (Samarasekera et al. 2012; Dhar et al. 2016a; Cullingham et al. 2019). The decrease in cold temperature spikes at critical timepoints in MPB development, late spring and early fall, promote the overwintering success of MPB larvae (Coggins et al. 2011). Additionally, warmer temperatures enable MPB success in higher latitudes and elevations (Howe et al. 2021). Along with the warming temperatures, even-aged stands provide more hosts to sustain MPB populations than mixed-age stands (Fettig et al. 2014). The direction and mechanism of MPB range expansion is important to understand in order to predict future progression and management resource allocation.

Molecular investigations are being used to determine the path of range expansion by MPB populations into a previously uninhabited range and potential paths for further expansion. Modelling using gene flow and connectivity indicates that the MPB populations in Hinton and Edson, Alberta, originated from Jasper (Wittische et al. 2019). Trevoy et al. (2019) applied a model using single-nucleotide polymorphism (SNP) datasets and identified that the MPB population in Jasper is an intermediate between the previously identified northern and southern population clusters in British Columbia. Despite clustering in the northern and southern Alberta populations, gene flow between them prevents population structure (Shegelski et al. 2021). Mixing between the northern and intermediate populations could increase genetic diversity in the beetles that expand eastward (Shegelski et al. 2021). Samarasekera et al. (2012) showed MPB at the eastern epidemic edge locations have evidence for expansion. Wittische et al. (2019) predicted routes of eastward expansion in Alberta and Saskatchewan that provide high genetic connectivity and little resistance from elevation or climate. While it is uncertain whether the genetic variation identified by Shegelski et al. (2021) will be advantageous or not to dispersing MPB, it highlights the complexity of MPB population structure. Despite geographical separation leading to genetic differences, MPB populations can respond similarly to management if other factors are also similar (Samarasekera et al. 2012; Cullingham et al. 2019).

The impacts of the increasing outbreaks and presence of MPB in previously unaffected areas is a concern for forest health and management. The MPB can be considered a native, invasive pest (Ainslie and Jackson 2011; Wittische et al. 2019; Koch et al. 2021; Wangen et al. 2024). Though native to western North America, MPB populations have spread to the east across Canada and the north in British Columbia and Alberta where it is an invasive species (Cullingham et al. 2019; Shegelski et al. 2021). Naïve forest stands are at risk for future MPB

infestation, including the boreal forest and pine stands at higher elevations (Wittische et al. 2019; Howe et al. 2021; Koch et al. 2021). With no acquired resistance to MPB, an infestation in these stands would have a consequential impact (Howe et al. 2021; Koch et al. 2021).

1.1.4 Impacts of the mountain pine beetle

1.1.4.1 Economic decreases

Mountain pine beetles have affected over 18 million hectares in North America, with approximately 71 thousand hectares of lodgepole pine mortality recorded in British Columbia in the 2010s (Morris et al. 2015; Chiu and Bohlmann 2022). From 2000 to 2020, the area impacted by the MPB outbreak was estimated at 20 million hectares (BCMFLNRO n.d.). Economically, this accounted for more than half of the merchantable pine (Morris et al. 2015). The annual allowable cut (AAC) is projected to decrease by over 30 per cent in the next 50 years, negatively impacting the economy due to decreases in employment and production. It is also predicted that there will be a loss of over $57 billion to the British Columbia Gross Domestic Product (BCGDP) by 2054 (Corbett et al. 2016). Not only is MPB damage costly, but so are management efforts. Mitigation of the MPB in Alberta has cost over $500 million, and restoration of forests in British Columbia has required large sums from both the provincial and federal governments (Burton 2006; Shegelski et al. 2021; Johnson 2024). Additionally, other forest products with high economic value, such as the pine's symbiont, the pine mushroom (*Tricholoma magnivelare*), will be negatively impacted from the loss of mature pine trees and cause further economic strain at a local scale (Dhar et al. 2016b).

1.1.4.2 Altered ecology

Ecologically, irruptive MPB outbreaks alter forest composition with the mortality of mature host trees. Space is created in the canopy, decreasing competition with remaining plant

species and allowing increased understory growth (McCambridge et al. 1982; Dhar et al. 2016a; Audley et al. 2020). After an outbreak, the dominant species in a stand is likely to change, even temporarily, due to the impacted forest succession from the released growth of seedlings and saplings (Andrus et al. 2020). Audley et al. (2020) reported a 47% recovery of pine growth which led to the reinstatement of pine as the dominant species, but the mean number of live trees 14 years post-outbreak remained lower than pre-outbreak numbers. The mature pine tree mortality caused by MPB also impacts stand age, decreasing average tree age (Hicke et al. 2013). Nutrient cycles and soil characteristics are also altered, largely due to falling needles (Griffin and Turner 2012; Reed et al. 2018). Wildfire probabilities are impacted, with a decrease in active crown fire probability but an increase in passive crown fire and fire spread probabilities (Jenkins et al. 2008; Simard et al. 2011). Mature *Pinus* mortality impacts the water cycle, causing groundwater levels to fluctuate and alters seasonal water availability, affecting the forest stands and the ecosystem services the forest provides (Dhar et al. 2016b).

1.1.4.3 Complex socio-ecological impacts

The socio-ecological impacts of MPB outbreaks show negative effects on recreation, aesthetics, and cultural heritage (Dhar et al. 2016b; Morris et al. 2017, Bleiker 2019). With the previous MPB outbreaks impacting 80% of the recreational trails and sites in central British Columbia, users shared negative impressions. While some sites reported increased usage and no closures, other sites reported short-term recreational losses due to the MPB outbreaks (Dhar et al. 2016b). While there are cumulatively minimal effects on the recreation in the forests after the MPB outbreaks, there are negative impacts on the resulting forest aesthetics, though public perception is complex (Dhar et al. 2016b). Additionally, property values are a large concern due to MPB-mediated tree mortality (Morris et al. 2017). Consideration of the numerous economic

and ecological impacts from MPB outbreaks present challenges to forest managers, which is further complicated by complex public perceptions.

1.1.5 Mountain Pine Beetle Management Methods

Managing MPB populations is difficult as the beetle life cycle occurs under the tree bark, and controlling large scale outbreaks or large populations is not feasible (Dhar et al. 2016a, Bleiker 2019). Current MPB management methods used in British Columbia are high in cost and often ecologically deleterious (Dhar et al. 2016a; Taylor et al. 1997). Management approaches are typically placed into two categories: direct or indirect control (Coggins et al. 2011; Fettig et al. 2014).

Direct control is a reactive approach, initiated after infestation and is used to treat symptoms and reduce the target MPB population (Coggins et al. 2011; Fettig et al. 2014). In British Columbia, pest reduction harvesting (formerly "sanitation harvesting") and salvage logging or single-tree treatments are the main direct control approaches. Effectiveness of pest reduction harvesting is limited due to the difficulty in quickly detecting infested trees. This method has been proven to slow MPB population growth, but is costly and labour-intensive, as over 50% of infested trees have to be removed annually to cease the MPB population increase (Coggins et al. 2011; Fettig et al. 2014; Taylor et al. 1997). Salvage logging, or removal of previously infested trees, does not impact MPB populations, but allows for the mitigation of economic losses by using the trees for lumber (Bleiker 2019; Fettig et al. 2014). This technique can have negative impacts on local wildlife (Dhar et al. 2016a). Single tree treatments used various techniques to remove MPB from a tree or small group of trees. One popular technique was the use of insecticides. While there was proof of decreased MPB survival, public perception

and off-target toxicity concerns have led to reduced applications and the discontinuation of some of the insecticides (Fettig et al. 2014; Dhar et al. 2016a; Taylor et al. 1997).

Indirect control is considered a preventative approach, and include use of pheromone baiting, stand thinning, and silvicultural techniques (Coggins et al. 2011). Stand thinning decreases the number of susceptible hosts for MPB, promotes tree health via decreasing nutrient competition, and increases wind speed within the stands, which impacts MPB dispersal (Coggins et al. 2011; Fettig et al. 2014). Not without risks, thinning can create favourable conditions for other woodboring insects and tree pathogens (Fettig et al. 2014). Clear cutting is another management technique that can stagger the age of trees within a stand, also decreasing the number of available hosts to MPB. One obstacle with this technique is the public perception that cleared blocks negatively impact aesthetics (Fettig et al. 2014). Creating mixed-species stands can lessen the number of host trees. This not only promotes a resilient forest, but also decreases the probability of a large-scale MPB attack as stands with high host tree concentrations are typically targeted (Coggins et al. 2011; Fettig et al. 2014). Though pheromones are listed as an indirect approach, they are often used in conjunction with a direct management technique. Attraction pheromones can concentrate MPB to a smaller area and cause fewer infested trees that can be removed via pest reduction harvesting. However, this has a risk of spillover infestations, where non-baited trees are colonized by MPB attracted to the area by the pheromones (Fettig et al. 2014; Klutsch et al. 2017). Anti-aggregation pheromones can protect host trees from attack while pest reduction harvesting removes infested trees. Slight improvement of results was observed when both types of pheromones were used along with pest reduction harvesting. The pairing of these tools is currently the most advised practice in the western United States of America (Fettig et al. 2014). Currently, British Columbia is in the recovery phase, mitigating the

impacts of the previous outbreak, but MPB outbreaks are expected to recur (Bleiker 2019). There is a need for improved methods to manage MPB populations, and molecular techniques show promise but the intraspecific genetic diversity in MPB needs to be considered (Kyre et al. 2020; Taylor et al. 1997).

1.2 Mountain pine beetle population genetics and structure

1.2.1 History of the taxonomic organization of the MPB species

Using molecular techniques for pest management introduces new obstacles, such as genetic differences between populations. For MPB, the population genetics is of particular concern as the species has shown post-zygotic isolation and the taxonomic structure of the species has been, and is still investigated (Wood 1963; Bracewell et al. 2011; Trevoy et al. 2019; CABI 2020). The history and genetic variation among populations could impact the responses to molecular techniques for management (Samarasekera et al. 2012).

Two previously separate species *Dendroctonus monticolae* and *Dendroctonus jeffreyi* were combined into the *D. ponderosae* species in 1963 (Wood 1963). Shortly after in 1968, *D. jeffreyi* was classified as a separate species (CABI 2020). The changes in the taxonomic groupings have led to investigations into the genetic variation within the *D. ponderosae* species, especially among populations in different geographical regions. Genetic differences were found to be highly correlated with geographical distances (Mock et al. 2007; Cullingham et al. 2012; Samarasekera et al. 2012). Notable variation was found in populations around large desert areas, and between northern and southern populations. However, there are exceptions to the geographic and genetic distance correlation. The genetic composition of populations in British Columbia are similar to the Idaho populations, despite the large geographical distance between them (Mock et al. 2007). Two populations geographically closest to each other, California and Arizona, show

the most genetic differences. The Arizona population is more divergent than expected from geography, and is along the previous speciation border between *D. monticolae* and *D. ponderosae* (Mock et al. 2007). This congruency between the suspected genetic discontinuity and the past speciation border, along with lack of geographical distance correlation, indicates that the different MPB populations might be progressing toward speciation once again.

1.2.2 Genetic Variation among Mountain Pine Beetle Populations

Additional studies show genetic differences among populations in western North America, with identification of distinct northern and southern clusters (Samarasekera et al. 2012). In Canada, there is a weak population structure signal between northern and southern populations, attributed to frequent gene flow between clusters (Janes et al. 2014). Genetic differentiation between the northern and southern clusters was low but significant (Samarasekera et al. 2012). There were also sub-clusters within the two main clusters (Samarasekera et al. 2012). Though molecular markers did not indicate large divergence or enough of a decrease in gene flow to support reproductive isolation, postzygotic isolation was found to occur via reduced egg hatch in hybrid males (Bracewell et al. 2011). There appears to be potential for a reproductive barrier between the Oregon and Idaho MPB populations, which was unexpected due to the close geographical distance, similar morphology, and small genetic distance. The early postzygotic barrier, male hybrid sterility, is thought to be recent and does not indicate that speciation has started (Bracewell et al. 2011).

The geographical influence on MPB populations also affects phenotypic traits, including body size and development time. Adult MPB size was found to decrease with increasing latitude on both sides of the Great Basin Desert, as adult beetles on the eastern side were larger on average than the beetles on the western side at the same latitudes (Bracewell et al. 2013).

Northern populations developed faster than southern populations, with clear genetic differences in development time and adult size showing local adaptations (Bracewell et al. 2013). However, despite the genetic and phenotypic differences, gene flow among populations is still frequent enough to obscure any molecular signal of population-level genetic structure (Mock et al. 2007; Bracewell et al. 2013; Janes et al. 2014). The increasing interest in the application of molecular biology techniques to assist in management practices can be limited due to gene expression and inter- and intra-population genetic differences (Kyre et al. 2020, 2024). These differences in gene expression and genetics can cause different responses to invading genetic material (antiviral response) and even pest management tactics. As such, genetic differences are important considerations when using genetic-based methods for pest management (Kyre et al. 2024).

1.3 RNA interference (RNAi) use and limitations

1.3.1 Gene expression analysis

Information in a cell is stored in deoxyribonucleic acid (DNA) with coding segments referred to as genes. The genes are turned into ribonucleic acid (RNA) via transcription. Specifically, proteins are coded from a class of RNA molecules called messenger RNA (mRNA). After the pre-mRNA strand is made, it undergoes modification via capping of the 5’ end, intron splicing, cleavage of the 3’ end, and polyadenylation of the 3’ end (Jurado et al. 2014). The mRNA strand is used as a template to create proteins through a process called “translation”. The resulting proteins carry out the necessary functions for cell life. This entire process converting information from DNA through to a functional product is referred to as gene expression (Anjum et al. 2016).

Gene expression can be altered, with the transcription and translation, or expression, of some genes being decreased or increased. This would also cause a decrease or increase in the

mRNA in the cell, and therefore an expected similar change in the functional gene product amount. Differential gene expression allows for different cell types to exist and adaptations to external and internal conditions (Gilbert 2000; Robert et al. 2016).

Gene expression is usually analyzed using real-time quantitative PCR (RT-qPCR) which measures cDNA made from extracted RNA (Taylor et al. 2017). These results can be variable, susceptible to contaminants, and require normalization to an internal control, often one or more housekeeping, or reference, genes. Reference gene expression optimally remains stable among cells and experimental conditions, and due to this stability, the selected reference genes often have essential roles in the cell (Kyre et al. 2024). Digital droplet PCR (ddPCR) is increasing in popularity for gene expression analysis due to improved efficiency. Compared to RT-qPCR, ddPCR is less sensitive to inhibiting factors, more specific to small fold-changes of expression, and does not require a standard curve to quantify the target gene copy number (Zmienko et al. 2015). Due to the improved efficiency and quantitative counts provided by ddPCR, normalization to a reference gene is not always required for gene expression analysis (Taylor et al. 2017). However, the use of at least one reference gene is recommended, optimally using two or three reference gene assays run in duplex with the target gene assays (Coulter 2018). Validation of a variety of reference genes to use in MPB gene expression analysis has been conducted, providing a catalogue of reference genes to select from for this project (Fraser et al. 2017; Horianopoulos et al. 2018).

1.3.2 Ribonucleic acid interference (RNAi)

The use of the RNAi pathway is being increasingly applied to pest management strategies due to target specificity and low production costs (Christiaens et al. 2020; Kyre et al. 2020). RNAi is a naturally occurring anti-viral pathway that degrades invading genetic material

(Dowling et al. 2016; Christiaens et al. 2020). The RNAi pathway can be initiated by exogenously introducing several variations of double-stranded RNA (dsRNA): open-ended short interfering dsRNA (siRNA), open-ended long dsRNA (dsRNA), hairpin-type RNA (hpRNA) with one closed end, or paperclip RNA (pcRNA) with two closed ends (Abbasi et al. 2020; Hashiro and Yasueda 2022). Long dsRNA and hpRNA are typically over 200 nucleotides long, much longer than the siRNA and pcRNA which range from 21-23 nucleotides (Abbasi et al. 2020). In order to cause gene suppression or knockout, the dsRNA or hpRNA must be an exact match to the target mRNA for a minimum of 16 consecutive nucleotides, or 25 nucleotides with few mismatches (Kyre and Rieske 2022; Hollowell et al. 2022).

There are two mechanisms for dsRNA that is ingested orally: clathrin-mediated endocytosis and the systemic RNA interference deficient-1 (SID-1) transmembrane channel-mediated uptake (Cappelle et al. 2016). One or both pathways have been demonstrated in insects (Cappelle et al. 2016, Yoon et al. 2016). In the SID-1 uptake, transmembrane channels facilitate the movement of dsRNA from the intestinal lumen into the cytosol (Winston et al. 2007; McEwan et al. 2012). In clathrin-mediated endocytosis, the dsRNA is brought into the cytoplasm by endosomes and released via acidification (Yoon et al. 2016). Eventually, in both pathways, the RNase III-type Dicer-2 (Dcr-2) cuts the dsRNA strand into smaller pieces, also known as small interfering RNA (siRNA). With help from other associated machinery components, like R2D2, the siRNA is moved to the RNA-induced silencing complex (RISC), of which Argonaute-2 is a critical component (Yoon et al. 2016; Christiaens et al. 2020; Kyre et al. 2020). Here, the double strands are separated and one complementary strand is degraded while the other is used as a template for the recognition of matching mRNA. Acting in a sequence specific manner, the Argonaute component degrades the mRNA that matches the template (Yoon et al. 2016; Kyre et

al. 2020). As a result, translation is ceased or decreased, decreasing the amount of protein that the mRNA codes for (Leelesh and Rieske 2020; Kyre et al. 2020). This process is highly sequence specific, which decreases the possibility of off-target effects (Christiaens et al. 2020; Kyre et al. 2020). This pathway can be co-opted to decrease or cease production of proteins to increase understanding of the protein's role within an organism. Alternatively, co-opting the RNAi pathway can be used to cause rapid, targeted mortality by introducing dsRNA that mimics mRNA, which codes for an essential protein, to the organism (Cooper et al. 2019). The RNAi pathway has been extensively co-opted in many insect species. Successful RNAi initiation has decreased target gene suppression in mosquitoes (Zhao et al. 2024; Girard et al. 2025), with associated mortality to some targets (Stewart et al. 2023). Gene suppression via RNAi was reported in Hemipteran insects like the brown planthopper (Li et al. 2025), the brown marmorated stink bug (*Halyomorpha halys*) (Ghosh et al. 2018; Finetti et al. 2023), and the Asian citrus psyllid (Ghosh et al. 2018). Additionally, RNAi was effective in crickets (*Modicogryllus siamensis*) (Tamaki et al. 2013) and *Drosophila* flies (Saleh et al. 2006; Murphy et al. 2016).

Gene expression analysis is an important part in RNA interference (RNAi) studies as it confirms that the RNAi pathway was initiated alongside the mortality observations (Wallace and Rieske 2023). For RNAi in bark beetles, most studies report suppressed gene expression combined with the observed phenotypical response (Rodrigues et al. 2018; Leelesh and Rieske 2020; Kyre et al. 2020; Liu et al. 2021, 2022, 2022; Kyre and Rieske 2022; Pampolini and Rieske 2023). Previous studies have reported rapid mortality of MPB after exposure to dsRNA combined with decreased target gene expression levels (Kyre et al. 2020; Kyre and Rieske 2022).

1.3.3 Transcriptome analyses have discovered and identified RNAi pathway components in insect species

Critical genes involved in the different RNAi pathways in insects have been identified, including a group of *Argonaute* genes, *dicer, Staufen*, *R2D2*, and *SID-1*, among others (Zhao et al. 2015; Yoon et al. 2016; Christiaens et al. 2020; Wallace and Rieske 2023). Important components of the specific RNAi pathway likely used in insects, the siRNA pathway, have been verified in Coleopteran beetles like the Colorado potato beetle (*Leptinotarsa decemlineata*) and the six-spined ips (*Ips calligraphus* Germar) (Yoon et al. 2016; Wallace and Rieske 2023). In the six-spined ips, the confirmation of the RNAi pathway components was in addition to beetle mortality and gene suppression as a response to RNAi (Wallace and Rieske 2023). In MPB, the RNAi machinery has not been investigated alongside bioassays testing the efficiency of RNAi. A search of a mapped MPB transcriptome (GCF_000355655.1 DendPond male 1.0 rna from genomic.fna) found the presence of some important RNAi components: *Argonaute-2*, *endoribonuclease dicer*, and *SID1*. With many previous studies showing the effectiveness of RNAi in MPB, and the presence of some critical components of the RNAi pathway confirmed in a general MPB transcriptome, it is expected that MPB should be sensitive to RNAi. However, the relationship between effective RNAi and the expression levels of core machinery in adult MPB has not been investigated.

1.3.4 Species-specificity of RNAi

Using dsRNA to target an mRNA strand is species-specific and limits off-target effects on other organisms that come into contact with the dsRNA (Kyre et al. 2019). This specificity is due to the dsRNA/mRNA sequence similarity requirements. The dsRNA and target mRNA sequences must be exactly the same for a minimum of 16 consecutive nucleotides, or with a few

differences in a longer sequence length, like 25 nucleotides (Kyre and Rieske 2022; Hollowell et al. 2022). Targeting less-conserved genes can further increase the species-specificity of this method (Whyard et al. 2009; Kyre et al. 2019). The species-specificity of RNAi has been shown in many studies.

One study investigated the impact of using dsRNA designed for *Halyomorpha halys* on *Rhodnius prolixus* (Finetti et al. 2023). There was no mortality or significant gene suppression observed in the off-target species when exposed to any of the three gene targets, supporting the species-specificity of the RNAi method in Hemiptera. Two genes, *vATPase* and highly conserved tubulin (*gTub23C*) were tested for species specificity among four species: fruit flies (*Drosophila melanogaster*), flour beetles (*Tribolium castaneum*), tobacco hornworms (*Manduca sexta*), and pea aphids (*Acyrthosiphon pisum*) (Whyard et al. 2009). For both genes, only the species for which the dsRNA sequence was designed for exhibited gene expression changes. For the more conserved tubulin gene, this was attributed to using a less conserved region of the gene (Whyard et al. 2009). Another study investigated the effects of dsRNA designed for southern pine beetle (*Dendroctonus frontalis*, SPB) on other species associated with the *Pinus* spp. hosts: pine sawfly (*Neodiprion lecontei*), eastern subterranean termite (*Reticulitermes flavipes*), and six-spined ips (*Ips calligraphus*) (Hollowell et al. 2022). The tested non-congeneric species transcriptomes showed little overlap with the SPB transcriptome. When treated with the SPB dsRNA in bioassays, only the six-spined ips (*Ips calligraphus*) showed a change in gene expression, but no difference in survival was observed (Hollowell et al. 2022). The other two species also showed no mortality from dsRNA exposure (Hollowell et al. 2022). These findings provide further support for the specificity of RNAi and the non-toxicity to off-target, non-congeneric species.

1.3.5 Limitations of RNAi

With variable outcomes from many different studies, it is difficult to predict what targets will work without considering the other factors affecting RNAi responses like the target species and life stage, the targeted genes, and the delivery method used to get the dsRNA to the insect (Poreddy et al. 2017; Cooper et al. 2019; Leelesh and Rieske 2020). For example, effective gene targets may vary depending on the study's targeted insect life stage (Bingsohn et al. 2017; Liu et al. 2021). Effective delivery methods can be species-specific, so that one method which elicits a RNAi response in one species may not cause a response in another species (Finetti et al. 2023). In this section, the rationale and specific examples behind these factors will be introduced.

1.3.5.1 Insect species

The increasing use of the RNAi pathway for insect and virus control, medicine, and to explore functional characterization of proteins comes with challenges. Generally, species within some insect orders like Diptera, Hemiptera, and Lepidoptera have variable RNAi responses. Among reported non-successful responses, there are studies with successful RNAi-mediated gene suppression in Lepidoptera species (Turner et al. 2006; Terenius et al. 2011). Other orders, like Coleoptera, are generally considered sensitive to RNAi, with less variation among the RNAi responses (Christiaens et al. 2020). However, there are exceptions to the generalization, often due to the additional factors affecting RNAi efficiency.

1.3.5.2 Gene targets

The targeted genes in RNAi studies can be a source of variation. Currently, there is a limited understanding about what makes certain gene targets more sensitive to RNAi compared to others (Cooper et al. 2019). The current knowledge is limited due to variation in experimental set up, limited knowledge about protein stability, and the gene target bias in experiments (Cooper

et al. 2019). Variation in the responses of insects to different gene target dsRNA is reported in numerous studies (Bingsohn et al. 2017; Kyre et al. 2019, 2020; Liu et al. 2021, 2022; Kyre and Rieske 2022). Other studies show gene suppression in all the targeted genes (Turner et al. 2006; Zhu et al. 2011; Zhang et al. 2016; Rodrigues et al. 2018; Leelesh and Rieske 2020; Sun et al. 2021; Pampolini and Rieske 2023).

In studies investigating the lethality of target gene suppression on the insect, the same targets are often tested. These studies aim to target essential genes that, when suppressed, should cause rapid mortality. A popular gene target for this type of study is the inhibitor of apoptosis (*iap*), a stress response gene that blocks cell death (Kyre et al. 2020). This target was used in previous RNAi experiments on MPB and SPB, along with two other stress response genes: heat shock protein (*hsp*) and shibire (*shi*) (Kyre et al. 2019, 2020; Kyre and Rieske 2022). One or more of those targets were tested on other Coleopteran species, including the Emerald Ash Borer (*Agrilus planipennis*, EAB) and the six-spined ips (Rodrigues et al. 2018; Leelesh and Rieske 2020; Wallace and Rieske 2023; Pampolini and Rieske 2023). While a few studies have looked at other essential gene targets in Coleopteran insects, including *Dendroctonus* spp., there is a need to further survey more gene targets to increase the knowledge and applicability of RNAi for targeted insect mortality.

Many of the commonly tested genes are highly conserved among species due to their critical roles (Reaume and Sokolowski 2011; Kyre and Rieske 2022). Silencing of homologous genes in a congeneric species have been reported (Poreddy et al. 2017; Kyre and Rieske 2022). Ingestion of material from a dsRNA-producing plant targeting a gene in the Lepidopteran insect *Manduca sexta* silenced the target gene, and the respective homologous gene, in both *M. sexta* and wild *Manduca quinquemaculata* (Poreddy et al. 2017). In the *Dendroctonus* genus, both

MPB and SPB exhibited silencing of the heat shock protein (*hsp*) gene when MPB-specific dsHSP was ingested (Kyre and Rieske 2022). While these results show promise for RNAi as a pest management tool for congeneric pests with similar niches, this could indicate that some off-target species may be affected.

1.3.5.3 Insect life stage

The life stage of the insect can cause varying responses to RNAi, especially among larvae and adults tested with the same gene target (Cooper et al. 2019; Leelesh and Rieske 2020). Often, the starting gene expression levels vary at different developmental stages, as shown in the Chinese White Pine beetle (*Dendroctonus armandi*) and the light brown apple moth (*Epiphyas postvittana*) (Turner et al. 2006; Liu et al. 2021, 2022). In common fruit flies (*Drosophila melanogaster*), larvae are not responsive to dsRNA delivered by injection or ingestion, whereas the adults and embryos are sensitive to injections (Cooper et al. 2019). In Coleopteran insects, the adult and larval responses to the same gene target often differ. In flour beetles (*Tribolium castenum*), the RNAi pathway initiation was found to be stage-specific, causing mortality when injected in larvae, but no mortality when injected in adults (Bingsohn et al. 2017). The varied RNAi effectiveness among life stages in another *Dendroctonus* species was shown when dsRNA was injected in *D. armandi*, resulting in the suppression of two out of three genes in adults, and all three genes in larvae (Liu et al. 2021). As shown in the above examples, the responses of the insect at different life stages can also be contingent on the dsRNA delivery method.

1.3.5.4 Delivery method for dsRNA

The delivery method of the dsRNA to the insect is yet another source of variability in RNAi efficiency. Insects in the Diptera and Orthoptera orders showed sensitivity, and resulting gene suppression, to RNAi when the dsRNA was injected, but not when it was fed (Baum et al.

2007; Cooper et al. 2019). In one study, the Hemipteran *Halyomorpha halys* nymphs showed gene suppression with dsRNA applied topically, but did not cause gene suppression in *Rhodnius prolixus* nymphs (Finetti et al. 2023). Rather, dsRNA had to be injected to elicit an RNAi response (Finetti et al. 2023). Species such as the EAB and the western corn rootworm (*Diabrotica virgifera virgifera*) in the Coleoptera order showed gene suppression after injection or oral uptake of dsRNA, further corroborating the sensitivity of Coleopteran insects to RNAi (Zhao et al. 2015; Rodrigues et al. 2018; Cooper et al. 2019). Another Coleopteran insect, the red flour beetle (*Tribolium castaneum*), has only been injected with dsRNA in RNAi studies (Linz et al. 2014; Bingsohn et al. 2017; Rodrigues et al. 2018). While the two main delivery methods are microinjection and oral ingestion, absorption of the dsRNA is another method found to be effective at initiating RNAi in Coleopteran insects (Baum et al. 2007; Leelesh and Rieske 2020; Kyre et al. 2020).

In order to transition from lab-based to *in situ*-based application, adaptations to these feeding methods are being tested and used. Some adaptations include expression in transformed plants and microbe-mediated delivery, which help to increase the stability of the dsRNA in natural settings (Cooper et al. 2019; Christiaens et al. 2020; Leelesh and Rieske 2020). Successful delivery and RNAi initiation has occurred when dsRNA was expressed by bacteria and fed to EAB larvae (Leelesh and Rieske 2020). Another microbe-mediated method with successful delivery and RNAi initiation is through the ingestion of dsRNA-expressing yeast (Duman-Scheel 2019). The dsRNA-expressing yeast shows promise for gene silencing and altering the immune system in mammals (Duman-Scheel 2019). In insects, ingestion of dsRNA-expressing yeast has resulted in gene silencing and decreased health (Murphy et al. 2016). The use of yeast progresses the applicability of RNAi as a pest management tool as it allows for an

inexpensive production and protects delivery of the dsRNA from degradation (Duman-Scheel 2019).

1.3.6 RNAi success in species closely related to MPB

Many species within the Coleoptera order are susceptible to RNAi with at least one method of dsRNA delivery, and they often exhibit a robust sensitivity to more than one dsRNA delivery method, such as injection, ingestion, or absorption (Zhao et al. 2015; Cooper et al. 2019; Pampolini and Rieske 2023). The sensitivity to RNAi in Coleopteran insects is promising as bark beetle (Coleoptera: Curculionidae: Scolytinae) population growth is supported by warming climate (Cullingham et al. 2012; Bentz and Powell 2014; Dhar et al. 2016a). Gene function studies have extensively used microinjection of dsRNA for to initiate RNAi-mediated gene suppression in *D. armandi* (Wang et al. 2016; Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022). For the SPB, lethality investigations have used ingestion of dsRNA droplets (Kyre et al. 2019, 2024; Kyre and Rieske 2022). In MPB, RNAi has been initiated in teneral adults through microinjection and in emerged adults with a combination of oral ingestion and absorption (Keeling et al. 2013; Kyre et al. 2020; Kyre and Rieske 2022). The ingestion of dsRNA targeting essential genes elicited a mortality response in emerged adult MPB (Kyre et al. 2020; Kyre and Rieske 2022). The combination of ingestion and absorption of dsRNA by MPB provides a step towards application as a MPB management tool in forests.

1.4 Proposed research

The MPB is an irruptive, native pest with important roles in forest development. Changes in climate, including increasing temperatures and drought, are providing ideal conditions for MPB population increases and consequential range expansion (Coggins et al. 2011; Howe et al. 2021). With naïve forest stands at risk, and increased tree mortality within the historical range,

outbreaks impact the ecosystem as well as the local and national economy (Morris et al. 2015; Corbett et al. 2016; Dhar et al. 2016a; Audley et al. 2020). Combinations of direct and indirect management strategies have shown success in restraining MPB population numbers, but are expensive and labor-intensive (Coggins et al. 2011; Fettig et al. 2014). Use of RNAi as a pest management tool is gaining momentum due to the cost-effective, species-specific nature (Kyre et al. 2020). Developing an additional tool for forest managers to assist with MPB management is crucial.

Results from RNAi studies can be impacted by factors such as the targeted insect species and life stage, the gene targets, and the dsRNA delivery method. Applying a genetic-based technique to MPB requires knowledge of population genetics due to the speciation history. At this point, evidence of MPB population structure in Canadian provinces has not been identified (Cullingham et al. 2012; Shegelski et al. 2021). This study aims to progress the development of RNAi as a MPB management tool by addressing some of the above-mentioned factors. Different delivery methods, separated into chapters, will be tested for proof-of-concept and advancing towards *in situ* application. This research is the first to test the efficacy of hpRNA-expressing yeast at initiating the RNAi pathway when orally ingested by adult MPB. In the fifth chapter, the genome-wide impact of the tested delivery methods on the MPB will be investigated with a transcriptome analysis. Novel gene targets will be tested throughout the study to identify more gene targets that can be used for MPB mortality.

This project includes using two different dsRNA sources, provided by Renaissance BioScience Corp. (RBSC). One source is an *in vitro* kit-synthesized dsRNA, hereafter referred to as dsRNA, and the other source is hairpin RNA, hereafter referred to as hpRNA, expressed in a proprietary RNAi production *Saccharomyces cerevisiae* strain. The dsRNA and hpRNA was

synthesized to individually target six essential genes in adult MPB. Two genes, the inhibitor of apoptosis (*iap*) and the heat shock protein (*hsp*), are stress response genes previously successful at RNAi initiation in MPB, along with other Coleopteran species (Kyre et al. 2019, 2020; Leelesh and Rieske 2020). Four additional genes were selected by RBSC: V-type proton ATPase subunit S1 (*vha19*), mesh protein (*mesh*), snakeskin protein (*ssk*), and tetraspanin 2a (*tsp2a*). These genes were selected for their essential functions in molecule transport and gut structure, as well as success in initiating RNAi in other Coleopteran insects (Baum et al. 2007; Hu et al. 2019). Three target genes: *ssk*, *mesh*, and *tsp2a* code for proteins that form a complex and localize in the midgut of *Drosophila* species. Suppression of either one of the genes leads to defects in the midgut structure and barrier function between cells (Jonusaite et al. 2020). This complex is a part of the smooth septate junction, which regulates the transfer of molecules in the intestinal and renal systems of arthropods (Hu et al. 2019). The *mesh* and *tsp2a* protein products are also essential for Malpighian tubule epithelium establishment and function in *Drosophila*, with mortality observed within one week of knockdown of *tsp2a* (Jonusaite et al. 2020; Beyenbach et al. 2020). The *ssk* protein product is a membrane protein also involved in barrier function in the intestines of insects (Hu et al. 2019). The *vha19* target gene is predicted to code a protein essential in the intestine and epidermal structure, with previous RNAi-mediated suppression causing mortality in *Caenorhabditis elegans* (Zawadzki et al. 2012). A random sequence, termed dsRS or hpRS that does not match any transcripts in the MPB transcriptome (NCBI 2022), was developed by RBSC as a control for dsRNA introduction to MPB.

Chapter 2 - Use and evaluation of microinjection as a delivery method for target dsRNA to initiate the RNAi pathway and test novel gene targets on emerged adult *Dendroctonus ponderosae.*

2.1 Introduction

One of the main factors contributing to variable RNA interference (RNAi) efficiency within and among insect species is the delivery method of the target double-stranded RNA (dsRNA) (Cooper et al. 2019). Some insect orders, such as Coleoptera, display sensitivity to orally ingested or absorbed dsRNA as well as to injected dsRNA. Other insect orders only show gene suppression after injecting the dsRNA (Joga et al. 2016; Singh et al. 2017; Cooper et al. 2019). Microinjecting target dsRNA allows for higher certainty in the amount of dsRNA delivered and often works with a smaller dose, approximately half of the lowest amount required in feeding trials, to achieve a phenotypic response (List et al. 2022). Microinjection is a common first step to test RNAi-mediated target gene suppression (Wang et al. 2016).

The initiation of RNAi using microinjection to deliver the target dsRNA has been used on adult *Dendroctonus* species to increase understanding of the roles of specific genes. In Chinese white pine beetles (*Dendroctonus armandi*), microinjecting dsRNA led to cytochrome P450 and an olfactory co-receptor gene suppression (Zhang et al. 2016; Liu et al. 2022; Liu and Chen 2022). Microinjecting dsRNA into *D. armandi* achieved partial knockdown of a gene assumingly unique to *D. armandi*, a type of olfactory receptor coreceptor, named DarmOrco, thought to allow the olfactory receptor (OR) to assemble into the proper structure (Zhang et al. 2016). The affected phenotype in the antennal response to odors was observed as *D. armandi* adults showed less response to monoterpenes associated with plant hosts (Zhang et al. 2016). Emerged adult *D. armandi* were injected with dsRNA for specific cytochrome P450 genes which led to knockdown

and increased sensitivity to (+)-α-pinene (Liu et al. 2022). In yet another microinjection study with *D. armandi*, dsRNA was injected for successful RNAi initiation and gene suppression (Sun et al. 2021). Beetle mortality in both injected and non-injected controls was less than in the treatment groups, indicating that microinjection does not impact *D. armandi* physiology (Liu et al. 2022).

The amount of dsRNA injected to achieve mortality or other phenotypic responses varies depending on the study. A review recently quantified the smallest and largest dsRNA amounts microinjected in different studies with different target insects that elicited a mortality response as 1.5E-05ng and 20μg (List et al. 2022). Injected amounts of 1.5E-05ng to 1.5E-03ng led to 75% and 90% mortality in *Tribolium castaneum*, respectively (Bingsohn et al. 2017). Other studies investigating the larval stage of *T. castaneum* injected a range of 500ng to 700ng delivered in 500nL (Linz et al. 2014). Wang et al. (2016) conducted a study with *D. armandi* larvae injected with 0.1μM in 50nL of target dsRNA, a volume similar to what is injected in adult beetles. With such a large difference between those extremes, most *Dendroctonus* spp. studies report amounts between 100 - 200ng (Zhang et al. 2016; Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022).

The targeted insect's life stage is another source of RNAi variability. Microinjection has successfully initiated the RNAi pathway in larval and adult insects (Bingsohn et al. 2017; Cooper et al. 2019; Liu et al. 2021). The effective amount of dsRNA required for insect injection depends on life stage. Larval *D. armandi* were injected with half the amount used for adult injections (Liu et al. 2021). Target gene expression often varies between the larval and emerged adult stages, impacting suppression success or quantity between the two life stages (Liu et al. 2021, 2022; Liu and Chen 2022). For example, adult *D. armandi* had suppressed gene expression

in two of the three targeted genes whereas larvae exhibited decreased gene expression in all three target genes (Liu et al. 2021). An RNAi study with the red flour beetle (*Tribolium castaneum*), showed similar results that varied with gene targets and beetle life stages (Bingsohn et al. 2017). Using the amounts and volumes previously successful at initiating RNAi-mediated gene suppression in the same life stage of a congeneric species as a guide is expected to help eliminate some of the variables impacting insect response to injected dsRNA.

Microinjections in MPB (*Dendroctonus ponderosae*) are not as common for RNAi initiation as they are in *D*. *armandi*, but have been successful. Keeling et. al (2013) reported successful RNAi initiation when teneral adults were injected with 200ng of dsRNA and allowed to complete development post-injection. The results showed 56–83% survival of injected beetles and suppressed target gene expression in three diphosphate synthases (Keeling et al. 2013). Teneral adults are a life stage right before emerged adults, and as such, emerged adult MPB may exhibit different responses to microinjection. Additionally, targeting a gene that is intended to have a lethal impact is another potential variable in MPB response compared to the previous knockdown study.

The first objective of this study was to use microinjection to deliver dsRNA to emerged adult MPB, testing the lethality of the six gene targets: inhibitor of apoptosis (*iap*), heat shock protein (*hsp*), V-type proton ATPase subunit S1 (*vha19*), tetraspanin 2a (*tsp2a*), mesh protein (*mesh*), and snakeskin (*ssk*). Two genes *iap* and *hsp* are stress response genes and have previously elicited a lethal response in adult MPB (Kyre et al. 2020). The gene targets *mesh*, *ssk*, and *tsp2a* form a protein complex in the midgut, and singular knockdown of *tsp2a* and *mesh* has proven lethal to *Drosophila melanogaster* (Jonusaite et al. 2020; Beyenbach et al. 2020). Suppressing the *D. melanogaster* gene *ssk* homolog in *Diabrotica virgifera virgifera* caused

larval mortality (Hu et al. 2019). Lastly, *vha19* gene suppression in *Caenorhabditis elegans* and *Haemonchus contortus* resulted in mortality (Zawadzki et al. 2012). The second objective is to use microinjection to test the lethal specificity of MPB-specific dsRNA that matches the sequences of MPB mRNA (MPB-dsRNA) on a congeneric species, the Douglas-fir beetle (*Dendroctonus pseudotsugae*, DFB). The final objective is to test different concentrations of dsIAP, termed a kill curve, in eliciting an RNAi response when microinjected into MPB. In all repetitions of microinjection assays testing the six individual gene targets and dsIAP kill curve, adult MPB and DFB mortality was high in the injection negative controls (water and dsRS) and the injected dsRNA treatment groups. This was further corroborated by higher survival observed in the non-injection control group. Gene expression analysis of one repetition showed a potentially suppressed target gene, but it was not statistically significant and this finding was not supported in the gene expression analyses of the other repetitions. The high rate of mortality in all injected beetles, regardless of the injected liquid, indicates that microinjection is too invasive and supports the use of other methods to test RNAi in emerged adult MPB.

2.2 Methods

Success of the microinjections will be based on the comparison of the survival of beetles in the target groups to the negative control groups. To ensure that the mortality can be linked to targeted gene suppression, gene expression will be quantified using digital droplet PCR (ddPCR).

2.2.1 Subject collection

Lodgepole pine (*Pinus contorta*) trees were baited to attract MPB by the British Columbia Ministry of Forests (BC MOF). Once successful colonization was confirmed, the infested trees were cut down in early spring and shipped to the University of Northern British Columbia (UNBC) campus in Prince George, British Columbia. The tree segments were then cut into smaller pieces, or bolts, with a length of roughly 120cm for easier management and storage. The ends of the bolts, and any areas where bark was missing, were covered with paraffin wax to maintain the phloem moisture levels. Once the wax was set, the bolts were placed in plastic totes with mesh-covered holes cut into the lids and sides to incorporate air flow while containing emerging MPB adults. Totes were stored in a non-heat regulated shed in the yard of the Enhanced Forestry Lab (EFL) on campus (Figure 2.1A-B). Exceptions to the storage were made for thinner trees received from the Kootenays, B.C., that were left in longer segments and placed in a mesh tent outside the shed due to space limits and the dry condition of the wood upon delivery. Bolts were monitored regularly before the expected flight period. During the flight period bolts were checked daily and emerged beetles collected, so collection dates are assumed to be the same as the emergence dates. In 2022, bolts from Smithers, B.C. were used for assays in sections 2.2.3, and 2.2.4. In 2023, bolts from Smithers, B.C., provided adult MPB for assays in sections 2.2.5, 2.2.6, and 2.2.7.

A congeneric bark beetle, the Douglas-fir beetle (*Dendroctonus pseudotsugae;* DFB) was live-trapped using pheromone-baited Lindgren funnel traps. Traps were set up at three locations on the University of Northern British Columbia Prince George campus. Traps were baited with the 3187 Douglas-fir beetle lures with three components: seudenol-frontalin, kairomone, and LR ethanol (Synergy Semiochemicals Corp., 2023). The traps and lures were set up in areas with low to no mature Douglas-fir trees around campus (Figure 2.1C). Traps were checked and DFB collected daily, so collection dates are assumed to be within a few days of emergence.

Figure 2.1 Subject collection methods. A) Mountain pine beetle-infested bolts in a modified plastic tote. B) Totes containing MPB-infested bolts stored in non-temperature regulated shed at the Prince George University of Northern British Columbia (UNBC) campus. C) Lindgren funnel trap with Douglas-fir beetle lure components (seudenol-frontalin, kairomone, and LR ethanol) to attract and collect live Douglas-fir beetles on UNBC Prince George campus.

2.2.2 Gene target dsRNA

This study tested two forms of dsRNA. The first was *in vitro* kit-synthesized long-dsRNA (hereafter referred to as dsRNA). The dsRNA was synthesized to individually target six essential genes (*iap*, *hsp*, *vha19*, *tsp2a*, *mesh*, and *ssk*) in adult MPB, with lengths of specific dsRNA ranging from 200-341 nucleotides long (Tabe 2.1). The random sequence dsRNA, dsRS, produced and provided by RBSC is 200bp long and does not match any transcripts in the MPB transcriptome. The location of the long dsRNA within the gene sequence is provided (see Appendix, Table S1).

Table 2.1 Information on the long dsRNA synthesized to target six essential genes in mountain pine beetle (*Dendroctonus ponderosae*).

dsRNA	Length (nucleotides)	Accession
dsIAP	341	XM_019910372.1
dsHSP	315	XM_019906797.2
dsVHA19	200	XM_019903780.2
dsTSP2A	231	XM_048663791.1
dsMESH	218	XM_048661387.1
dsSSK	200	XM_048663913.1
dsRS	200	NA

The second form was heat-inactivated, hpRNA-expressing yeast (hereafter referred to as hpRNA). The *Saccharomyces cerevisiae* strain used is a RBSC proprietary RNAi production strain, and the hpRNA is genetically encoded through a sense-loop-anti-sense design which is cloned between a constitutive yeast promoter and terminator in the pRS426 vector. The yeast biomass, with the hpRNA encapsulated, was harvested by centrifugation, washed, heat killed (75^{o}C for 5 minutes) and freeze dried. The stem of the hpRNA is the same length as the dsRNA (Table 2.1) with the loop being approximately 80 nucleotides long between the inverted repeats

of the stem. Only the double-stranded (stem) region will be processed by dicer in the RNAi pathway.

2.2.3 Microinjection Delivery Trial Method

All microinjections were performed using the UltraMicroPump3 with MICRO2T and Microliter syringes (Hamilton; World Precision Instruments). We tested two different needles, angled and side-delivery (Hamilton 7758-04: (26/2”/2)S and 7784-07: (26s/2”/5)S) to determine the better needle for liquid delivery into adult MPB. The side delivery needles dispense the liquid from a small hole in the side of the needle rather than at the tip like in the angled needles. Ten beetles were assigned to each treatment group: control (no injection), angled needle, and side-delivery needle. The age of beetles, based on collection dates, were kept as even as possible among groups. Beetles were placed in a petri dish on ice prior to injection to anesthetize them (Liu et al. 2021). Entomology forceps were used to immobilize one MPB at a time on an ice block placed under a dissection microscope (Figure 2.2). The beetles were injected between the second and third abdomen compartments, in-between the rear-most pair of legs, with 50nL of distilled water (Zhang et al. 2016; Wang et al. 2016). To ensure room for the liquid, the needle was inserted and slightly drawn back before injection (Linz et al. 2014). After injection, beetles were placed in petri dishes with moistened filter paper and kept in a refrigerator at approximately 10°C to slightly reduce beetle activity after injection, aiming to allow the injection site to heal. Beetles in the no-injection control group were placed directly into a petri dish with moistened filter paper at approximately 10°C. MPB mortality was monitored daily, allowing beetles to acclimatize to room temperature for approximately 20 minutes before checking mortality.

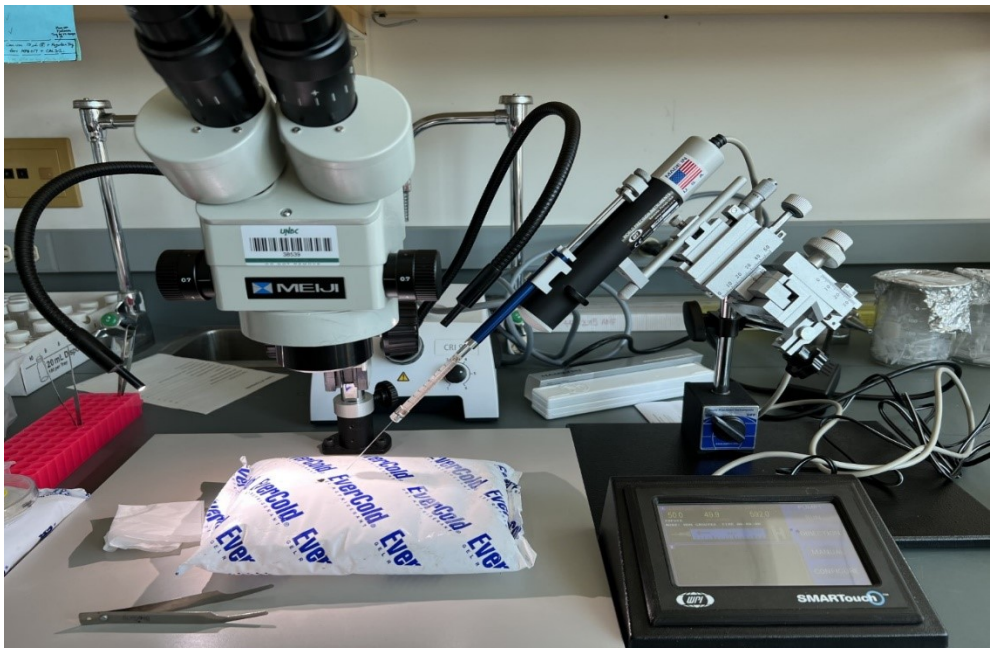

Figure 2.2 Microinjection set up using UltraMicroPump3 with MICRO2T and Microliter syringes (Hamilton; World Precision Instruments). Emerged adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) are placed on an ice block, immobilized with entomology forceps, and injected between the second and third abdomen compartments.

2.2.4 Microinjection to Test Six Targets on Emerged Adult Mountain Pine Beetles (2022)

Following the microinjection trial (section 2.2.3), target *in vitro* synthesized dsRNA was injected into adult mountain pine beetles (MPB) using the angled needles (Hamilton 7758-04: (26/2"/2)S). Fifteen adult MPB were assigned to each treatment or control group: no-injection control (beetles are not injected with anything), injected with distilled water, double-stranded random stem (dsRS; does not match any transcript in the MPB transcriptome), dsIAP, dsHSP, dsMESH, dsSSK, dsVHA19, and dsTSP2A. Beetle emergence dates in each group were kept as close as possible, with each group composed of beetles from a three-week emergence range. Within each experimental group, ten beetles were randomly selected for mortality observations after treatment, and five beetles were assigned to be flash frozen 24h post-injection for gene expression analysis. Each target dsRNA was diluted in RNase-free water to a concentration of 1µg/µl. Syringes and needles were changed between treatment groups, when possible, but due to

limited materials syringes were re-used after rinsing with water five times. Injection followed the procedure outlined in the trial: beetles were placed on ice prior to injection, then immobilized on an ice pack under a dissection microscope and injected between the second and third abdominal segments. Each beetle was injected with 50nl, equating to 50ng of target dsRNA. After injection, beetles were placed in a petri dish with moistened filter paper on ice. Once the fifteen beetles for one target gene were injected, the petri dishes were placed in a fridge at approximately 10°C to slightly reduce activity after injection. Beetles frozen for the gene silencing subset were flash frozen in liquid nitrogen 24h post-injection and stored at -80°C. The mortality subset of beetles was monitored daily; the moistened filter paper was changed every 48h or as needed (Kyre et al. 2020). Beetles were left at room temperature for approximately 10 minutes before mortality counts. All adult MPB still alive 15 days post-injection were flash frozen in liquid nitrogen and stored at -80°C.

2.2.5 Repetition of Microinjection to Test Six Targets on Emerged Adult Mountain Pine Beetles (2023)

Following the microinjection trial (section 2.2.3), emerged adult mountain pine beetles (MPB) were injected with 50nl of 1µg/µl target *in vitro* synthesized dsRNA within a week of emergence. Prior to injection, adult MPB were sexed using auditory examination, where males chirp when pressure is applied to the margins of the pronotum with thumb and forefinger (Rosenberger et al. 2016). For increased accuracy, all beetles that did not chirp were re-tested to catch formerly silent males (Rosenberger et al. 2016). Adult MPB were assigned to each treatment group: no-injection control, dsRS, dsIAP, dsHSP, dsMESH, dsSSK, dsVHA19, or dsTSP2A, keeping the collection dates of beetles in each group as similar as possible with each group having an emergence day range of four days (four days for repetition one, two days for

repetition two, three days for repetition three). Within each group, ten beetles were randomly assigned to mortality observations after injection, while five beetles were randomly assigned to gene expression analysis. Males and females within each group were kept separately. Unlike in the previous assay, after injection, beetles were placed in petri dishes lined with moistened filter paper and stored vertically at 4°C for 72h, in an attempt to reduce activity during healing, then moved to 11°C for the remaining duration of the assay. Beetle mortality was monitored daily and filter paper changed every 48h or as needed. Beetles in the gene expression analysis group were flash frozen in liquid nitrogen 72h after injection and stored at -80°C. This assay was repeated two more times for a total of three repetitions.

2.2.6 Mountain Pine Beetle Microinjection *iap* Kill Curve

Optimal dosage of target dsRNA to elicit an RNAi response in injected adult mountain pine beetles (MPB), will be determined using a "kill curve" assay with a three-step concentration gradient of injected dsIAP. Beetles were sexed by auditory examination and assigned to one of six treatment groups: no-injection control, H_2O, dsRS (1.9µg/µl), dsIAP1 (1µg/µl), dsIAP2.5 (2.5µg/µl), dsIAP5 (5µg/µl). Within each treatment group, five males and five females were assigned for mortality observation while three males and three females were assigned for gene expression analysis. Males and females in the mortality observation groups were kept in separate petri dishes. The previously outlined microinjection protocol was followed (section 2.2.3). Beetles were injected with 50nl of liquid, equating to 50ng of dsRNA in dsIAP1, 125ng in dsIAP2.5, and 250ng in dsIAP5. Beetles in the gene expression analysis group were flash frozen in liquid nitrogen 48h after injection. Each assay was repeated three times and each repetition consisted of beetles collected on the same day (repetition 1: June 25, repetition 2: June 26, repetition 3: June 27) with testing done seven days after collection.

2.2.7 Microinjection of Douglas-fir Beetles, an Off-target Species, with Mountain Pine Beetle Targets

The efficacy of the MPB-dsRNA was tested on an off-target, congeneric bark beetle species, the Douglas-fir beetle (DFB). A BLASTn search was performed, but not informative as to the sequence similarity among the MPB targets and DFB sequences as only cytochrome c oxidase subunit I (COI) sequences are represented for DFB in NCBI (NCBI 2025). The collected adult DFB were sexed based on elytral declivity (Jantz and Johnsey 1964). Beetles were assigned to one of eight treatment groups: no-injection control, dsRS, dsIAP, dsHSP, dsSSK, dsMESH, dsVHA19, or dsTSP2A. Within each treatment group, ten beetles (6 females, 4 males) were assigned to the mortality observation group and five beetles (3 females, 2 males) were assigned for gene expression analysis. Beetles were treated no longer than seven days after collection, typically within 3-4 days. *In vitro* synthesized dsRNA was diluted to a concentration of 1µg/µl. Adult DFB were injected with the same method and amount of dsRNA as adult MPB (section 2.2.3). Once injected, or immediately for the no-injection group, beetles were placed in a petri dish lined with moistened filter paper and stored vertically at approximately 4°C for 72h, then moved to approximately 11°C. Beetles were monitored daily for mortality, and the filter paper replaced every 48h or as needed. Beetles in the gene expression group were flash frozen 72h after microinjection and stored at -80°C. The assay was repeated three times. Entomopathogenic fungal growth became a challenge, and impacted beetles were isolated into a separate petri dish to try and prevent the spread to other beetles. The start of the fungal outbreak was recorded.

2.2.8 Gene expression analysis

2.2.8.1 RNA Extraction and cDNA synthesis

RNA was extracted from silencing beetles (n=5-6) using the RNeasy Plus Mini Kit (Qiagen, Toronto, ON). The kit protocol was followed, using the entire adult beetle for extraction. As an additional step, the QIAshredder (Qiagen, Toronto, ON) was used to further homogenize the sample before the gDNA eliminator step. RNA was eluted twice with 50µl of water each time. The first elute was used for downstream applications.

Eluted RNA was quantified with the Qubit 4 Fluorometer Broad Range RNA assay (Thermo Fisher Scientific Inc., 2015) using 2-3µl of sample in 197-198µl of working solution. The integrity of the RNA samples was determined using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). This software allows for a quantification of RNA integrity with an RNA Integrity Number (RIN), based on 18S and 28S peaks. However, this value is not reliable for insect RNA as there is an endogenous break in the 28S rRNA (Fabrick and Hull 2017). Heat-denaturing the samples at 70°C prior to running the RNA 6000 Nano chip causes the hydrogen bonds in the 28S break to dissociate, creating two peaks of similar size that migrate closely to the 18S rRNA (Fabrick and Hull 2017). Thus, the RNA quality was visually assessed using the electropherogram for each sample, showing little to no degradation and the two peaks close together.

Samples that were assessed to be of good RNA quality were selected for the downstream applications, starting with cDNA synthesis. The cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON), following the kit protocol. Input RNA, normalized to 500ng, was added to 4µl of the supermix, and the reaction volume topped off to 20µl with nuclease-free water. Each treatment group

included a no-reverse transcriptase control (no-RT), where a supermix without RT replaced the normal supermix in the protocol above, to ensure there was no DNA contamination in this step. The samples were run through the following incubation steps: 25°C for five minutes, 46°C for 20 minutes, and 95°C for one minute. After the incubation, the synthesized cDNA was diluted 1:10 with TE buffer and stored at -20°C.

2.2.8.2 qPCR Assay and gBlock design

PrimeTime Std qPCR assays were designed for all six gene targets (*iap, hsp, mesh, vha19, tsp2a,* and *ssk*) and for dsRS. These sequence-specific primers were designed using PrimerQuest®, the Integrated DNA Technologies (IDT) primer design tool (IDT 2023). The output of primers and probes were subjected to a BLASTn search to further confirm specificity to the MPB transcriptome (NCBI 2023). Assays were designed outside of the dsRNA region, spanned an exon-intron boundary, with the exception of *iap* that did not contain introns, and were optimized for 60°C. Assays for the housekeeping or reference genes RNA Polymerase II (*RPII*) and porphobilinogen deaminase (*PBD*), as internal controls were designed using the same method as the six gene target assays (Table 2.2). Each target assay was resuspended to a concentration of 20X and the reference gene assays were resuspended to a concentration of 40X as per supplier protocol (IDT 2023).

A gBlock is a DNA sequence containing the primer and probe sequences for each of the six gene target assays. The gBlock acts as a positive control for the digital droplet polymerase chain reaction (ddPCR). The designed gBlock (IDT gBlocks™ Gene Fragments Entry 2023) was resuspended as per the provided protocol. From the suspended concentration, the theoretical copy number was used in a serial dilution. The diluted target gBlock was combined with the diluted gBlock containing the primer and probe sequences for the two reference genes. For each

theoretical copy number value in the serial dilution of the target gBlock, the corresponding same theoretical copy number value for the reference gBlock was mixed in.

Table 2.2 Primer and probe sequences for target genes and reference genes used in digital droplet PCR (ddPCR) for expression analysis in adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) after dsRNA exposure.

Gene	Primer	Sequence
RPII	RPII - F	GACGTTGGAGCAGTTCAAAGAG
	RPII - R	GGAAGAACACGAACATCTGGTC
	Probe	/5HEX/CGACAAACC/ZEN/CAGCGAGAAGAGCG/3IABkFQ/
PBD	PBD - F	GGCTTCAATGTGTGTCCAGTG
	PBD - R	CACCAAACCAACGAAAAGATGTTC
	Probe	/5HEX/CGCCAATCT/ZEN/TATCACCGTTGCCG//3IABkFQ/
dsRS	dsRS - F	GGCAGTTAAAGTCGGGAGAATAG
	dsRS - R	TATCTAAGAGCCAGAGGGATGG
	Probe	/56-FAM/CCGCAACAC/ZEN/ACAGTTTACCGCATT/3IABkFQ/
iap	iap -F	GGCCTTATTGGAGGCATCTT
	iap - R	TTGGCGATCTCTCGGTTTG
	Probe	/56-FAM/ATCCGGGTA/ZEN/GATTGCGGACATTCG/3IABkFQ/
hsp	Hsp -F	GAAACTGCCGAGGCTTACTT
	Hsp – R	ACGGTCATACCAGCAATGATAC
	Probe	/56-FAM/AGCGTCGTT/ZEN/GAAATAAGCAGGCAC/3IABkFQ/
tsp2a	Tsp2a - F	CCATGCTCGTCGCCTTTATTA
	Tsp2a - R	GGTAGGTGTCAGCTTGTTCTTC
	Probe	/56-FAM/TGCTTGGAT/ZEN/GAGGACAATAGAAACGACC/3IABkFQ/
vha19	vha19 – F	AGTTCAACCAACGTCCTACAG
	vha19 – R	AGCCCAGACCAAATTGGAATA
	Probe	/56-FAM/CGACTCCCA/ZEN/GGACCAGGTCATCTA/3IABkFQ/
mesh	Mesh - F	GAAGGAGGAGTTCCAGCTTATG
	Mesh - R	GCGTAACCTCGTCCAGATAAAT
	Probe	/56-FAM/ACGGCACAA/ZEN/GAAGTTGGGAGTACA/3IABkFQ/
ssk	Ssk - F	GCACTACTGGCATGGTTATCT
	Ssk - R	ATGACGCTATCAACGAGGTAAA
	Probe	/56-FAM/AAGACAGGT/ZEN/TGGTCTCGCTTTGGG/3IABkFQ/

2.2.8.3 Linear Dynamic Range Determination and Assay Optimization

All six assays were run on a temperature gradient to confirm the optimal annealing temperature. The temperature range used to test the assays was 52°C -62°C. An initial test was performed to ensure that each of the six gene target assays worked well when duplexed with each

reference gene. Each gene assay was run twice, duplexed once with each housekeeping gene. The annealing temperature for future assays was set to 58°C.

To set the linear dynamic range (LDR) of digital droplet polymerase chain reaction (ddPCR), both reference genes were run individually with serial dilutions of the mixed gBlock containing primer and probe sequences for all six gene targets and the two reference genes. A range of 400,000copies/µl to 0.4copies/µl in 10-fold dilutions were run with the iap assay duplexed with each reference gene in duplicate.

2.2.8.4 Digital Droplet PCR Conditions

With the ddPCR conditions tested and optimized, the frozen beetle cDNA for gene expression analysis was run with the reference genes. Assays were run in duplex, pairing each target assay with a reference gene. A no-template control (NTC) and positive control using water and the mixed gBlock, respectively, was run with each assay combination. Each beetle's cDNA was run twice for the target of interest, with a different reference gene each time. This allowed for duplicated runs of the gene of interest for each biological repetition, and for each reference gene to be used once on each biological repetition.

A master mix was made for each assay combination, consisting of reagent amounts for a total volume of 24µl per well for as many wells as planned. Each well contained 12µl 2X Supermix for probes with no dUTPs (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON), 1µl of the respective gene target, 0.5µl of the respective reference gene, 2.4µl of sample, and 8.1µl of water to reach 24µl total volume. A no-template control (NTC) was included to ensure that there is no contamination in the reagents and a positive control was included to ensure that the reagents were working. For the NTC and positive controls, the sample was replaced with water and gBlock, respectively.

Once loaded, the plate was sealed and placed in the Automated Droplet generator (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON). About 20,000 droplets are made per well, and the new plate was sealed and placed in a thermocycler at the following conditions: 95°C for 10 minutes to activate the polymerase, followed by 45 rounds of 95°C for 30 seconds (denaturation step) and 58°C for one minute (annealing step). The 45 rounds were followed by 98°C for 10 minutes to deactivate the enzymes and remained at 4°C until removed from the thermocycler. The droplet reader was used to obtain the end-point quantification of the amplification, and results were viewed with QX Manager Software Standard version 1.1 (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON).

2.2.8.5 Statistical Analysis

The droplet count data was processed, visualized, and initially analyzed by the QX Manager Software and the manual baseline set around 2000 to ensure negative or incomplete amplification droplets were not included in the positive droplet count. Once all wells were clearly quantified, data was exported in a CSV file. For each sample, each value of the target gene was normalized to the reference genes: the average of the two target gene counts was divided by the geometric mean of the reference gene counts (Livak and Schmittgen 2001; Vandesompele et al. 2002; Zmienko et al. 2015). The resulting relative gene expression data was log2 transformed before statistical analysis for improved data distribution, symmetry, and easier interpretation (Rieu and Powers 2009; Taylor et al. 2017). Once transformed, data was checked for normality. If data was normally distributed, a parametric test comparing means was performed. If only two means, or two treatment conditions (i.e. one control and one test) were being compared, a Student's two-sided t-test was performed. This allowed detection of up-regulation as a potential response to dsRNA delivery (Zawadzki et al. 2012; Haller et al. 2019;

Wallace and Rieske 2023). If more than two means, or treatment groups (i.e. two controls and two tests) were being compared, a one-way ANOVA test was used, followed by a Tukey HSD test if the ANOVA test was significant ($p < 0.05$). For the experiments where the data was not normally distributed, the Student's t-test was replaced with the Mann-Whitney U test and the ANOVA test was replaced with the non-parametric Kruskal-Wallis test followed by the Dunn's test if significant ($p < 0.05$). Data analysis was performed in R Statistical Software (v4.3.1; R Core Team 2023). Data was visualized using a boxplot showing the gene expression in a log2 base scale of the target gene relative to the geometric mean of the two reference genes with the "ggplot" package (Wickham 2016).

2.3 Results

2.3.1 Microinjection Trial

In the microinjection trial, the angled needles were more effective than side-delivery needles for adult MPB injections. The side-delivery needles were relatively blunt, making it difficult to penetrate the exoskeleton. The side-delivery needles also needed to be injected farther into the beetle to ensure liquid delivery, which increased the invasiveness of the procedure.

Survival of the beetles was 70% at seven days post-injection for the angled needles, like the no-injection control group, and 40% for the needles with a side-delivery. However, after seven days, the mortality rate increased for the angled needles, with no survival by 13 days post-injection. In contrast the survival rate of the beetles injected with the side-delivery needle and the no-injection control groups were at 20% survival 13 days post injection (See Appendix, Figure S1).

2.3.2 Six targets on emerged MPB adults (2022)

All targets showed high mortality 14 days after microinjection; dsVHA19, dsSSK and dsIAP showed 100% mortality by day 14. However, the negative controls showed high levels of mortality by day 14 as well, with 50% survival in the no-injection control, 100% mortality in the beetles microinjected with water, and 10% survival in those microinjected with dsRS (Figure 2.3).

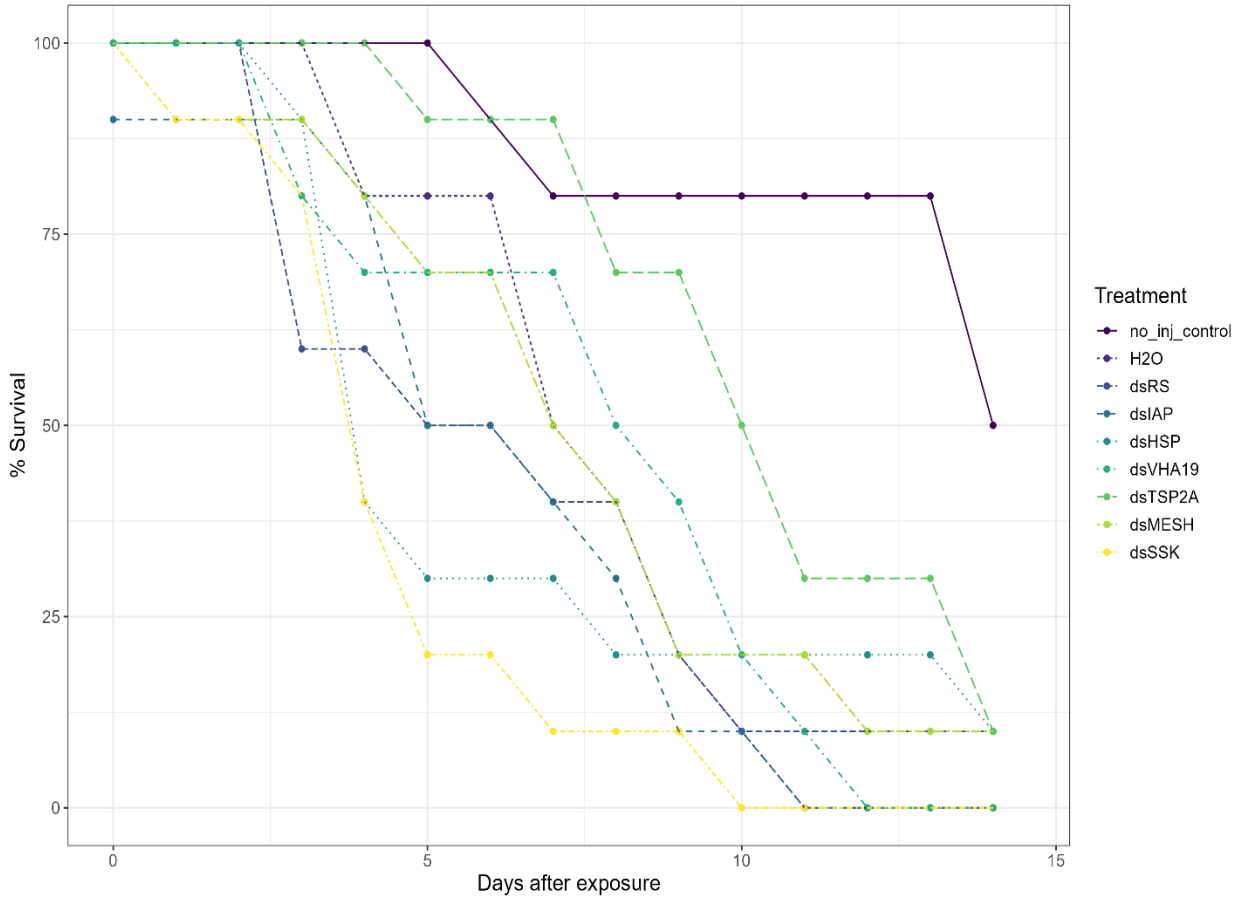

Figure 2.3 Percent survival over 14 days of emerged adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) microinjected with 50ng of target double-stranded RNA (dsRNA) for six gene targets (n=10): dsIAP, dsHSP, dsVHA19, dsTSP2A, dsMESH, and dsSSK. The survival of three negative controls: no-injection control (no_inj_control), water (H2O), and a double-stranded random stem (dsRS) was plotted alongside for comparison.

2.3.2.1 Gene Expression Analysis: RNAi pathway initiation for the *iap* gene target

The mean relative *iap* gene expression of the dsRS injected group is higher than the other three treatment groups (Figure 2.4). The data conformed to a normal distribution after a $\log_2$ transformation. A one-way ANOVA test was conducted to statistically compare the means among the different treatment groups, and results show there is significant difference between the mean of at least one of the treatment groups ($p = 0.00242$, $df = 3$, $F = 7.455$). Using Tukey's post hoc test, the dsRS injected group mean $\log_2$ relative *iap* expression was significantly higher than the non-injection control, the water, and the dsIAP groups ($p = 0.009$, $p = 0.04$, and $p =$

0.002, respectively) (Figure 2.4). There are no significant differences of the mean *iap* gene expression between beetles in the non-injected control group, the water group, or the dsIAP group.

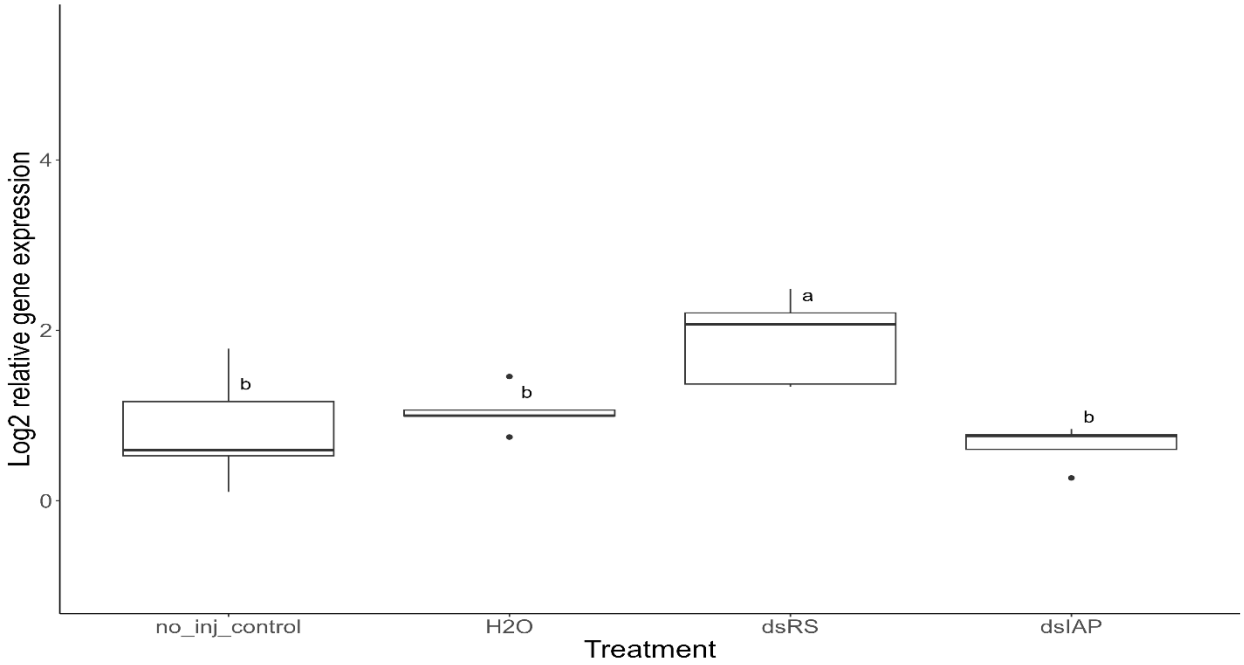

Figure 2.4 Comparison of the mean (n=5) of the log_2 transformed relative *iap* gene expression between the dsIAP and control groups 24h post-microinjection. Statistical analysis using a one-way ANOVA indicated that the *iap* gene expression in the double-stranded random stem (dsRS) treatment group is significantly different compared to the other three group means ($p = 0.00242$, $df = 3$, $F = 7.455$).

2.3.3 Six targets on emerged MPB adults (2023)

The percent survival remained high for the first seven days post-microinjection. The no-injection survival rate remained above 80% for the duration of the assay, while the other negative control, dsRS injection, showed survival rates similar to the gene target treatments (Figure 2.5). At the end of the assay, the lowest survival rate was observed in the dsTSP2A treated beetles. In all other targets there was over 50% survival of beetles at ten days post-microinjection.

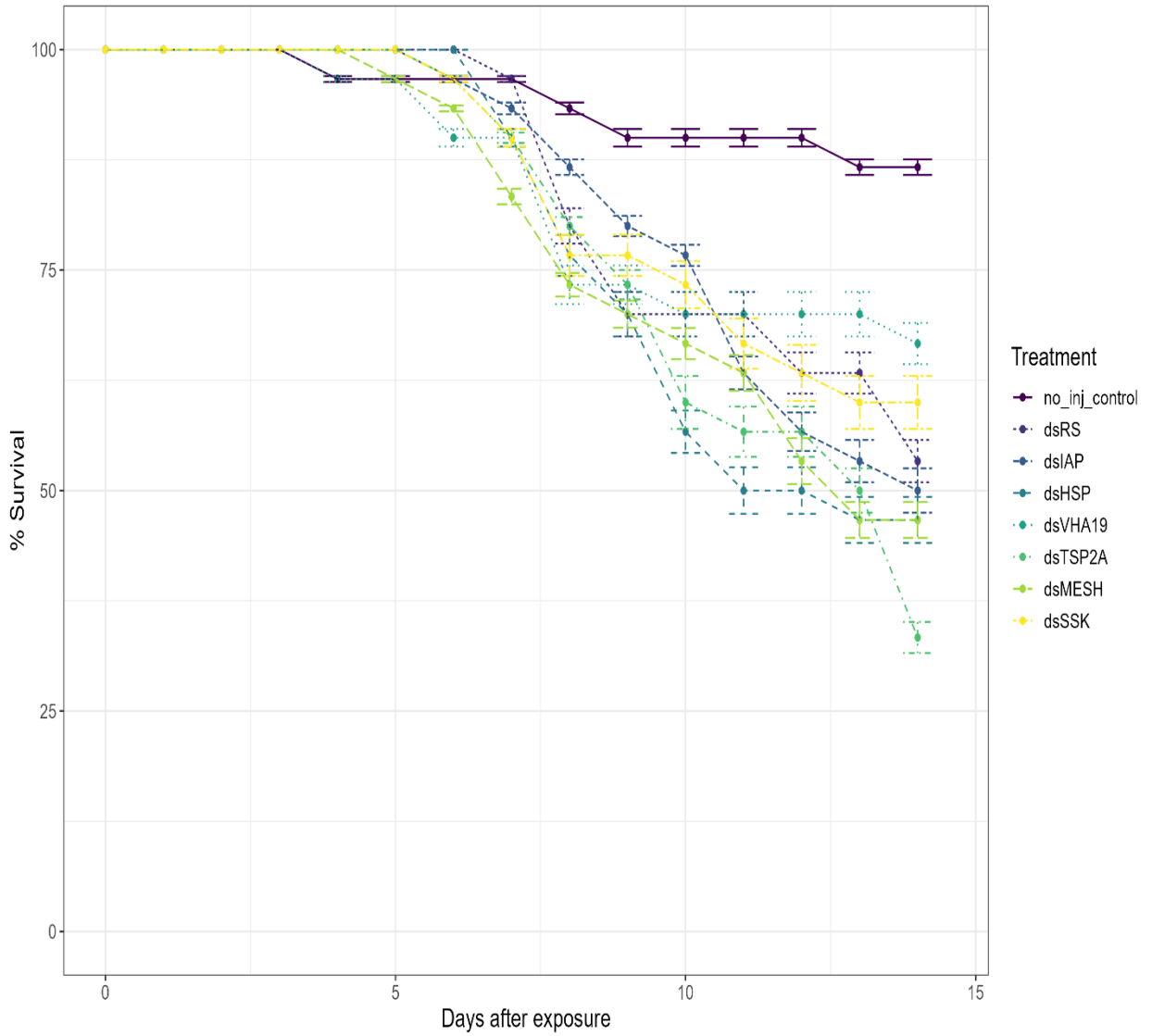

Figure 2.5 Mean average percent survival (± 1SE) over 14 days of adult mountain pine beetle (*Dendroctonus ponderosae*, MPB) microinjected with 50ng of double-stranded RNA (dsRNA) for six gene targets (n=10): dsIAP, dsHSP, dsVHA19, dsTSP2A, dsMESH, and dsSSK. The survival of the negative controls (n=10), no-injection control (no_inj_control) and double-stranded random stem (dsRS), were recorded and plotted alongside for comparison.

2.3.3.1 Gene Expression Analysis: RNAi pathway initiation for *iap* and *tsp2a* gene targets

After a $\log_2$ transformation, the data was normal allowing for use of the parametric one-way ANOVA test to compare the group means. There was no significant difference in *tsp2a* gene expression among treatment and control groups ($p= 0.581$, $df = 2$, $F = 0.569$) (Figure 2.6A).

Similarly, there was no statistically significant difference among the group means for *iap* gene expression (p= 0.916, df = 2, F = 0.088) (Figure 2.6B).

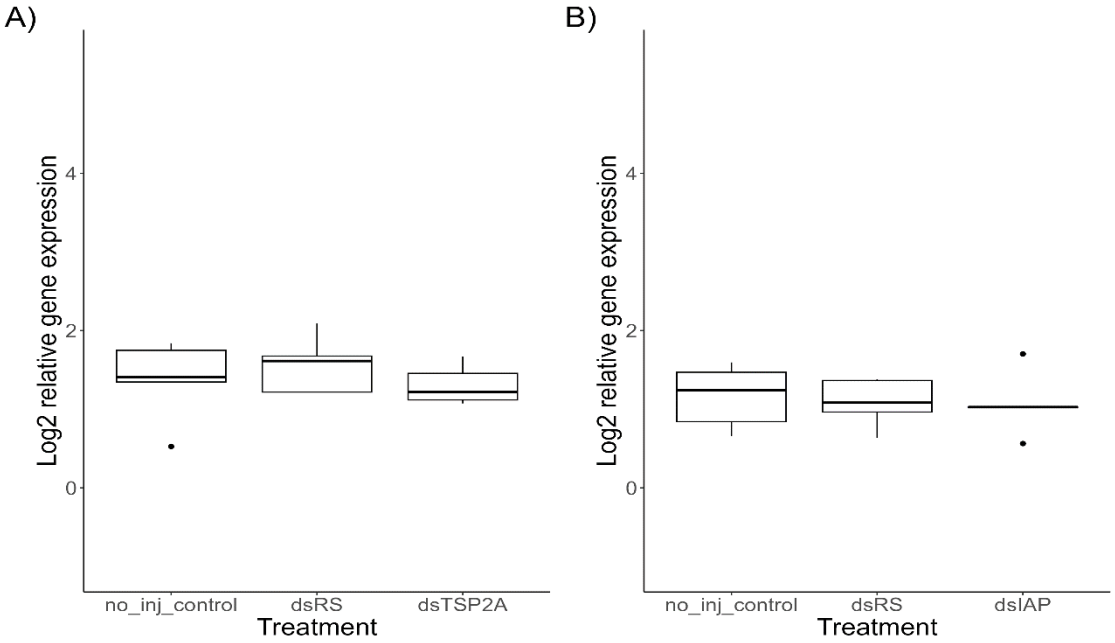

Figure 2.6 Comparison of the mean (n= 5) log_2 difference of log_2 target gene expression between treatment and control groups 72h post-microinjection. Statistical analysis using the one-way ANOVA test showed no significant difference among group means. A) *tsp2a* gene target, p= 0.581, df = 2, F = 0.569 B) *iap* gene target, p= 0.916, df = 2, F = 0.088

2.3.4 MPB *iap* Kill Curve

By day seven, the percent survival in all injected groups (H2O, dsRS, dsIAP1, dsIAP2.5, and dsIAP5) was below 25% (Figure 2.7). The no-injection control group had an average percent survival above 50%. The group with the lowest survival rate was dsIAP5, with 250ng of dsIAP delivered. This was followed by the two negative control groups of water and dsRS. The two negative control groups that were injected with non-target liquids had low mean adult MPB survival (Figure 2.7).

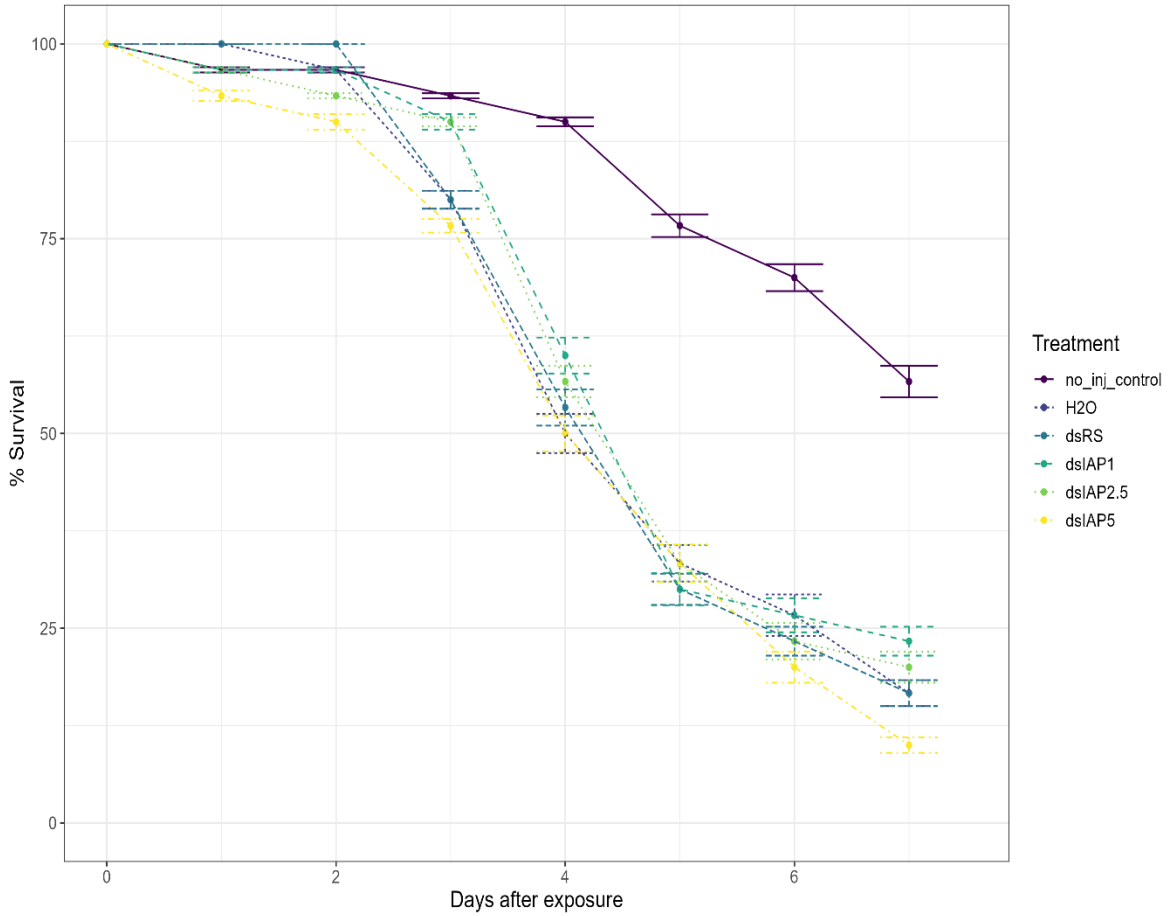

Figure 2.7 Average percent survival (± 1SE) over seven days of adult mountain pine beetle (*Dendroctonus ponderosae,* MPB) microinjected with dsIAP at three concentrations (n=10): 50ng (dsIAP1), 125ng (dsIAP2.5), or 250ng (dsIAP5). The survival of the negative controls, no-injection control (no_inj_control), double-stranded random stem (dsRS), and water (H2O) were plotted alongside for comparison

2.3.5 Microinjection of Douglas-fir Beetles, an Off-target Species, with Mountain Pine Beetle Targets

After seven days, no large amount of mortality was observed in any of the treatment groups (Figure 2.8). The survival of the non-injected DFB is higher than all the other treatment groups, remaining above 75% for 16 days. The dsRS negative control survival is similar to the survival in the gene target treatment groups. The gene target groups dsIAP, dsSSK, dsVHA19,

dsMESH, and dsTSP2A show survival rates of 10% or lower by 18 days post-injection (Figure 2.8). Around day eight, entomopathogenic fungi began to appear on some beetles.

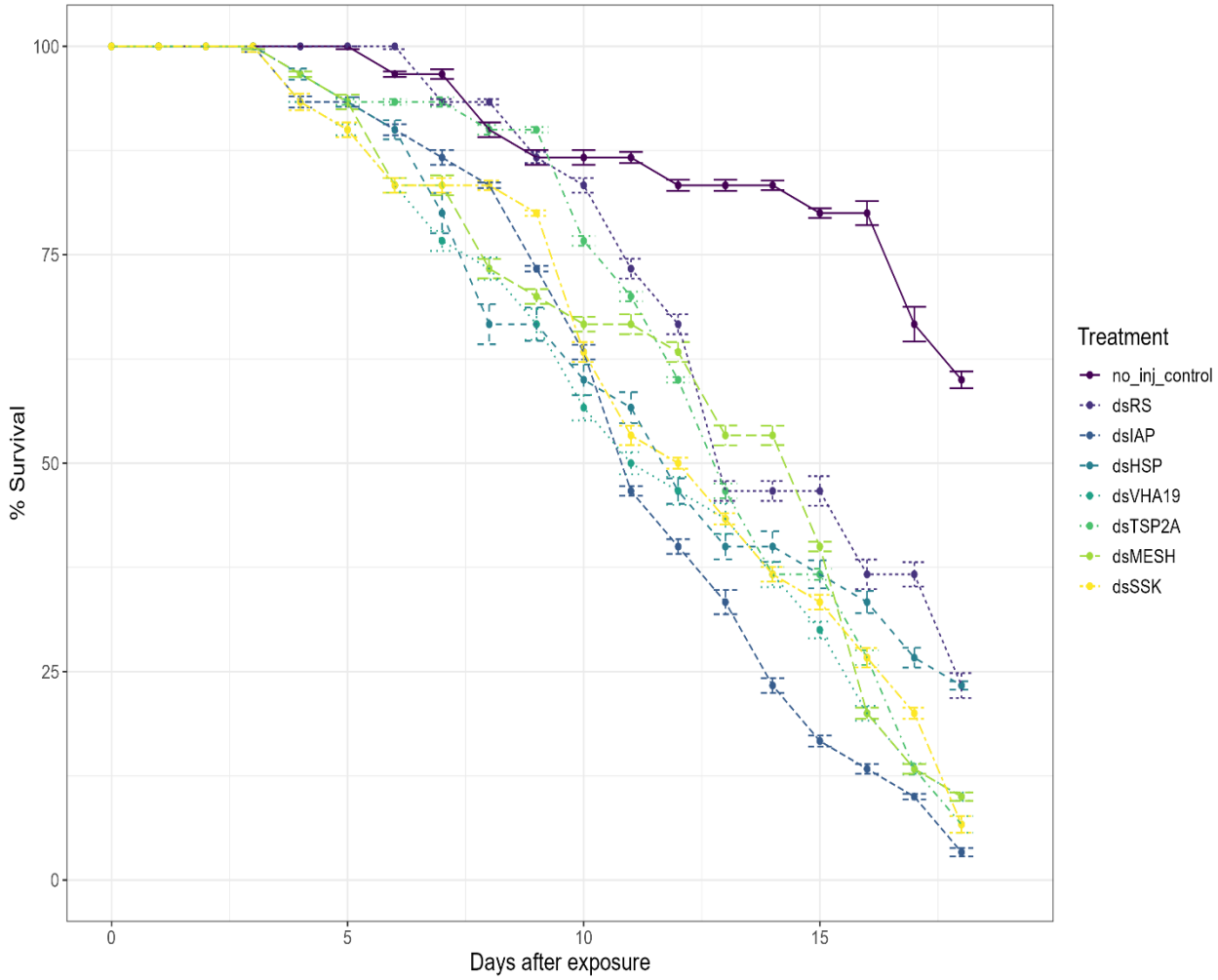

Figure 2.8 Average percent survival (± 1SE) of adult Douglas-fir beetles (*Dendroctonus pseudotsugae*, DFB) over 18 days microinjected with 50ng of dsRNA for six gene target MPB-dsRNA (n=10): dsIAP, dsHSP, dsVHA19, dsTSP2A, dsMESH, and dsSSK. Average percent survival of the negative controls, no-injection control (no_inj_control) and double stranded random stem (dsRS, does not match any transcript in the mountain pine beetle transcriptome), was plotted for comparison.

2.4 Discussion

2.4.1 Microinjection Trial: needle selection

Needle type affects microinjection success. In the first eight days post-injection, the beetles injected with the angled needle showed similar survival with the non-injected control beetles, but after eight days exhibited increased mortality. Meanwhile the beetles injected with the side-delivery needle matched the non-injected control mortality starting after eight days (See Appendix, Figure S1). However, the side-delivery needle caused more mortality than the other two treatments in the first eight days. With a blunt end, the side-delivery needle did not easily pass through the exoskeleton of the beetles, making the injection longer and more difficult. Additionally, the liquid delivery was variable because the opening was not at the tip; this required invasive insertion of the needle into the beetle. The angled needle was therefore selected for subsequent target dsRNA delivery as it caused less initial mortality and allowed a less invasive injection compared to the blunt side-delivery needle (Linz et al. 2014).

2.4.2 Mountain pine beetle microinjections

2.4.2.1 Adult MPB mortality obscured by negative control mortality

The first adult MPB injection experiment showed high mortality in all beetles injected, regardless of whether they were injected with target dsRNA or the negative controls of water (H2O) and dsRS (Figure 2.3). The only negative control group that maintained high survival was the group that was not injected at all. These mortality observations may be influenced by random mortality, although the same mortality trend was observed in subsequent repetitions (Figure 2.5). The mean mortality of the control group injected with dsRS is once again right in the middle of all the treatment groups, with just above 50% survival (Figure 2.5). Microinjecting 50ng of dsRNA into adult MPB is lower than the 150-200ng used in *D. armandi* microinjections in

previous literature, but any evidence of a potential phenotypic response or gene suppression was obscured by the high mortality.

2.4.2.2 Gene expression analysis: target gene suppression is not apparent in most repetitions

As with mortality, gene expression analyses did not reveal a difference among the treatment and control groups. Visually, the *iap* gene expression in the beetles injected with water looks slightly higher than the non-injection and dsIAP group, indicating that the injection method may be a stressor for the beetles. The significant increase of mean log2 *iap* expression in the dsRS group was unexpected, and can provide support for a MPB stress response to microinjection. Another explanation could be that dsRS is impacting MPB physiology. In a study on *Adalia bipunctata* and *Coccinella septempunctata*, mortality has been observed in beetles treated with a dsRNA-based control (dsGFP) that does not match any transcripts, attributed to a general immune stimulation or overwhelmed RNAi system (Haller et al. 2019). Increases in gene expression have been shown in target genes after dsRNA delivery (Turner et al. 2006; Haller et al. 2019). Though the reported increase in gene expression responses in the previous studies were in insects treated with target dsRNA, and not a negative control dsRNA, this may indicate that the expression of *iap* is increased after injection with any dsRNA sequence. The inclusion of a water control shows that the injection of dsRNA is not the main factor causing mortality, as beetles injected with just water show similar mortality. Therefore, it is possible that the gene expression increase in the dsRS group was not enough to cause a phenotypic response, but highlights a possible interaction. As a stress response gene, *iap* inhibits apoptosis, or cell death by blocking specific enzymes involved in the process (Verhagen et al. 2001). Up-regulation of *iap* in MPB larvae has been found as a response to stressful stimuli, such as overwintering

(Robert et al. 2016). If the dsIAP group is assumed to have had the same initial increase in *iap* expression as the dsRS group, the *iap* gene may have been suppressed, thus reducing the relative gene expression back to baseline levels (Figure 2.4). An increase followed by a decrease in gene expression after dsRNA exposure has been reported when targeting the *EposPBP1* gene in light brown apple moths (*Epiphyas postvittana*) (Turner et al. 2006). Additionally, fluctuating gene expression levels over a five day duration, with a decrease from day one to day three and increase from day three to day five was reported in *Coccinella septempunctata* when targeting *C. septempunctata vATPase A* and *Diabrotica virgifera virgifera vATPase A* (Haller et al. 2019).Though the gene expression responses occurred over a longer time period than captured in our study, an *iap* gene allele in northern MPB populations has efficient up-regulation, and could account for the fast response in *iap* expression (Horianopoulos et al. 2018). The *iap* gene target was selected for gene expression analysis as it was previously suppressed in adult MPB (Kyre et al. 2020) and, as such, *iap* has been treated as a positive control for the assays in this study.

The findings from the second bioassay quantifying *tsp2a* and *iap* expression disagree with the *iap* assay response in the first bioassay. The mean relative $\log_2$ *iap* expression looks fairly similar among the three treatment groups (Figure 2.6B). These new repetitions do not show increased *iap* gene expression in the dsRS group, contradicting the inferred results from the initial assay. Sampling time point may affect gene expression analysis (Zhao et al. 2015; Sun et al. 2021; Liu et al. 2022; Liu and Chen 2022). In previous studies on *Agrilus planipennis*, no gene suppression was observed 24h post-injection, but was found from day two to day six (Zhao et al. 2015). Other studies in *D. armandi* found the RNAi mediated gene suppression 24h post-injection, but saw further decreases in gene expression by 72h post-injection (Liu et al. 2022). In *D. ponderosae*, the suppression has been detected 17 days after teneral adults were injected

(Keeling et al. 2013). In this study, no difference in gene expression between control and treatment groups was observed at 24h or 72h post microinjection.

2.4.3 Increased dosage of dsRNA showed similar responses to previous assays

The kill curve tested the amount of dsRNA for *iap* in a range previously successful at initiating an RNAi response both in relative gene expression and phenotype in *D. armandi* (Sun et al. 2021; Liu et al. 2022; Liu and Chen 2022). The resulting average mortality from the three kill curve assays corroborates the previous mortality observations with all targets. The mortality rate of the beetles in this kill-curve microinjection experiment appeared more rapid in all treatment and control groups than what was observed in the other microinjection bioassays. All injected beetles, regardless of treatment, showed 75-90% mortality. It is important to note that the non-injected beetle survival was also lower in these assays than in previous ones. This indicates that there may have been other variables impacting the mortality of the injected beetles as well. Physiological differences between reared and caught insects have been noted, including body size and energy reserves (Huho et al. 2007). Although the MPB in the bioassays were sourced from wild population larvae, they were captured upon emergence and did not have a flight period, much like laboratory-reared insects. However, these physiological differences would be impacting all the tested MPB, and survival of the non-injected control in the initial microinjection bioassay remained high (Figure 2.5). A major difference between the bioassay trials is that the adult MPB used in these bioassays emerged weeks later in the emergence period than the MPB in the previous microinjection bioassays. This suggests that delayed emergence could be connected to decreased fitness levels in adult MPB. Increased dosage in the kill curves produces similar mortality to previous injection assays.

2.4.4 Microinjection of DFB with MPB targets elicits mortality in negative control and target dsRNA groups.

Microinjecting adult DFB with six MPB targets was used to investigate the species-specificity of the gene targets. Douglas-fir beetle mortality showed high survival rates for the initial seven days post-injection. While this was also the case in the 2023 MPB injection assays, the average mortality of the DFB assays differs from the MPB assays as the dsRS negative control mortality rate is closer to the no-injection group mortality rates (Figure 2.8). Therefore, the microinjection method may not be as lethal to adult DFB as for MPB. The average survival of the target groups also remains high in the initial week after microinjection, indicating that the delivery of MPB dsRNA targets is not lethal in an off-target, congeneric species.

At day eight post-injection, a white fungus tentatively identified as an entomopathogenic fungi, *Beauvaria* spp., appeared on the Douglas-fir beetles (Zimmermann 2007). The identification was made using the appearance of the white colonies coming from within the adult beetles as well as the fact that this fungus is commonly found on soil and indoor plants (Zimmermann 2007). This was not entirely unexpected as some *Beauvaria* spp. are pathogens to Coleopteran species and *Dendroctonus* spp. are associated with many microbes, including fungus (Paine et al. 1997; Klepzigl et al. 2001; Zimmermann 2007). Both MPB and the southern pine beetle (*Dendroctonus frontalis*) phoretically carry fungi in the mycangia (Klepzigl et al. 2001; Chiu and Bohlmann 2022). This suggests that trapped emerged DFB adults also carry associated fungi. Due to these factors, fungal infections in bioassays with beetles is a concern (Whitney and Spanier 1982). The fungus may have impacted the health of the treated beetles, acting as a confounding variable to the impacts of microinjection and MPB-dsRNA on the adult DFB mortality.

2.4.5 Feasibility of microinjection as a delivery method for MPB

Our results shed new light on the effectiveness of microinjection in adult *Dendroctonus* species. Effectiveness of RNAi via microinjection delivery may be dependant on a myriad of factors including the insect's life stage, species, amount of dsRNA, and target genes (Cooper et al. 2019; List et al. 2022). Microinjection has been used extensively to knockdown genes in order to understand their role in *D. armandi* adults, with high survival rates from the method (Zhang et al. 2016; Liu et al. 2021, 2022; Liu and Chen 2022). One possible explanation for the difference in beetle survival in these studies compared to this study is the needle size used for injection. We used a large, 26-gauge needle, whereas the *D. armandi* injections used a 32-gauge needle (Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022). With the 26-gauge needle having an outer diameter (OD) twice as large as the 32-gauge needle, this would cause more damage to the injected beetles. Within our study, there were differences in the survival of adults from two congeneric species, *D. ponderosae* and *D. pseudotsugae*, after microinjection. This might indicate that different bark beetle species could have a better response to microinjection than *D. ponderosae*.

Successful RNAi initiation via microinjection has been achieved in the teneral adult life stage of *D. ponderosae* (Keeling et al. 2013). The teneral adult life stage precedes the adult stage when MPB emerge from the phloem (Chiu and Bohlmann 2022). At this life stage, the exoskeleton is not as hard, making microinjection easier (Chiu and Bohlmann 2022, C.I. Keeling, personal communication, 2024). Keeling et al (2013) used smaller needles, suggesting that the large needles used in our study increased injected MPB mortality. The needles used on teneral adult MPB are much smaller than the needles used in *D. armandi* microinjection, which may indicate that MPB are more susceptible to injury from microinjection than congeneric

species (Keeling et al. 2013; Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022). While this study may have found a potential indication of *iap* suppression in one repetition of MPB injections, this was not found in subsequent injections.

Another difference in this study compared to previous studies that can cause variation in the RNAi response is the amount of dsRNA delivered. For the target survey, 50ng of dsRNA was delivered using a concentration of 1µg/µl. Though *D. armandi* studies typically delivered more dsRNA to the target insect, smaller amounts have been successful at causing decreased gene expression and showing the phenotypic response in other insects (Bingsohn et al. 2017). When the small dsRNA doses were increased, the observation of the phenotypic response occurred faster after microinjection and had slightly increased mortality from 75% to 90% (Bingsohn et al. 2017). The microinjection assays in this study were monitored longer to account for the possibility of a delayed phenotypic response. The use of a kill curve with the most promising gene target, *iap*, based on previous success in MPB, accounted for the possibility of an inadequate amount of dsRNA delivered in initial assays. With 50ng, 125ng and 250ng delivered, these amounts were now in the range of even more studies that found successful RNAi initiation (Keeling et al. 2013; Zhao et al. 2015; Sun et al. 2021; Liu et al. 2022; Liu and Chen 2022).

Lastly, a large factor credited with causing variable RNAi responses are the genes that are targeted for suppression or knockdown (Cooper et al. 2019). To address that variable in this study, two of the six tested gene targets were selected due to previous success at RNAi initiation resulting in gene suppression and rapid mortality in MPB and other insects (Leelesh and Rieske 2020; Kyre et al. 2020; Wallace and Rieske 2023). The other four targets were selected based on RNAi initiation success in other insect species, and having essential roles in the cell, aiming to broaden the applicable gene targets for MPB mortality (Zhao et al. 2015; Yoon et al. 2016; Hu et

al. 2019). As such, two of the four targets are expected to elicit a response in MPB. The other four show promise to elicit a response, but have not been previously tested in *D. ponderosae*. With no indication of the RNAi response in the phenotype, and confounding death likely attributed to the invasive delivery via microinjection, the data suggests that the RNAi pathway is not being initiated and that the microinjection method is too invasive.

2.5 Conclusion

With four repetitions testing six gene targets via microinjection in emerged adult MPB, the mortality of injected negative control groups and target dsRNA groups were similar. High survival was only observed in the non-injected control groups, indicating that the method of microinjection, rather than the specific target dsRNA, caused the observed mortality. Gene expression analysis showed a potential, though obscured, successful suppression of the *iap* gene in one repetition. However, no further evidence was found in the gene expression analysis for subsequent repetitions, subverting the possible RNAi success. The lack of RNAi-mediated mortality was further corroborated by the MPB kill curve that investigated higher amounts of dsIAP delivered. The mortality trend was the same, with injected groups, including the negative controls of water and dsRS, showing high mortality. Lastly, microinjection of MPB-dsRNA into an off-target, congeneric species, DFB, led to better initial survival in the injected negative control groups. No rapid mortality was observed in treatment groups, indicating that there was no RNAi initiation. Despite being a gold standard for RNAi initiation in many insects, microinjection is not often used in *D. ponderosae*, especially in emerged adults. Therefore, the data from this study supports the use of alternative methods to investigate RNAi as a pest management tool in MPB.

Chapter 3 - Testing novel targets for RNAi-mediated gene suppression and mortality in emerged adult *Dendroctonus ponderosae* via oral delivery.

3.1 Introduction

Co-opting the naturally occurring, anti-viral RNA interference (RNAi) pathway shows promise as a cost-effective and species-specific insecticide for pest management (Cooper et al. 2019; Christiaens et al. 2020). Studies have reported successful RNAi-initiation and mortality in agricultural and forest pests (Rangasamy and Siegfried 2012; Kyre et al. 2019, 2020; Haller et al. 2019; Pampolini and Rieske 2023). The species-specificity allows the researcher to target a single species for RNAi-mediated mortality (i.e. targeted mortality) due to the double-stranded RNA (dsRNA) and messenger RNA (mRNA) requirements (see Chapter 1, section 1.3.4). Delivering dsRNA via microinjection and oral ingestion are the main methods used by researchers to investigate RNAi responses in different species to different gene targets (Baum et al. 2007; Leelesh and Rieske 2020; Kyre et al. 2020; List et al. 2022).

Microinjection allows for direct delivery and certainty in the dsRNA dose to an insect (List et al. 2022). Microinjecting *Dendroctonus armandi* has initiated an RNAi response to elucidate gene functions. However, results from adult MPB microinjection assays suggest that it is an invasive procedure (see Chapter 2, section 2.3.2 and 2.3.4). Additionally, microinjection is constricted to laboratory application due to the method requirements (see Chapter 2, section 2.2.1) and requires trapping and removing adult MPB from the forest stands. Removing MPB from the stands negates the purpose and cost-effective benefits of applying RNAi as a pest management tool.

Laboratory-based oral ingestion is less invasive than microinjection and has previously initiated RNAi in Coleopteran insects (Rangasamy and Siegfried 2012; Rodrigues et al. 2018;

Haller et al. 2019). Western corn rootworm (*Diabrotica virgifera virgifera*, WCR) adults were fed an artificial diet containing 0.5µg or 1µg of dsRNA targeting vacuolar ATPase (*vATPase*) (Rangasamy and Siegfried 2012). Mortality was higher in WCR adults that ingested *vATPase* dsRNA and significant *vATPase* suppression was observed at 24h, 48h, and 72h when compared to water and dsRNA controls (Rangasamy and Siegfried 2012). There was no significant difference in gene suppression or mortality between beetles fed 0.5µg or 1µg of dsRNA (Rangasamy and Siegfried 2012). In another study, larvae from two different ladybird species, *Adalia bipunctata* and *Coccinella septempunctata*, ingested 4µl of a sucrose solution containing 2µg/µl or 0.25µg/µl of respective species-specific dsRNA targeting *vATPase A* (Haller et al. 2019). Increased mortality and decreased *vATPase A* expression was observed in the 2µg/µl groups when compared to non-dsRNA fed and nonsense dsRNA fed controls (Haller et al. 2019). Oral ingestion of dsRNA targeting shibire (*shi*) and heat shock protein (*hsp*) genes by emerald ash borer (*Agrilus planipennis*, EAB) neonates led to dosage-dependent mortality (Rodrigues et al. 2018). Higher mortality occurred in neonates after feeding on 10µg/µl than neonates that fed on 1µg/µl (Rodrigues et al. 2018). In another study, spraying 500µg of *in vitro* synthesized dsRNA onto seedlings infested with EAB eggs decreased gallery area and cambial ingestion in the seedlings and caused a decrease in *hsp* expression in larvae (Pampolini and Rieske 2023).

Delivering dsRNA orally initiated systemic RNAi responses in *Dendroctonus* species. Adult southern pine beetles (SPB, *Dendroctonus frontalis*) ingested a vertically suspended droplet of a sucrose solution containing 10µg of dsRNA (Kyre et al. 2019; Kyre and Rieske 2022). A significant decrease in heat shock protein (*hsp*) and shibire (*shi*) expression, but not inhibitor of apoptosis (*iap*) expression, was observed after dsRNA droplet ingestion by SPB. Higher mortality was observed in all three treatment groups compared to the control groups

(Kyre et al. 2019). Mountain pine beetle (MPB, *Dendroctonus ponderosae*) adults were submerged up to the posterior end of the pronotum in 2.5µg/µl dsRNA, allowing for dsRNA ingestion and absorption (Kyre et al. 2020). After dsRNA ingestion/absorption, MPB mortality was higher, and the *hsp*, *shi*, and *iap* expression decreased significantly, in the dsHSP, dsSHI, and dsIAP groups compared to the control (Kyre et al. 2020). Successful RNAi initiation via oral ingestion of dsRNA is critical, as dsRNA ingestion will be required for future *in situ* applications. However, using *in vitro* synthesized dsRNA for oral ingestion can be limited by dsRNA instability (Cooper et al. 2019). After successful RNAi initiation is achieved ("proof-of-concept"), finding a more stable dsRNA delivery method for *in situ* application is an essential step towards using RNAi as a pest management tool.

Stability can be improved using microbial vectors, like bacteria and yeast (Cooper et al. 2019; Christiaens et al. 2020; Leelesh and Rieske 2020). A strain of *Escherichia coli* expressing dsRNA caused successful mortality and gene suppression compared to the control (non-dsRNA expressing bacteria) in Emerald Ash Borer (*Agrilus planipennis* Fairmaire) neonates when targeting *hsp* and *shi* genes (Leelesh and Rieske 2020). Successful RNAi initiation via dsRNA-expressing bacteria was also achieved in Colorado potato beetle larvae (*Leptinotarsa decemlineata* Say, CPB) larvae (Zhu et al. 2011). Decreased expression of β-Actin (*Actin*), Protein transport protein sec23 (*Sec23*), Vacuolar ATP synthase subunit E (*vATPaseE*), Vacuolar ATP synthase subunit B (*vATPaseB*), and Coatomer subunit beta (*COPβ*) was observed after CPB larvae ingested 200µl of dsRNA-expressing bacteria. Knockdown of *Actin*, *Sec23*, and *COPβ* was more efficient after dsRNA-expressing bacteria ingestion than after *in vitro* synthesized dsRNA ingestion, but not in *vATPaseE* or *vATPaseB* (Zhu et al. 2011). Gene suppression in all five treatment groups was associated with decreased survival when compared

to the control (Zhu et al. 2011). Another microbial vector for dsRNA delivery is yeast, usually a *Saccharomyces cerevisiae* strain (Duman-Scheel 2019). In Spotted Wing Drosophila (*Drosophila suzukii*), dsRNA-expressing *S. cerevisiae* targeting vacuolar H+ ATPase 26 kD subunit *(Vha26)* and y-tubulin 23C *(yTub23C)* caused a decrease in movement, indicating a decrease in health (Murphy et al. 2016). A lethal response was observed in *D. suzukii* after *in vitro* synthesized v*ha26* and *yTub23C* dsRNA was ingested but not after dsRNA-expressing yeast ingestion (Murphy et al. 2016). In addition to microbial vectors, other production platforms have been successful.

A proprietary cell-free platform used to produce dsRNA has been approved in the United States of America for use as a biopesticide for CPB (Rodrigues et al. 2021). Significant suppression of *PSMB5*, a gene that codes for a subunit of the proteasome beta molecular machine, was observed 24h after ingestion of dsPSMB5 (255×10−5g/L) on a potato leaf. Expression of *PSMB5* and the amount of the coded protein, was consistently decreased at 24h through to 72h post-ingestion (Rodrigues et al. 2021). While RNAi has been previously approved in transgenic corn targeting the western corn rootworm (Reinders et al. 2023), recent United States Environmental Protection Agency (EPA) approval for foliar application of RNAi is groundbreaking for the progression of RNAi as a pest management tool for other insects.

Oral delivery using the submergence method will be used to test lethality of six gene targets using dsRNA: inhibitor of apoptosis (*iap*), heat shock protein (*hsp*), V-type proton ATPase subunit S1 (*vha19*), tetraspanin 2a (*tsp2a*), mesh protein (*mesh*), and snakeskin (*ssk*). In addition, vector-mediated hpRNA delivery to MPB via a strain of *S. cerevisiae* will be tested for the first time. Two genes, *iap* and *hsp*, are stress response genes and have previously elicited a lethal effect on adult MPB through the RNAi pathway (Kyre et al. 2020). The other four targets

are novel targets for RNAi in MPB, with three forming a complex critical to epithelial structure in the gut and *vha19* is involved in proton transport (Izumi et al. 2012, 2016; Zawadzki et al. 2012; Hu et al. 2019; Jonusaite et al. 2020; Beyenbach et al. 2020).

3.2 Methods

3.2.1 Subject Collection

Subject collection was previously described (see Chapter 2, section 2.2.1). The majority of MPB used in the following assays were from Smithers, BC and emerged naturally, except where otherwise noted (section 3.2.2). In 2022, bolts from Smithers, B.C., provided adult MPB for assays in sections 3.2.2, 3.2.3, and 3.2.5. In 2023, emerged MPB were collected from bolts from Smithers, B.C. and used in the assays described in 3.2.4, 3.2.6, and 3.2.7. Lastly, in 2024 bolts were received from Smithers, B.C., and emerged MPB were used in the section 3.2.8 assay. All emerged MPB were collected, and MPB exhibiting lower health (missing appendages, extremely lethargic) were excluded from assays.

3.2.2 Sucrose Bioassay – Method Test

Adult MPB were collected as they emerged and some extracted from galleries to randomly assort the beetles into three groups of ten beetles. Each group was assigned a treatment: no treatment (negative control), submergence in 1% sucrose solution, and droplet suspension of the 1% sucrose solution. The no-treatment group was placed in a petri dish lined with moistened filter paper and stored at 23°C in the dark.

Beetles in the submergence group were placed in enough 1% sucrose solution in a 0.6ml microcentrifuge tube to reach the posterior end of the pronotum (Kyre et al. 2020), a range of 5-7µl, depending on MPB size. Beetles were held in place with a compressed piece of Kimwipe, preventing them from backing out of the sucrose solution. Beetles were left submerged for 12h at 23°C in the dark. After the 12h period, beetles were removed from the tubes and placed in a petri dish lined with moistened filter paper and placed back in the dark at 23°C.

Beetles in the droplet suspension group were held to a 1µl drop of the 1% sucrose solution that was on a vertical plastic material (weigh boat held vertically). Beetles were held in place with entomological forceps until the drop was gone. After feeding, beetles were placed in a petri dish lined with moistened filter paper and stored at 23°C in the dark. Beetles in all three treatment groups were monitored daily for mortality.

3.2.3 Blue-dyed Sucrose Bioassay

To qualitatively determine the ingestion efficiency of the submergence and droplet suspension delivery methods, the 1% sucrose solutions were dyed with blue food colouring. Two drops of dye were added to 1ml of 1% sucrose solution. Emerged adult MPB were randomly assorted into two groups of ten: one group was submerged in the dyed sucrose solution and the second group was fed using the droplet suspension method. For each group, the previously outlined methods (section 3.2.2) were followed.

At 24h after initial exposure, beetles were dissected to record the presence or absence of blue dye in their guts. A few untreated beetles were dissected to act as a negative control for the colour of the guts. This 24h assay was independently performed twice.

Further refinement of the submergence method was tested, using a 0.2ml PCR tube rather than the 0.6ml tube (Figure 3.1). Other than this change, previous protocols were followed, though less liquid was needed to reach the posterior end of the pronotum in the smaller tube (section 3.2.2).

Figure 3.1 A submerged adult mountain pine beetle (*Dendroctonus ponderosae*, MPB) in blue-dyed 1% sucrose solution. Beetles were submerged in liquid to the posterior end of the pronotum for 12h.

After 12h of submergence, the beetles were removed and placed in a petri dish lined with a moistened filter paper and put in a dark incubator at 23°C. Beetles were dissected 24h after initial exposure to the blue-dyed sucrose solution, and the presence or absence of blue dye in the gut was recorded.

3.2.4 Submergence Trial – feeding status

After observations that beetles ingest the moistened filter paper, the potential effect on uptake of target liquids was investigated. Twenty-four emerged adult MPB collected four and three days before the assay were distributed among treatments to ensure that both collection dates were represented in each group. Twelve beetles were assigned to the "starved" group, while the other twelve beetles were assigned to the "fed" group. Within each group, there were three treatments: water, 0.05mg/ml and 0.1mg/ml heat-inactivated, hpRS-expressing yeast.

Beetles in the “starved” group were placed in petri dishes without filter paper. These petri dishes were placed in a box with moistened paper towel to provide moisture for MPB without direct contact. Beetles in the “fed” group were placed in petri dishes lined with moistened filter paper. Beetles were kept either “starved” or “fed” for five hours before submergence. Beetles were submerged in the three assigned treatments to the posterior end of the pronotum for 12h. Beetles were removed and placed into petri dishes with moistened filter paper and stored in the dark at 23°C. The presence or absence of liquid remaining in the tube after12h was recorded. Mortality of the beetles was tracked for 16 days.

3.2.5 Submergence target dsRNA Assay

The kit-synthesized dsRNAs for six gene targets were tested on adult MPB using the submergence method. Concentrations of the dsRNA were as close to 2.5µg/µl as possible: *IAP* (2µg/µl), *HSP* (1.8-2.1µg/µl), *mesh* (1.99-2.1µg/µl), *tsp2a* (1.9µg/µl), *vha19* (1.9-2µg/µl), *ssk* (2-2.1µg/µl), and dsRS (2-2.1µg/µl). Emerged adult MPB collected from four different dates within a week of the assay were evenly distributed among the seven treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented. Within each treatment group, ten beetles were assigned to the mortality observation group and five beetles were assigned to the gene expression analysis group. The previously outlined protocol (section 3.2.3) was followed for the submergence of MPB. The volume of the liquid added ranged from 5-6µl. Gene expression beetles were flash frozen in liquid nitrogen 24h after initial dsRNA exposure and stored at -80°C. Beetles in the mortality observation groups were monitored daily until 100% mortality was observed in all treatment groups. Filter paper in the petri dishes was changed every 48h.

3.2.6 Female Submergence Assay

Female adult MPB emerged from bolts kept in cold storage at 6°C for about four months to delay beetle emergence were assigned to one of six different treatments: dsIAP (2.5µg/µl), dsVHA19 (2.5µg/µl), hpRNA-expressing yeast solutions (heat-inactivated yeast mixed with distilled water) of 0.05mg/ml hpIAP, 0.1mg/ml hpIAP, 0.05mg/ml hpVHA19, 0.1mg/ml hpVHA19. Three controls were also included: 0.1mg/ml hpRS, dsRS (2.5µg/µl), and water. Emerged adult MPB collected three and four days before the assay were evenly distributed among treatments to ensure that both collection dates were represented in each group. Within each treatment group, ten beetles were assigned to the mortality observation group and six were assigned to the gene expression analysis group. The previously described submergence protocol was followed (section 3.2.3/3.2.4). The volume of the liquid added ranged from 3-5µl. Gene expression beetles were flash frozen in liquid nitrogen 72h after the initial dsRNA exposure and stored at -80°C. Beetles in the mortality observation group of each treatment were monitored daily and mortality recorded until 100% mortality was achieved in all treatment groups.

3.2.7 Male Submergence Assays

Male adult MPB emerged from bolts previously cooled to delay beetle emergence (see section 3.2.6) were assigned to one of seven treatments: dsMESH (2.5µg/µl), 0.05mg/ml hpIAP, 0.1mg/ml hpIAP, 0.05mg/ml hpMESH, 0.1mg/ml hpMESH, and 0.1mg/ml hpRS; and water. Emerged adult MPB collected from four different dates within five days of the assay were evenly distributed among the seven treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented for the first repetition. In the second repetition, emerged adult male MPB collected from nine different dates within nine days of the assay were evenly distributed among the seven treatments. Within each treatment group, ten beetles were

assigned to the mortality observation group and six were assigned to the gene expression analysis group. The previously described submergence protocol was followed (section 3.2.3/3.2.4). The volume of the liquid added ranged from 3-5µl. Gene expression beetles were flash frozen in liquid nitrogen 72h after initial dsRNA exposure and stored at -80°C. Beetles in the mortality observation group of each treatment were monitored daily and mortality recorded until 100% mortality was achieved in all treatment groups.

3.2.8 Submergence Assay to test three targets on emerged adult mountain pine beetle (2024)

Thirty-four emerged female adult MPB were assigned to one of the control or treatment groups: no-submergence control, water (H_2O), dsRS (2.5µg/µl), dsIAP (2.5µg/µl), dsVHA19 (2.5µg/µl), and dsMESH (2.5µg/µl). Within each treatment group, ten beetles were assigned to the mortality observation group and six beetles were assigned to each of the following gene expression analysis groups: 24h, 48h, 72h, and 96h post-submergence. Emerged adult MPB collected one and two days before the assay were evenly distributed among treatments to ensure that both collection dates were represented in each group. Beetles were submerged in the assigned target (or not submerged for the no-submergence control group) as described in previous methods (section 3.2.3/3.2.4). The volume of the liquid added ranged from 3-6µl. However, some alterations were made to the storage conditions of the beetles for the assay duration are as follows. Once submerged, beetles were placed under a cardboard box at room temperature (approximately 20°C). After 12h, beetles were removed and placed on petri dishes as previously detailed (section 3.2.3/3.2.4). Petri dishes were stored vertically and returned to the dark at room temperature (approximately 20°C). Filter paper was changed daily. Gene expression beetles in each group within each treatment were flash frozen in liquid nitrogen at the

set post-submergence time and stored at -80°C. Beetles in the mortality observation group of each treatment were monitored and mortality recorded daily for 14 days. Below is a summary of the submergence assays performed in this chapter for review (Table 3.1).

Table 3.1 Brief summary of the submergence assay methods performed on emerged adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) in this study.

Assay	Section	dsRNA/hpRNA targets	MPB sex (M/F)	MPB age
Blue-dyed sucrose method test	1.2.2 and 1.2.3	NA	NA	< 3 days
Feeding status trial	1.2.4	Water, 0.05mg/ml, and 0.1mg/ml hpRS-expressing yeast solutions	mixed	3-4 days
Submergence assay	1.2.5	dsIAP, dsHSP, dsTSP2A, dsVHA19, dsMESH, dsSSK	NA	4-7 days
Female Submergence assay	1.2.6	dsIAP, dsVHA19, hpIAP, hpVHA19	F	3-4 days
Male Submergence assay	1.2.7	dsMESH, hpIAP, hpMESH	M	2-5 days
2024 Submergence assay	1.2.8	dsIAP, dsVHA19, dsMESH	F	1-2 days

3.2.9 Gene Expression Methods

The gene expression analysis method follows the protocol outlined (see Chapter 2, section 2.2.8). Briefly, RNA was extracted from selected samples using the RNeasy Plus Mini Kit (Qiagen, Toronto, ON). The use of a QIAshredder (Qiagen, Toronto, ON) step was added to

the kit protocol, prior to the gDNA eliminator step. The resulting extractions were quality checked using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). From samples with good quality (for definition of good quality: Chapter 2, section 2.2.8.1), cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON) following kit protocol and normalizing RNA input to 500ng. The cDNA was used in ddPCR where each sample was run twice, once with each housekeeping gene (Chapter 2, section 2.2.8.2). Each reaction consisted of: 12µl 2X Supermix for probes with no dUTPs (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON), 1µl of the respective gene target assay, 0.5µl of the respective reference gene assay, 2.4µl of the sample cDNA, and 8.1µl of water to reach 24µl total volume. For the no-template control (NTC) and positive control, the cDNA was replaced with water and gBlock, respectively (Chapter 2, section 2.2.8.2). The Thermocycler conditions were: 95°C for 10 minutes to activate the polymerase, followed by 45 rounds of 95°C for 30 seconds for the denaturation step and 58°C for 1 minutes for the annealing step. The 45 rounds were succeeded by a step of 98°C for 10 minutes to deactivate the enzymes and remained at 4°C until removed from the thermocycler. The droplet reader was used to obtain the end-point quantification of the amplification, and results were viewed with QX Manager Software Standard version 1.1 (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON).

For each sample, each target gene value was normalized to the reference genes by dividing the average target gene counts by the geometric mean of the reference gene counts, similar to the principle of the double delta Ct method with RT-qPCR (Livak and Schmittgen 2001; Bio-Rad Laboratories 2007; Zmienko et al. 2015). The resulting normalized data was $\log_2$ transformed and data was checked for normality using the Shapiro-Wilk test alongside visual

confirmation using histograms and Q-Q plots. For normally distributed data, the parametric one-way ANOVA test comparing means was performed, followed by a Tukey HSD test if the ANOVA test was significant ($p < 0.05$). Data was visualized using a boxplot showing the relative gene expression in a $\log_2$ base scale of the target gene.

3.3 Results

3.3.1 Sucrose bioassay test

Survival of adult MPB in the second and third groups remained high for eight days after treatment with a 1% sucrose solution. The no treatment control group (group_1) showed earlier mortality, with 50% survival by day eight compared to the 100% and 90% survival in the second and third groups, respectively (see Appendix, Figure S2). After the ninth day, survival in all three groups decreased rapidly.

3.3.2 Blue-dyed sucrose bioassay test and feeding status results

No uptake using the vertical droplet suspension was observed in two trials (see Appendix, Table S2 and S3). Uptake in the first submergence trial was 20%. Uptake increased to 40% in the second trial. With an uptake in 60% of beetles in the last trial, the submergence method was used for assays testing target genes for RNAi-mediated gene suppression (see Appendix, Table S4).

3.3.3. Impact of feeding status on target liquid uptake

Uptake of the target liquid was not observed to change whether the moistened filter paper was present or absent. After 12h of submergence, most of the liquid was gone from the tubes and any remaining liquid was a very small amount behind the head of the beetle. Some subjects had even more liquid remaining, but this occurred in both yeast treatments in “starved” and “fed” groups. All beetles were alive for 72h post submergence, and by day 16, MPB mortality was similar among the “starved” and “fed” groups (see Appendix, Table S5).

3.3.4 Submergence target dsRNA Assay – Six targets (2022)

The adult MPB mortality in the treatment groups was gradual, with all groups showing 50% or higher survival six days after dsRNA exposure (Figure 3.2). Survival rates declined after seven days, with dsMESH showing 20% survival. Survival in the dsRS group remained 50% or

higher for seven days, but decreased beginning on day eight. In all treatment groups 100% mortality was reached by day 11.

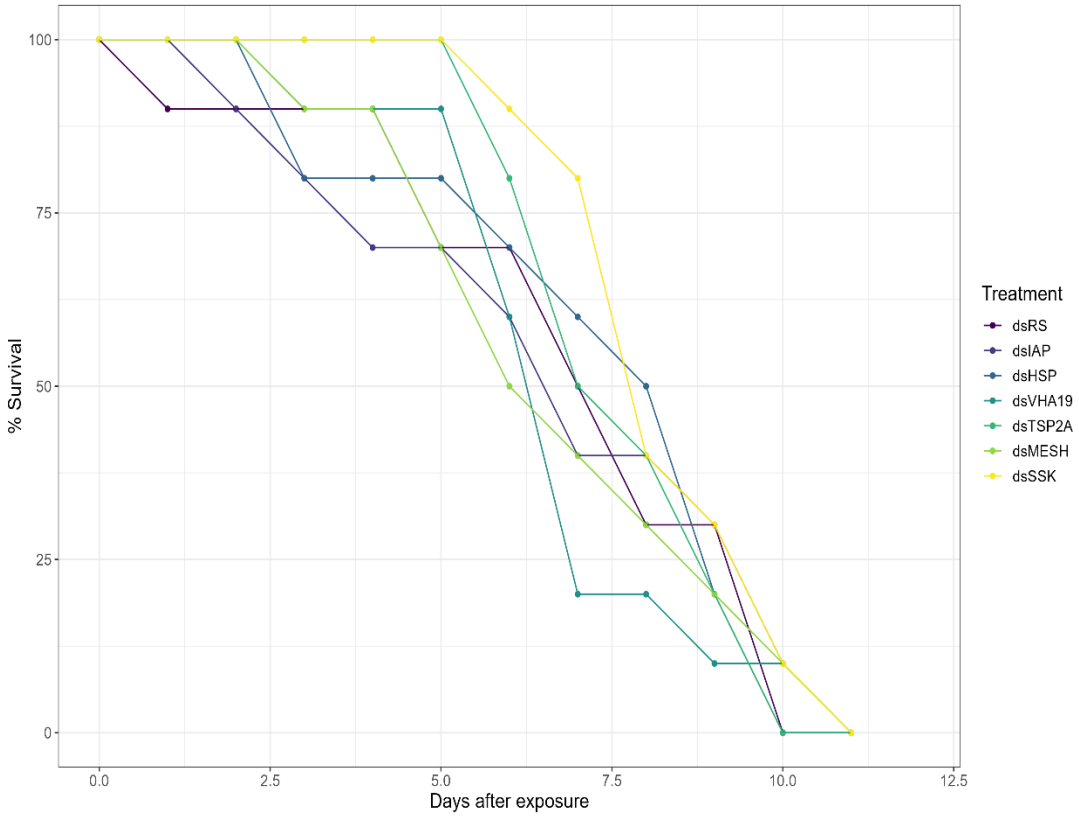

Figure 3.2 Percent survival over 11 days of adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) (n = 10) submerged in dsRNA for six gene targets: dsIAP, dsHSP, dsVHA19, dsTSP2A, dsMESH, and dsSSK and the control dsRS.

3.3.4.1 Gene expression analysis: Submergence target dsRNA Assay – Six targets (2022)

Two gene targets, dsHSP (t = -1.1787, df = 7.3002, p = 0.2755) and dsTSP2A (W = 9, p = 0.5476), showed no statistically significant difference than the negative control (Figure 3.3B&D). Beetles treated with dsIAP showed a lower mean *iap* expression than the beetles treated with dsRS, the negative control (t = -3.2484, df = 6.9886, p = 0.01412) (Figure 3.3A). In the three other treatment groups, dsMESH (t = 4.4964, df = 4.2431, p = 0.009468), dsVHA19 (t = -5.5759, df = 5.834, p = 0.00155), and dsSSK (t = -5.0566, df = 5.3101, p = 0.003299), the

treated beetles showed a higher mean target gene expression than the beetles in the negative control group (Figure 3.3C&E-F).

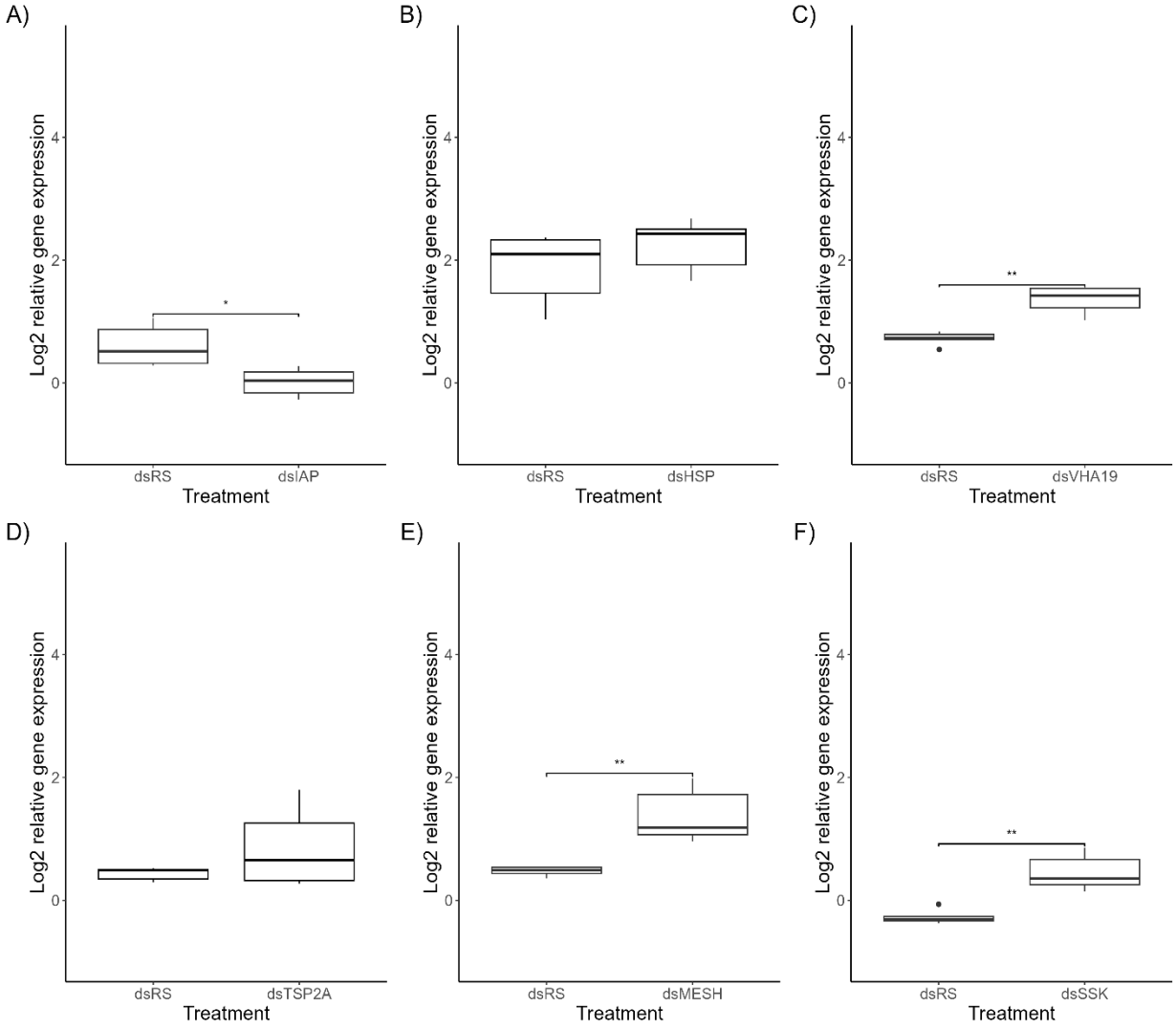

Figure 3.3 Comparison of the mean (n = 5) of the $\log_2$ transformed relative target gene expression between the treatment and negative control (dsRS) group 24h after submergence in dsRNA. Statistical analysis was performed using a Student's t-test (A-C, E-F) or a Mann-Whitney-Wilcoxon Test (D). Significance codes are: '***' < 0.001; '**' < 0.01; '*' < 0.05 A) *iap* gene (t = -3.2484, df = 6.9886, p = 0.01412), B) *hsp* gene (t = -1.1787, df = 7.3002, p = 0.2755), C) *vha19* gene (t = -5.5759, df = 5.834, p = 0.00155), D) *tsp2a* gene (W = 9, p = 0.5476), E) *mesh* gene (t = 4.4964, df = 4.2431, p = 0.009468), and F) *ssk* gene (t = -5.0566, df = 5.3101, p = 0.003299).

3.3.5 Female Submergence Assay

The mortality of adult female MPB in each group occurred quickly, with 50% or higher mortality by day five post-submergence (Figure 3.4). Complete mortality was observed in all groups by day ten. The 0.05mg/ml hpVHA19 (vha19_0.05) treatment group had 100% mortality six days after submergence. The mortality of female MPB in the three negative control groups showed similar patterns as the dsRNA treatment groups (Figure 3.4).

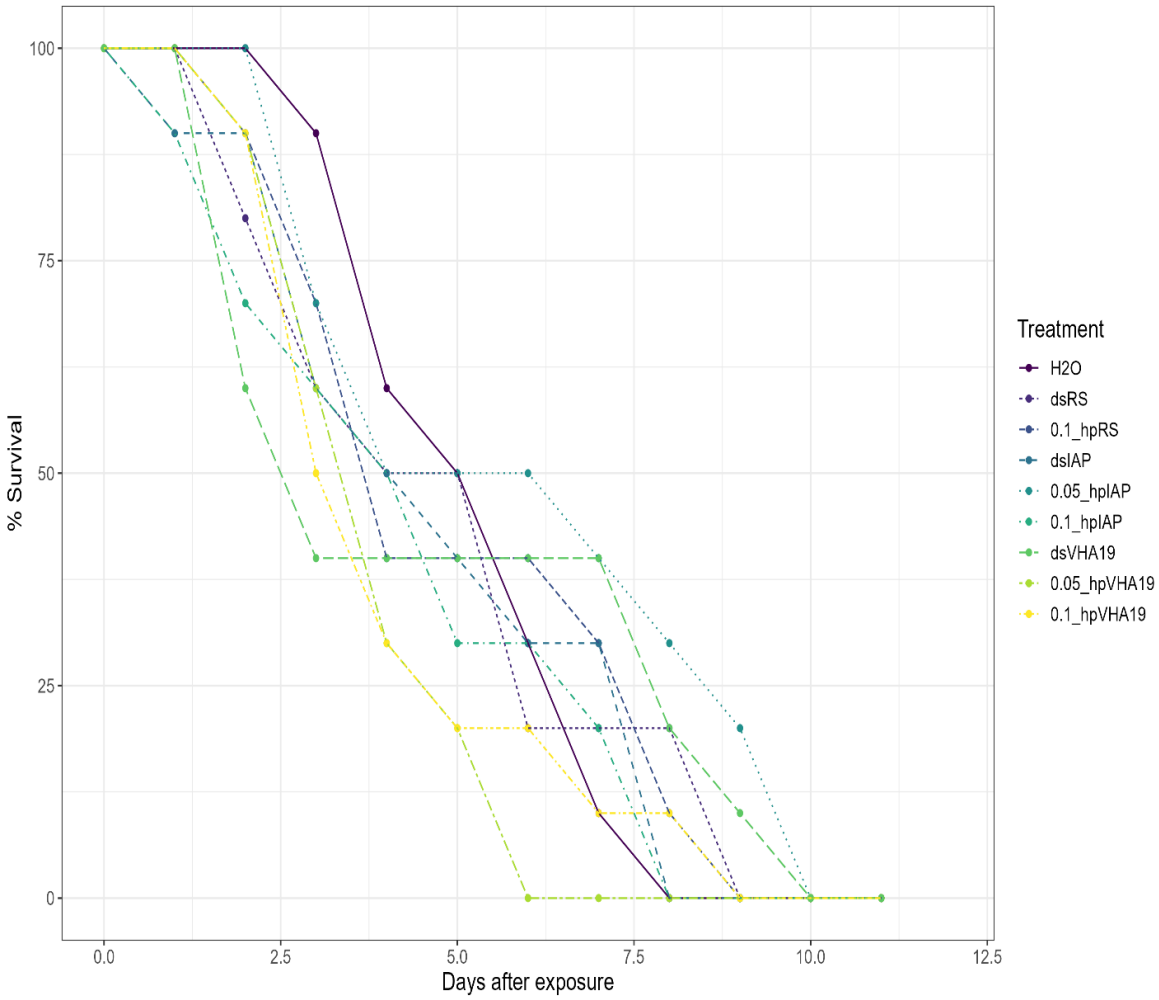

Figure 3.4 Percent survival over 11 days of adult female mountain pine beetles (*Dendroctonus ponderosae,* MPB) (n = 10) submerged in kit-synthesized dsRNA or heat-deactivated, dsIAP- and dsVHA19-expressing yeast solutions of different concentrations: 0.05mg/ml hpIAP (0.05_hpIAP), 0.1mg/ml hpIAP (0.1_hpIAP), 0.05mg/ml hpVHA19 (0.05_hpVHA19), 0.1mg/ml hpVHA19 (0.1_hpVHA19) and kit-synthesized dsIAP (dsIAP) and dsVHA19 at 2.5µg/µl (dsVHA19). The survival of two negative controls: water (H2O) and a random sequence expressed in a 0.1mg/ml yeast solution (0.1_hpRS) and kit-synthesized (2.5µg/µl (dsRS) were recorded and plotted alongside for comparison.

3.3.5.1 Gene expression analysis: Female submergence assay – 2023

A one-way ANOVA test found no statistically significant differences in the mean $\log_2$ relative gene expression between the treatment and control groups for both target genes: *iap* ($p = 0.581$, $F = 0.7777$, $df = 5$) and *vha19* ($p = 0.485$, $F = 0.903$, $df = 4$) (Figure 3.5). For both gene targets, there was no difference in the mean $\log_2$ relative gene expression between different

concentrations of the hpRNA-expressing yeast solutions or between the dsRNA or hpRNA. After quality control measures, the kit-synthesized dsVHA19 (2.5µg/µl (dsVHA19)) treated group was down to two biological repetitions and was excluded from the statistical analysis but plotted for visual observation (Figure 3.5).

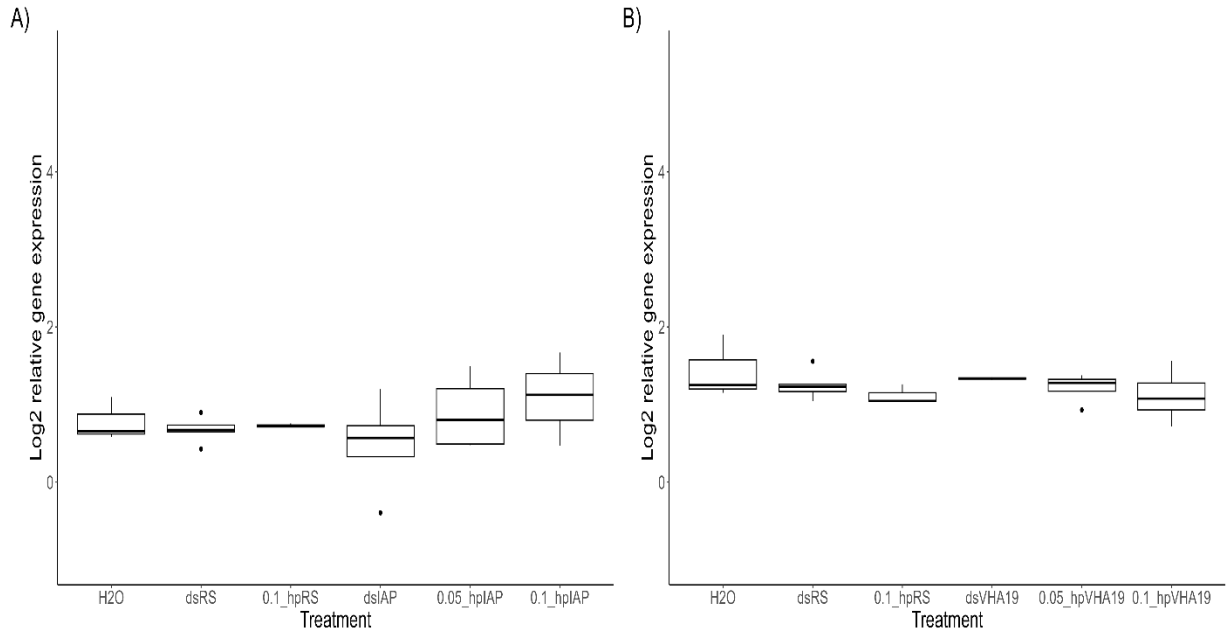

Figure 3.5 Comparison of the mean log_2 transformed relative target gene expression between the treatment controls and the negative controls 72h after submergence in dsRNA or hpRNA. Statistical analysis was performed using a one-way ANOVA test. A) Comparison of 0.05mg/ml hpIAP (0.05_hpIAP, n=4), 0.1mg/ml hpIAP (0.1_hpIAP, n=3), dsIAP at 2.5µg/µl (dsIAP, n=4), and the negative controls water (H2O, n=3), dsRS (dsRS, 2.5µg/µl, n=5), 0.1mg/ml hpRS (0.1_hpRS, n=3): *iap* expression is not significantly different (p = 0.581, F= 0.7777, df= 5) B) Comparison between 0.05mg/ml hpVHA19 (0.05_hpVHA19, n=4), 0.1mg/ml hpVHA19 (0.1_hpVHA19, n=6), dsVHA19 at 2.5µg/µl (dsVHA19, n=2*), and the negative controls water (H2O, n=3), dsRS (dsRS, 2.5µg/µl, n=5), and 0.1mg/ml hpRS (0.1_hpRS, n=3): *vha19* expression is not significantly different (p = 0.485, F= 0.903, df= 4). * With only two biological repetitions, this treatment group was excluded from the statistical analysis but plotted for visual observation.

3.3.6 Male Submergence Assays

Adult male MPB mortality was observed in the treatment and negative control groups. By day seven post-submergence, all groups showed 50% or less survival (Figure 3.6). By day fourteen, 100% mortality was observed in all the groups. In 0.1mg/ml hpMESH (0.1_hpMESH),

100% mortality was observed by day nine, followed by complete morality in the water (H2O) and 0.05mg/ml hpMESH (0.05_hpMESH) groups by day 11.

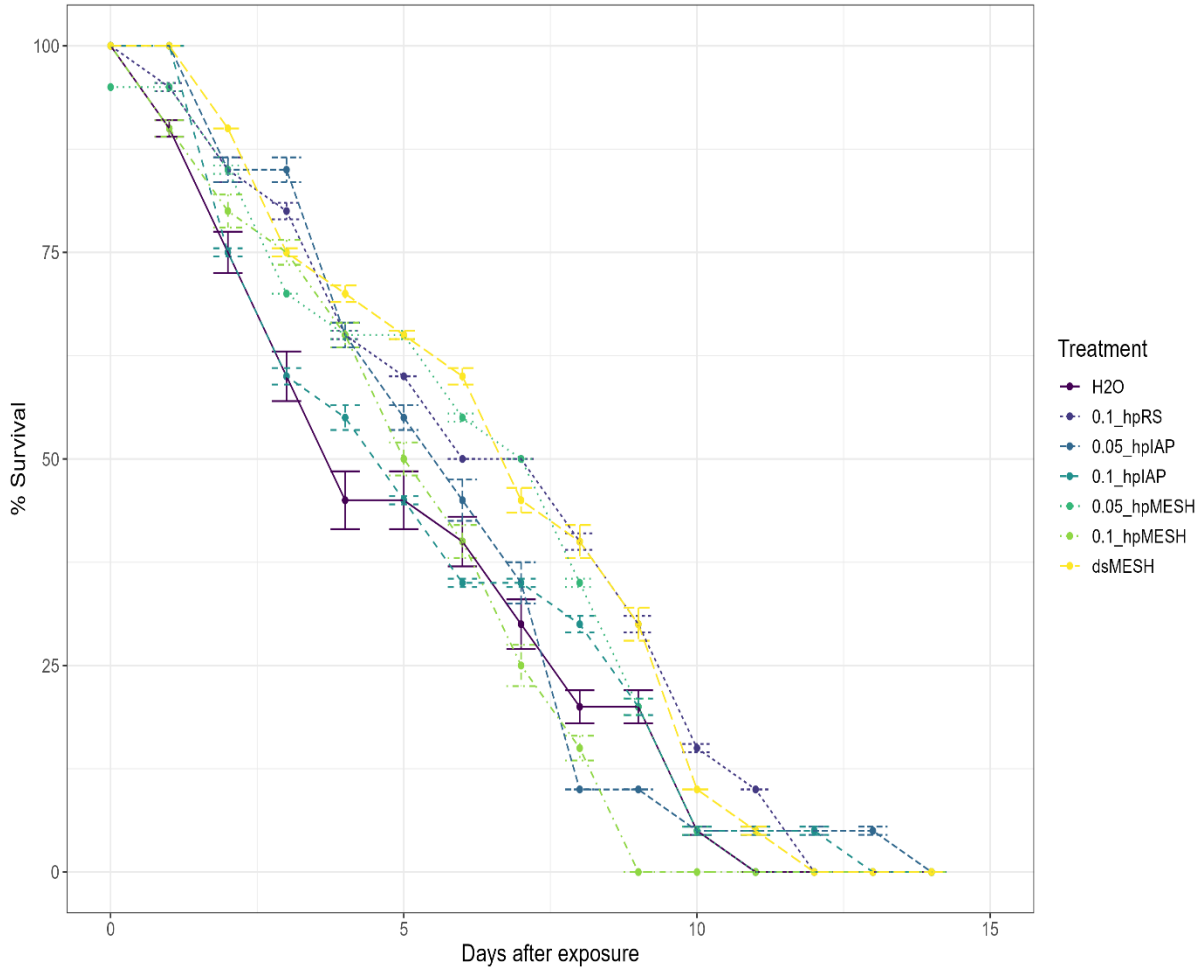

Figure 3.6 Percent survival over 14 days of adult male mountain pine beetles (*Dendroctonus ponderosae*, MPB) (n = 10) submerged in: 0.05mg/ml hpIAP (0.05_hpIAP), 0.1mg/ml hpIAP (0.1_hpIAP), 0.05mg/ml hpMESH (0.05_hpMESH), 0.1mg/ml hpMESH (0.1_hpMESH), or dsMESH at 2.5µg/µl (dsMESH). The survival of two negative controls: water (H2O) and 0.1mg/ml hpRS yeast solution (0.1_hpRS; does not match any transcript in the mountain pine beetle transcriptome) was recorded and plotted alongside for comparison.

3.3.6.1 Gene expression analysis: Male submergence assay – 2023

A comparison of group means using the Kruskal-Wallis test showed that there was no statistically significant difference in the mean $\log_2$ relative gene expression between the treatment and control groups for both gene targets: *iap* ($p = 0.5977$, $\chi^2 = 1.88$, df= 3) and *mesh* ($p = 0.5663$, $\chi^2 = 2.9495$, df= 4) (Figure 3.7). There was also no difference between the varying

concentrations of the hpRNA-expressing yeast solutions within each target. For the mesh target, there was no statistically significant difference between beetles submerged in dsRNA or hpRNA (Figure 3.7).

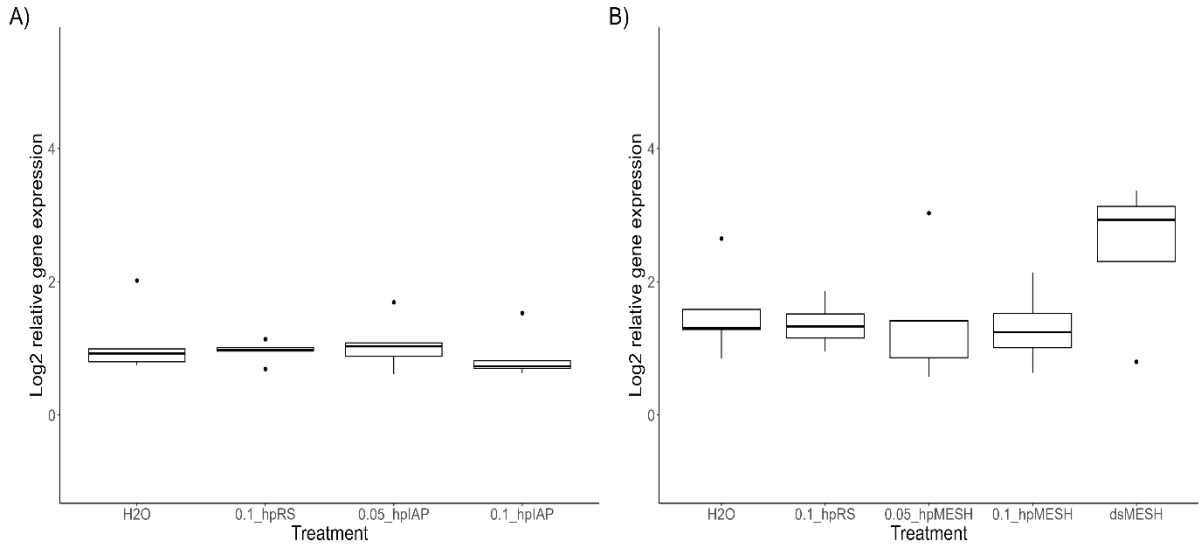

Figure 3.7 Comparison of the mean $\log_2$ transformed relative target gene expression between the treatment controls and the negative controls 72h after submergence in dsRNA. Statistical analysis was performed using the Kruskal-Wallis test. A) Comparison between 0.05mg/ml dsIAP (0.05_dsIAP, n=5), 0.1mg/ml dsIAP (0.1_IAP, n=5) and the negative controls water (H2O, n=5) and 0.1mg/ml dsRS (0.1_dsRS, n=5): *iap* expression is not significantly different ($p = 0.5977$, $\chi^2 = 1.88$, df= 3). B) Comparison between yeast solutions 0.05mg/ml dsMESH (0.05_dsMESH, n=5), 0.1mg/ml dsMESH (0.1_mesh, n=6) and kit-synthesized dsMESH at 2.5µg/µl (ndsMESH, n=4) to the negative controls water (H2O, n=5) and 0.1mg/ml dsRS (0.1_dsRS, n=5): *mesh* expression is not significantly different ($p = 0.5663$, $\chi^2 = 2.9495$, df= 4).

Though obscured by variable data, the mean $\log_2$ relative *mesh* expression does appear slightly higher in the kit-synthesized treatment group (dsMESH), similar to the previous submergence results from 2022 (see section 3.3.4.1, Figure 3.3E).

3.3.7 Submergence assay testing three targets on emerged adult mountain pine beetle (2024)

The mortality of adult female MPB in the dsRNA-treated groups dsVHA19 and dsMESH was higher than the adult female MPB mortality in the negative control groups and the dsIAP treatment group (Figure 3.8). The lowest survival was observed in the dsMESH group with only

10% survival eight days post-submergence. This was followed by dsVHA19 with 30% survival and dsIAP with 40% survival. Between day seven and eight in the no-submergence control (no_sub_control), survival dropping from 70% to 40%. Similarly, survival in the dsRS control group decreased from 80% to 50% between day seven and eight. High survival, 90%, was observed in the water control group throughout the assay (Figure 3.8). Mortality after eight days post-submergence was likely impacted by fungus visible on deceased beetles.

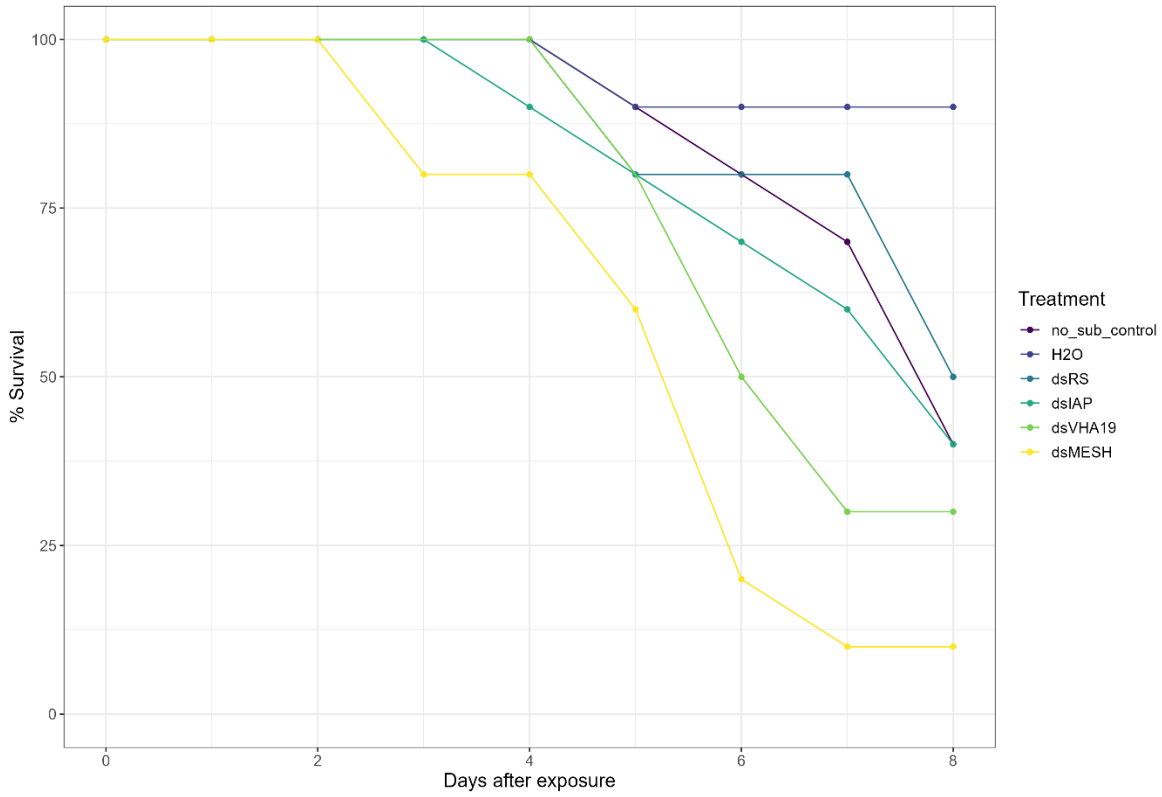

Figure 3.8 Percent survival over eight days of adult female mountain pine beetle (*Dendroctonus ponderosae*, MPB) (n = 10) submerged in kit-synthesized dsRNA for three gene targets: *iap* (dsIAP), *vha19* (dsVHA19), and *mesh* (dsMESH) at a concentration of 2.5µg/µl. The survival of three negative controls: no-submergence control (no_sub_control), water (H2O), and 2.5µg/µl double-stranded random stem (dsRS; does not match any transcript in the mountain pine beetle transcriptome) was recorded and plotted alongside for comparison.

3.3.7.1 Gene expression analysis - submergence assay testing three targets on emerged female adult mountain pine beetle (FS24)

3.3.7.1.1 Relative *iap* gene expression

A comparison between dsIAP and the control groups at 24h post-submergence using a one-way ANOVA indicated that the mean of at least one group was significantly different than the others ($p < 0.0001$, $F = 22.41$, $df = 3$) (Figure 3.9A). Tukey's HSD test identified that the mean $\log_2$ relative *iap* expression in two controls, water ($p = 0.01$) and dsRS ($p = 0.00013$), was significantly lower than in the no-submergence control. In dsIAP, the mean $\log_2$ relative *iap* expression was significantly lower than the no-submergence control and water groups ($p < 0.0001$ and $p = 0.001$, respectively), but not the dsRS group ($p = 0.104$).

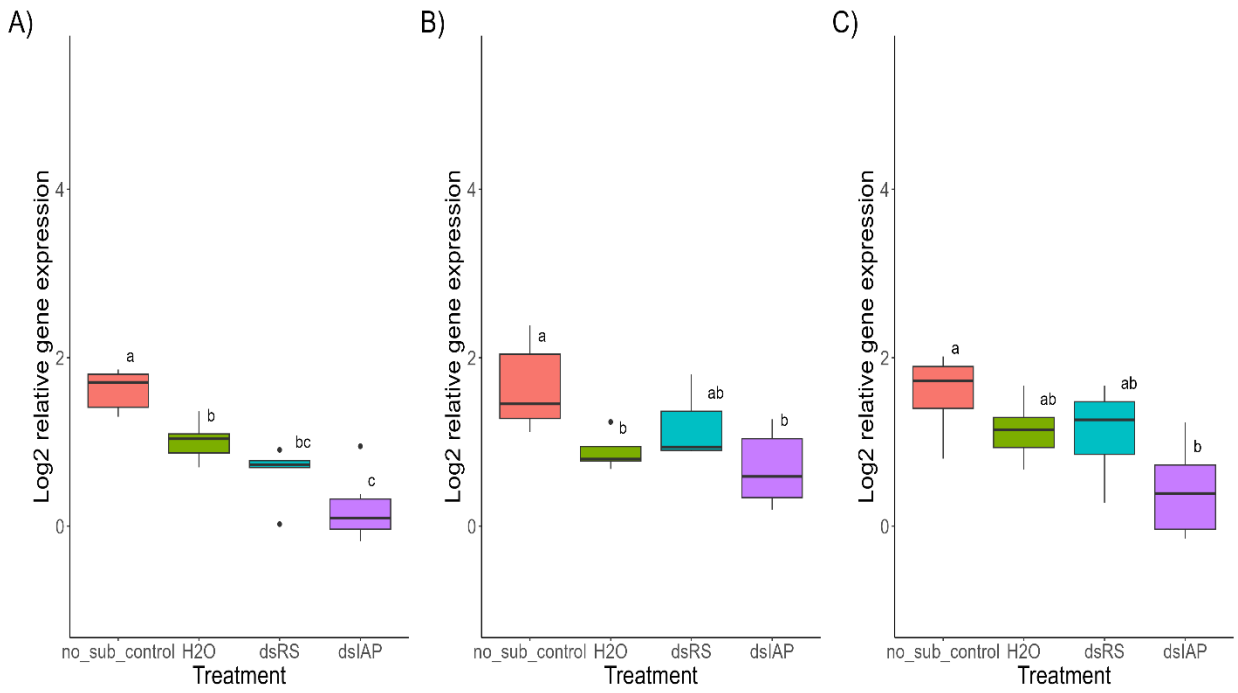

Figure 3.9 Comparison of the mean $\log_2$ relative *iap* gene expression between the dsIAP and control groups: no-submergence control (no_sub_control), water (H2O), and 2.5µg/µl double-stranded random stem (dsRS; does not match any transcript in the mountain pine beetle transcriptome). Statistical analysis using the one-way ANOVA test showed significant differences among at least one of the group means at each time point. A) At 24h (n = 6), *iap* gene expression in H2O (p = 0.01) and dsRS (p = 0.00013) groups were significantly lower than the no-submergence control group. Expression of *iap* in dsIAP was significantly lower than no-submergence control and H2O (p < 0.0001 and p = 0.001, respectively). B) At 48h (n=6), *iap* expression in the H2O (n=5) and dsIAP groups was significantly lower than the no-submergence control group (p = 0.03 and p = 0.004, respectively). C) At 72h (n = 6; dsRS n=5), *iap* expression was significantly lower in the dsIAP group than the no-submergence control group (p = 0.0024).

The same method at 48h post-submergence calculated that at least one of the group's mean relative $\log_2$ *iap* expression was different than the others (ANOVA; p = 0.0053, F = 5.836, df= 3) (Figure 3.9B). The subsequent Tukey's HSD test identified a significantly lower *iap* expression in two groups, H2O (p = 0.03) and dsIAP (p = 0.004), compared to the no submergence control. The *iap* expression in the dsIAP group was not significantly different from the other two control groups: H2O or dsRS (p = 0.851 and p = 0.232, respectively).

The one-way ANOVA comparison of means between treatment groups 72h post-submergence also showed that there was a difference in at least one of the group means from the

other groups ($p = 0.0045$, $F = 6.054$, $df = 3$) (Figure 3.9C). Tukey's HSD test reported that the relative $\log_2$ *iap* expression in the dsIAP group is significantly lower than in the no-submergence control ($p = 0.0024$). Once again, the *iap* expression in the dsIAP group is not significantly different from the other two controls: H2O and dsRS ($p = 0.0759$ and $p = 0.117$, respectively).

A one-way ANOVA comparing the relative mean *iap* log2 expression within each treatment group over the three time points indicated no significant difference: no submergence control ($p = 0.97$, $F = 0.031$, $df = 2$), water ($p = 0.348$, $F = 1.139$, $df = 2$), dsRS ($p = 0.107$, $F = 2.638$, $df = 2$), and dsIAP ($p = 0.254$, $F = 1.505$, $df = 2$).

3.3.7.1.2 Relative *vha19* gene expression

A one-way ANOVA comparison of mean relative $\log_2$ *vha19* expression between the treatment groups 24h post-submergence reported that at least one group's mean was different than the others ($p = 0.0243$, $F = 3.889$, $df=3$) (Figure 3.10A). Tukey's HSD test showed a significant decrease in the relative $\log_2$ *vha19* gene expression in the dsRS and dsVHA19 groups when compared to the no-submergence control group ($p = 0.03$ and $p = 0.044$, respectively). There is no statistical difference between the *vha19* expression in dsVHA19 compared to H2O ($p = 0.922$) or dsRS ($p = 0.998$). At 48h post-submergence, a Kruskal-Wallis test showed no significant difference in the mean relative $\log_2$ *vha19* expression between the different treatment groups ($p = 0.09$, $\chi^2 = 6.49$, $df = 3$) (Figure 3.10B).

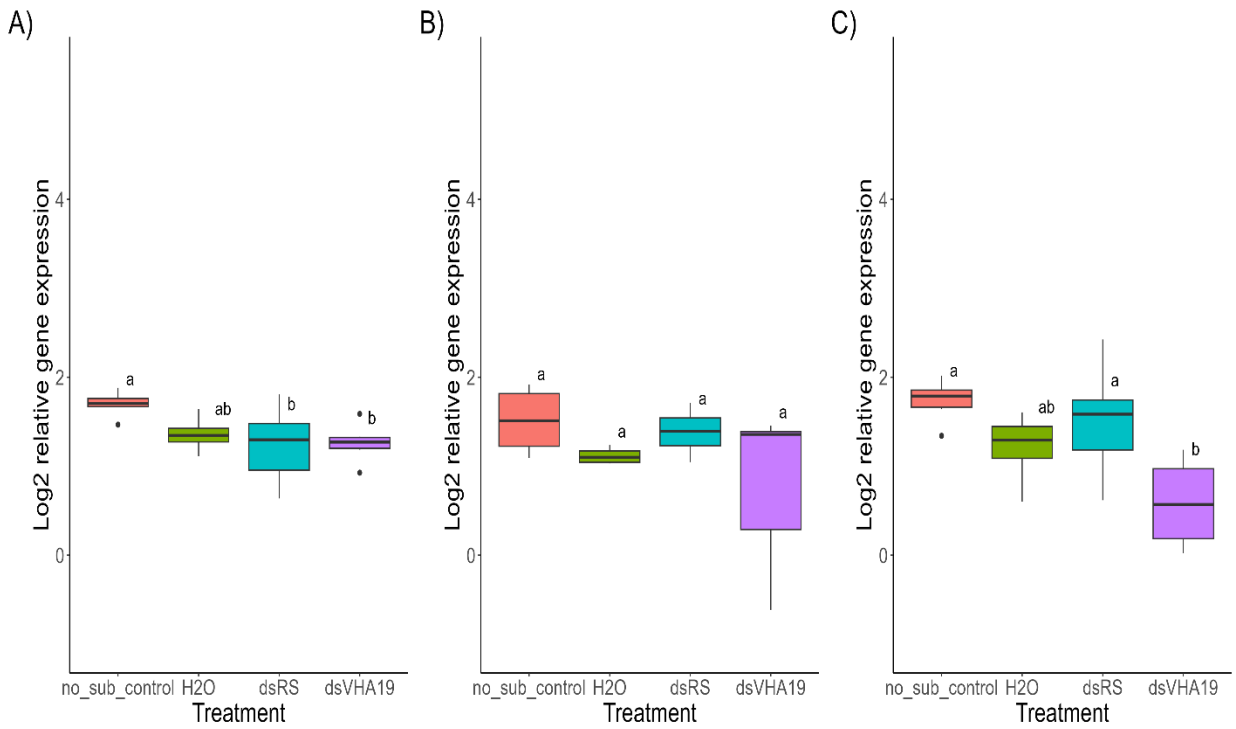

Figure 3.10 Comparison of the mean $\log_2$ relative *vha19* gene expression between the dsVHA19 and control groups: no-submergence control (no_sub_control), water (H2O), and 2.5µg/µl double-stranded random stem (dsRS; does not match any transcript in the mountain pine beetle transcriptome). A) At 24h (n=6), a one-way ANOVA calculated that *vha19* gene expression was significantly lower in the dsRS and dsVHA19 groups compared to the no-submergence control ($p = 0.03$ and $p = 0.044$, respectively). B) At 48h (n=6), a Kruskal-Wallis test showed no significant differences in relative *vha19* gene expression between any of the group means ($p = 0.09$, $\chi^2 = 6.49$, df= 3). C) At 72h (n= 6), a one-way ANOVA test output showed that the *vha19* relative expression was statistically significantly lower in the dsVHA19 group (n=4) than the no-submergence control and dsRS groups (n=5) ($p = 0.006$ and $p = 0.037$, respectively).

The comparison at 72h post-submergence revealed that at least one group's mean was significantly different than the other's (ANOVA; $p = 0.00855$, $F = 5.397$, $df = 3$) (Figure 3.10C). A Tukey's HSD test showed the mean relative $\log_2$ *vha19* expression was significantly lower in the dsVHA19 group than in the no-submergence and dsRS controls ($p = 0.006$ and $p = 0.037$, respectively), but not from the H2O control ($p = 0.186$).

A Kruskal Wallis test comparing the mean $\log_2$ relative *vha19* expression at the three time points in the dsVHA19 treatment group and a one-way ANOVA comparison for the control

groups showed no significant differences: no submergence control ($p = 0.311$, $F = 1.263$, $df = 2$), water ($p = 0.24$, $F = 1.573$, $df = 2$), dsRS ($p = 0.628$, $F = 0.481$, $df = 2$), dsVHA19 ($p = 0.1881$, $\chi^2 = 3.3419$, $df = 2$).

3.3.7.1.3 Relative *mesh* gene expression

Expression of the third target gene, *mesh*, between the dsMESH and control groups compared at different times post-submergence showed variable results (Fig 3.11). The one-way ANOVA test at 24h post-submergence showed no significant difference in the mean relative $\log_2$ *mesh* expression between any of the treatment and control groups ($p = 0.502$, $F = 0.813$, $df = 3$) (Figure 3.11A). At 48h, a Kruskal-Wallis comparison of means also showed no significant differences in the mean relative $\log_2$ *mesh* expression between any of the treatment and control groups ($p = 0.8881$, $\chi^2 = 0.6364$, $df = 3$) (Figure 3.11B). There is large variation in the *mesh* expression in the dsMESH group at 48h and 72h.

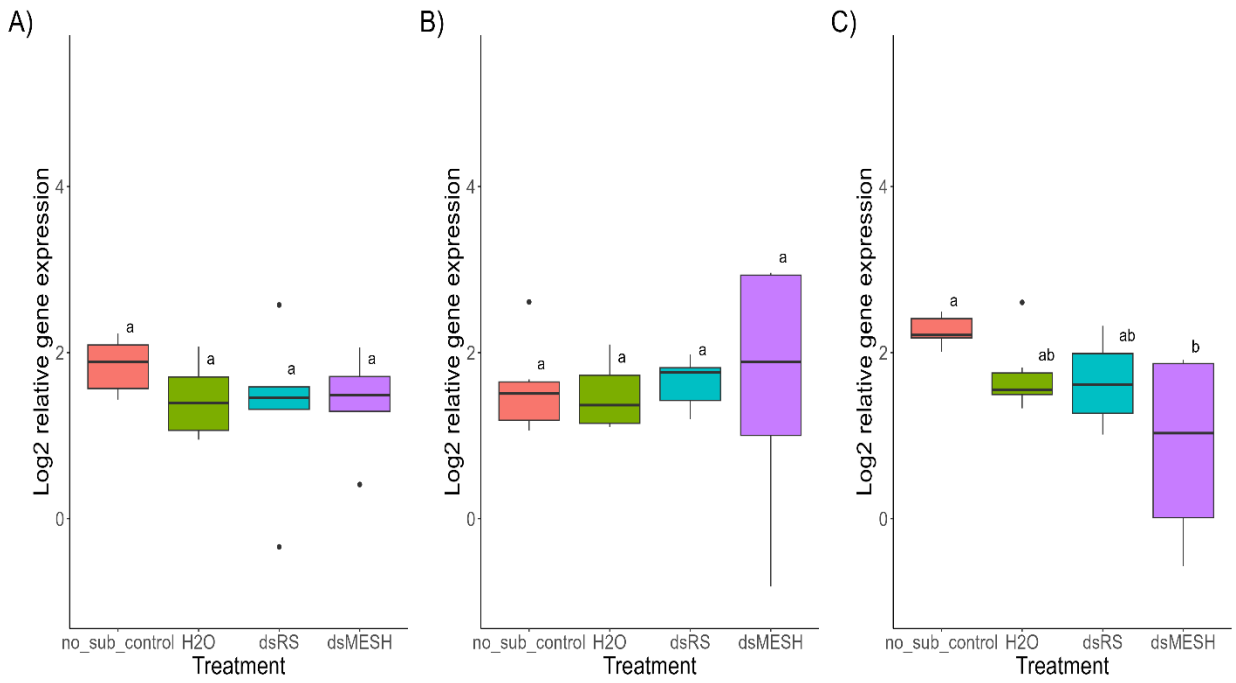

Figure 3.11 Comparison of the mean $\log_2$ relative *mesh* gene expression between the dsMESH and control groups: no-submergence control (no_sub_control), water (H2O), and 2.5µg/µl double-stranded random stem (dsRS; does not match any transcript in the mountain pine beetle transcriptome). A) At 24h (n=6), a one-way ANOVA analysis reported no significant differences between any of the groups ($p = 0.502$, $F = 0.813$, $df = 3$). B) At 48h (no_sub_control & H2O n=6; dsRS & dsMESH n=5), a Kruskal-Wallis test calculated no statistically significant differences between any of the group means ($p = 0.8881$, $\chi^2 = 0.6364$, $df = 3$). C) At 72h (n=6; dsRS n=4), a Kruskal-Wallis test followed by a Dunn's test indicated that the mean *mesh* expression was significantly lower in the dsMESH group (n=5) than the no-submergence control group ($p = 0.024$).

At 72h post-submergence, a Kruskal-Wallis test indicated that the mean relative log2 *mesh* expression in at least one group is different from the others ($p = 0.03193$, $\chi^2 = 8.8095$, $df = 3$) (Figure 3.11C). The post-hoc Dunn's test further identified that the *mesh* expression in dsMESH is significantly lower than the no-submergence control ($p = 0.024$). However, it is not significantly different than the other two controls: water ($p = 1.00$) or dsRS ($p = 1.00$).

Comparing the mean $\log_2$ relative *mesh* expression in each treatment or control group at the three different time points using a one-way ANOVA showed a significant difference in at least one time point in the no submergence control ($p = 0.0276$, $F = 4.603$, $df = 2$). A Tukey's

post hoc test revealed a significant difference in the mean $\log_2$ relative *mesh* expression in the no-submergence control group at 48h compared to the 72h ($p = 0.0225$). In the other treatment and control groups, no statistically significant differences over time were reported: water ($p = 0.482$, $F = 0.767$, $df = 2$), dsRS ($p = 0.0729$, $F = 0.324$, $df = 2$), dsMESH ($p = 0.289$, $F = 1.366$, $df = 2$).

3.4 Discussion

3.4.1 Submergence is the optimal method for orally delivering liquid to adult MPB

Feeding emerged adult MPB a 1% sucrose solution increased their survival compared to adult MPB that were not fed. After emergence, MPB are not fed before assays, thus the ingestion of sugar may have boosted survival over the testing period, further supported by metabolic responses this delivery method (see Chapter 5, section 5.4.5.1). In natural settings, recently emerged MPB have increased lipid stores for flight that can be broken down for energy when food sources are not available (Evenden et al. 2014; Jiang et al. 2019a; Chaudhari et al. 2025).

The method of ingestion affects uptake success in adult MPB. Two methods have been used for laboratory-based feeding in *Dendroctonus* spp.: vertical droplet suspension for southern pine beetles (*D. frontalis*) and submergence for MPB (Kyre and Rieske 2022). After testing each method on emerged adult MPB, the submergence method had the most uptake, 60%, in beetles (see Appendix, Table S3). The uptake efficiency is not impacted by the presence or absence of moistened filter paper, despite observations that MPB chew and potentially ingest the paper. In both "starved" and "fed" beetles, most or all of the liquid was gone from the tubes after 12h. Tubes with remaining liquid were observed in both "fed" and "starved" scenarios. These trials indicate that not every beetle submerged in the target liquid may absorb or ingest the liquid. Uptake of the target liquid can be monitored by the observation of whether any liquid is left in the tube after 12h of MPB submergence. However, this is not always accurate as MPB that did not have blue dye in their guts came from tubes with no liquid remaining. This makes considering the uptake success when interpreting results complicated. Without more information on this variation, sample removal is not reasonable. Therefore, the uptake success is likely

impacting the RNAi initiation results. Investigations into specific uptake success are recommended with future use of this delivery method.

3.4.2 Successful RNAi initiation in four of six gene targets

For six days after exposure to target dsRNA, adult MPB mortality was lower than expected. In a previous study, mortality in adult MPB exposed to target dsRNA showed 50% mortality after 4 days (Kyre et al. 2020). Kyre et al. (2020) also reported that adult MPB mortality remained low in the negative control group. In our study, MPB mortality in the negative control (dsRS) group was similar to the treatment groups (Fig 3.2). A study delivering dsIAP, dsHSP, and dsSHI orally to *Ips calligraphus* showed similar mortality with a negative control, dsGFP, causing more than 50% mortality by day six (Wallace and Rieske 2023). However, dsIAP and dsSHI showed higher mortality than the control and dsHSP, indicating the lethality of those two targets (Wallace and Rieske 2023). In this assay, we were unsuccessful in identifying target genes as the absence of rapid mortality in the MPB exposed to the six individual targets and similar mortality in the negative control suggests that these targets were not lethal to adult MPB.

An RNAi response in treated MPB led to a small, but statistically significant decrease in *iap* expression in the dsIAP group compared to the dsRS group (Figure 3.3A). The difference between the dsIAP and control group is similar to the changes reported in previous MPB RNAi studies as well as in *Ips calligraphus* (Kyre et al. 2020; Wallace and Rieske 2023). Interestingly, Kyre et al. (2020) reported MPB mortality caused by the gene expression change, which was not observed in our study (Figure 3.2). A BLASTn search (NCBI 2024) resulted in a 99.71% match between the dsRNA sequence introduced to the adult MPB and all five *iap* gene transcript variants. These sequence matches exceed the minimum 16-25 exact nucleotide match

requirement for RNAi initiation (Kyre and Rieske 2022; Hollowell et al. 2022). Therefore, the absence of a lethal effect from an RNAi-mediated decrease in *iap* gene expression can likely be attributed to an impartial knockdown.

The increase in target gene expression 24h after adult MPB were exposed to dsVHA19, dsMESH, and dsSSK when compared to the dsRS control was unexpected, but not unprecedented. An RNAi study on *Ips calligraphus* reported increased *shibire* gene expression 72h post-dsSHI exposure and decreased *iap* expression after dsIAP exposure, similar to our study (Wallace and Rieske 2023). These two genes have different essential functions in the cell, as *iap* is a stress response gene and *shibire* is involved in communication through the central nervous system in insects (Kyre and Rieske 2022). The three target genes showing increased relative gene expression in our study, *mesh*, *ssk*, and *vha19* are also not involved in stress response but rather in smooth septate junction structure of the endodermally-derived epithelia and molecule transport in insects, respectively (Baum et al. 2007; Izumi et al. 2016; Hu et al. 2019). Contrary to our study, successful reduction in a *ssk* homolog's expression and associated beetle mortality was achieved 48h after western corn rootworm (*Diabrotica virgifera virgifera*) were exposed to dsRNA (Hu et al. 2019). Additionally, RNAi-mediated *vha19* suppression was achieved in *Haemonchus contortus* larvae, though this study reported an increase in other gene expression after dsRNA delivery (Zawadzki et al. 2012). The increase was reported in the two genes *mitr-1* and *noah-1*, with roles in the mitochondria (energy metabolism and respiration) and an extracellular matrix component homolog, respectively (Zawadzki et al. 2012). Similarly, another study investigating a different vATPase in *Adalia bipunctata* and *Coccinella septempunctata* reported increased target gene expression after exposure to dsRNA and the expression levels varied among different time points (Haller et al. 2019).

Unexpectedly, the remaining two genes tested in our study, *hsp* and *tsp2a*, did not exhibit any significant gene expression differences from the controls. In a different study testing RNAi via oral delivery of dsRNA to MPB, *hsp* caused mortality and significant gene suppression (Kyre et al. 2020). Feeding MPB-specific dsHSP to a congeneric species, the southern pine beetle (*Dendroctonus frontalis*, SPB), also resulted in *hsp* suppression (Kyre and Rieske 2022). The opposite, feeding SPB-specific dsHSP to MPB, also resulted in a reduction in *hsp* expression (Kyre and Rieske 2022). Successful *hsp* suppression was reported in SPB, and when different populations were tested, two of three populations showed significant reductions in *hsp* expression with one population showing no difference in *hsp* expression compared to the control (Kyre et al. 2019, 2024). These different responses among different populations suggests that the lack of *hsp* suppression in our study might be due to population sensitivity. In another forest pest, *Ips calligraphus*, there was also no difference in *hsp* expression between the dsHSP and control groups, similar to our study's findings (Wallace and Rieske 2023). As this study is the first to test *tsp2a* as a target for MPB mortality via RNAi, there are no direct studies to compare our results to. However, *tsp2a* has been suppressed in *Drosophila* using RNAi and, as it forms a complex with *mesh* and *ssk*, *tsp2a* was predicted to be a lethal target in MPB (Izumi et al. 2016; Xu et al. 2019). With no gene expression response, this could be another population-sensitive target, or confounding factors could be responsible, like inefficient dsRNA uptake (see section 3.3.2/3.4.1).

3.4.3 Absence of an RNAi response when submerging adult female mountain pine beetles in hpRNA-expressing yeast solutions

The mortality in all groups, including the three control groups of water (H2O), heat-inactivated, hpRS-expressing yeast (0.1_hpRS) and *in vitro* dsRS (dsRS) was high. Mortality

started between day one to day three in all groups which is earlier than expected for both the negative controls and for an RNAi response in MPB or SPB (Kyre et al. 2019, 2020). A similar mortality trend was reported in *Ips calligraphus*, where the beetle mortality in the controls was also high early on in the assay and successful RNAi initiated gene suppression was still reported (Wallace and Rieske 2023). While similar, the mortality from our study shows more mortality earlier on in the bioassay than the *Ips calligraphus* bioassay. Comparable mortality in all groups indicates that the dsRNA and hpRNA was not lethal to adult female MPB. The observed mortality was likely caused by other confounding variables.

One possible variable impacting the results is the female adult MPB condition. First, larvae were kept at cooler temperatures in the spring/early summer to delay development and emergence, essentially manifesting quiescence (Bentz and Hansen 2018). Development temperature is an important variable when rearing insects, and can impact insect performance (Huho et al. 2007; Sørensen et al. 2012). Beetles acclimatized to cooler temperatures tend to have decreased performance at higher temperatures (Sørensen et al. 2012). During the cooling period, MPB were stored at approximately 6°C and were allowed to develop at ambient summer temperatures after, then kept at a mid-range temperature of 23°C during the bioassay. Thus, it is not expected that the cooling period should have a large physiological impact on emerged MPB as this manipulated a naturally occurring dormancy. Second, there is a negative correlation between the time since emergence from the bolts and MPB flight fitness (Evenden et al. 2014). While these females recently emerged, they did emerge from the bolts later in the season, and this may also negatively affect the fitness, and therefore the survival of MPB during the assay. Ultimately, the cause of mortality of MPB in all the groups in the bioassay is likely due to poor beetle condition.

No significant differences in gene expression between the treatment and control groups corroborates the absence of an RNAi response predicted from the mortality trend. The group treated with *in vitro* dsIAP (dsIAP) looks to have a lower mean relative $\log_2$ *iap* expression than the other groups, but this is not significant. Unexpectedly, the group treated with hpIAP-expressing yeast (0.1mg/ml hpIAP, 0.1_hpIAP) appears to have a higher mean relative $\log_2$ *iap* expression than the other groups, but once again this is not a significant difference. No difference between the relative *iap* expression between the treatment and control groups has also been reported in other studies on SPB (Kyre et al. 2019). Success of *iap* suppression in SPB varies by population as well, with no difference in *iap* expression between the treated and control groups in the New York, USA population and statistically significant differences in *iap* expression between treatment and control groups in the other two populations from Mexico (Kyre et al. 2024). Suppression of *iap* appears to be species-specific, as dsRNA designed for MPB is ineffective at initiating successful RNAi in SPB, and the same was reported for SPB-designed dsRNA in MPB (Kyre and Rieske 2022). Possible explanations for the lack of *iap* suppression in our study could once again be linked to the use of a northern MPB population compared to southern populations. This is further supported by the presence of an *iap* allele with efficient up-regulation in northern populations, which may affect the *iap* knockdown success (Horianopoulos et al. 2018).

The relative $\log_2$ *vha19* expression looks very close among the treatment and control groups. In a previous submergence bioassay in this study, *vha19* expression in the *in vitro* dsVHA19 group was significantly higher than in the control *in vitro* dsRS group (see section 3.3.4.1/3.4.2). However, *vha19* has been successfully suppressed using RNAi in two worm species: *Haemonchus contortus* larvae and *Caenorhabditis elegans* (Zawadzki et al. 2012).

Targeting *vATPase A* in *Adalia bipunctata* and *Coccinella septempunctata* had variable results, with gene suppression and in one instance an increase in gene expression (Haller et al. 2019). Further investigations into *vha19* as a lethal target for adult MPB are required to reach an improved conclusion.

3.4.4 No RNAi initiation when submerging adult male mountain pine beetles in hpRNA-expressing yeast solutions

As the first trial using heat-inactivated, hpRNA-expressing yeast on adult male MPB, this experiment focused on two gene targets: *iap* and *mesh*. The average adult male MPB mortality from two repetitions was higher than expected in the two control groups: water (H2O) and 0.1mg/ml of hpRS-expressing yeast (hpRS_0.1). Both hpIAP treatment groups did show more mortality than the hpRS group by day six, but two out of the three dsMESH/hpMESH groups showed less mortality than both control groups by day six. Beetles in the 0.1mg/ml hpMESH-expressing yeast treatment had more mortality than the hpRS control. Considering the mortality data, the hpRNA expressed and delivered to the adult male MPB by heat-inactivated yeast did not cause targeted mortality. Additionally, the MPB mortality in the water control group suggests that the observed mortality could be due to confounding factors, such as cold storage and late emergence, similar to observations in the female submergence bioassays (see section 3.4.5).

Gene expression analysis further confirmed that all treatments were unsuccessful at achieving an RNAi response. The mean relative $\log_2$ expression in the hpRNA-expressing yeast groups for each target are similar to one another and to the controls. Interestingly, the mean relative $\log_2$ expression of the *mesh* gene in the dsMESH group is higher than the mean relative $\log_2$ expression in the hpMESH-expressing yeast and control groups (Fig 3.5). While not statistically significant, this pattern matches what was reported in the previous submergence

assay where exposure to dsMESH via submergence led to a statistically significant increase in *mesh* expression in adult MPB (section 3.3.4.1, see Fig 3.3E). In the first submergence bioassay, the gene expression was quantified at 24h post-submergence, whereas in the male MPB bioassay the gene expression was quantified after 72h. Therefore, this suggests that the pattern of higher mean *mesh* expression is consistent over a 72h period post-submergence. Increases in gene expression has been observed after 72h in *Ips calligraphus* (Wallace and Rieske 2023). The expectation that increased *mesh* expression is stable over time is plausible, but fluctuating gene expression levels over the duration of a bioassay have been reported in *Adalia bipunctata* and *Coccinella septempunctata* (Haller et al. 2019). With the non-statistically significant increase in *mesh* gene expression in the male MPB bioassay, it is also possible that *mesh* expression decreases over time after the initial increase at 24h post-submergence. Without quantifying the gene expression in the same bioassay over consecutive timepoints, the actual pattern of gene expression cannot be determined.

3.4.5 Successful RNAi initiation in female adult mountain pine beetles submerged in *in vitro* dsRNA with gene expression quantified at consecutive time points

The mortality of adult female MPB remained low for the first three days in all control groups and in the dsIAP and dsVHA19 groups, more akin to expectations based on mortality observations from other MPB studies (Kyre et al. 2020). Beetle mortality in the dsIAP group was similar to the no submergence control group, once again suggesting that the dsIAP treatment is not lethal to adult female MPB. With higher survival percentages in two out of three controls, this bioassay better displays the effects of target dsRNA than previous bioassays that appeared to have confounding variables impacting MPB mortality (section 3.3.5/3.3.6). With rapid mortality

evident in two treatment groups, it appears that the dsMESH and dsVHA19 had a lethal effect on adult female MPB.

3.4.5.1 RNAi-mediated *iap* suppression

Our study is the first to incorporate multiple negative controls with MPB submergence, and the results show the importance as it can influence the interpretation. For example, *iap* expression in the dsIAP group was significantly different than in the dsRS group after 24h in an earlier bioassay (see section 3.3.4.1, Figure 3.3) but is only significant from the no submergence control and water groups, not dsRS, after 24h in this assay. This study is the first to quantify *iap* gene expression in treatment groups over three time points. The mean $\log_2$ relative *iap* expression in the dsIAP group does not significantly change over time and neither do any of the controls, contradicting an earlier assumption that *iap* expression was changing over 24h (See Chapter 2, section 2.4.2.2). Though there is a significant decrease in *iap* expression, it is not a complete knockdown and is not associated with MPB mortality. Additionally, the variability in gene expression among the four bioassays in our project suggests that *iap* may not be a reliable lethal target for RNAi in adult female MPB.

3.4.5.2 RNAi-mediated vha19 suppression

The pattern of relative $\log_2$ *vha19* expression in the dsVHA19 group is not significantly different over the 72h quantification period, but when compared to the controls the significance does change over time. Interestingly, the *vha19* expression in each of the control groups over time is also not different. The lower *vha19* expression in the dsVHA19 treatment compared to the no submergence control at 24h, no difference from any controls at 48h, and lower *vha19* expression in the dsVHA19 treatment compared to the no submergence control and dsRS at 72h indicates that the differences are significant but slight. It does appear that the slight *vha19*

suppression changes are contributing to MPB mortality, unlike what was observed in dsIAP. Conversely, in an earlier bioassay, dsVHA19 treatment led to increased *vha19* expression in MPB at 24h (section 3.3.4). No significant difference was found between female MPB in the dsVHA19 groups and control groups in yet another previous submergence assay (section 3.3.5), adding to the variation in MPB gene expression response to dsVHA19.

Other studies investigating a different vATPase gene also showed varying levels at different time points. In *Adalia bipunctata* the expression of *vATPase A* was stable over time when measured every 48h, and in *Coccinella septempunctata*, *vATPase A* was stable for low dsRNA doses (0.25µg/µl) and more variable with higher (25µg/µl) doses (Haller et al. 2019). In some cases, with a high or low dose, the variation among time within the same treatment group was not significant, like in our study. However, in other target and dose combinations, the variation within a treatment group over time was significant (Haller et al. 2019). In our study, only one dosage with no off-target species testing was performed, which are factors that likely impacted the *vha19* expression variation over time. In MPB, dsVHA19 appears to be a possible lethal target, but still shows variation among bioassays and even within the bioassays at different time points.

3.4.5.3 Delayed response in adult female MPB to dsMESH delivery

The absence of a difference in the mean relative $\log_2$ *mesh* expression at 24h and 48h post dsMESH exposure was unexpected when compared to the rapid mortality of MPB treated with dsMESH. By 72h, the mean $\log_2$ relative *mesh* expression was lower than the no-submergence control. This gene expression difference coincides with the first MPB mortalities in the dsMESH group; however, gene suppression is expected to have been detected before. This is based on Kyre et al. (2020) observing gene suppression in adult MPB 24h post-dsRNA exposure when

mortality began days later, though *mesh* was not one of the tested targets. No response in MPB *mesh* expression at 24h contradicts the findings of a previous bioassay in this study that reported increased *mesh* expression after 24h (section 3.3.4.1). However, in a male MPB assay, no *mesh* expression changes were reported after 72h (section 3.3.6).

Our study is the first to test dsMESH on adult MPB, but *mesh* has been targeted in other studies investigating Coleopteran insect larvae (Hu et al. 2016; Petek et al. 2020). In *Diabrotica virgifera virgifera*, reducing mesh expression via ingestion of *dvssj2* (*mesh* homolog) in larvae caused mortality (Hu et al. 2016). Similarly, a study testing *mesh* as a target for *Leptinotarsa decemlineata* reported increased larvae growth impairments and mortality after dsMESH ingestion in both laboratory and field trials (Petek et al. 2020). These findings support our study with observed adult MPB mortality when treated with dsMESH. However, Petek et al. (2020) did not report *mesh* expression quantification, there is no way to compare the trend observed in the time series. While there appears to be a correlation between adult female MPB mortality and reduced *mesh* expression, the results are variable like in the other two targets in this bioassay.

3.4.6 dsRNA uptake success

The findings from this study show variable results in all gene targets that were tested in more than one bioassay and over time in the last bioassay. It also showed MPB mortality when treated with dsMESH but *mesh* suppression was only observed starting at 72h post-submergence. One explanation for the variable results is that the quantified gene expression changes observed are due to chance. This is plausible in the *vha19* target as repeated testing showed variation with no discernable pattern, and gene expression was found to increase in one assay and slightly decrease in another. However, another explanation for the variable gene expression, and even mortality, is the dsRNA or hpRNA uptake efficiency by MPB when submerged. In early trials

using a coloured sucrose solution, only 60% of MPB had confirmed uptake. This means that gene expression can be skewed as most beetles are still alive when frozen, and a subset of these may have not successfully ingested the dsRNA. Additionally, when observing mortality, treatments with survival that is higher than expected might be due to some MPB not ingesting the treatment. Future studies should compare gene expression in the deceased MPB to the alive ones to see if this theory holds merit.

3.5 Conclusion

This study tested two previously successful targets and four novel targets on emerged adult mountain pine beetles (*Dendroctonus ponderosae*, MPB) by submerging the beetle in liquid dsRNA. This study is also the first to test heat-inactivated, hpRNA-expressing yeast as a delivery method for dsRNA to MPB. Four out of six targets tested on adult MPB showed significant gene expression differences. The first, dsIAP, caused decreased gene expression in two of the four bioassays it was tested in. The next three, dsMESH, dsSSK, and dsVHA19 showed up-regulation in adult MPB in an initial submergence assay. In two subsequent assays for each target, dsMESH only triggered any gene suppression in one and dsVHA19 only decreased gene expression in one assay. The yeast-expressed hpMESH and hpVHA19 did not elicit and RNAi response when tested in assays alongside dsMESH and dsVHA19. The remaining two targets: dsHSP and dsTSP2A did not cause gene expression changes. As a first for MPB responses to RNAi, the last bioassay quantified gene expression patterns over time in adult females. With no significant changes in gene expression within treatment groups over time, target gene knockdown appears stable over three days. The submergence method elicits an RNAi response, with success impacted by beetle condition and uptake efficiency.

Chapter 4 - Investigating the effectiveness of delivering heat-inactivated, hpRNA-expressing yeast through phloem to adult mountain pine beetles.

4.1 Introduction

Laboratory-based studies providing support for RNAi efficacy in insects have mainly used microinjection or forced oral ingestion (Turner et al. 2006; Keeling et al. 2013; Zhao et al. 2015; Zhang et al. 2016; Wang et al. 2016; Rodrigues et al. 2018; Kyre et al. 2019; Haller et al. 2019; Kyre et al. 2020; Liu and Chen 2022; Wallace and Rieske 2023). Microinjection and some oral ingestion methods, like submergence, are not applicable for field-based tests or, ultimately, use in *in-situ* applications due to the method protocols and treatment of individual beetles (see Chapter 2, section 2.3.2 and Chapter 3, sections 3.2.2/3.2.3). Switching from these strictly laboratory-based methods to methods that can be applied *in situ* is important for the next step of testing RNAi as a pest management tool. However, the dsRNA delivery method can affect RNAi initiation, and further testing would be required in this intermediate step before *in situ* application (Cooper et al. 2019). Studies have started to investigate the RNAi response when dsRNA is delivered by associating it with a natural or artificial diet (Hu et al. 2016; Ghosh et al. 2018; Pampolini and Rieske 2023). Additionally, the response of insects fed microbe-expressed dsRNA associated with a diet have been studied (Zhu et al. 2011; Murphy et al. 2016; Leelesh and Rieske 2020).

Association of dsRNA to either a natural or artificial diet has been shown to successfully initiate an RNAi response in insects. Green beans were used to entice ingestion of dsRNA by the brown marmorated stink bug (*Halyomorpha halys*) nymphs as 0.017µg/µL *vitellogenin* or 0.067µg/µL of *juvenile hormone acid O-methyltransferase* dsRNA was moved through the beans using capillary action (Ghosh et al. 2018). After feeding on green beans for five days, with diet

replacement at day three, a significant decrease in the expression of both targeted genes was reported (Ghosh et al. 2018). Additionally, dsRNA delivered to Asian citrus psyllid (*Diaphorina citri*) and glassy-winged sharpshooter leafhopper (*Homalodisca vitripennis*) via foliar sprays, translocation into tissues from root soaks, tissue absorption, or trunk injections of cultivated *Citrus sinensis* x *Poncirus trifoliata* crosses led to RNAi-mediated gene suppression (Ghosh et al. 2018).

Associating dsRNA with diets has also been effective for RNAi initiation in Coleopteran insects. Successful RNAi was reported after using a foliar spray on the natural diet of the emerald ash borer (*Agrilus planipennis*) (Pampolini and Rieske 2023). Green ash seedling stems and leaves were sprayed with 500µg of dsHSP, which caused decreased *hsp* expression when ingested by EAB larvae. Decreased gallery area and tissue consumption were also reported (Pampolini and Rieske 2023). An artificial diet was used to entice ingestion of dsRNA (1.5µg/µl) targeting *Drosophila ssk* and *mesh* orthologs in western corn rootworm (*Diabrotica virgifera virgifera*) first instar larvae (Hu et al. 2016). As a result, significant gene suppression in the *ssk* ortholog was reported, while the gene expression analysis results for the *mesh* ortholog were not reported. In a second study using an artificial diet and dsRNA (167ng/µl) targeting the *ssk* homolog feed to western corn rootworm (*Diabrotica virgifera virgifera*) first instar larvae, successful RNAi response was achieved, as determined by decreased mRNA and protein levels associated with increased mortality compared to the control treatment (Hu et al. 2019). Another study reported vacuolar ATPase (*vATPase*) suppression and increased mortality in western corn rootworm adults fed 0.5µg or 1µg of dsRNA associated with an artificial diet (Rangasamy and Siegfried 2012). While the use of a natural or artificial diet in laboratory-based settings appears

to be effective, the instability of *in vitro* synthesized dsRNA in *in situ* applications remains a concerning variable.

Many RNAi studies use microbes to produce and deliver the dsRNA, increasing dsRNA stability and reducing production costs (Duman-Scheel 2019; Hashiro and Yasueda 2022). Some vehicles that have been successful at RNAi initiation in insects include bacteria and yeast. Transformation of a vector in *Escherichia coli* led to successful production of dsRNA and downstream RNAi response when fed to neonate emerald ash borer larvae (Leelesh and Rieske 2020). An increase in larval mortality alongside decreased target gene expression was reported (Leelesh and Rieske 2020). Similar success was also reported in Colorado potato beetle larvae after eating potato leaves covered in heat-killed, dsRNA-expressing bacteria (Zhu et al. 2011). Gene expression of all five targets, β-Actin (*Actin*), Protein transport protein sec23 (*Sec23*), Vacuolar ATP synthase subunit E (*vATPaseE*), Vacuolar ATP synthase subunit B (*vATPaseB*), and Coatomer subunit beta (*COPβ*), was significantly decreased and increased mortality observed compared to the controls (Zhu et al. 2011). Additionally, the heat-killed, dsRNA-expressing bacteria was cheaper to produce and caused larger gene suppression than *in vitro* synthesized dsRNA for three, *Actin*, *Sec23*, and *COPβ*, of the five targets (Zhu et al. 2011). In contrast, for *Drosophila suzukii* larvae, *in vitro* synthesized dsRNA was more efficient than Brewer's yeast (*Saccharomyces cerevisiae*) expressing the dsRNA when ingested (Murphy et al. 2016). However, the dsRNA-expressing yeast still impacted *D. suzukii* fitness (Murphy et al. 2016). Two of these studies used *in vitro* or microbe-mediated dsRNA associated with a natural diet for delivery to targeted insects (Zhu et al. 2011; Pampolini and Rieske 2023).

Using dsRNA delivered to MPB via a natural or artificial diet has not been investigated. One obstacle is that emerged and flying adult MPB typically select large pine trees (Dhar et al.

2016a), which are not optimal for a laboratory study of this nature. However, MPB have been reared post-injection using simulated tree cortex conditions (Keeling et al. 2013). Keeling et al. (2013) reared teneral adult MPB to full adults using layers of phloem punches in the wells of a 24-well plate, with a small amount of baker's yeast and one beetle added to each well (Keeling et al. 2013). This method was coined "phloem homes" (C.I. Keeling and C.C Chiu, personal communication, 2022) and provided natural diet and living conditions for MPB ingestion and development.

In this study, the phloem homes method was adapted to deliver heat-inactivated, hpIAP-expressing yeast to MPB. The objective of the study was to test the efficacy of heat-inactivated, hpRNA-expressing yeast associated with lodgepole pine (*Pinus contorta*) phloem to initiate an RNAi response in adult MPB. Only one target, *iap*, was chosen based on the previous gene suppression observed in the submergence assays (Chapter 3, section 3.3.4 and 3.3.7.1). Three different yeast solutions and phloem associations were used: yeast solutions painted on to phloem (PHP), yeast solutions translocated through the phloem (PHT), and phloem soaked in yeast solutions (PHS). Within each association, different hpIAP-expressing yeast solution concentrations were tested, along with two negative controls for comparison. After initially testing each association type, no MPB mortality or *iap* expression changes were observed between the treatment and control groups in the gene expression analysis. A second attempt at painting higher concentrations of the hpIAP-expressing yeast solutions onto the phloem (PHP24) yielded MPB mortality, but was later obscured by fungus. With high MPB survival in the negative controls, and the start of a lethal response in MPB to targets, this method shows promise for dsRNA delivery to MPB.

4.2 Methods

4.2.1 Subject Collection

Subject collection was previously described (see Chapter 2, section 2.2.1). The majority of MPB used in the following assays were from Smithers, BC and all emerged naturally. In 2023, emerged MPB were collected from bolts from Smithers, B.C. and used in the assays described in 4.2.3.1, 4.2.3.3, and 4.2.3.4. Lastly, in 2024 bolts were received from Smithers, B.C., and emerged MPB were used in the section 4.2.3.2 assay. All emerged MPB were collected, and MPB exhibiting obvious physical ailments or lower health (missing appendages, extremely lethargic) were excluded from assays.

4.2.2 Ground Phloem Feeding Trails

4.2.2.1 Phloem Preparation

Phloem was removed from a young (approximately 10 years old) lodgepole pine (*Pinus contorta)* and the outer bark was removed, limiting the contamination from microorganisms present in the bark in the final ground phloem (Whitney and Spanier 1982). Phloem sheets were frozen at -25°C overnight and cut into small pieces the following morning. The pieces were further processed in a coffee grinder for approximately 30s. The resulting ground phloem was moved to a mortar with liquid nitrogen and a pestle was used to further grind the phloem. The ground phloem was stored in the freezer at -25°C.

4.2.2.2 Feeding Assay Trial

A feeding assay, developed by modifying the submergence assay (see Chapter 3, section 3.2.3/3.2.5) was tested using ground phloem to entice ingestion of dsRNA targets. Emerged adult MPB were assigned to one of three treatment groups (n=10): (1) submergence in a blue-dyed 1% sucrose solution, (2) ground phloem soaked with blue-dyed 1% sucrose solution, or (3) blue-

dyed 1% sucrose solution-soaked filter paper placed between the beetle and the phloem. Beetles in the first treatment group were submerged for 12h following the previously described protocol (see Chapter 3, section 3.2.3) and stored in the dark at 23°C.

In the second treatment group, ground phloem was compacted to the bottom of 0.2ml PCR tubes. The compacted phloem was then soaked with 6µl of the blue-dyed 1% sucrose solution and an adult MPB was added to the tube and kept confined with a piece of compressed Kimwipe. The tube was kept horizontal and beetles were left in the tubes for 12h in the dark at 23°C.

The third treatment group of soaked filter paper between the beetle and the phloem involved compacting phloem into the bottom of a 0.2ml PCR tube. A punch of filter paper was soaked with 6µl of dyed sucrose solution and placed atop the compacted phloem, ensuring it covered the top of the phloem and was snug to the tube all around with no phloem on top of or showing beside the filter paper. An adult MPB was then added to the tube and its movement constricted with a compressed piece of Kimwipe. The beetle was left in the tube, with the tube placed horizontally, for 12h in the dark at 23°C.

In all three treatment groups, beetles were removed after 12h, placed in petri dishes lined with moistened filter paper, and returned to the dark for two hours. After the two hours, beetles were dissected to observe the presence or absence of blue dye within the gut.

The third method, soaked filter paper on top of ground phloem, was repeated once more, with modification to the composition of the phloem and filter paper. Once the phloem was compressed to the bottom of a 0.2ml PCR tube, 4µl of water was added to the phloem for greater adherence to the punched-out piece of filter paper that was placed on top. Blue food colouring was then directly added to the filter paper and a light layer of phloem placed on top of the filter

paper to weigh it down and further entice the beetle to ingest the dyed filter paper. Beetles (n=10) were placed horizontally in the dark at 23°C and checked twice at 2h intervals. After 4h, beetles were dissected to observe the presence or absence of blue dye in the beetle midgut.

The soaked phloem method was also repeated once more, incorporating similar modifications as the filter feeding method. Ground phloem was compacted into a 0.2ml PCR tube and 2µl of blue food colouring dye added directly onto the phloem. Beetles (n=10) were placed in the horizontal tubes and left for 4h in the dark at 23°C. Beetles were removed from the tubes and placed in a petri dish with moistened filter paper. After 0.5h, beetles were dissected to observe the presence or absence of blue dye.

4.2.2.3 Feeding Assay – dsRNA targets IAP and HSP

Following the methods previously outlined (see section 3.2.2), ground phloem was compressed into the bottom of a PCR tube, with one side slightly higher than the other for an angled finish. The *in vitro* synthesized dsIAP, dsHSP, and control dsRS were normalized to 5µg and the calculated volume of dsRNA, a range of 2.5-2.7µl, was added to the top of the phloem. To the controls, 2.5µl of distilled water was added to the phloem. Forty emerged adult MPB collected four to nine days before the assay were distributed among treatments, and the two sub-groups within each treatment, to ensure that all collection dates were represented in each treatment. In each treatment group, ten beetles were assigned to the mortality observation group, and five beetles were assigned to the gene expression analysis group. Beetles were kept in the horizontally-placed tubes, in the dark at 23°C for 4h. After, beetles were removed and placed on moistened filter paper in petri dishes in the dark. Beetles in the gene expression groups were flash frozen 24h after initial exposure to the dsRNA targets, and stored at -80°C. Beetles in the mortality observation groups were checked daily, with the filter paper changed every 48h.

4.2.3 Phloem home feeding trials

To set up phloem homes, phloem was harvested from a lodgepole pine tree and the outer bark was removed from some pieces of phloem. Phloem was punched with a 12mm leather punch, and bottom layers had a small crescent removed to allow room for the beetle and avoid squishing the beetle with the top layer. Twelve wells of a 24-well plated were filled with two pieces, or layers, of phloem. This phloem still had the bark attached, and pieces were stacked so that the phloem faced the middle. The other 12 wells were filled with three pieces, or layers, of phloem, with two pieces having the outer bark removed. These two were placed in the well first, and topped with the third piece containing outer bark with the bark facing the top, or outside, of the stack (Figure 4.1).

Figure 4.1 Arrangement of lodgepole pine (*Pinus contorta*) phloem punches in a sterile 24-well plate creating "phloem homes" (C.I. Keeling and C.C Chiu, personal communication, 2022). The two-layered adaption is shown with the top layer removed and placed below the well plate.

To investigate MPB survival in the phloem homes, twelve adult MPB (six females and six males) were assigned to each phloem composition type and placed under the top layer of phloem in the assigned well. Adult MPB were sexed using auditory examination, where males

chirp when pressure is applied to the margins of the pronotum with thumb and forefinger (Rosenberger et al. 2016). For increased accuracy, all beetles that did not chirp were re-tested to catch formerly silent males (Rosenberger et al. 2016). A moistened Kimwipe was placed on top of the well plate under the lid. The plate was placed in the dark at 20°C and checked daily for 14 days, recording beetle mortality, wood quality, and beetle chewing progress. The Kimwipe was changed daily.

4.2.3.1 Phloem home – yeast solutions painted on phloem (PHP)

Freshly harvested phloem with the bark still attached was punched with a 15mm leather punch, to better fit the wells than in the trials (see section 4.2.2), making enough phloem discs for two per well in a 24-well plate per treatment. Using solutions consisting of heat-inactivated, hpRNA-expressing yeast and water, a total of five treatments were tested: water and 0.1mg/ml hpRS as controls, 0.01mg/ml hpIAP, 0.05mg/ml hpIAP, and 0.1mg/ml hpIAP. Each treatment was painted on to 48 pieces of phloem twice, for a total of two coats of assigned solution. Adult MPB were added on top of the first piece of phloem in the plates, with one beetle per well. Emerged adult MPB collected from five different dates within a week of the assay were evenly distributed among the five treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented. Within each treatment, 18 beetles (14 females, four males) were assigned to the mortality observation group, and six (three females, three males) were assigned to the gene expression analysis group. The top piece of phloem was gently placed on top of the first piece and the beetle, with the bark facing out. A dry Kimwipe was placed on top of the wells, under the plate lid. Plates were stored in a dark incubator at 21-23°C. Beetle mortality, wood quality, and chewing progress of beetles were checked daily for 15 days. The

beetles assigned to gene expression analysis were flash frozen in liquid nitrogen 72h after initial exposure to the treated phloem.

4.2.3.2 Phloem home – yeast solutions painted on phloem 2024 (PHP24)

The previously outlined procedure for painting yeast solutions onto phloem was followed, with a few alterations (refer to section 4.2.2.1). Concentration of the heat-inactivated, hpRNA-expressing yeast and MilliQ water solutions were increased for the control and treatment solutions: MilliQ water, hpRS (5mg/ml), hpIAP50 (0.5mg/ml), hpIAP100 (1mg/ml), and hpIAP500 (5mg/ml). Emerged adult MPB collected from three different dates within four days of the assay were evenly distributed among the five treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented. Male beetles were used within two days and females were used within five days. Beetles were sexed as previously described (see section 4.2.3.1) and a pair of beetles (a male and a female) were added to each well. All males were removed after 48h. Twelve females were assigned to the mortality observation group, six were assigned to the 72h post-exposure gene silencing group, and six were assigned to the 120h post-exposure gene silencing group. Plates were stored under a cardboard box (i.e. in the dark) at room temperature, fluctuating around 20°C. Beetle mortality, wood quality, and chewing progress of beetles were checked daily for 14 days. The beetles assigned to gene expression analysis groups were flash frozen in liquid nitrogen at the designated time (72h or 120h) after initial exposure to the treated phloem. The treated phloem was replaced three days into the assay with fresh treated phloem.

4.2.3.3 Phloem home – phloem soaked in yeast solutions (PHS)

A quick trial to determine the optimal soaking time of phloem punches in liquid was performed with water. Freshly harvested phloem was punched into 15mm diameter discs and

fully submerged in water. Punches were checked after 1, 4, 7, 24, and 48h of soaking. The thoroughness of the soaking into the middle of the disc, as well as the phloem integrity (firm or flaccid) was checked and recorded.

After the optimal soaking time for phloem integrity and thoroughness of soaking into the disc was determined to be 24h, four solutions of heat-inactivated, hpRNA-expressing yeast at different concentrations were made and enough phloem punches for one plate (48 plus extra for insurance) soaked in the solutions. Enough punches for one plate were soaked in water as a control. In total, five treatments were applied to the phloem for 24h: water, 0.1mg/ml hpRS, 0.01mg/ml hpIAP, 0.05mg/ml hpIAP, and 0.1mg/ml hpIAP.

After soaking for 24h, phloem punches were placed into the wells of the 24-well plates for each treatment. Phloem was briefly air dried to remove excess moisture before a beetle was added to each well. Emerged adult MPB collected from three different dates within a week of the assay were evenly distributed among the five treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented. Beetles were sexed as outlined previously (section 4.2.3.1) and within each treatment, 18 beetles (14 females, four males) were assigned to the mortality observation group and six (females) were assigned to the gene expression analysis group. Plates were stored in the same conditions as PHP assay (section 4.2.2.1). Beetle mortality, wood quality, and chewing progress of beetles were checked daily for 14 days. The beetles assigned to gene expression analysis were flash frozen in liquid nitrogen 72h after initial exposure to the treated phloem.

4.2.3.4 Translocated yeast solutions through the phloem

4.2.3.4.1 Translocation Trials – water and yeast

Phloem removed from a young lodgepole pine (*Pinus contorta*) tree was used in a modification of "phloem sandwiches". Phloem pieces were placed between two pieces of damp paper towel lining the inner sides of 6-inch x 6-inch glass plates. All components were put together and held in place with large binder clips. These "sandwiches" were placed upright in a Tupperware container with the bottom edge of the phloem submerged 1cm into the water dyed with blue food colouring (Figure 4.2A). A fan was used to move air over the sandwiches, inducing capillary uptake. The phloem was checked and blue dye progress up the strip measured after 4h and after soaking overnight.

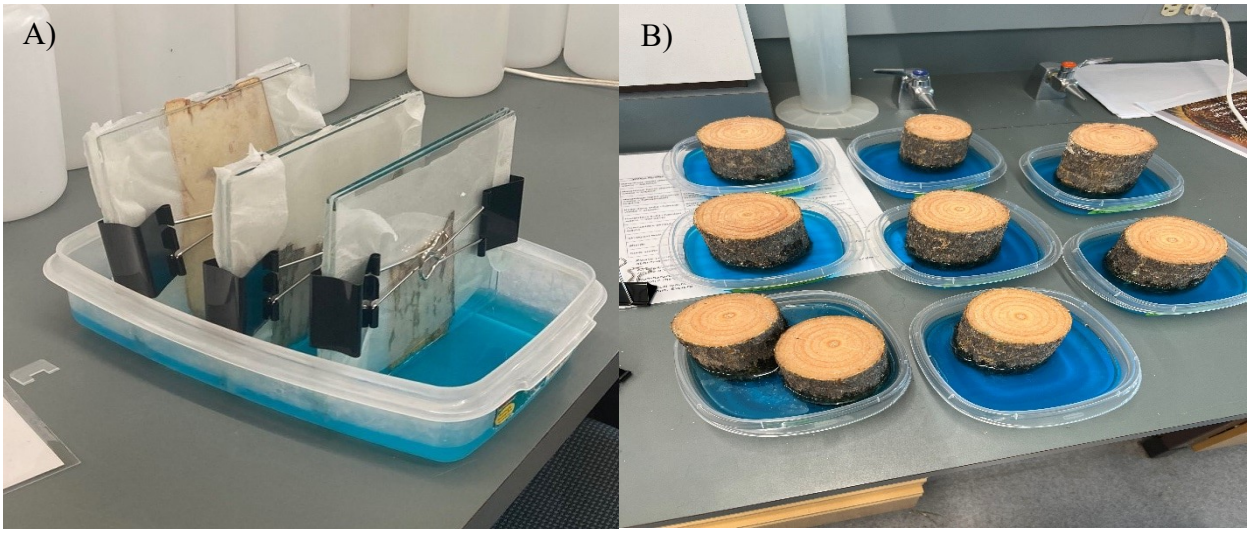

Figure 4.2 Set up for blue-dyed water translocation through lodgepole pine (*Pinus contorta*) phloem trials. A) "Phloem sandwiches" in blue dyed water. Pieces of phloem are placed between two moistened paper towels on the inside of glass squares and clipped together. B) Lodgepole pine rounds placed in blue-dyed water immediately after being cut.

A second method tested translocation through a young pine tree trunk cut into rounds and the freshly cut edge placed immediately into blue-dyed water to prevent sap sealing the phloem (Figure 4.2B). A fan was used to move air above the rounds, promoting capillary action. The

rounds were checked daily for three days for presence of dye at the top or along the bark on the side.

After trials with blue-dyed water, new rounds were used to test the translocation of heat-inactivated, hpRNA-expressing yeast solutions through the phloem. Rounds were placed in one of four different yeast solutions: 0.1mg/ml hpRS, 0.01mg/ml hpIAP, 0.05mg/ml hpIAP, and 0.1mg/ml hpIAP, with one or two rounds per treatment. The rounds were left in the solutions under a fan for four days, topping up the solutions daily.

Digital droplet PCR (ddPCR) was used to confirm the presence of hpRNA in the bottom, middle, and top portions of the rounds. Total RNA was extracted from the lodgepole pine (*Pinus contorta*) punches using the Qiagen RNeasy PowerPlant kit with on-column DNase digestion following manufacturer protocols (Qiagen, Toronto, ON). There were some exceptions to the protocol. Lysis buffer was prepared and aliquoted into bead tubes before adding the sample. Samples were homogenized using a bead beater for two minutes in one-minute intervals. The on-column DNase treatment was performed after the PM5 wash step (step 10) of the RNeasy PowerPlant kit protocol and digestion time was increased from 15 to 18 minutes. Extracted RNA was quantified with the Qubit 4 Fluorometer High Sensitivity Assay (Thermo Fisher Scientific Inc., 2015) and the integrity was checked using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). All samples were of acceptable quality for downstream applications. Next, cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories (Canada) Inc., Mississauga, ON), following the kit protocol. Input RNA was normalized to 300ng. Each section (top, middle, and bottom) included a no-reverse transcriptase control (no-RT), where a supermix without RT replaced the normal supermix, to ensure there was no DNA contamination in this step. The cDNA was used

for ddPCR with the designed dsRS assay (see Chapter 2, section 2.2.8.2, Table 2.2). Positive and negative controls were included using gBlock and water, respectively.

4.2.3.4.2 Phloem home – yeast solutions translocated through the phloem (PHT)

The treated phloem with the bark still attached was carefully removed and punched with a 15mm leather punch, making enough phloem discs for two per well in a 24-well plate per treatment. Punches taken from the bottom of the rounds were the bottom layer in each well, and the top pieces in the wells were taken from the top of the round. Sample phloem punches from the bottom, middle, and top of the rounds were frozen for later analysis to confirm the success of translocation of the yeast through the phloem.

Using solutions consisting of heat-inactivated, hpRNA-expressing yeast and water, a total of five treatments were tested: water and 0.1mg/ml hpRS as controls, 0.01mg/ml hpIAP, 0.05mg/ml hpIAP, and 0.1mg/ml hpIAP. Adult MPB were added on top of the first piece of phloem in the plates, with one beetle per well. Emerged adult MPB collected from eight different dates within ten days of the assay were evenly distributed among the five treatments, and the two sub-groups within each treatment, to ensure that each collection date was represented. Beetles were sexed as previously described (section 4.2.3.1) and in each treatment group, 18 beetles (water, 0.1mg/ml hpRS, and 0.01mg/ml hpIAP: 14 females, four males; 0.05mg/ml hpIAP and 0.1mg/ml hpIAP: 13 females, five males) were assigned to the mortality observation group and six (three females and three males) were assigned to the gene expression analysis group. Plates were prepared and stored in the same conditions as previously outlined (see sections 4.2.2.2/4.2.2.3*).* Beetles assigned to gene expression analysis were flash frozen in liquid nitrogen 72 hours after initial exposure to the treated phloem.

4.2.4 Gene expression analysis

The gene expression pipeline has been described in Chapter 2 (section 2.2.8) and summarized in Chapter 3 (section 3.2.9).

4.3 Results

4.3.1 Phloem Preparation and Feeding Trial Results

Trials conducted to study the uptake of target fluid show more MPB were ingesting the target liquid in the soaked phloem method compared to the other two methods (Table 4.1). Refinement of the method to soak phloem with the target liquid had an uptake of 90%, similar to prior results and showing the highest uptake percentage.

Table 4.1 A tally of the best uptake success among the different trials (determined by the presence or absence of blue dye in MPB adult (n = 10) guts) 12h after exposure to the blue-dyed 1% sucrose solution.

	Blue-dye present	Blue-dye absent
Submerged	5	5
Soaked phloem	9	1
Soaked filter on phloem	6	4

4.3.2 Mortality of MPB fed dsRNA-soaked ground phloem

Mortality of MPB fed 5µg of each target dsRNA, including the controls, was similar over the 11 days (Figure 4.3). For the first six days, mortality in all groups was less than 50%, but increased greatly after this.

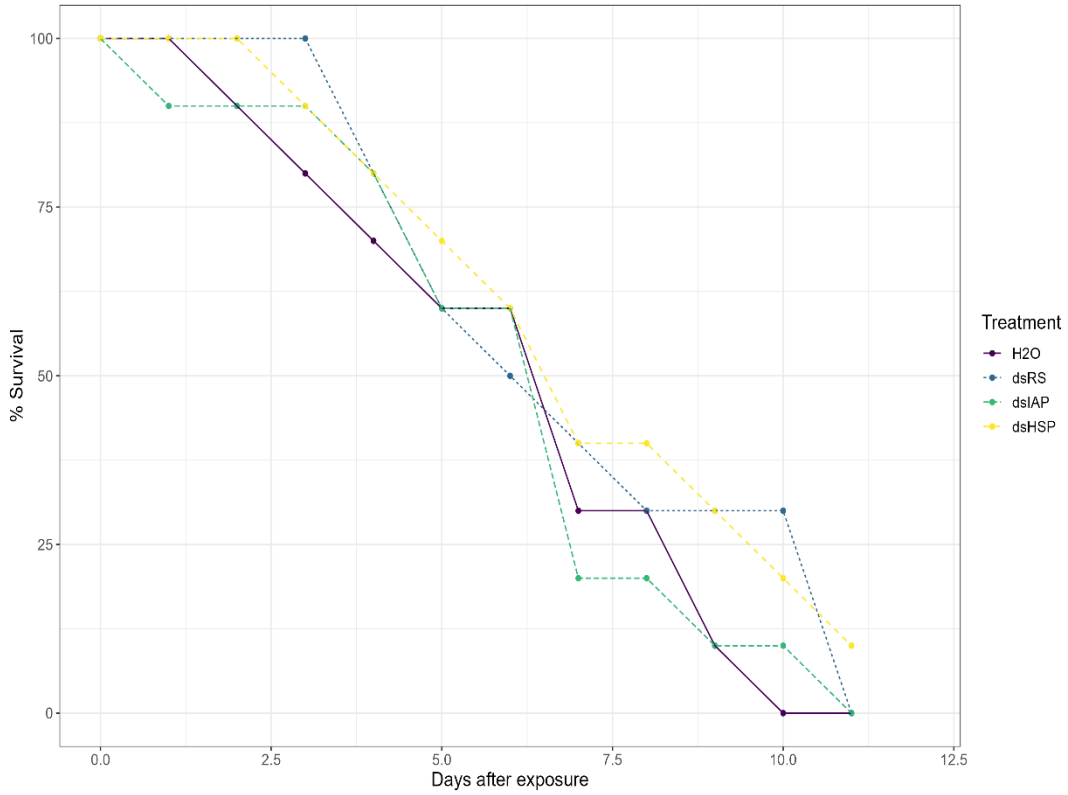

Figure 4.3 Percent survival over 11 days of adult MPB fed 5µg of target dsRNA for two gene targets (n=10): dsIAP and dsHSP. The survival of two negative controls: water (H2O) and a double-stranded random stem (dsRS; does not match any transcript in the mountain pine beetle transcriptome) was plotted alongside for comparison.

4.3.3 Translocation trials

Translocation of water and yeast solutions in tree rounds was a better method than translocation through phloem sandwiches. After four hours, blue dye was observed 2-6cm up the phloem pieces in the sandwiches, though the presence was inconsistent along the width of the piece. The translocation distance also appeared to differ among the different pieces. After 96h, it appeared that the translocation had moved entirely up the height of the phloem piece, though the progress still appeared inconsistent based on the blue dye (Figure 4.4).

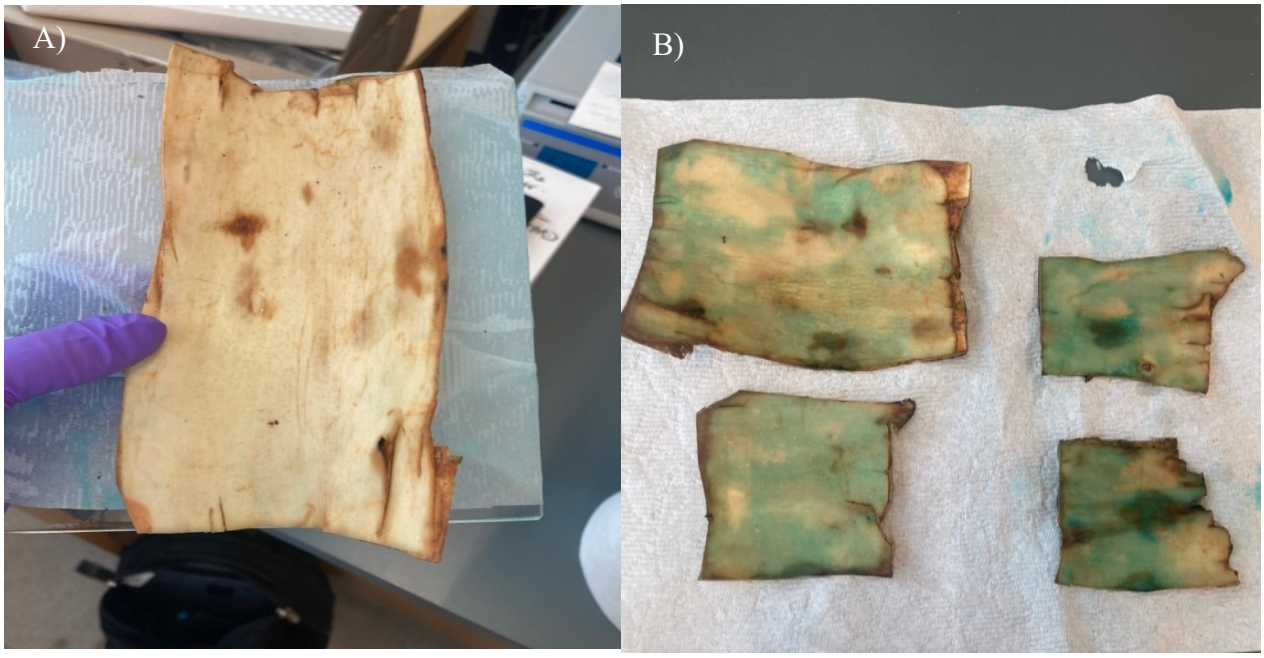

Figure 4.4 Progress of blue-dyed water translocated through lodgepole pine (*Pinus contorta*) phloem in "phloem sandwiches" after A) 4h and B) 96h.

Pine rounds showed more consistent translocation when placed in four different yeast solutions: 0.1mg/ml hpRS, 0.01mg/ml hpIAP, 0.05mg/ml hpIAP, and 0.1mg/ml hpIAP (Fig 4.5). Successful translocation in the rounds was determined by blue dye showing around the circumference on the tops of the rounds. Blue dye was present at the top of the rounds after 96h, and presence of hpRNA was detected with ddPCR in cut outs from the bottom, middle, and top of the phloem after translocation.

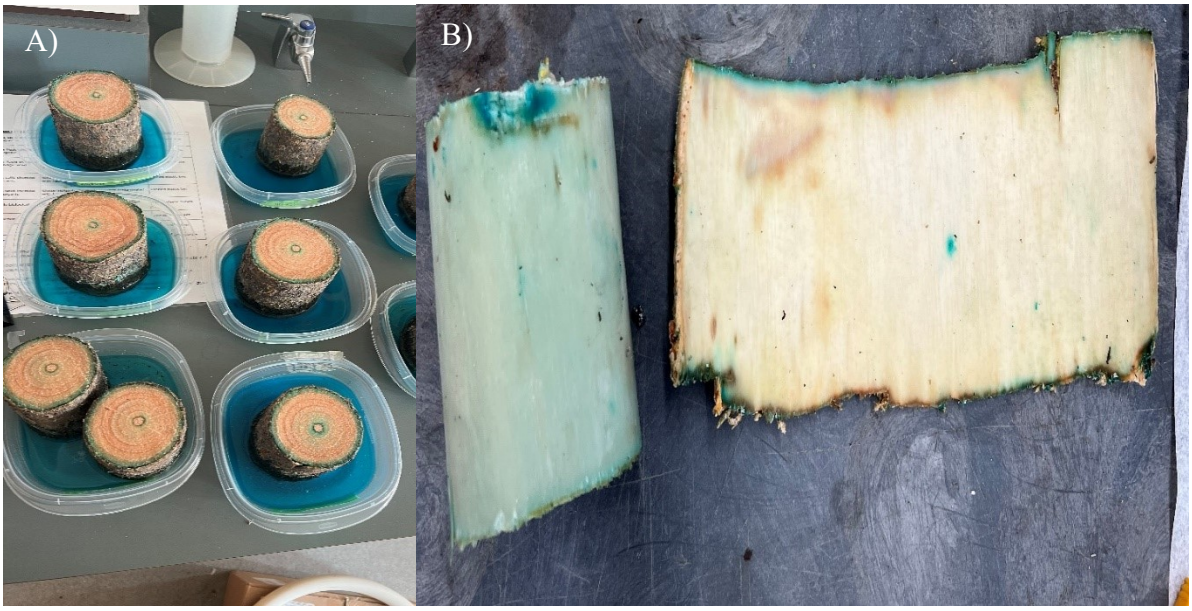

Figure 4.5 Translocation success of blue-dyed hpRNA-expressing yeast solutions through lodgepole pine (*Pinus contorta*) phloem in the cut trunk after 96h. A) Presence of blue dye around the circumference at the top B) Phloem separated from the rest of the tree round, showing the presence of blue dye at the top and bottom.

4.3.4 Phloem home trial

An initial trial was conducted to determine the MPB survival and phloem longevity in the phloem homes. When placed in the phloem homes, most females had burrowed into the phloem after two days. After about ten days, the wood quality declined, appearing slimy. However, no fungus was present on the MPB. Some beetles escaped from the designated well, indicating that the phloem punches should be larger.

Mortality of MPB in the three or two layered wells was similar with four and five deaths over 13 days, respectively. The two layered set up was used for further phloem homes as it appeared to promote burrowing, and therefore chewing, among female MPB (Figure 4.6).

Figure 4.6 Phloem home trial after 13 days. A) Wells marked with white had the phloem removed after MPB mortality. Lots of frass in wells indicates successful MPB chewing. B) Removed top layer shows MPB burrow in the bottom layer.

4.3.5 Phloem home – yeast solutions painted on phloem (PHP)

4.3.5.1 Repetition one: mortality and *iap* expression analysis

All targets and controls showed high survival rates over the 14-day duration of the assay (Figure 4.7). Mortality was highest, with approximately 23%, in the beetles treated with the lowest concentration of hpIAP-expressing yeast solution: 0.01mg/ml hpIAP (0.01_hpIAP). There was no MPB mortality in the control group 0.1mg/ml hpRS (0.1_hpRS), and approximately 6% mortality in the water (H2O) group.

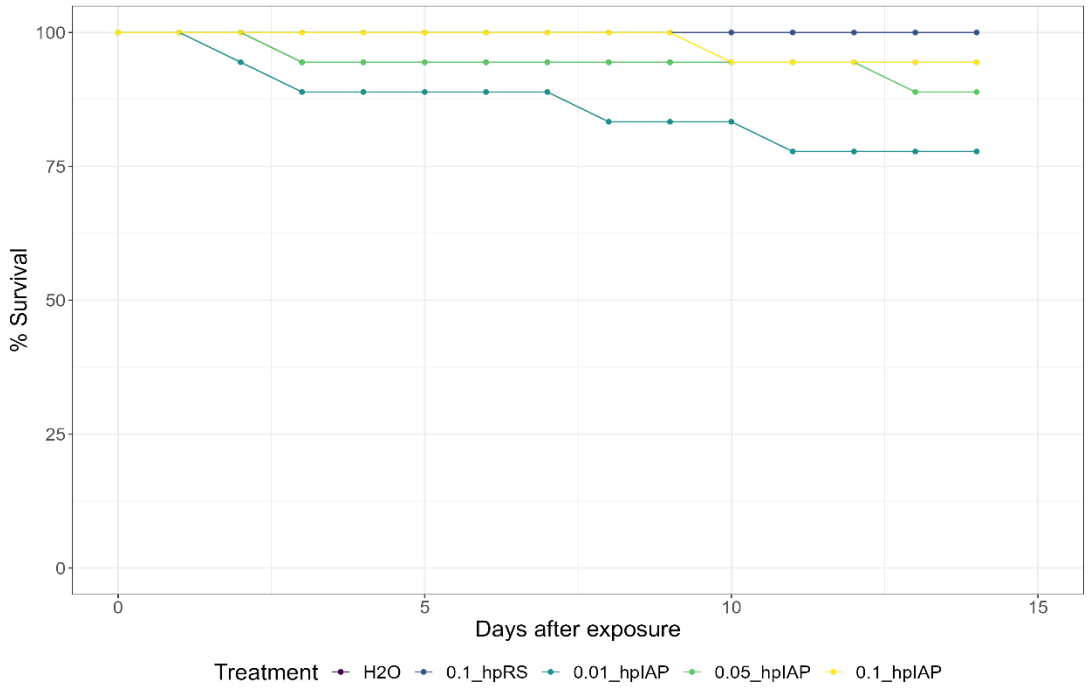

Figure 4.7 Percent survival of adult MPB fed lodgepole pine (*Pinus contorta*) phloem painted with different amounts of dsIAP- expressing yeast (n=18): 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), and 0.1mg/ml hpIAP (0.1_hpIAP). The survival of MPB in two negative controls: water (H2O) and 0.1mg/ml hpRS (0.1_hpRS; does not match any transcript in the mountain pine beetle transcriptome) was included for comparison.

A one-way ANOVA test reported that there was no statistically significant difference in the mean $\log_2$ relative *iap* gene expression between any of the treatment and control groups ($p = 0.277$, $F = 1.359$, $df = 4$) (Figure 4.8).

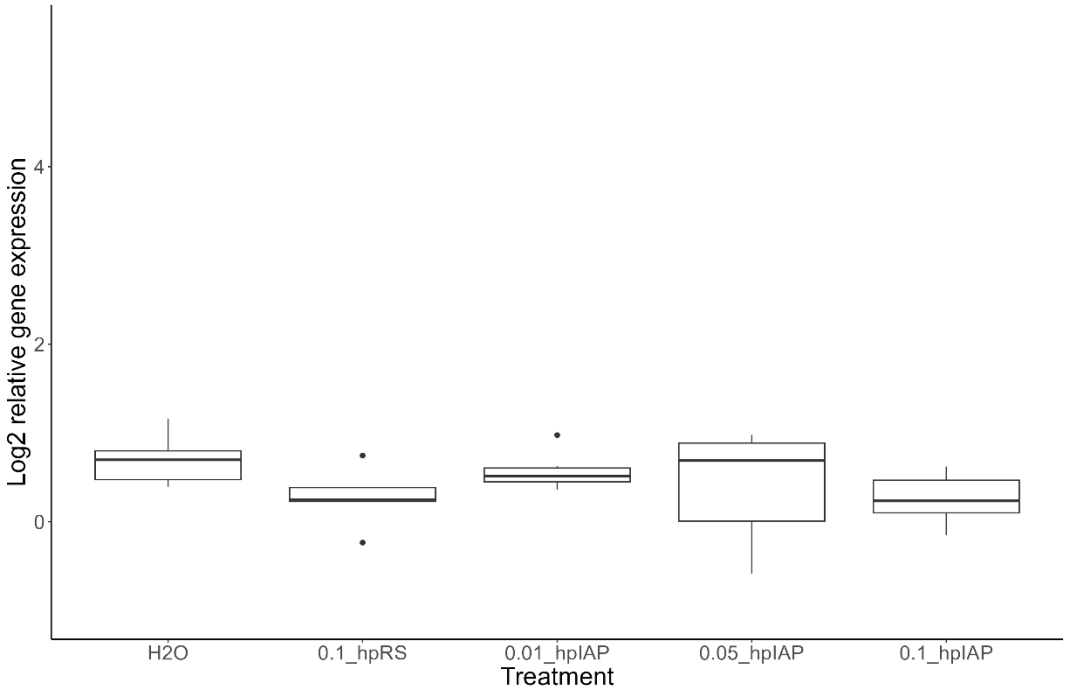

Figure 4.8 Comparison of the mean (n=6) of the log_2 transformed relative *iap* gene expression between 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), 0.1mg/ml hpIAP (0.1_hpIAP) and the control groups: water (H2O) and 0.1mg/ml hpRS (0.1_hpRS, n = 5) 72h after initial exposure. Statistical analysis using a one-way ANOVA reported no significant difference between the mean log_2 transformed relative *iap* gene expression among groups ($p = 0.277$, $F = 1.359$, $df = 4$).

4.3.5.2 Second repetition: mortality and *iap* expression analysis

Mortality of female adult MPB was higher in the second PHP assay that tested higher hpIAP-expressing yeast concentrations (Figure 4.9). After three days, all three hpIAP treatment groups showed mortality, along with the hpRS group (5.0_hpRS). On day four, MPB mortality in the three treatment groups was higher than in the control groups. The difference in MPB mortality between the treatment and control groups increased on day five. By day six, fungus was present on MPB and MPB mortality in the controls increased. The mortality observations were stopped on day eight due to the fungal growth.

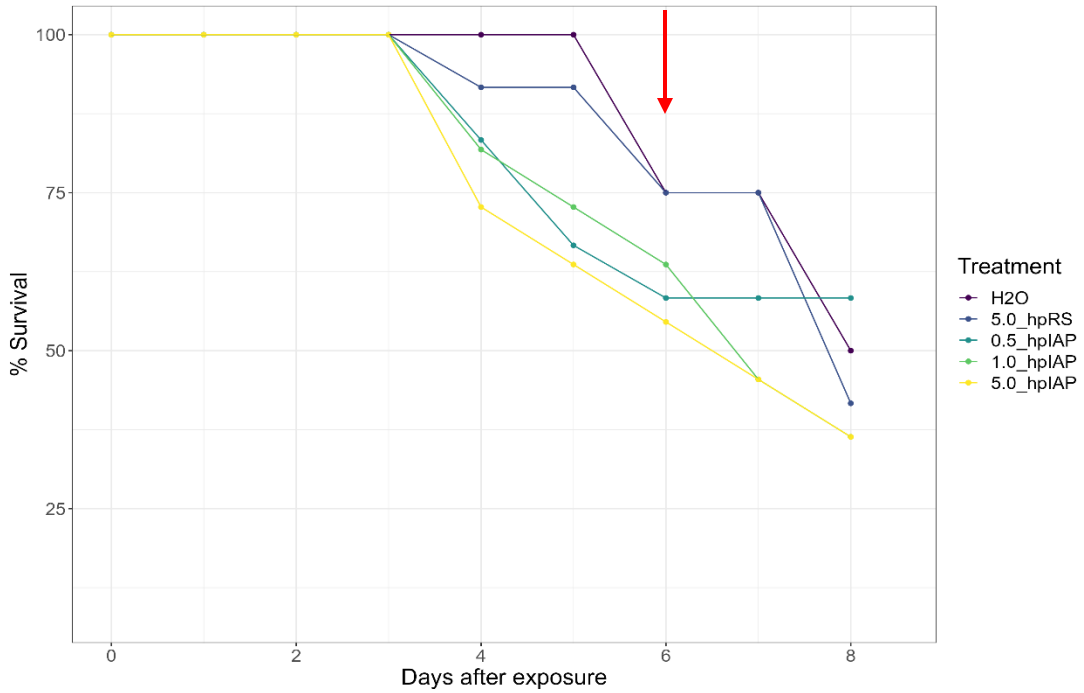

Figure 4.9 Percent survival of adult female MPB fed lodgepole pine (*Pinus contorta*) phloem painted with different amounts of hpIAP-expressing yeast (n=18): 0.5mg/ml hpIAP (0.5_hpIAP), 1.0mg/ml hpIAP (1.0_hpIAP), and 5.0mg/ml hpIAP (5.0_hpIAP). The survival of MPB in two negative controls: water (H2O) and 5.0mg/ml hpRS (5.0_hpRS; does not match any transcript in the mountain pine beetle transcriptome) was included for comparison. Red arrow indicates the first observation of fungus.

The mean relative $\log_2$ transformed relative *iap* gene expression in a small subset of tested adult female MPB did not show any gene expression changes at two different time points after the initial exposure (Figure 4.10). After quality control measures, the 0.5mg/ml hpIAP (0.5_hpIAP*)* treated group was down to two biological repetitions and was excluded from the statistical analysis but plotted for visual observation. A one-way ANOVA test reported that there was no statistically significant difference in the mean $\log_2$ transformed relative *iap* gene expression between any of the treatment and control groups at 72h ($p = 0.674$, $F = 0.589$, $df = 4$) and 120h ($p = 0.616$, $F = 0.678$, $df = 4$) after female adult MPB were initially exposed to hpIAP-expressing yeast. Additionally, none of the groups show significant differences in mean $\log_2$ transformed relative *iap* gene expression between the two time points as reported by a Student's

t-test: water (t = 0.27954, df = 7.1547, p-value = 0.7877), 5.0 mg/ml hpRS (t = 0.22968, df = 4.9837, p-value = 0.8275), 1.0 mg/ml hpIAP (t = 1.3946, df = 5.397, p-value = 0.2178), and 5.0 mg/ml hpIAP (t = 0.14672, df = 7.6486, p-value = 0.8871).

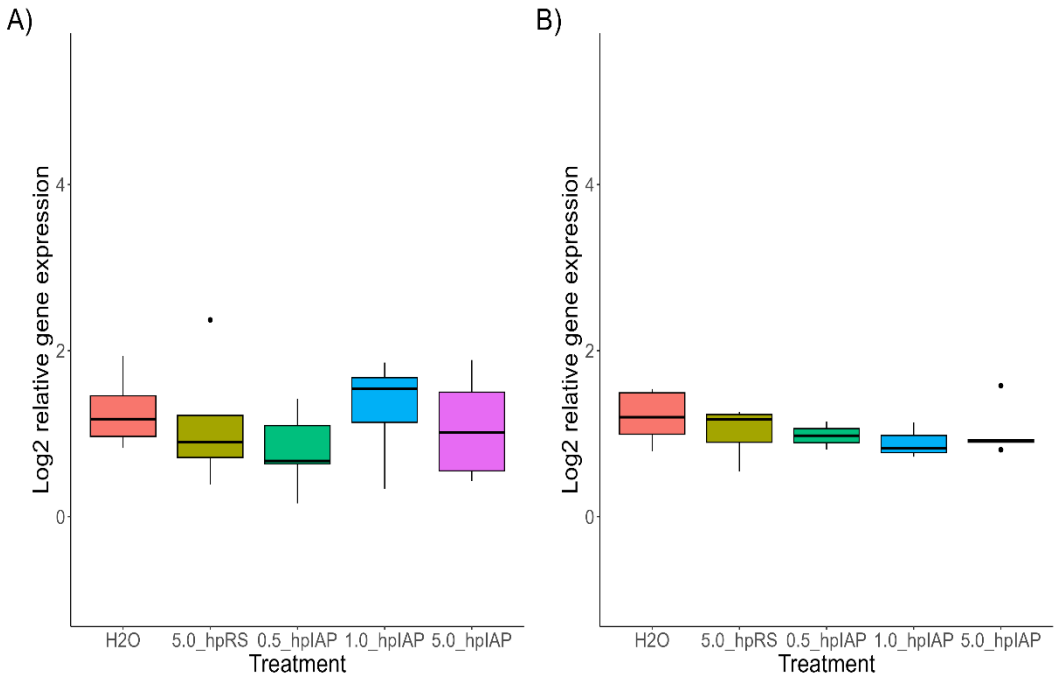

Figure 4.10 Comparison of the mean log_2 transformed relative *iap* expression between the treatment controls and the negative controls at two different timepoints after initial exposure to hpRNA. Statistical analysis was performed using a one-way ANOVA test. A) After 72h, comparison of 0.5mg/ml hpIAP (0.5_hpIAP, n=5), 1.0mg/ml hpIAP (1.0_hpIAP, n=5), 5.0mg/ml hpIAP (5.0_hpIAP, n = 6) and the negative controls water (H2O, n=5) and 5.0mg/ml hpRS (5.0-_hpRS, n=5): *iap* expression is not significantly different (p = 0.674, F = 0.589, df = 4). B) After 120h, comparison of 0.5mg/ml hpIAP (0.5_hpIAP, n=2*), 1.0mg/ml hpIAP (1.0_hpIAP, n=4), 5.0mg/ml hpIAP (5.0_hpIAP, n = 5) and the negative controls water (H2O, n=6) and 5.0mg/ml hpRS (5.0-_hpRS, n=6): *iap* expression is not significantly different (p = 0.495, F = 0.834, df = 3). * With only two biological repetitions, this treatment group was excluded from the statistical analysis but plotted for visual observation.

4.3.6 Phloem home – translocated yeast solutions through the phloem (PHT)

The presence of hpRNA throughout the phloem was confirmed with ddPCR (see Appendix, Table S5). After ingestion of hpRNA translocated through phloem, adult MPB survival remained above 50% in all groups for the duration of the assay (Figure 4.11). The survival of MPB in the 0.1mg/ml hpIAP (0.1_hpIAP) group remained the highest at 83%.

Beetles in the control groups showed similar survival to the other two treatment groups, 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), with a range of 61-66% survival.

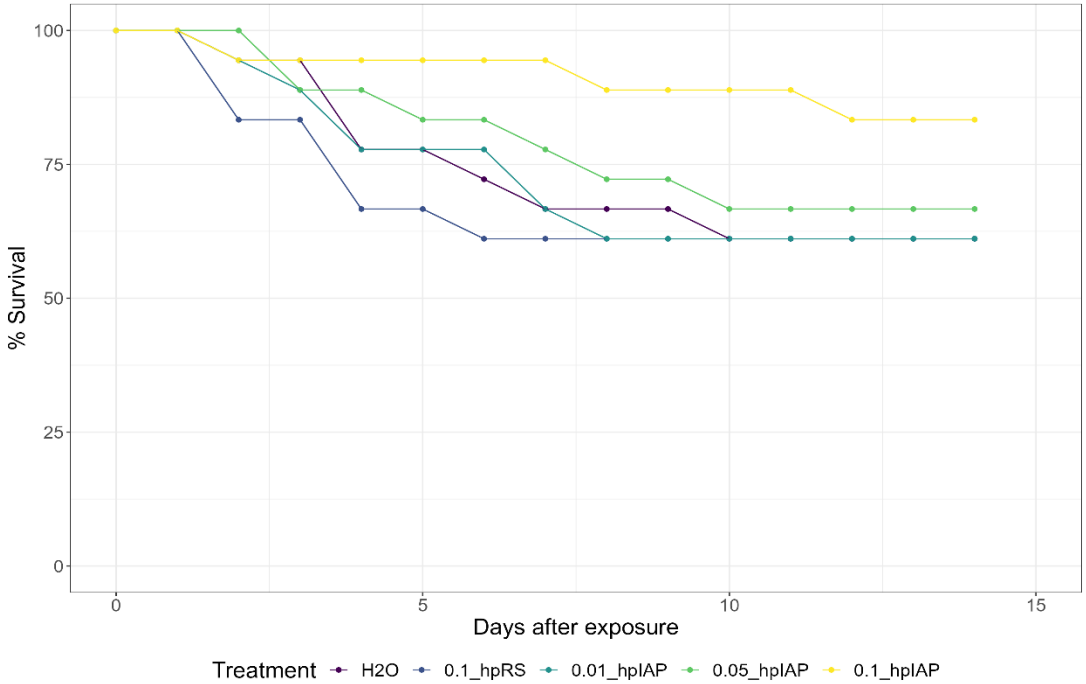

Figure 4.11 Percent survival of adult MPB fed different amounts of hpIAP- expressing yeast translocated through lodgepole pine (*Pinus contorta*) phloem (n=18): 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), and 0.1mg/ml hpIAP (0.1_hpIAP). The survival of MPB in two negative controls: water (H2O) and 0.1mg/ml hpRS (0.1_hpRS; does not match any transcript in the mountain pine beetle transcriptome) was included for comparison.

Gene expression analysis did not show any relative *iap* gene expression differences between the groups (Figure 4.12). The Kruskal-Wallis test determined that there was no statistically significant difference in the mean $\log_2$ relative *iap* gene expression between any of the treatment and control groups ($p = 0.4437$, $\chi 2 = 3.731$, $df = 4$).

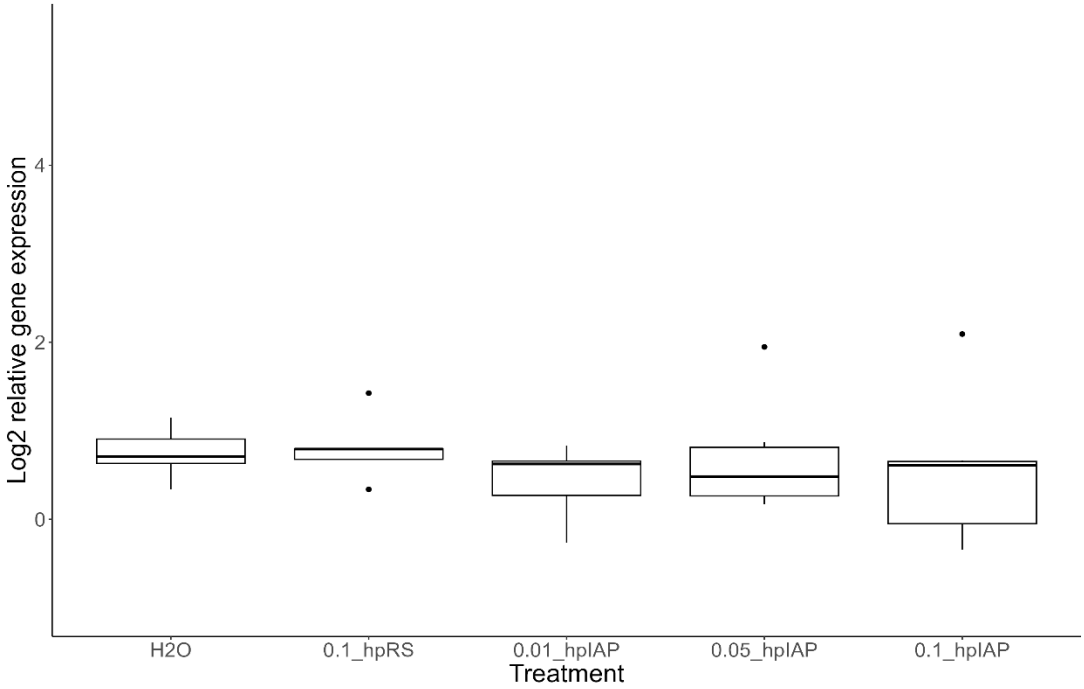

Figure 4.12 Comparison of the mean (n=6) of the log_2 transformed relative *iap* gene expression between 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), 0.1mg/ml hpIAP (0.1_hpIAP) and the control groups: water (H2O) and 0.1mg/ml hpRS (0.1_hpRS, n = 5) 72h after initial exposure. Statistical analysis using the Kruskal-Wallis test reported no significant difference between the mean log_2 transformed relative *iap* gene expression among groups ($p = 0.4437$, $\chi 2 = 3.731$, $df = 4$).

4.3.7 Phloem home – phloem soaked in yeast solutions (PHS)

Adult MPB survival in the treatment and control groups was more variable in this assay; however, MPB survival was still 50% or higher for the duration of the assay (Figure 4.13). Survival was lowest in the 0.05mg/ml hpIAP (0.05_hpIAP) treatment group at 50%, and was highest in the 0.1mg/ml hpRS (0.1_hpRS) control group at 83%. Beetle survival in the water group was 66%, similar to the 0.01mg/ml hpIAP (0.01_hpIAP) and 0.1mg/ml hpIAP (0.1_hpIAP) groups (Figure 4.13).

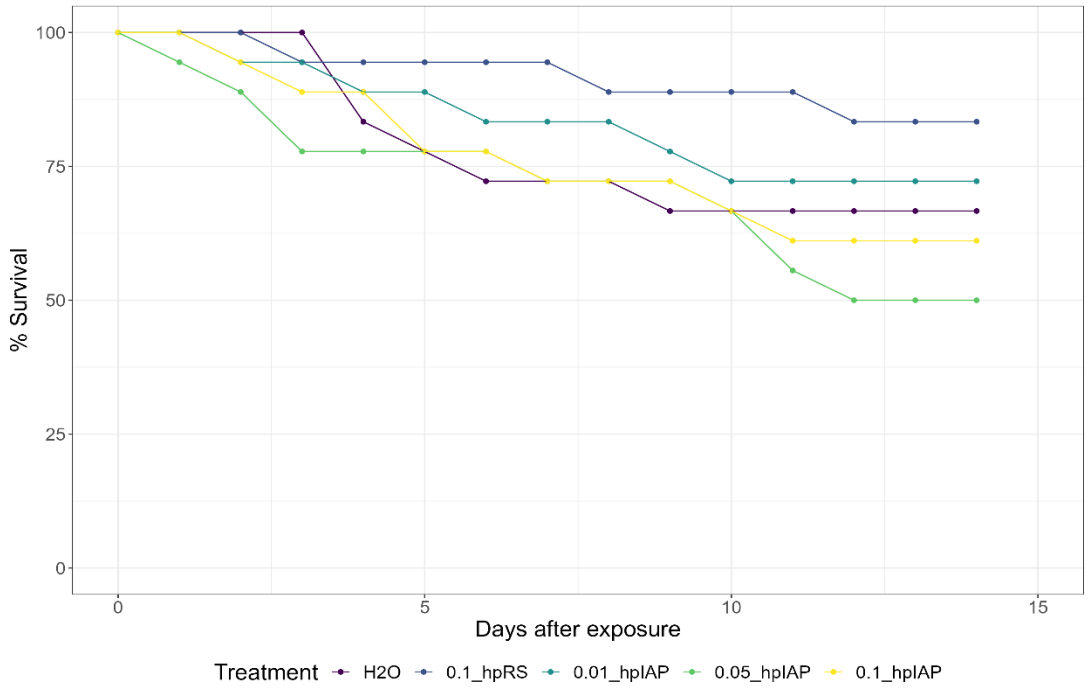

Figure 4.13 Percent survival of adult MPB fed lodgepole pine (*Pinus contorta*) phloem soaked in different amounts of hpIAP- expressing yeast (n=18): 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP), and 0.1mg/ml hpIAP (0.1_hpIAP). The survival of MPB in two negative controls: water (H2O) and 0.1mg/ml hpRS (0.1_hpRS; does not match any transcript in the mountain pine beetle transcriptome) was included for comparison.

Gene expression analysis reported no statistically significant difference in mean $\log_2$ relative *iap* gene expression among the treatment and control groups (Figure 4.14). The mean $\log_2$ transformed relative *iap* gene expression is slightly higher in the 0.1mg/ml hpIAP (0.1_hpIAP) than in the other groups (Figure 4.14). The Kruskal-Wallis test reported no statistically significant difference in the mean $\log_2$ relative *iap* gene expression between any of the treatment and control groups ($p = 0.0822$, $\chi 2 = 8.269$, $df = 4$).

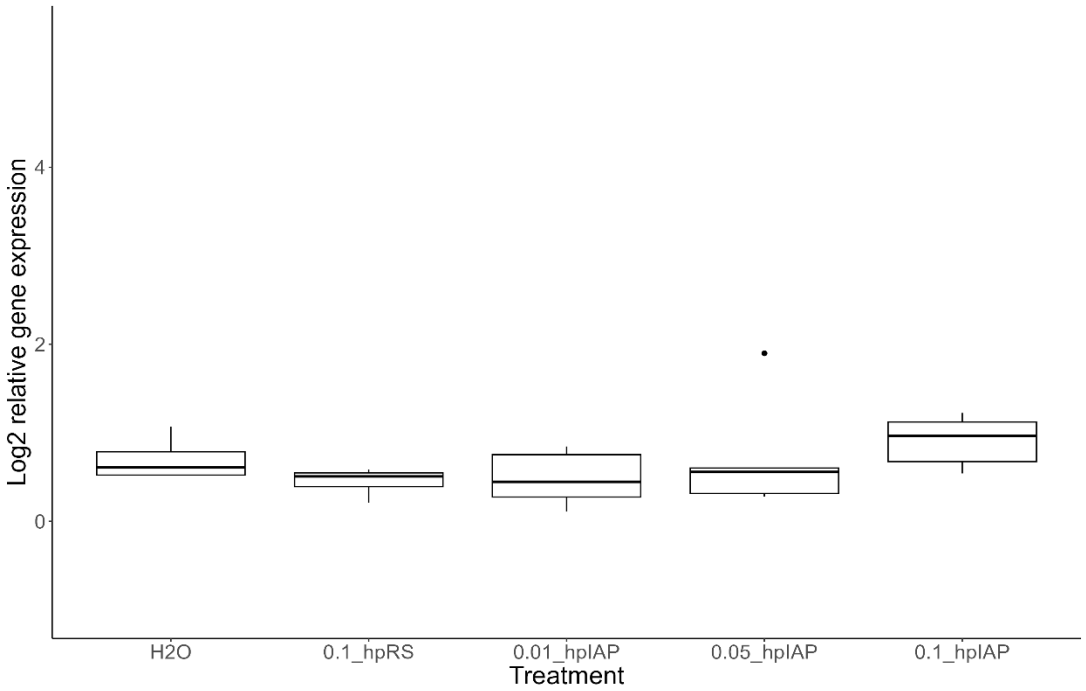

Figure 4.14 Comparison of the mean (n=6) of the log_2 transformed relative *iap* gene expression between 0.01mg/ml hpIAP (0.01_hpIAP), 0.05mg/ml hpIAP (0.05_hpIAP, n = 5), 0.1mg/ml hpIAP (0.1_hpIAP) and the control groups: water (H2O, n = 4) and 0.1mg/ml hpRS (0.1_hpRS) 72h after initial exposure. Statistical analysis using the Kruskal-Wallis test reported no significant difference between the mean log_2 transformed relative *iap* gene expression among groups ($p = 0.0822$, $\chi2 = 8.269$, $df = 4$).

4.4 Discussion

4.4.1. Phloem entices target liquid ingestion by MPB

Blue-dyed 1% sucrose solution uptake by adult MPB was higher when phloem was used to entice ingestion. Blue dye was found in 90% of MPB, an increase of 30% compared to the submergence method. The uptake success in the soaked phloem group shows promise for testing dsRNA delivery effectiveness using phloem, which can also progress the applicability of RNAi for MPB.

When soaked phloem was used to test the efficacy of dsIAP and dsHSP on adult MPB, there was no observable RNAi initiation from the mortality observations (Figure 3.4). Based on previous RNAi assays (see Chapter 3, section 3.4.2) in our study and from other studies, mortality in the dsIAP and dsHSP groups should have started around day three (Kyre et al. 2020). In emerald ash borer larvae, *hsp* silencing was detected 7 days after a foliar spray of dsHSP was applied to seedlings (Pampolini and Rieske 2023). Though it is likely that the ingestion of dsRNA-soaked phloem delays the response time before mortality, similar mortality in the negative controls did not provide evidence for RNAi initiation. Based on similar observations in previous chapters, mortality of MPB was likely caused by decreased beetle health (see Chapter 6, section 6.2.1).

4.4.2 Phloem homes show phloem ingestion and high survival

The number of phloem layers and presence of bark had no effect on MPB survival. With 62.5% survival after 13 days in both the two- and three-layered wells, the phloem home showed higher survival than previously observed in phloem ingestion studies. More MPB were observed burrowing through the phloem when it was attached to bark in the two-layered wells, an adaptation of the three-layered set up (Keeling et al. 2013). The presence of burrows and frass

indicated ingestion, and due to no impact on MPB survival, the two-layer adaptation was used in subsequent phloem home assays.

4.4.3 Successful dsRNA translocation through lodgepole pine

In all three segments (bottom, middle, and top) of the cut lodgepole pine rounds, hpRNA-expressing yeast was detected using ddPCR (see Appendix, Table S5). Translocation of dsRNA has been successful when applied as a root soak to White oak (*Quercus alba*) and Loblolly pine (*Pinus taeda*) seedlings (Bragg and Rieske 2022a, 2022b). Adapting the translocation through the phloem of lodgepole pine rounds for MPB ingestion was an important proof-of-concept for potential application of RNAi to manage MPB populations.

4.4.4 No RNAi in dsRNA-expressing yeast translocated or soaked phloem home

4.4.4.1 Unsuccessful RNAi initiation in the phloem home – translocated (PHT) assay

The MPB survival was high in all groups, indicating that the hpIAP-expressing yeast translocated through the lodgepole pine phloem was not lethal. This was further supported by higher MPB survival in two of the treatment groups, 0.05mg/ml hpIAP (0.05_hpIAP), and 0.1mg/ml hpIAP (0.1_hpIAP), than in the water (H2O) and 0.1mg/ml hpRS (0.1_hpRS) negative control groups. Inconsistent chewing by individual adult MPB were observed between and within treatment groups. This may have affected the amount of dsRNA ingested, impacting the reliability of this method.

Gene expression analysis confirmed that RNAi was not initiated in adult MPB. There was no significant difference in the mean relative $\log_2$ *iap* expression among any of the groups. Visually, the mean relative $\log_2$ *iap* expression in the hpIAP-expressing yeast treatment groups appears to be slightly lower than in the two control groups, but the already slight difference is obscured by variation in the data.

4.4.4.2 No RNAi response in the Phloem home soaked (PHS) assay

Similar to the PHT assay, MPB survival was above 50% for the duration of the PHS assay (Figure 4.13). More in line with expectations, MPB survival was highest in one of the negative controls, 0.1mg/ml hpRS (0.1_hpRS). Different from the PHT assay, lowest MPB survival was in the 0.05mg/ml hpIAP (0.05_hpIAP), and 0.1mg/ml hpIAP (0.1_hpIAP) groups (Figure 4.13). However, the water (H2O) control survival was similar to the survival of the hpIAP-expressing yeast treatment groups. Therefore, it is unlikely that RNAi initiation was successful, and that the observed mortality was random or due to confounding factors like beetle condition or age from delayed emergence (see Chapter 3, section 3.4.3 and Chapter 6, section 6.2.1) as this assay was performed later in the summer than the PHT and PHP assays.

Gene expression analysis provided further evidence that an RNAi response was not achieved. The mean relative $\log_2$ *iap* expression was similar among all groups with no statistically significant difference. Interestingly, *iap* expression in the 0.1mg/ml hpIAP (0.1_hpIAP) group was higher than the others, which was the first time an increased *iap* expression after treatment has been observed in our assays. While non-significant reduction of *iap* gene expression has been reported in southern pine beetles (*Dendroctonus frontalis*), neither a non-significant or significant increase has been reported in southern pine beetles or MPB (Kyre et al. 2019, 2020; Kyre and Rieske 2022).

4.4.5 No RNAi initiation in dsRNA-expressing yeast solution painted on phloem home (PHP) assay

With MPB survival above 75% in all groups, ingestion of hpIAP-expressing yeast painted on phloem does not appear to be lethal to adult MPB. The high MPB survival in the control groups suggests that this specific association method holds potential for future testing

due to the decrease in confounding factors impacting MPB mortality and more control over delivered dosage to MPB than in PHT assays.

No statistically significant differences were reported in the mean relative $\log_2$ *iap* expression between the groups, further supporting that RNAi initiation was not achieved. One such explanation could be the inconsistent chewing progress of individual MPB adults within each treatment (see section 4.4.4.1). This would impact the mortality and the gene expression analysis results. Another possible explanation is that the actual dose of hpIAP ingested by the adult MPB was unknown, due to individual differences in chewing and general ingestion ambiguity (List et al. 2022).

4.4.5.1 Increased dsRNA-expressing yeast solution concentration and MPB pairing in second phloem home painted (PHP24) assay indicates RNAi-initiation potential

The MPB mortality in all groups was higher than the previous phloem home assays. Higher MPB mortality in treatment groups than in the control groups suggests that the hpIAP-expressing yeast was eliciting a lethal response in MPB. However, the effect of RNAi-mediated mortality is likely confounded by fungus beyond day six. While the increased concentrations of hpIAP-expressing yeast started to show a lethal response in adult female MPB, more yeast may be contributing to the increased growth of fungus in the phloem homes, as previous assays were not affected by fungus. This could be due to the increased amount of yeast providing more nutrients for MPB-associated fungi to increase (Paine et al. 1997; Klepzigl et al. 2001; Zimmermann 2007). For future studies, the confounding effect of fungal growth on mortality can likely be prolonged with the addition of sorbic acid (Rangasamy and Siegfried 2012; Costa and Reeve 2012; Vélez et al. 2016). The addition of sorbic acid has previously acted as a preservative and increased the longevity of the diet in *Thanasimus dubius*, a predator of southern pine beetle

(Costa and Reeve 2012). Adding 0.12% sorbic acid into an artificial diet for adult western corn rootworm (*Diabrotica virgifera virgifera)* minimizes fungal contamination (Vélez et al. 2016). This preventative measure was not used in our studies due to the previously fungus-free phloem home assays and the potential impact of the sorbic acid on beetle fitness. Adding a low percentage of sorbic acid will not impact the already heat-inactivated yeast, and the hpRNA is protected within the yeast cell walls (C. Snowdon, personal communication, February 7, 2025). However, the effect of ingesting sorbic acid on adult MPB would need to be determined and the sorbic acid dosage adjusted accordingly.

The higher concentrations of hpIAP-expressing yeast did not cause statistically significant differences in mean relative $\log_2$ *iap* expression between any of the groups or within groups over time. It is interesting to note that the median relative $\log_2$ *iap* expression in the 1.0mg/ml hpIAP (1.0_hpIAP) group is higher at 72h post-exposure compared to 120h post-exposure. There are subtle shifts in the 0.5mg/ml hpIAP (0.5_hpIAP) and 1.0mg/ml hpIAP (1.0_hpIAP) groups. However, the variation in the data is large, impacting the analysis and preventing further interpretation of these small shifts in the data. Gene expression analysis at 120h accounted for the possibility of a delayed gene suppression response, and the *iap* expression pattern does appear to mirror the mortality observations. Significant gene suppression was detected in Colorado potato beetles after six days of feeding on dsRNA-expressing bacteria (Zhu et al. 2011). In emerald ash borer larvae, gene suppression was detected seven days after dsRNA exposure (Pampolini and Rieske 2023). In a study on Western corn rootworm larvae, no sign of RNAi was initially present, but activity was detected after 12 days (Baum et al. 2007). In our study, gene expression was not analyzed after 120h, but would likely have been affected by the fungal presence.

The pairing of a male and female into the one well did appear to increase burrowing, and therefore phloem ingestion in the females. Naturally, female MPB are the first to burrow into a host tree, and after mating, continue burrowing to create oviposition galleries (Chiu and Bohlmann 2022). Simulating the host attack and mating situations in the phloem home increased ingestion of the phloem, and associated hpIAP-expressing yeast, by the target female adult MPB.

4.4.6. General absence of response in adult MPB to dsRNA-expressing, heat-inactivated yeast

All three different applications of hpIAP-expressing, heat-inactivated yeast solutions to lodgepole pine phloem did not lead to an RNAi response when ingested by adult MPB. In submergence trials, hpRNA-expressing, heat-inactivated yeast solutions were also unsuccessful at initiating an RNAi response in adult MPB (see Chapter 3, section 3.3.5/ 3.3.6). In the phloem homes, some MPB ingested only small amounts of treated phloem, which may have skewed the mortality and gene expression results. The yeast solutions were all gone from the tube in the submergence assays, though the ingestion percentage in submergence was previously found to be 60%. However, when the yeast solution concentrations were increased, and ingestion enticed, in the second phloem home painted (PHP24) assay there appeared to be a lethal effect. Thus, one possible explanation for no RNAi response is the inefficient and inconsistent ingestion of the dsRNA-expressing yeast in the assays.

Another possible factor impacting the RNAi response is the dose of hpRNA. In the initial assays, concentrations were between 0.01mg/ml and 0.1mg/ml. This range is similar to *in vitro* dsRNA dosages of 0.017µg/µL and 0.067µg/µL that initiated an RNAi response when translocated through a green bean and ingested by brown marmorated stink bug nymphs (Ghosh et al. 2018). This value increased when used for injections, sprays, or cuts in *Citrus sinensis* x

Poncirus trifoliata trees to target the Asian citrus psyllid. Concentrations ranged from 0.5mg/ml for foliar spray, 0.2-1.0mg/ml for soil and root drenches, and 1.7mg/ml for injections (Ghosh et al. 2018). In each of those methods, the volume used varied, impacting the total dose of dsRNA. Concentrations of hpRNA-expressing yeast solutions were increased in the latter phloem home – painted assay (PHP24) to: 0.5mg/ml, 1.0mg/ml, and 5.0mg/ml. With a potential lethal response but no gene silencing response, this suggests that the dose of dsRNA is an important factor in the lack of an RNAi response.

In previous chapters, significant gene suppression has been detected in previous assays but with small foldchanges (See Chapter 3, section 3.3.4 and 3.3.7.1). Our RNA extraction method uses the entire adult MPB to measure and compare target gene copies. This is the same as previous studies in adult MPB and congeneric southern pine beetles (Keeling et al. 2013; Kyre et al. 2019, 2020). However, this is the first study to investigate lethality caused by gene targets that likely have a localized effect in the structure of MPB intestines, and using RNA from the whole body may be diluting the RNAi effect in gene expression (C. Snowdon, personal communication, 2023). In studies testing the efficacy of RNAi targeting *ssk* and *mesh* orthologs in Western corn rootworm larvae, RNA was extracted from whole larvae (Hu et al. 2016, 2019). While adults are likely larger than larvae, whole adults were also used for gene expression analysis when reproductive genes were targeted in western corn rootworm (Niu et al. 2017). Therefore, while extracting RNA from a whole adult MPB may dilute the response of a localized gene, it is unlikely to be a large effect as adult MPB are small and many studies have used whole-beetle RNA for localized gene expression quantification. In our study, the gene targets are expressed in more than one tissue type and expression appears to be important in each of those tissues, like *tsp2a* in the Malpighian tubules and midgut epithelia (Jonusaite et al. 2020).

Neither submergence nor phloem associations delivering hpRNA-expressing yeast to MPB caused an RNAi response. The second phloem home painted (PHP24) assay showed that hpIAP-expressing yeast is potentially lethal to adult female MPB, but a fungus outbreak impacted the assay and obscured any further inferences. While it is a possibility that adult MPB were not breaking down the heat inactivated yeast vectoring the hpRNA in the stomach, the mortality trend in the PHP24 assay suggests this was unlikely. Additionally, the lack of a response by MPB when submerged in hpRNA-expressing yeast coincided with a lack of response when submerged in *in vitro* synthesized dsRNA, and as such the response is likely due to beetle-related factors rather than dsRNA factors, like MPB emergence dates or beetle health (see Chapter 3, section 3.3.5/3.3.6). Yeast has been successfully used to produce and deliver dsRNA to *D. suzukii* larvae and initiate decreased fitness via RNAi (Murphy et al. 2016). Yeast is a part of the natural diet of *D. suzukii*, and therefore can be ingested as expected. Fungal associates, including yeasts, of MPB are a nutrient source for larvae and teneral adults (Paine et al. 1997; Chiu and Bohlmann 2022). In MPB, yeast was added to the phloem homes when rearing teneral adults into emerged adults, supporting that yeast is ingested by MPB (Keeling et al. 2013). Therefore, MPB are expected to successfully ingest and breakdown the heat-inactivated, hpRNA-expressing yeast, and the absence of responses are likely connected to decreased beetle condition and low dosages.

4.5 Conclusion

This study was the first to investigate the effectiveness of hpRNA-expressing, heat-inactivated yeast associated with lodgepole pine phloem at initiating an RNAi response when ingested by adult MPB. The *iap* gene was targeted in all assays for a proof-of-concept gene due to previous gene suppression success in adult MPB. Two of the three yeast-phloem association methods, phloem soaked in hpRNA-expressing yeast solutions and hpRNA-expressing yeast solutions translocated through phloem, did not elicit an RNAi response. The other association, hpRNA-expressing yeast solutions painted on phloem, showed the beginnings of a lethal response in adult female MPB with increased yeast concentrations and adapted methods. In all assays, however, there was no significant differences in mean *iap* expression among groups. Producing hpRNA in heat-inactivated yeast and delivering it via associations with phloem shows promise for future RNAi assays with MPB, with increased concentrations and method refinement, as survival was often high in the controls.

Chapter 5 Preliminary transcriptomic analysis comparing negative controls to one another within each of the three dsRNA/hpRNA delivery methods to investigate the genomic response of adult MPB.

5.1 Introduction

Applying RNA interference (RNAi) as a pest management tool for insects is gaining momentum (Christiaens et al. 2020; Lü et al. 2022). Variation in the effectiveness of RNAi are attributed to target insect species and life stage, gene targets, and the dsRNA delivery method (Poreddy et al. 2017; Cooper et al. 2019; Leelesh and Rieske 2020). Robust RNAi initiation has been reported in many Coleopteran insects, including *Dendroctonus* spp. (Cooper et al. 2019; Christiaens et al. 2020). As an important pest in North American forests, the mountain pine beetle (*Dendroctonus ponderosae,* MPB) has shown responsiveness to RNAi in laboratory-based trials (Keeling et al. 2013; Kyre et al. 2020; Kyre and Rieske 2022). A congeneric species, the southern pine beetle (*Dendroctonus frontalis,* SPB) has also exhibited RNAi-mediated gene suppression and associated mortality (Kyre et al. 2019; Kyre and Rieske 2022). The SPB response to gene targets varies among populations, providing another variable to consider for RNAi application (Kyre et al. 2024). As RNAi advances toward bark beetle management, attention to the genetic diversity among populations of the target insect is needed.

Transcriptome analyses have been performed on MPB for the identification of overwintering genes and increased understanding of detoxification gene families (Robert et al. 2013, 2016). An investigation into overwintering larvae and a study comparing starved and fed adult MPB used a type of transcriptome analysis called RNAseq (Robert et al. 2013, 2016). The RNAseq method can identify differential gene expression using the full length of transcripts, making it a useful tool for comparing transcript levels between different experimental or natural

conditions (Lohman et al. 2016). Using RNAseq, detected expression patterns of gene families provided further understanding of their roles in host colonization by adult MPB (Robert et al. 2013). This method also allowed for identification of critical genes for overwintering MPB larvae (Robert et al. 2016). While the general RNASeq methods are transferable to relative differential gene expression analyses, a more focused method can also be used.

The RNAseq output is redundant and costly for relative gene expression, where expression is normalized to a reference gene (Lohman et al. 2016). An alternative to the full RNAseq analysis is TagSeq. TagSeq analysis uses the 3' end of the RNA molecules to create a single initial library molecule per transcript (DNA Technologies Core). TagSeq generates low-cost and low-noise data from fewer sequencing reads, making it ideal for gene expression profiling when compared to RNAseq (Lohman et al. 2016).

Further analyses can be performed on TagSeq results to provide functional interpretations. An analysis becoming more frequently used to represent gene functions is gene ontology (Andersson et al. 2013; Wei et al. 2017). The gene ontology analysis was used to identify genes with olfactory function in MPB and the European spruce bark beetle (*Ips typographus*) (Andersson et al. 2013). These previous transcriptome analyses on MPB were used to identify changes in transcript levels and identify protein functions.

Gene ontology (GO) analysis is commonly incorporated with differential gene expression investigations. Using a protein identification and associated GO terms, the analysis results provide the functional annotation (Wei et al. 2017; Tehrani et al. 2024). This analysis also sorts the GO terms associated with a list of proteins or genes into one of three categories: cellular component (CC), biological process (BP), and molecular function (MF) (Wei et al. 2017). One regularly used analysis method is the gene ontology enrichment analysis (Xin et al. 2022). The

enrichment of a gene ontology term refers to the measured frequency of the gene ontology term compared to the expected frequency relative to the genome (Wei et al. 2017). The functional annotation, categorizations, and the enrichment measurement of the GO terms can assist with inferences about what pathways are involved in experimental conditions or observed phenotypes.

Previous RNAi initiation success in adult MPB demonstrates the presence of RNAi machinery in MPB (Keeling et al. 2013; Kyre et al. 2020; Kyre and Rieske 2022). This assumption is supported by successful RNAi in assays within this study (see Chapter 3, section 3.3.4 and 3.3.7.1). An initial search of an MPB transcriptome from an overwintering population (Thompson et al. 2020; B. Murray, unpublished results) confirmed the presence of some transcripts known to be involved in the RNAi pathway: Argonaute-2, endoribonuclease dicer-like, and SID1 transmembrane family member 1-like (Yoon et al. 2016; Haller et al. 2019). The identification of RNAi machinery transcripts has been provided alongside RNAi mediated gene suppression in the six-spined ips (*Ips calligraphus*) (Wallace and Rieske 2023), but an associated transcriptome analysis with RNAi initiation in MPB has not been reported.

Variable results from the assays conducted throughout our study remain unexplained from the experimental results. In negative control groups the MPB mortality has often been higher than expected, suggesting that the methods are inducing adult MPB mortality. The ingestion of dsRNA can alter the physiology of insects, and could be causing unexpected, off-target responses (Lü et al. 2022). Additionally, the variation in RNAi initiation success may be linked to genetic differences in different MPB populations, as was observed in SPB (Kyre et al. 2024).

In this chapter, a bioinformatic study was performed to investigate the possible factors impacting assay results. The first objective of the TagSeq analysis was to evaluate the genomic

response of adult MPB to the dsRNA and hpRNA delivery methods by comparing adult MPB treated with negative controls to non-treated adult MPB. The second objective of the TagSeq analysis was to confirm the presence of critical RNAi components in the MPB population used for this study. Lastly, a positive control group of MPB with suppressed *iap* expression was included to compare to MPB in the negative control group of the same delivery method for an increased understanding of the genomic responses.

5.2 Methods

5.2.1 Sample Preparation

Total RNA samples from the negative controls in all three different delivery methods, microinjection, submergence, and phloem homes, were quality checked to meet the requirements for 3'Tag-Seq analysis (https://dnatech.genomecenter.ucdavis.edu/sample-requirements/). The three samples, comprised of mixed sex or unsexed MPB, within each treatment with the best quality were selected for submission. Most of the quality control was performed before gene expression analysis using digital droplet PCR (ddPCR), as previously described (see Chapter 1, section 1.2.8.1). Briefly, samples were quantified with the Qubit 4 Fluorometer Broad Range RNA assay (Thermo Fisher Scientific Inc., 2015) and the sample integrity was determined using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). Though the RIN assigned to the samples is not reliable for insect RNA as there is an endogenous break in the 28S rRNA, the RNA quality was visually assessed using the electropherogram for each sample (Fabrick and Hull 2017). Lastly, the ND-1000 Nanodrop (v.3.8.1, NanoDrop Technologies 2024) was used to measure the 260/280 and 260/230 ratios. Selected samples were diluted to 60ng/µl at a volume of 25µl and submitted for analysis at the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center.

5.2.2. 3'Tag-Seq Library Preparation and Sequencing

The library preparation and sequencing were carried out at the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center, supported by NIH Shared Instrumentation Grant 1S10OD010786-01. Barcoded 3'Tag-Seq libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the recommendations of the manufacturer. The fragment size distribution of the libraries was

verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The libraries were quantified by fluorometry on a Qubit instrument (LifeTechnologies, Carlsbad, CA), and pooled in equimolar ratios. Twenty-four libraries were sequenced on one lane of an AVITI sequencer (Element Biosciences, San Diego, CA) with single-end 100 bp reads. The sequencing generated more than 4 million reads per library.

5.2.3 Differential Gene Expression (DGE) Analysis

The data analysis on 3'TagSeq sequencing was performed by the Bioinformatics Core at UC Davis Genome Center. Single-end 151bp AVITI reads were subjected to quality control. Adapter sequences and low-quality bases ($q < 30$) were removed using trim_galore/v0.6.7 (Krueger et al. 2021). Reads that were less than 30bp in length were discarded. Reads that passed quality control were aligned to the mountain pine beetle genome (GCF_020466585.1) using STAR/v2.7.11b (Dobin et al. 2013). Annotation of the MPB genome (GCF_020466585.1) includes 12,777 protein-coding genes and 23,095 mRNAs, of which 22,374 are fully supported (NCBI 2022). Raw counts generated from STAR aligner was normalized using TMM method in edgeR (Robinson et al. 2010). Differential expression analysis was carried out using limma and voom (Law et al. 2014; Ritchie et al. 2015), that adjusted for a hidden variable identified that was most likely sex-linked based on the sex information available for a subset of samples. Genes were filtered to require a two counts-per-million in at least two samples. The p-value was adjusted using the Benjamini-Hochberg procedure to control the false discovery rate.

5.2.4 Annotation and Gene Ontology of DGE results

From the list of significantly up-regulated genes (adjusted p-value < 0.05) from each comparison (Table 5.1), a 1.5 foldchange cut off was applied. For genes with a foldchange greater than 1.5, a UniProt ID and respective gene ontology (GO) terms were obtained where

possible (UniProt 2024). This process was repeated with the down-regulated genes, though only those with a foldchange below -1.5 were included. A two-fold up- or down-regulation is commonly used as a cut off, but by using a cut off of 1.5, the annotated genes are likely to have consequences in function of the beetles (Silva and Vogel 2016). The GO terms were uploaded for GO Enrichment analysis to the NaviGO website (Wei et al. 2017; https://kiharalab.org/navigo/views/goenrich.php), where the proteins and associated GO terms are arranged by their calculated counts compared to the expected counts based on the genome. Information on the biological processes, molecular function, and/or cellular function that the up- or down-regulated genes were involved in will also be provided by NaviGO (Wei et al. 2017). For GO terms with no domain assigned were searched using QuickGo (EMBL-EBI, https://www.ebi.ac.uk/QuickGO/term). Visualization of the gene ontology results was performed using "ggplot2" (Wickham 2016) in R Statistical Software (v4.3.1; R Core Team 2023).

Table 5.1 Treatment comparisons within each dsRNA/hpRNA delivery method used for differential gene expression analysis to investigate the response to RNAi and the delivery methods in adult mountain pine beetle (*Dendroctonus ponderosae*).

dsRNA/hpRNA delivery method	Comparison
Microinjection	A16M_D vs.C22 (dsRS vs not-injected)
	A16M_W vs.C22 (water vs not-injected)
	A16M_D vs. A16M_W (dsRS vs water)
Submergence	J22S_D vs.C22 (dsRS vs not-submerged)
	J22S_I vs.C22 (dsIAP vs not-submerged)
	J22S_D vs. J22S_I (dsRS vs dsIAP)
Phloem-fed	PHPF_D vs.C23 (hpRS vs not-fed)
	PHPF_W vs.C23 (water vs not-fed)
	PHPF_D vs. PHPF_W (hpRS vs water)

5.3 Results

5.3.1 Sample Selection

Quality check results of the three samples with the best quality measurements from each negative control group, dsIAP treatment group, and no-treatment group from each year that met requirements are listed (see Appendix, Table S7). Only one sample did not meet one of the quality requirements due to a low 260/230 ratio, but with a good quality electropherogram from the Agilent measurements, it was included in the analysis (see Appendix, Table S7, sample A16M_D5). Quality of the samples upon receival by the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center showed slightly decreased values but were still of a satisfactory level for downstream analysis.

5.3.2 Differential Gene Expression analysis results

After quality control, reads from each sample were uniquely mapped with rates ranging from 46-65% (see Appendix, Table S8). The other reads were either non-uniquely mapped (multi-mapped/mapped to too many loci) or unmapped (too short/other). The first output from the differential gene expression showed a large gap along the first dimension in a multi-dimensional scaling (MDS) plot (Figure 5.1A). After further investigation using the recorded sex for some of the samples along with patterns from previous studies, it was determined that the difference was likely a sex-linked effect. Once added to the formula as a fixed unknown variable, the resulting MDS plot showed clusters more aligned to the treatments (Figure 5.1B). Additionally, the number of genes that were significantly different in the comparisons decreased.

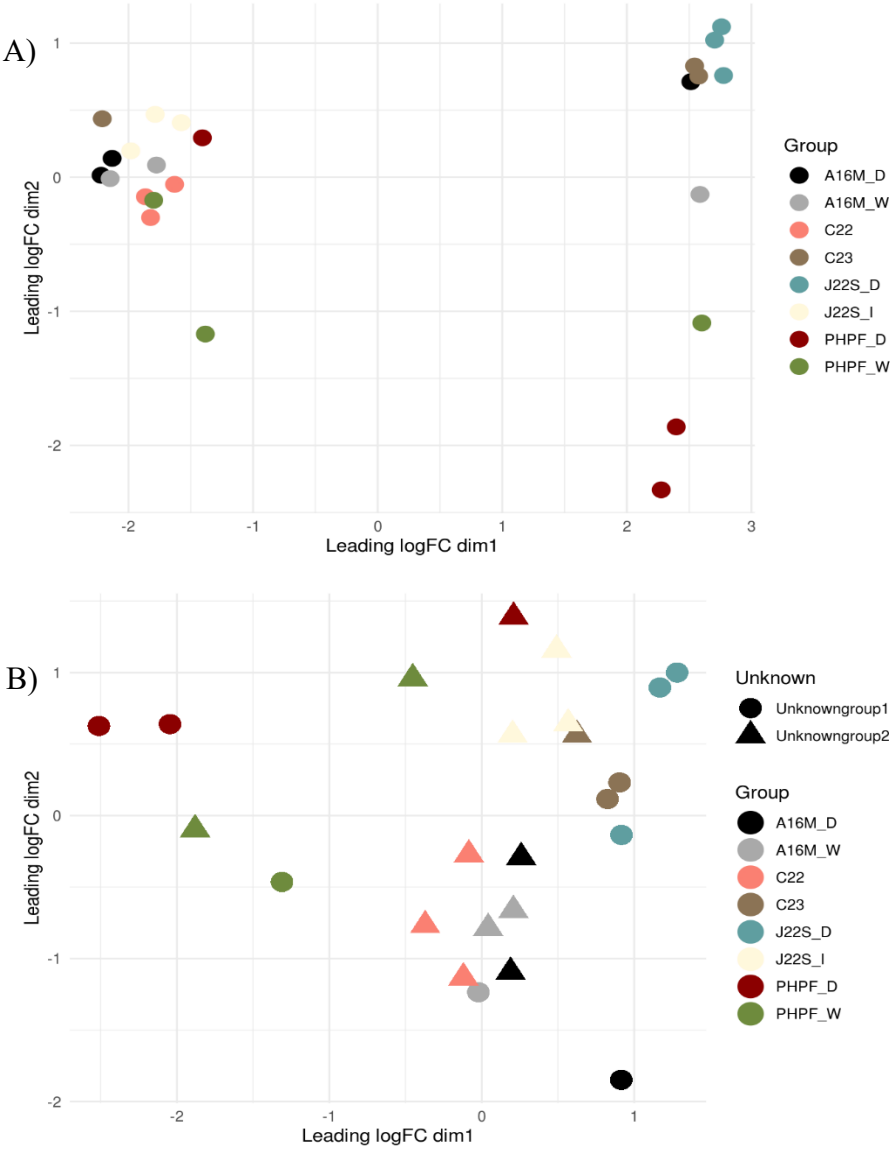

Figure 5.1 Multidimensional scaling (MDS) plots performed by the Bioinformatics Core at UC Davis Genome Center. (A) The initial MDS plot shows a large separation along the first dimension, which was determined to be a sex effect. (B) This sex effect was added as an unknown variable to the formula. Females are represented by circles and males represented by triangles. Microinjection samples are represented by A16M prefix, submergence samples by the J22S prefix, and phloem-fed samples by PHPF prefix. Controls are listed with 'C' followed by the last two digits of emergence year. Lastly, dsRS-treated samples are represented by the suffix 'D',water-treated samples by the suffix 'W', and dsIAP-treated samples with the suffix 'I'.

5.3.2.1 Expression of RNAi machinery did not differ significantly between comparisons

Significant differential gene expression results did not include any RNAi machinery. Transcripts of the RNAi machinery, like protein argonaute-2, endoribonuclease dicer, and SID1 transmembrane family member were found in the unfiltered (did not meet the 1.5/-1.5foldchange cutoff) data in each microinjection, submergence and phloem home comparison, showing small, insignificant differences (Table 5.2). One protein-coding gene, SID-2, which has been identified to be involved in dsRNA uptake for environmental RNAi was not found in these comparisons because it was also not identified in the MPB genome (Keeling et al. 2021).

Table 5.2 Presence or absence of genes coding for RNAi machinery components in the differential gene expression analysis comparing the negative controls in different delivery methods.

Gene	Annotation	Present or absent
LOC109543529	protein argonaute - 2	present
LOC109533193	protein argonaute - 2	present
LOC109546978	endoribonuclease Dicer	present
LOC109540929	endoribonuclease Dcr -1	present
LOC109537029	SID1 transmembrane family member	present
LOC109533632	SID1 transmembrane family member 1	present
-	SID-2 systemic RNA interference defective protein 2	absent
LOC109538703	V-type proton ATPase 16 kDa proteolipid subunit c	present
LOC109537913	double-stranded RNA-binding protein Staufen homolog	present

5.3.2.2 Microinjection comparisons

Comparisons of the transcripts in adult MPB microinjected with water (A16M_W) or dsRS (A16M_D) to those not injected (C22) yielded no significant differences in gene expression. Only one significant gene (padj < 0.05), *venom allergen 5*, was significantly down regulated in MPB microinjected with dsRS compared to when microinjected with water.

5.3.2.3 Submergence comparisons

Differential gene expression analysis comparing adult MPB submerged in dsRS (J22S_D) or submerged in dsIAP (J22S_I) to beetles not submerged at all (C22) yielded 305 and 265 genes, respectively, with significantly different expression (padj < 0.05). After applying a log foldchange limit of >1.5 for upregulated and <-1.5 for downregulated genes, 182 and 117 genes were differentially expressed between J22S_D and J22S_I compared to C22, respectively. In J22S_I compared to C22, stress response genes like heat shock proteins (*hsp*) were up-regulated. Gene expression in J22S_I compared to J22S_D indicated no significantly differentially expressed genes.

5.3.2.4 Phloem-fed comparisons

Differential gene expression analysis comparing MPB fed phloem with hpRS (PHPF_D) or water (PHPF_W) painted on to phloem to MPB not fed anything (C23) resulted in 1003 and 567 significant genes (padj < 0.05), respectively. After the log foldchange cut offs, there were 344 and 231 significantly different genes for PHPF_D and PHPF_W, respectively. There were 249 genes significantly differentially expressed between PHPF_D and PHPF_W, and 82 after log foldchange cut-offs.

5.3.3. Gene ontology

5.3.3.1 Microinjection control comparisons

No significant gene ontology enrichment terms were obtained for any of the following microinjection control comparisons: injected with water (A16M_W) to no injection (C22), injected with dsRS (A16M_D) to no injection (C22), and A16M_D to A16M_W.

5.3.3.2 Submergence comparisons

The gene ontology enrichment analysis for J22S_D compared to C22 yielded 98 GO terms associated with the up-regulated genes and 68 GO terms associated with the down-regulated genes. Within the list, 40 of the up-regulated and 23 of the down-regulated GO terms were enriched ($p < 0.05$, see Appendix, Tables S11 and S12). The top 10 enriched (i.e. 10 GO terms with the smallest p value) GO terms from each category were selected for visualization (Figure 5.2).

Three GO term annotations in the cellular component domain from the up-regulated genes were the mitochondrion, chitin-based extracellular matrix, and intraciliary transport particle B. The top three GO term annotations from the biological process domain were: isoprenoid biosynthetic process, tricarboxylic acid, and glyoxylate cycle. The top three GO term annotations from the molecular function domain are: acyl-CoA dehydrogenase activity, diphosphomevalonate decarboxylase activity, and structural constituent of chitin-based larval cuticle.

The GO term annotation in the cellular component domain from the down-regulated genes is the myosin II complex. The top four GO term annotations from the biological process domain are: cell wall modification, pectin catabolic process, fatty acid elongation (monounsaturated fatty acid), and fatty acid elongation (polyunsaturated fatty acid). The top four GO term annotations from the molecular function domain are: chitin binding, odorant binding, rhamnogalacturonan endolyase activity and L-galactose dehydrogenase activity.

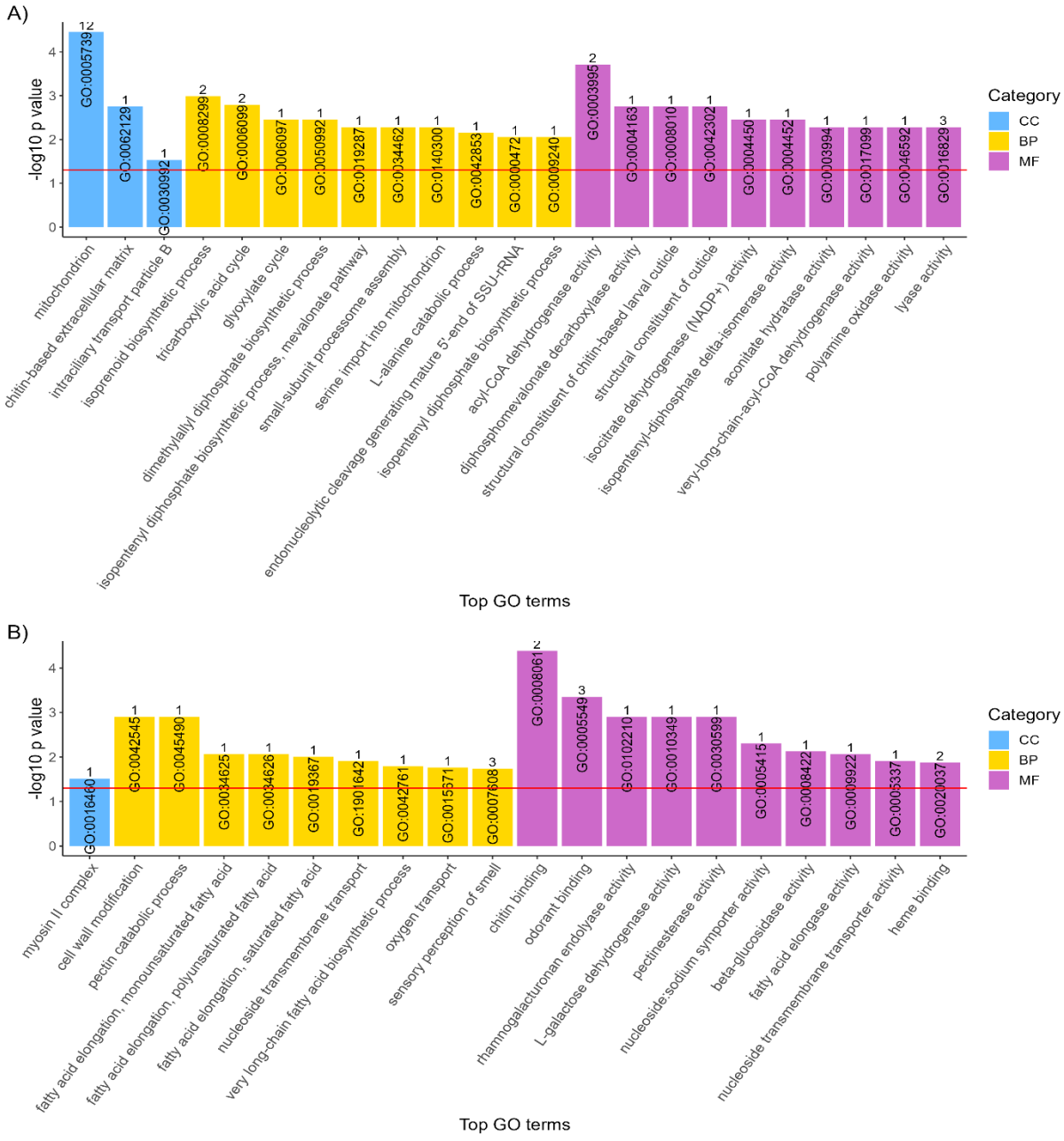

Figure 5.2 Enriched ($p < 0.05$) GO terms from the comparison between adult MPB submerged in dsRS and adult MPB not submerged in anything. Categories are: cellular component (CC), biological process (BP), and molecular function (MF). Significance level is indicated by the horizontal red line, any bar above the line indicates $p < 0.05$. A) Enriched GO terms associated with up-regulated genes. B) Enriched GO terms associated with down-regulated genes.

The gene ontology enrichment analysis for J22S_I compared to C22 yielded 70 GO terms associated with the down-regulated genes and 78 GO terms associated with the up-regulated genes. Within the list, 24 of the up-regulated and 19 of the down-regulated GO terms were enriched ($p < 0.05$, see Appendix, Tables S9 and S10). The top 10 enriched GO terms (i.e. the 10 GO terms with the smallest p value) from each category were selected for visualization (Figure 5.3).

Three GO term annotations in the cellular component domain from the up-regulated genes were the chitin-based extracellular matrix, mitochondrion, and the A band. The top three GO term annotations from the biological process domain were: L-alanine catabolic process, macromolecule depalmitoylation, and nucleoside monophosphate phosphorylation. The top three GO term annotations from the molecular function domain were: scavenger receptor activity, ATP-dependent protein folding chaperone, and beta-fructofuranosidase activity.

The GO term annotation in the cellular component domain from the down-regulated genes was the mediator complex. The top three GO term annotations from the biological process domain were: phagosome-lysosome fusion, lipid catabolic process, and zinc II ion transmembrane transport. The top eight GO term annotations were: MAP kinase serine/threonine phosphatase activity, histone H2AXS140 phosphatase activity, five different RNA polymerase II CTD heptapeptide phosphatase repeats, and L-galactose dehydrogenase activity.

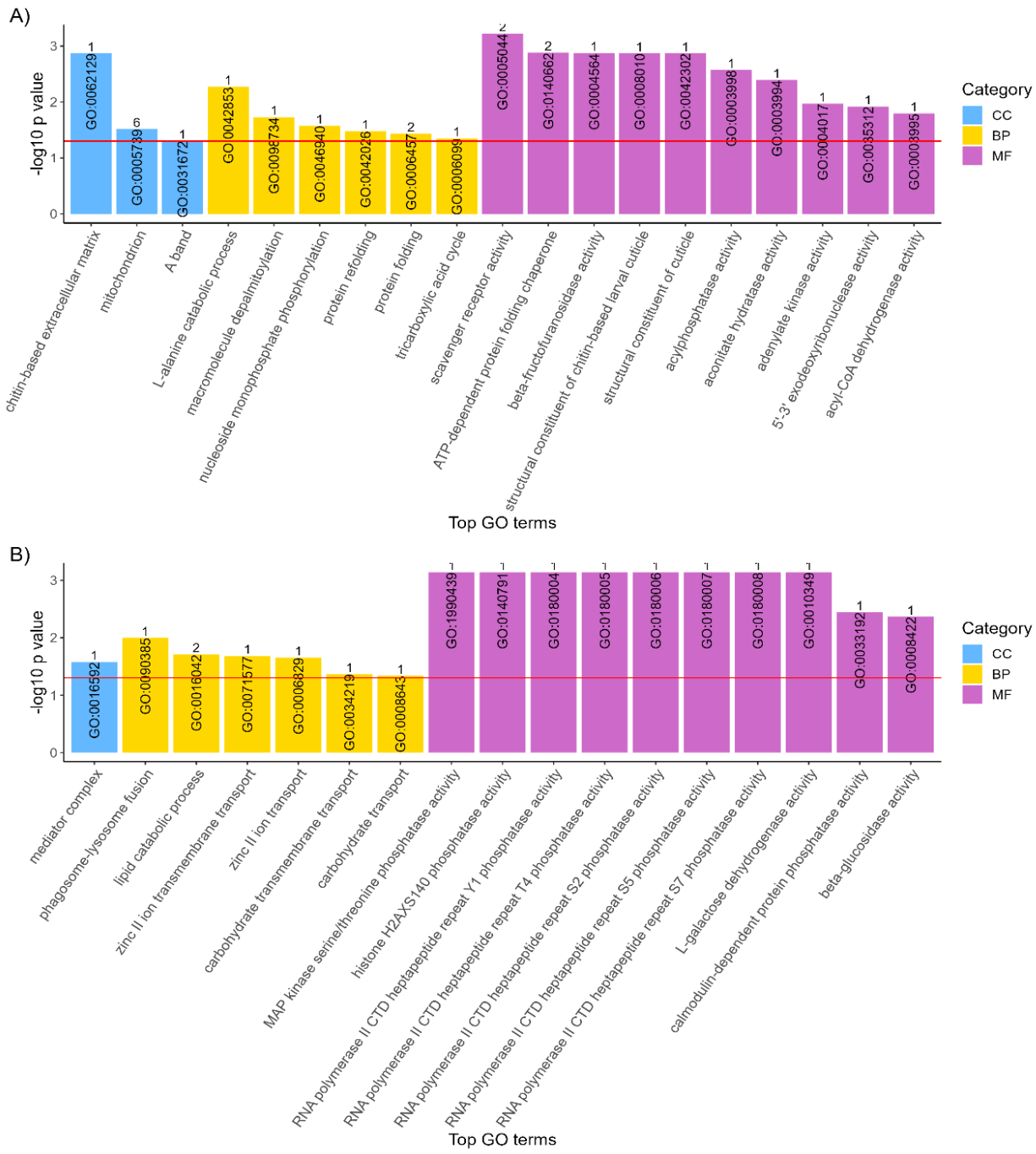

Figure 5.3 Enriched ($p < 0.05$) GO terms from the comparison between adult MPB submerged in dsIAP and adult MPB not submerged in anything. Categories are: cellular component (CC), biological process (BP), and molecular function (MF). Significance level is indicated by the horizontal red line, any bar above the line indicates $p < 0.05$. A) Enriched GO terms associated with up-regulated genes. B) Enriched GO terms associated with down-regulated genes.

Among both comparisons, there were some GO terms that are up-regulated and down-regulated regardless of what dsRNA (J22S_D or J22S_I) when compared to C22. Genes associated with mitochondria and chitin-based extracellular matrix were upregulated in both treatments. The L-alanine catabolic process, protein refolding, and tricarboxylic acid cycle were enriched biological processes in both treatments. Genes involved in the acyl-CoA dehydrogenase activity, aconitate hydrolase activity, scavenger receptor activity, structural constituent of chitin-based larval cuticle, structural constituent of cuticle, aconitate hydratase activity, carbonate dehydratase activity, lyase activity, and glutathione transferase activity molecular functions were also significantly enriched in both dsRNA conditions.

The GO terms associated with the down-regulated genes did not have any overlap between J22S_D compared to C22 and J22S_I compared to C22 in the cellular component or biological processes categories. The molecular functions of L-galactose dehydrogenase activity, beta-glucosidase activity, and sugar transmembrane transporter activity were significantly enriched in both dsRNA treatments.

In the comparison between J22S_D and C22, genes involved in oxygen binding and transport as well as odorant binding and smell were down-regulated. These genes were not significantly down-regulated in the dsIAP-submerged MPB when compared to MPB not submerged.

5.3.3.3 Phloem-fed control comparisons

The gene ontology enrichment analysis for PHPF_D compared to C23 yielded 53 GO terms associated with the down-regulated genes and 104 GO terms associated with the up-regulated genes. Within the list, 41 of the up-regulated and 22 of the down-regulated GO terms were enriched ($p < 0.05$, see Appendix, Tables S15 and S16). The top 10 enriched GO terms (i.e.

the 10 GO terms with the smallest p value) from each category were selected for visualization (Figure 5.4).

The enriched GO term annotation in the cellular component domain from the up-regulated genes was chitin-based extracellular matrix. The top three GO term annotations from the biological process domain were: cellulose catabolic process, fatty acid biosynthetic process, and unsaturated fatty acid biosynthetic process. The top three GO term annotations from the molecular function domain were: cellulase activity, stearoyl-CoA 9-desaturase activity, and transmembrane monodehydroascorbate reductase activity.

The three enriched GO term annotations in the cellular component domain from the down-regulated genes were the chitin-based extracellular matrix, origin recognition complex, and nuclear origin of replication recognition complex. The top three GO term annotations from the biological process domain were: dUMP biosynthetic process, dUTP catabolic process, and regulation of synaptic transmission (cholinergic). The top three GO term annotations from the molecular function domain were: structural constituent of chitin-based larval cuticle, structural constituent of cuticle, and dUTP diphosphatase activity.

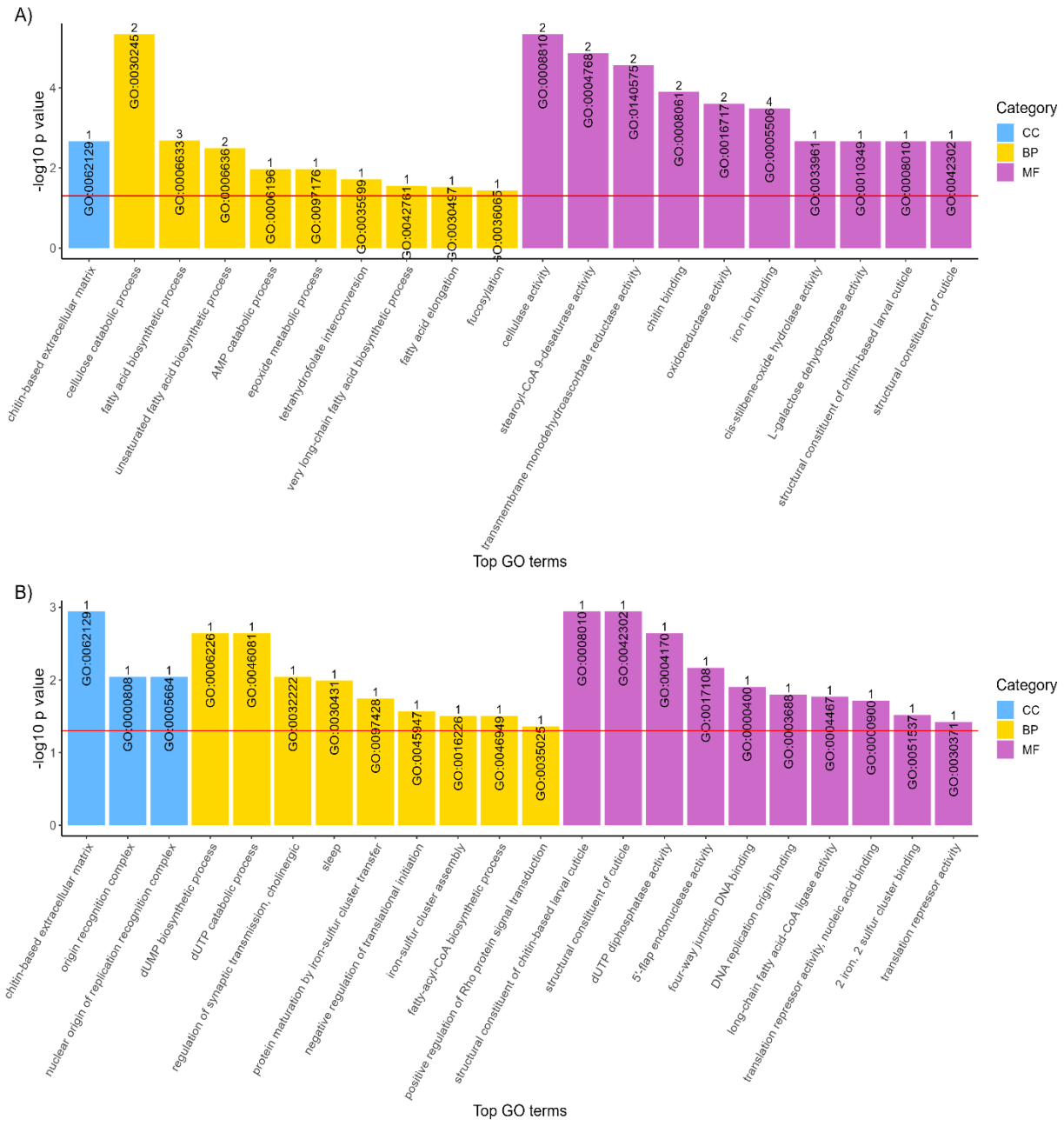

Figure 5.4 Enriched ($p < 0.05$) GO terms from comparison between adult MPB fed dsRS-treated phloem (PHPF_D) and adult MPB not fed (C23). Categories are: cellular component (CC), biological process (BP), and molecular function (MF). Significance level is indicated by the horizontal red line, any bar above the line indicates $p < 0.05$. A) Enriched GO terms associated with up-regulated genes. B) Enriched GO terms associated with down-regulated genes.

The gene ontology enrichment analysis for PHPF_W compared to C23 yielded 83 GO terms associated with the down-regulated genes and 92 GO terms associated with the up-

regulated genes. Within the list, 43 of the up-regulated and 32 of the down-regulated GO terms were enriched ($p < 0.05$, see Appendix, Tables S13 and S14). The top 10 enriched GO terms (i.e. the 10 GO terms with the smallest p value) from each category were selected for visualization (Figure 5.5).

The enriched GO term annotation in the cellular component domain from the up-regulated genes was the peroxisome. The top three GO term annotations from the biological process domain were: cellulose catabolic process, pectin catabolic process, and carbohydrate metabolic process. The top three GO term annotations from the molecular function domain were: cellulase activity, hydrolase activity acting on glycosyl bonds, and hydrolase activity hydrolyzing O-glycosyl compounds.

The enriched GO term annotation in the cellular component domain from the down-regulated genes was the mitochondrion. The top three GO term annotations from the biological process domain were: fatty acid beta-oxidation, sucrose biosynthetic process, and isopentenyl diphosphate biosynthetic process (mevalonate pathway). The top three GO term annotations are: citrate (Si)-synthase activity, diphosphomevalonate decarboxylase activity, and acetate CoA-transferase activity.

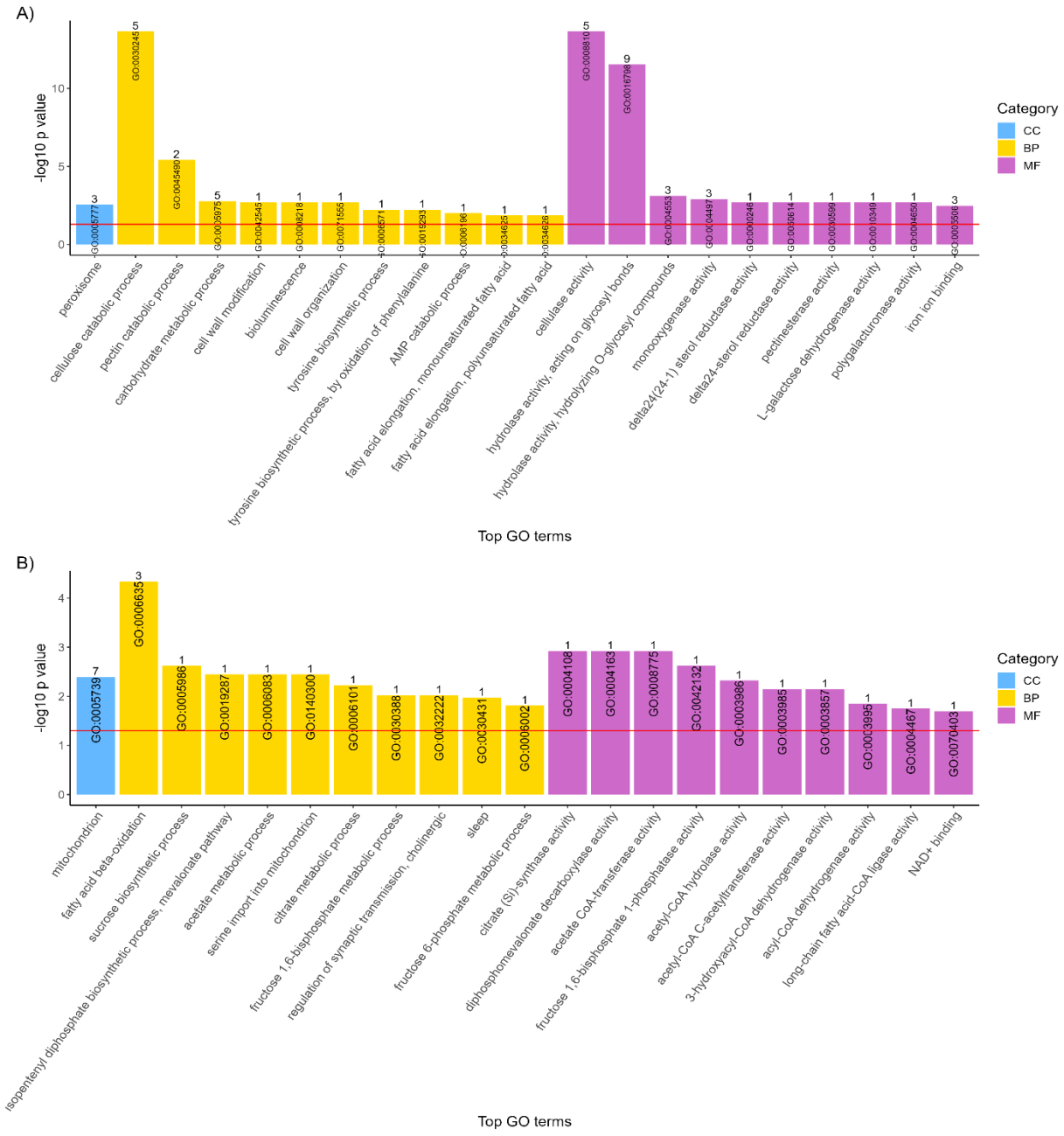

Figure 5.5 Enriched ($p < 0.05$) GO terms from comparison between adult MPB fed water-treated phloem (PHPF_W) and adult MPB not fed (C23). Categories are: cellular component (CC), biological process (BP), and molecular function (MF). Significance level is indicated by the horizontal red line, any bar above the line indicates $p < 0.05$. A) Enriched GO terms associated with up-regulated genes. B) Enriched GO terms associated with down-regulated genes.

The gene ontology enrichment analysis for PHPF_D compared to PHPF_W yielded 29 GO terms associated with the down-regulated genes and 21 GO terms associated with the up-

regulated genes. Within the list, seven of the up-regulated and 15 of the down-regulated GO terms were enriched ($p < 0.05$, see Appendix, Tables S17 and S18). The top 10 enriched GO terms (i.e. the 10 GO terms with the smallest p value) from each category were selected for visualization (Figure 5.6).

The one enriched GO term annotation in the cellular component domain from the up-regulated gene was the chitin-based extracellular matrix. The only enriched GO term annotation from the biological process domain was tissue regeneration The top three GO term annotations from the molecular function domain were: structural constituent of chitin-based larval cuticle, structural constituent of cuticle, and glucuronosyltransferase activity.

There were no enriched GO term annotations in the cellular component domain from the down-regulated genes. The top three GO term annotations from the biological process domain were: cellulose catabolic process, dUMP biosynthetic process, and the dUTP catabolic process. The top three GO term annotations were: cellulase activity, dUTP diphosphatase activity, and phenylalanine 4-monooxygenase activity.

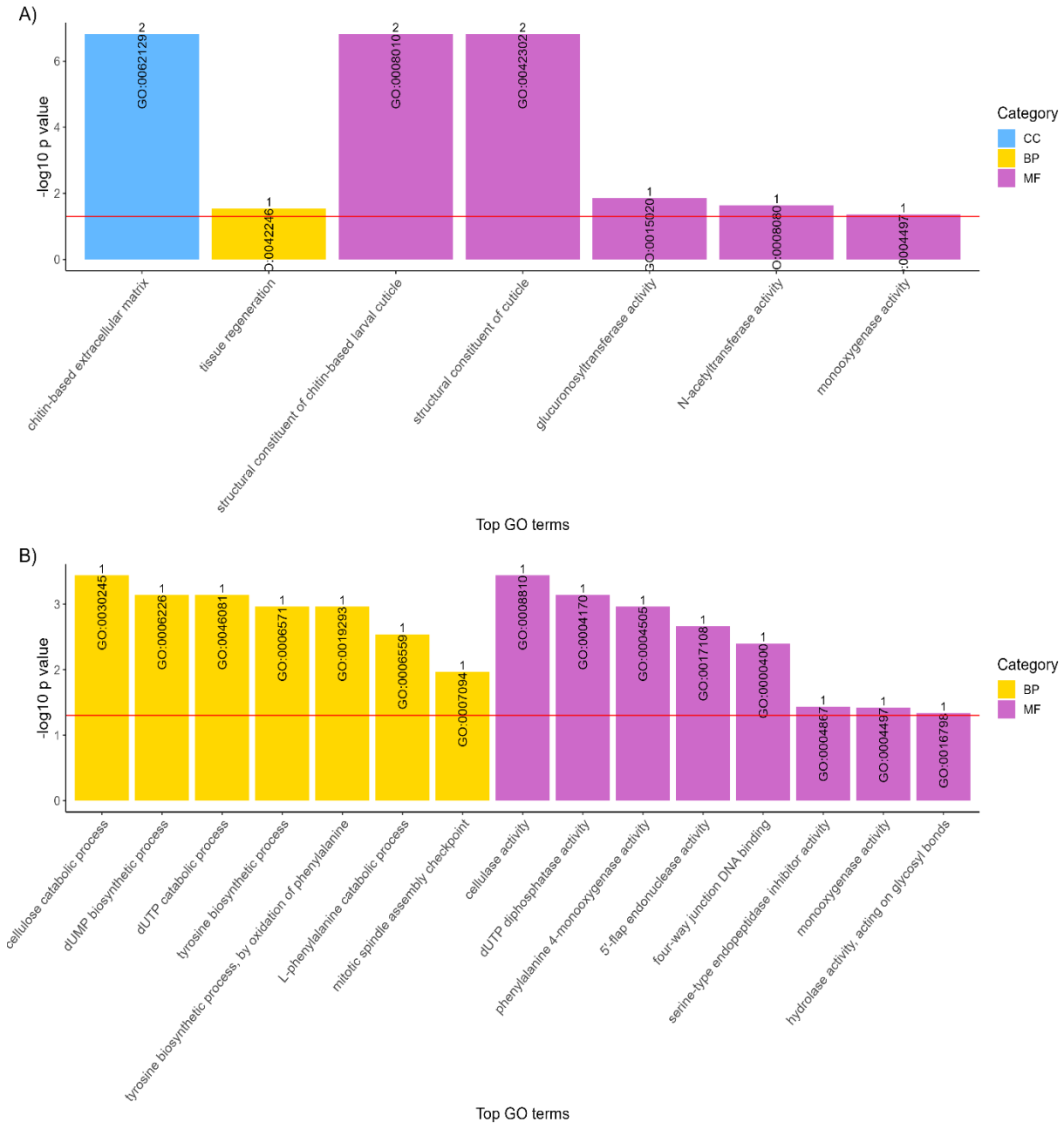

Figure 5.6 Enriched ($p < 0.05$) GO terms from comparison between adult MPB fed dsRS-treated phloem (PHPF_D) and adult MPB fed water-treated phloem (PHPF_W). Categories are: cellular component (CC), biological process (BP), and molecular function (MF). Significance level is indicated by the horizontal red line, any bar above the line indicates $p < 0.05$. There are no enriched cellular components associated with the up- or down-regulated genes. A) Enriched GO terms associated with up-regulated genes. B) Enriched GO terms associated with down-regulated genes.

Among the three different phloem-fed comparisons, there were some GO terms that were up-regulated and down-regulated when PHPF_D or PHPF_W were compared to C23. The GO

terms associated with the up-regulated genes in PHPF_D compared to C23 and PHPF_W compared to C23 both included the chitin-based extracellular matrix in the cellular component category. There were five enriched biological processes that were associated with the up-regulated gene lists: cellulose catabolic process, fatty acid biosynthetic process, L-galactose dehydrogenase activity, AMP catabolic process, very long-chain fatty acid biosynthetic process. There were 10 enriched terms that overlapped in the two up-regulated lists for molecular function (see Appendix, Tables S13 and S15).

Some molecular function terms overlap between the PHPF_D-versus-PHPF_W up-regulated gene list, the PHPF_D-versus-C23, and PHPF_W-versus-C23 lists. The structural constituent of chitin-based larval cuticle and structural constituent of cuticle are enriched in the PHPF_D-versus-PHPF_W and PHPF_D-versus-C23 comparisons. One GO term, the monooxygenase activity molecular function was enriched in all three comparison up-regulated gene lists.

The GO terms associated with the down-regulated genes in the PHPF_D-versus-C23 list and the PHPF_W-versus-C23 list did not have any overlap in the cellular component category. The regulation of synaptic transmission (cholinergic), sleep, protein maturation by iron-sulfur cluster transfer, iron-sulfur cluster assembly, fatty-acyl-CoA biosynthetic process, and positive regulation of Rho protein signal transduction were enriched biological processes. Enriched molecular functions included long-chain fatty acid-CoA ligase activity and 2 iron, 2 sulfur cluster binding. The PHPF_D-versus-PHPF_W list of GO terms associated with the down-regulated genes did not have any shared terms with the PHPF_W-versus-C23 comparison list. There were shared GO terms between the PHPF_D-versus-PHPF_W list and the PHPF_D-versus-C23 list. Three biological processes were downregulated in both lists, the: dUTP

diphosphatase activity, dUMP biosynthetic process, and dUTP catabolic process. Two molecular functions, 5'-flap endonuclease activity and four-way junction DNA binding were shared among the two down-regulated lists.

Two molecular functions, the structural constituent of chitin-based larval cuticle and structural constituent of cuticle were both up- and down-regulated in PHPF_D. Up-regulation of these functions occurs when compared to PHPF_W and C23. These functions were simultaneously down-regulated in the PHPF_D compared to C23. The chitin-based extracellular matrix in the cellular component category is also up- and down-regulated in PHPF_D compared to C23.

5.4 Discussion

5.4.1 Presence of genes coding for critical RNAi machinery was confirmed in all MPB

Genes that code for proteins involved in the RNAi machinery were previously identified in Colorado potato beetles (*Leptinotarsa decemlineata*) and the six-spined ips (*Ips calligraphus*) (Yoon et al. 2016; Wallace and Rieske 2023). These include *argonaute – 1* and *2*, *aubergine*, v-ATPase 16 kDa subunit (*vha16*), and *dicer*. An additional component, the SID1 transmembrane protein, is known to be involved in dsRNA uptake and systemic spread (Haller et al. 2019). The *staufen* gene is involved in siRNA formation from dsRNA and has only been identified in Coleopteran insects (Yoon et al. 2018; Wallace and Rieske 2023). In nematodes, like *Caenorhabditis elegans*, *SID-2* is required for dsRNA uptake from the environment (Winston et al. 2007; McEwan et al. 2012). Orthologs of this gene have been identified in birds and mammals, listed as SID1 transmembrane family member 2, but not in Coleopteran insects (NCBI, 2025). However, dsRNA uptake via the SID-1 transmembrane channel-mediated pathway was supported in Colorado potato beetles, and along with successful environmental RNAi in other studies, provides support for the presence of a SID-2 like protein (Zhu et al. 2011; Cappelle et al. 2016; Rodrigues et al. 2021) Five of the eight genes were found in the mountain pine beetle genome, with no results for the *aubergine*, *argonaute-1*, and *SID-2* genes (Keeling et al. 2021). Two loci were found for the protein argonaute-2, SID1 transmembrane family member 1, and dicer-coding genes (Table 5.2). All eight loci belonging to previously identified genes were present, but not significantly up-regulated or down-regulated in this TagSeq study, supporting the assumption that MPB contain the necessary components for an RNAi response (Yoon et al. 2016; Wallace and Rieske 2023).

The absence of SID-2 might explain some of the variability in RNAi success within this study. In SID-2 knockdown *C. elegans*, resistance to ingestion or absorption of dsRNA was observed (McEwan et al. 2012). After injection, these knockdown worms were able to show a systemic response due to *SID-1* function. Therefore, dsRNA uptake from the lumen requires *SID-2* and *SID-1* facilitates the dsRNA entry into cells (McEwan et al. 2012). In most insects, *SID-2* homologs are not present (Capelle et al. 2016; Cooper et al. 2019). Orthologs of *SID-2* were present in mammals, like humans, and annotated as SID-1 transmembrane family member 2. A BLASTn search of the second SID1 transmembrane family member 1 sequence in MPB (see section 5.3.2.1, Table 5.2, LOC109533632) was a 92% match to a SID1 transmembrane family member 2- like gene in the Southeast Asian Ambrosia Beetle (*Euwallacea similis*) (BLASTn, NCBI 2025). Due to SID-2 orthologs in mammals and birds annotated as SID1 transmembrane family member 2- like, this may indicate that the second SID1 transmembrane family member 1 gene in MPB has a role similar to *SID-2* for environmental uptake. In the absence of *SID-2*, nanoparticles can increase dsRNA uptake, and have improved RNAi efficiency in *Anthonomus grandis* (Christiaens et al. 2020). Further investigation is required to determine whether a *SID-1* gene functions like a *SID-2* gene, or if an alternative dsRNA delivery method using nanoparticles or paperclip RNA should be used.

5.4.2 No significant differential gene expression when adult MPB injected with compared to no-injection controls

High mortality was observed in all injected adult MPB, even when injected with the water and dsRS negative controls, compared to the low mortality in non-injected control group (See Chapter 2, section 2.3.2-2.3.4). There were no significant differentially expressed genes when the TagSeq analysis was used to compare transcript abundance in C22 compared to

A16M_W or A16M_D. These results are unexpected as mortality is typically associated with gene expression changes (Leelesh and Rieske 2020; Kyre et al. 2020). One likely explanation is that the beetles tested were alive, and therefore not lethally wounded from the injection compared to the deceased beetles. One gene, *venom allergen 5*, was found to be significantly down-regulated between A16M_D and A16M_W. Differential gene expression of a single gene does not support the genomic response to microinjection that was predicted based on the MPB mortality observations.

5.4.3 Differentially expressed genes in the comparisons show similar response to dsRS and dsIAP.

Many genes were significantly up-regulated or down-regulated in J22S_I compared to C22. The targeted gene, *iap*, was not among this list. A search into the non-significant results showed a very small down-regulation of the *iap* gene. This was unexpected due to the ddPCR quantifying a statistically significant decrease in *iap* expression in J22S_I. The absence of a lethal response in the treated beetles might be explained by the small decrease in *iap* expression. However, the up-regulation of stress response genes, like *hsp*, with similar roles to *iap* may indicate that other genes were compensating for the down-regulation of *iap*. Additionally, the J22S_I and C22 beetles emerged at different times, and though they were subject to the same storage conditions, the emergence times may be reflective of physiological differences (see Chapter 6, section 6.2.1). Therefore, it is possible that the *iap* expression comparison was not as exact as comparing J22S_D to J22S_I. No significant differentially expressed genes were identified between J22S_D to J22S_I, which were from the same bioassay. These results do not support the ddPCR results and suggest the samples size for this TagSeq analysis was too small

and a larger sample size, as are typically used in this cost-effective analysis, is needed (Lohman et al. 2016).

5.4.4 Differential gene expression indicates a genomic response in MPB when ingesting phloem.

Adult MPB fed phloem associated with hpRS-expressing yeast (PHPF_D) or water (PHPF_W) had the largest number of significant genes when compared to beetles that were not fed or treated with any hpRNA (C23). This was expected due to the previously identified up-regulation of metabolic genes, especially for carbohydrate metabolism (Robert et al. 2013; Tittiger et al. 2005). These include cytochrome P450s, beta-glucosidase, endopolygalacaturonase, and esterases, among others. Down-regulated genes are also mostly as expected based on previous studies, like actin (Tittiger et al. 2005). Interestingly, peritrophin 1, a gene previously down-regulated in feeding bark beetles was up-regulated in PHPF_D (Tittiger et al. 2005). Peritrophins are the main constituent of the peritrophic membrane in insects (Dias et al. 2019). The peritrophic membrane has an important role in digestion, which can explain the up-regulation of this gene in MPB ingesting phloem.

Differential gene expression as a response to ingestion is further supported by the lower number of significant genes that were up- or down-regulated when comparing PHPF_D and PHPF_W. These results provide confirmation that the beetles were ingesting the phloem, and presumably the target liquid as well. With the long list of up-regulated and down-regulated genes, further annotation is needed to identify possible patterns or responses to this hpRNA delivery method and ingestion of the negative control liquids.

5.4.5 Submergence comparison gene ontology reveals unexpected gene regulation

5.4.5.1 Functional annotation of up-regulated genes shows an increase in glycolysis

Many of the enriched GO terms for the up-regulated genes in J22S_D or J22S_I to C22 comparisons were associated with biological processes and molecular functions involved in metabolism. The only cellular component that was enriched in both submergence treatments was the mitochondrion, which was up-regulated in comparison to C22. Mitochondria have a role in many important processes, including immune response, stress sensing, signaling, cell survival and metabolism (Lubawy et al. 2022). In both J22S_I and J22S_D, the tricarboxylic acid (TCA) cycle was an enriched biological process, along with L-alanine catabolic process. Components of fatty acid metabolism were also up-regulated. Up-regulation of glycolysis was expected, due to the starvation of the adult MPB before treatment. In an agricultural pest, *Helicoverpa armigera*, similar up-regulation of glycolysis and fatty acid metabolism was reported after 48h of starvation (Jiang et al. 2019b). These processes allow for conversion of stored energy into glucose for insect energy requirements (Jiang et al. 2019b).

Functions related to the exoskeleton were up-regulated in beetles within both J22S_I and J22S_D treatments relative to C22. While the exoskeleton undergoes alterations with insect development, allowing for insect growth, emerged adults are not expected to be undergoing molting (Huang et al. 2025). It is speculated that the specific up-regulation of larval cuticle and cuticle structural constituents might be due to the recent emergence of the tested MPB. The soft cuticle, like that found in developing insects, is also found in more flexible body parts, like wings (Lodhi et al. 2024). The up-regulation of these functions may be preparation for the flight period usually occurring right after emergence (Bleiker and Van Hezewijk 2016; Chiu and Bohlmann 2022). This could also be linked to the peritrophic membrane, which is similar to the

cuticle, and it has been identified that chitin synthases are involved in peritrophic membrane formation (Dias et al. 2019). This is likely connected to the up-regulation of the chitin-based extracellular matrix. This is further supported by the up-regulation of peritrophin-1, a component of the peritrophic membrane in phloem-fed beetles (see section 5.4.4).

Beetles in J22S_I showed up-regulation of processes and functions that were involved in stress responses relative to C22. Biological processes of protein folding and refolding and the ATP-dependent protein folding chaperone molecular function were enriched. Under stress conditions, protein misfolding increases and can impact signalling pathways (Lanneau et al. 2008). Heat shock proteins have ATP-dependent chaperone capabilities and can assist in protein refolding (Lanneau et al. 2008). This process would rely on ATP production from the mitochondria (Lubawy et al. 2022). Additionally, scavenger receptor and glutathione transferase activities have been previously identified as immune responses. Scavenger receptors are involved in the endocytosis of apoptotic cells (Robert et al. 2013). Up-regulation of glutathione transferase activity, which metabolizes toxins, in response to feeding has been reported in MPB (Robert et al. 2013). It is likely that the glutathione transferase activity was responding to the presence of terpenoids in the phloem (Chiu and Bohlmann 2022). Speculation that the stress may be in response to dsIAP, as it is not in the J22S_D comparison, is negated by the absence of any significant differentially expressed genes between J22S_I and J22S_D treatment groups.

Processes and functions involved in the mevalonate pathway, like isopentyl diphosphate biosynthesis and delta-isomerase activity were up-regulated in J22S_D. The mevalonate pathway produces frontalin, an anti-aggregation pheromone that was previously determined to be produced upon feeding (Robert et al. 2013; Keeling et al. 2016; Sun et al. 2021). This is the second response in submerged beetles to show similar responses as feeding MPB, as was

observed in the glutathione transferase activity (Robert et al. 2013; Keeling et al. 2016). However, up-regulation of products in this pathway have been linked to the typical increase in pheromone biosynthesis after adult emergence in other insects (González-Caballero et al. 2014).

5.4.5.2 Down-regulated gene functional annotation supports starved conditions in MPB submergence

In both J22S_I and J22S_D treatments, synthesis of sugars and fatty acids were down-regulated relative to the C22 group. Previous up-regulation of glycolysis, indicating the use of energy stores, suggests that the decrease in synthesis was also linked to the starved condition of the MPB (Jiang et al. 2019b). Expression of genes associated with the GO annotations relating to sugar, protein, and nucleoside transport was also decreased. Specifically, in J22S_I, the zinc II ion transport was down-regulated relative to no submergence. Transport of molecules, like sugars, is often energy-dependent (Chaudhari et al. 2025). In a state of starvation where limited energy stores are required for survival, molecule transport might be decreased to conserve energy.

Eight different phosphatases related to gene transcription in J22S_I compared to C22 were significantly down-regulated. Five act on carboxyl-terminal domain (CTD) repeats in RNA Polymerase II, and are involved in transcription regulation (Schüller 2013). Alternative phosphorylation states are required for different transcription stages (Phatnani and Greenleaf 2006). It can be speculated that the down-regulation of transcription may be linked to dsIAP uptake and RNAi initiation, as it is expected that *iap* transcription would decrease. Along with increases in stress response in the J22S_I group, this adds to the speculation that some sort of RNAi response occurred, especially when considering the digital droplet PCR (ddPCR) results (see Chapter 3, section 3.3.4). However, with no significant difference between J22S_I and

J22S_D, this was not supported by this preliminary transcriptomic analysis. Rather, the down-regulation may be due to ingestion of dsRNA liquid, as alternative food can cause transcription changes (Huang et al. 2017). This was also observed in the PHPF_D compared to PHPF_W and compared to C23 (see section 5.4.6.2).

In J22S_D, genes related to smell and oxygen were downregulated relative to C22. One possible explanation is reduced respiration due to the starved condition of the MPB. In *Helicoverpa armigera*, oxygen consumption was decreased in starved larvae compared to fed larvae (Jiang et al. 2019b). However, it would be expected to appear down-regulated in both of the submergence treatments, like the up-regulated processes involved in glycolysis. Another possible explanation is the impact of submerging the adult MPB up to the posterior end of the pronotum, and sealing the tube for 12h. Oxygen may have been too low, eliminating the need for oxygen transport. Many insects, including Coleopteran beetles, are tolerant to hypoxic or anoxic conditions for a determined period of time (Hoback and Stanley 2001). In Lepidopteran insects, adults are among the most sensitive of the life stages to hypoxia conditions, perhaps accounting for the gene regulation response (Hoback and Stanley 2001). Once again, since both groups of dsRNA-treated beetles were subject to the same submergence method, it would have been expected to be observed in both. These results showed the starved conditions for beetles in J22S_D and J22S_I compared to in C22. With no significant differentially expressed genes in the J22S_I compared to the J22S_D, no definitive conclusions can be drawn about the RNAi response.

5.4.6 Phloem-fed gene ontology

5.4.6.1 Phloem-fed up-regulated gene functional annotation confirms the ingestion of phloem.

Most of the up-regulated processes and functions in the fed adult MPB compared to the MPB that were not fed were involved in metabolic processes, with lipid production and regulation. In insects, energy obtained from diet is stored as triglycerides, and other lipids (Chaudhari et al. 2025). Up-regulation of their synthesis suggests successful phloem ingestion by adult MPB in the phloem home method. This was further supported by the up-regulation of processes and functions related to the breakdown of pectin, a large component of plant cell walls, and cellulose (Kirsch et al. 2016). The absence of stress response genes, and the up-regulation of genes previously found to be associated with feeding, supports using the phloem home method for hpRNA delivery to MPB in future RNAi testing.

Interestingly, one gene involved in the bioluminescence biological process was up-regulated in PHPF_W. This has been reported before in feeding MPB, and was theorized to be involved in detoxification and other physiological roles during host colonization (Robert et al. 2013). It was also proposed that this might indicate a form of low-light communication in MPB (Robert et al. 2013).

Other studies have demonstrated that genes involved in the mevalonate pathway were up-regulated upon feeding (Aw et al. 2010; Robert et al. 2013). This was expected due to aggregation pheromones used to attract MPB for a mass attack on a new host, and later anti-aggregation pheromones to prevent over-crowding in the host tree (Robert et al. 2013; Keeling et al. 2016; Chiu and Bohlmann 2022). This was not observed in our study. This could be affected by the longer starvation period in our study (typically up to seven days) than in the other studies

(24-48h) (Aw et al. 2010; Robert et al. 2013). In *Lutzomyia longipalpis*, genes involved in the mevalonate pathway were up-regulated after adult emergence and not directly impacted by the act of feeding (González-Caballero et al. 2014). This information can be used to speculate that a prolonged starvation period may decrease the up-regulation of genes in the mevalonate pathway in MPB upon feeding compared to non-fed MPB, but this is not a direct comparison.

When the MPB response in PHPF_D and PHPF_W were compared to each other, up-regulated genes were involved in toxin tolerance and detoxification, wound healing and exoskeleton components, and protein regulation. The toxin tolerance and detoxification, specifically in the form of glucuronosyltransferase activity and monooxygenase activity, could have been due to the terpenoids in the pine phloem, which are toxic to MPB (Chiu and Bohlmann 2022). This was supported by the absence of other genes involved in stress response, like *hsp* proteins. The absence of a large impact from hpRS delivery via phloem when compared to water supports using hpRS as a negative control in future RNAi studies.

5.4.6.2 Phloem-fed down-regulated gene functional annotation supports metabolic response to feeding.

Among the PHPF_D and PHPF_W beetles, most of the down-regulated genes were involved in metabolic processes, like glycolysis. This was expected as MPB were no longer in a starved state and did not need to use stored energy for maintenance (Jiang et al. 2019b). Rather, the stores were being filled by ingestion of phloem. One other large group of down-regulated genes were involved in DNA replication and metabolism, mostly in the PHPF_D beetles. Down-regulation of DNA replication can indicate a less favourable diet, as was observed in *Oedaleus asiaticus* fed one type of grass compared to other, optimal grasses (Huang et al. 2017). This

suggests that adult MPB were physiologically impacted by the ingestion of the hpRS along with the phloem.

Genes involved in DNA replication and metabolism were also down-regulated in PHPF_D compared to PHPF_W, providing further support that the hpRS, or presence of hpRNA, is impacting MPB. Some metabolic processes, such as cellulase, hydrolase, and monooxygenase activity, were represented by the down-regulated genes as well. This was unexpected, as these are typically associated with ingestion of plant material. However, if the hpRS is having physiological impacts on the MPB, the amount ingested was likely decreased (Huang et al. 2017). Despite down-regulation of genes associated with DNA replication indicating a negative physiological response to the ingested hpRS-expressing yeast solutions, up-regulation of genes involved in phloem breakdown and lipid storage indicates phloem ingestion and provides support for using phloem-feeding to deliver hpRNA in future investigations.

5.5 Conclusion

The 3'TagSeq and subsequential differential gene analysis comparing the different delivery methods provided insight to the MPB genomic response. Despite high mortality in all injected beetles, there was no significant gene expression between injected and non-injected beetles. The differential gene expression results showed that submerged MPB were in starved conditions, breaking down energy stores for survival. There was no significant *iap* silencing, contradicting the ddPCR results. Up-regulation of *hsp* genes was observed, showing the potential for compensation of any *iap* loss of function. Ultimately, there was no evidence for an RNAi machinery response due to no significant differences between J22S_I and J22S_D beetles. In the phloem-fed MPB, less feeding occurred when yeast was present compared to water-associated phloem, but ingestion of phloem was confirmed in both treatment groups. Out of the three delivery methods, the differential gene expression shows less stress in MPB ingesting hpRNA-associated phloem, which is promising for future RNAi trials with MPB.

Chapter 6 – Discussion and Conclusion

This study has identified promising novel gene targets and laboratory-based dsRNA and hpRNA delivery methods for RNAi initiation in emerged adult mountain pine beetles (*Dendroctonus ponderosae*, MPB). This study has also provided new insights into previously established dsRNA delivery methods. In our hands, microinjecting dsRNA into emerged adult MPB was too invasive, despite being a successful delivery method in teneral adult MPB and congeneric species. The maximum uptake success in the submergence method trials was 60%. This provides some explanation for the variation observed, potentially obscuring any suppression obtained when using this method. To advance the laboratory-based delivery method and provide support for RNAi use in lethal trap trees, we introduce a phloem-based hpRNA delivery method, adapted from Keeling et al. (2013) that led to successful ingestion, high MPB survival in controls, and the beginnings of RNAi-induced mortality. Lastly, an investigative transcriptome analysis provided more details in the MPB responses to the delivery methods and dsIAP exposure. This thesis includes four data-comprising chapters, summarized below. Chapter summaries are followed by important factors that were discovered during this thesis and suggested future directions for RNAi in MPB.

6.1 General chapter conclusions

6.1.1 Chapter two summary: microinjection is too invasive for adult mountain pine beetles

In the second chapter, microinjection was used to test six gene targets for MPB mortality using the RNAi pathway and to test the MPB-designed dsRNA on a congeneric species. Later, microinjection delivered cascading dsRNA concentrations, creating a kill-curve, to determine the optimal concentration for rapid MPB mortality. Microinjection has been used extensively in *Dendroctonus armandi* adults, resulting in RNAi initiation and no physiological stress from the

injection method (Zhang et al. 2016; Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022). Microinjection for dsRNA delivery in MPB is not as common. One study reported successful RNAi initiation after dsRNA injection into teneral adults, with a 56–83% survival of injected beetles (Keeling et al. 2013). In our study, all injected MPB showed high mortality, likely due to lethal injuries caused by using a larger needle than was used in *D. armandi* and teneral adult MPB injections (Keeling et al. 2013; Sun et al. 2021; Liu et al. 2021, 2022; Liu and Chen 2022). The congeneric species, the Douglas Fir beetle (*Dendroctonus pseudotsugae*, DFB), showed less initial mortality in response to microinjection and no physiological response to the MPB-dsRNA. Our results show that our microinjection delivery method, using needles that are approximately twice as large as those in *D. armandi* and much larger than those used for teneral adult MPB, is too invasive for emerged adult MPB and support other methods for dsRNA delivery to initiate RNAi for emerged adult MPB.

6.1.2 Chapter three summary: submergence irregularly elicits an RNAi response in mountain pine beetles

A method that involves dsRNA ingestion is more practical than microinjection for pest management applications. Submerging MPB in a liquid solution of dsRNA has previously been successful at initiating the RNAi pathway (Kyre et al. 2020). This method was used in the exhaustive third chapter to test the six gene targets. Our study added another variable and tested two different dsRNA sources: kit-synthesized dsRNA (over 200 nucleotides, see Chapter 2, section 2.2.2) and hpRNA produced and expressed in a *Saccharomyces cerevisiae* strain. Successful RNAi-mediated suppression was achieved in four of the six targets in an assay using *in vitro* synthesized dsRNA. Decreased *iap* expression as a response to submergence in dsIAP was initiated in two of four assays. This was less than expected as *iap* was a previously

successful target in MPB and *Ips calligraphus* (Kyre et al. 2020; Wallace and Rieske 2023). In the same assay, *mesh*, *ssk*, and *vha19* up-regulation was observed in response to *in vitro* synthesized dsMESH, dsSSK, and dsVHA19, respectively. In a subsequent assay, submergence in dsMESH and dsVHA19 caused gene suppression.

This study represents the first to test hpRNA-expressing yeast for RNAi initiation in MPB. There was no successful RNAi initiation when both female and male MPB were submerged in hpRNA-expressing yeast solutions. However, the results were likely impacted by factors such as experimental pre-emergence cold storage and emergence timing, as there was no response to the *in vitro* synthesized dsMESH, dsIAP, and dsVHA19 in these assays, either. Colder temperatures during the development can impact beetle health upon emergence into warmer temperatures (Huho et al. 2007; Sørensen et al. 2012).

Our study was also the first to incorporate consecutive time points, 24h, 48h, and 72h, for MPB gene expression quantification after dsRNA exposure. The results show that target gene expression does not change significantly over the three time points. However, the difference between the relative gene expression in the treatment group compared to the relative gene expression in the control groups does change over time. This shows the variability in the RNAi results obtained in the submergence assays performed, which is likely explained by random chance or the incomplete uptake success from this method (see Chapter 3, section 3.3.2). When comparing the target gene expression in the treatment group to three negative controls, the gene expression was only significantly decreased in the treatment group compared to one or two control groups, never all three. Generally, target gene expression in the dsRS group, which controls for the most experimental conditions out of the three controls, was not significantly different from gene expression in the target dsRNA group.

6.1.3 Chapter four summary: ingestion of phloem associated with dsRNA-expressing yeast solutions shows promise for further testing

A novel method for hpRNA delivery to MPB was tested in the fourth chapter. This phloem-feeding method, adapted from Keeling et al. (2013), entices dsRNA ingestion by associating it with phloem. Termed "phloem home" (C.I. Keeling and C.C. Chiu, personal communication, 2022), MPB were placed one-per-well into a 24-well plate. Each well contained two phloem layers, and each layer was treated with hpIAP-expressing yeast or the negative control solutions of water and hpRS-expressing yeast. Initial trials testing three phloem and yeast solution associations: yeast solutions painted on phloem (PHP), yeast solutions translocated through the phloem (PHT), and phloem soaked in yeast solutions (PHS) yielded no MPB response via mortality or gene expression. However, MPB survival in the negative controls was high. Upon increasing the concentration of the hpIAP-expressing yeast solutions painted onto the phloem, the start of a mortality response was observed but interrupted by the presence of fungus. Our results show that this method has potential to be a lab-based method for hpRNA delivery to MPB. On a larger scale, this method shows the potential for effective RNAi use in trap trees to cause targeted MPB mortality.

6.1.4 Chapter five summary: identification of the metabolic processes involved in the delivery methods provides support for future directions

In the fifth chapter, an investigative transcriptomic analysis was used to clarify the variable results in the three previous chapters. Genes identified to be critical to the RNAi pathway, *argonaute -2*, *dicer*, SID1 transmembrane family member, and double-stranded RNA-binding protein Staufen homolog (Yoon et al. 2016; Wallace and Rieske 2023) were found in the adult MPB in all experimental conditions. In both dsRNA submerged treatments, differentially

expressed genes were mostly involved in metabolism. Genes involved in glycolysis were up-regulated, indicating mobilization of energy stores due to the starved conditions (Jiang et al. 2019b). In the dsIAP-treated MPB, genes involved in stress response were up-regulated. The stress response was not observed in the negative control dsRNA. In the dsRS treatment group, genes involved in odorant reception and oxygen transport were down regulated. While it would be expected to be down-regulated in both treatments, this could be an effect of hypoxia conditions.

In MPB fed water-treated phloem (PHPF_W) and hpRS-expressing yeast associated phloem (PHPF_D) relative to unfed controls (C23) (see Chapter 5, section 5.3.2.4), up-regulated genes demonstrated successful phloem ingestion due to the genes being involved in lipid synthesis, the main form of storage in MPB, and plant material breakdown (Chaudhari et al. 2025). The absence of up-regulated genes involved in the mevalonate pathway in our study was unexpected, but could indicate that these genes were up-regulated upon emergence, like in Dipteran insects (González-Caballero et al. 2014), rather than by feeding alone (Robert et al. 2013).

Down-regulated genes were involved in glycolysis, supporting the hypothesis that feeding MPB were storing energy rather than mobilizing energy stores. In the PHPF_D compared to PHPF_W and C23, genes involved in DNA replication and metabolism were down-regulated. This has been connected to less favourable diet ingestion in grasshoppers (*Oedaleus asiaticus*), and could point to a negative response to dsRNA. However, the up-regulation of feeding-related genes does confirm that ingestion of the hpRS-treated phloem occurred.

6.2 Important factors to consider for MPB bioassays

6.2.1 Beetle emergence timing

Conducting bioassays using three delivery methods with beetles from 3 annual emergence events, I have observed that when MPB emerged later in the season compared to the initial emergences, the MPB exhibited poor health. Previous studies have mentioned a short storage time of emerged adult MPB before use in RNAi bioassays (Kyre et al. 2020). Post-emerged MPB have also been categorized in a previous study where after three days, MPB were considered middle-aged until day nine when they were categorized as old (Evenden et al. 2014). These categorizations are based on dispersal patterns in the wild and the maturation of MPB (Evenden et al. 2014). In this study, I observed that the time of MPB emergence impacts their health, potentially more than the post-emergence storage time. Examples of this are provided by comparing submergence bioassays in this study. The terms early and late emergence are relative to the season and based on when the first MPB emerges. In 2022 and 2024, emergence started in early July, but in 2023 emergence started in early June. Emergence is considered early within a two-week period of that initial emergence, and the middle ground is up to a month from first emergence. Emergence is categorized as late a month or more after the initial emergence. Beetle health becomes more crucial in submergence experiments, as it uses starvation conditions. Beetle survival in the controls was higher in the assays performed with early-emerged MPB, like the submergence assay in 2024 with more than 50% MPB survival on day seven (see Chapter 3, section 3.3.7). The MPB survival in the negative controls for the male and female submergence assays from 2023, using late-emerged MPB, was low and indistinguishable from the gene target groups, with 50% or less MPB alive by day seven (see Chapter 3, sections 3.3.5 and 3.3.6). The 2022 submergence assay was in the middle ground regarding emergence time, and MPB survival

in the negative control was above 75% on day seven, but it rapidly decreased and was at 100% mortality by day ten (see Chapter 3, section 3.3.3). This theory is also supported when comparing the MPB survival of the non-injected MPB between the 2022 and 2023 microinjection assays (see Chapter 2, sections 2.3.2 and 2.3.3, respectively). The 2022 microinjection experiment was performed using late-emerged MPB and MPB survival in the non-injected group was at 50% on day 14. In the 2023 microinjections, in which early emerged MPB were used, MPB survival in the non-injected group was above 80% by day 14. The decreased health of MPB that emerge later in the flight period may be due to the collection method used in this study (see Chapter 2, section 2.2.1). The use of small bolts may cause the phloem to lose moisture as time progresses. Therefore, MPB that emerge early are reared in conditions similar to nature. Later emergence exposes MPB to drier phloem and increased monoterpenes, as moisture was noted to decrease to 64% or lower after six months in jack pine (*Pinus banksiana*) bolts (Guevara-Rozo et al. 2019). Reduced moisture was predicted to impact larval development in MPB (Guevara-Rozo et al. 2019). The impact of emergence timing on MPB health in this study is likely influenced by moisture levels and other differences in bolts compared to living trees.

6.2.2 Beetle sex in assays

Another important factor in assays was the sex of MPB. This is important due to possible differences in relative gene expression of the target gene between males and females before dsRNA exposure as well as the potential different responses to RNAi between sexes. In a population of MPB from Utah, USA, relative gene expression of *hsp*, *iap*, and *shibire* (*shi*) was lower in males than females, but the only significant difference was in *hsp* (Kyre et al. 2020). After dsRNA exposure, only female MPB showed significant *hsp* suppression (Kyre et al. 2020).

Investigations into *iap* expression show that this sex-linked gene is expressed less on the neo-Y alleles, but this is compensated for by the neo-X allele, allowing similar *iap* expression between males and females (Horianopoulos et al. 2018). Horianopoulos et al. (2018) also discovered evidence that a northern neo-X allele showed *iap* up-regulation earlier in the fall, and the presence or absence of this allele could impact *iap* expression in male MPB, an important factor to consider in future dsIAP assays.

Our study was the first to test V-type proton ATPase subunit S1 (*vha19*), mesh protein (*mesh*), snakeskin protein (*ssk*), and tetraspanin 2a (*tsp2a*) for targeted mortality in MPB. Previous RNAi bioassays testing *ssk*, *mesh*, and *v-Atpase A* homologs in *Diabrotica virgifera virgifera* larvae did not include sexing of the insects. Investigation of *tsp2a* as a knockdown target in *Drosophila melanogaster* used only females, and reported mortality due to prevention of fluid excretion (Beyenbach et al. 2020). Knockdown of *mesh* was also observed in only female *Drosophila*, and only had a lethal impact in larvae, due to the importance of mesh in Malpighian tubule (Jonusaite et al. 2020). Lastly, knockdown of *vha19* in *Haemonchus contortus* larvae did not include identification of the larvae sex, and caused changes in larvae development and viability (Zawadzki et al. 2012).

Initial microinjection and submergence assays did not consider MPB sex (see Chapter 2, section 2.3.2 and Chapter 3, section 3.3.3, respectively). Based on previous literature, this likely impacted *hsp* silencing, but is not expected to have affected the other five gene targets (Kyre et al. 2020). Subsequent bioassays in our study focused mostly on females, with a few males used when needed for numbers. Submergence assays included only one sex, with partial knockdown successful in females and eliminating concerns for sex-specific responses to the dsRNA (see Chapter 3, section 3.3.6). In the microinjection assays that followed, MPB were sexed, and males

and females used were kept as even as possible. Most of the phloem homes were predominantly female, but males were needed to increase the number of MPB (see Chapter 4, sections 4.2.6, 4.2.8, and 4.2.9). In the silencing groups, males and females were kept even, but noted. The only gene target was *iap*, which was not found to have significant differences in gene expression between male and female MPB (Horianopoulos et al. 2018; Kyre et al. 2020). Therefore, this is not expected to impact results, and the final phloem home only tested females to remove this potential variable (see Chapter 4, section 4.2.7).

Due to the identification of MPB sex used in the phloem home bioassays, the component explaining the highest amount (17%) of the differential gene expression in the TagSeq analysis was determined to be sex based. This variable was removed as it obscured other components that were predicted to explain the impacts of the delivery methods or dsRNA exposure (see Chapter 5, section 5.3.2). Therefore, for RNAi bioassays with MPB, the sex of tested beetles should at least be determined and recorded, though I recommend to use single-sex bioassays while in the target identification stages.

6.2.3 Sample size

The variability in the gene expression analysis results may also indicate that there is not enough statistical power. With higher power, the Type II error probability, in which the null hypothesis is falsely accepted, is decreased (Gupta et al. 2016). For many studies, power within the range of 80-90% is standard (Gupta et al. 2016). Here, I used a small set of bioassays for statistical power and sample size calculations on the gene expression analyses to determine if power could be contributing to the variable results. All tests were performed using the “pwrss” package (Bulus 2022) in R Statistical Software (v4.3.1; R Core Team 2023).

The first analysis was performed on two genes with no significant gene expression changes in the 2022 submergence assay (see Chapter 3, section 3.3.4.1, Figure 3.3 B&D). Previous mortality was observed with approximately a 50% decrease in gene expression (Kyre et al. 2020). This study would have 80% power to detect significance with a 50% decrease or increase in *hsp* expression in the dsHSP group compared to dsRS. With a small increase observed in the *hsp* expression in the dsHSP group, it is expected that this indicates no significant difference. In order to observe a 50% decrease or increase in *tsp2a* expression in the dsTSP2A group, a slightly larger sample size of n = 6 would give a power of 80%. However, as a decrease in *tsp2a* is expected, a one-sided Student's t-test would require the current sample size, n=5, to have 80% power.

In order to have the statistical power to reduce Type II errors with small differences in gene expression, a sample size of 15-30 beetles is required. While these sample sizes are manageable, the purpose of the research should be considered. In our study, the goal is to achieve a lethal response in MPB, which is not occurring with small decreases in mean gene expression. Gene expression decreases of 50% or larger were statistically significant and associated with emerald ash borer larvae, SPB adult, and MPB adult mortality (Kyre et al. 2019, 2020; Leelesh and Rieske 2020). Therefore, future studies should aim for a sample size providing 80-90% power for a change in gene expression around 50%.

When more controls are included, and thus more statistical comparisons performed, a larger sample size is required for statistical power. Sample size calculations from the 2024 female submergence assay gene expression analysis indicate a range of 10-40 beetles would be the required sample size to have 80% power in the ANOVA with the current variances (see Chapter 3, section 3.3.7.1). Two exceptions to the sample size range are the dsMESH groups at

24h and 48h, where a much larger and unobtainable required sample size was calculated (see Chapter 3, section 3.3.7.1.3). A sample size range of 80-120 beetles would be needed for power to reach 80% in the PHP24 gene expression analysis by ANOVA (see Chapter 4, section 4.3.5.2). The larger sample size required likely indicates one of two things. First, it may indicate that there is no significant difference. This is likely the case in some comparisons, like the FSA (see Chapter 3, Figure 3.5B) and MSA (see Chapter 3, Figure 3.7A) *vha19* and *iap* gene expression analyses, respectively (see Chapter 3, sections 3.3.5.1 and 3.3.6.1). Second, a large required sample size could suggest that the variation in the data is too large, and impacted by other factors, like beetle health. Variation in the data due to other factors is likely the case in the dsMESH groups in the 2024 female submergence assay.

Ultimately, the sample sizes appear to be too small for some of the questions being asked in our study. Our results have limited power in the more complex gene expression analyses, and there could be significant gene expression changes that went undetected. Due to time and resource constraints, increasing the sample sizes to the required numbers is not always feasible. Additionally, the statistical power provided by our sample sizes was able to detect a 50% or greater difference in gene expression, and smaller changes in gene expression were not associated with MPB mortality. Other variables besides power and sample size were likely impacting the gene expression analysis, like beetle health (see section 6.2.1). For future RNAi investigations, it is recommended to increase sample sizes for gene expression analysis to within the range of 10-20 beetles to decrease the impact of other variables on the gene expression analysis.

6.3 Future Directions

The ambiguity of the results from this study leads to many new questions, which require more experiments. While a comprehensive list of all recommended or potential future directions for RNAi studies with MPB is not practical, a refined list is presented here.

6.3.1 Gene targets

A large variable in our study, along with many other RNAi studies, is the gene target (Cooper et al. 2019). While we have shown promise for the targets tested in this study, further investigations should be conducted using *iap*, *mesh*, *vha19*, and *ssk*. Additionally, testing different dsRNA constructs within the target gene sequences used in this study should be tested for improved effectiveness. With the results from the TagSeq analysis suggesting gene compensation when *iap* suppression was observed, combining similar gene targets, like *iap* and *hsp*, may reduce compensation and increase the efficiency of RNAi in MPB.

Another possible explanation for the absence of a mortality response in MPB after exposure to dsIAP is gene paralogs. Paralogs are duplications of a gene, some of which are functional (Deem and Brisson 2024). These paralogous genes contribute to evolution and have roles in the genetic pathways in an organism, though identification of these genes can be difficult (Dunivant et al. 2024). Therefore, it is likely that the presence of paralogous genes in an organism can impact the RNAi response, and therefore efficiency. Paralogs can be different enough to avoid RNAi-mediated suppression and may compensate for the suppression of the original gene. For example, individual targeting of paralogs was achieved in *Mimulus lewisii* (Phrymaceae) flowers (Dunivant et al. 2024). With 5.6% of the genome identified as duplicated, or paralogous, genes (NCBI 2022), investigating the specific MPB gene paralogs in future studies may provide insights to better gene targets.

Responses to the same target can be influenced by geographical factors as well. Genetic differences exist between MPB populations, especially the northern and southern populations in western Canada. Large genetic differences also exist between MPB populations around the Great Basin Desert in the United States of America (Bracewell et al. 2011, 2013; Janes et al. 2014). Different responses to the same dsRNA target were reported among different southern pine beetle (*Dendroctonus frontalis*, SPB) populations (Kyre et al. 2024). Future studies should investigate the variation in the target genes and the response in MPB from different populations in Canada and the United States of America, as the results will be crucial for the application of this method as a MPB management tool.

6.3.2 Delivery method

Our results support the effectiveness of submerging MPB in dsRNA to elicit an RNAi response, as was originally shown by Kyre et al. (2020). Our study aimed to advance the lab-based delivery to a method comparable to proposed *in situ* uses. A novel delivery method using phloem and hpRNA-expressing heat-inactivated yeast associations was tried. Upon increasing the hpIAP-expressing yeast solutions, this method showed promise for RNAi-mediated mortality, with high MPB survival in the negative controls. High MPB survival suggests that this method might also boost MPB vitality, allowing MPB that emerged later in the season to be tested and increasing productivity during the flight season. It is my recommendation to include this method in future MPB RNAi investigations, but to incorporate an anti-fungal agent, like sorbic acid (Rangasamy and Siegfried 2012; Costa and Reeve 2012; Vélez et al. 2016), to prevent fungal infections.

With the assumed absence of SID-2 in MPB, dsRNA uptake from ingestion-based delivery methods is likely limited. However, one of the SID-1 transporters could function

similarly to SID-2, allowing for environmental RNAi (see Chapter 5, section 5.4.1). Increasing the dsRNA delivered could increase the dsRNA successfully delivered to cells. Alternatively, delivering the dsRNA via nanoparticles shows promise for increasing dsRNA uptake in insects (Christiaens et al. 2020). Using a "paperclip" dsRNA structure, where both ends are closed, has increased dsRNA uptake via the clathrin-mediated pathway in *Aedes aegypt* (Abbasi et al. 2020). It is possible that using this dsRNA structure could improve RNAi sensitivity in MPB, and should be investigated in future studies.

6.4 Final remarks

It is my hope that the chapters in this thesis provide details that assist future MPB bioassays. At the beginning of this project, I was advised that bioassay planning was crucial, and I reiterate that advice here. While planning, I recommend prioritizing the more promising, and any non-feeding, bioassays for the first wave of MPB. Using early emerged MPB, as they appear to be the healthiest and will hopefully have high survival in the controls, enables any mortality response from target dsRNA delivery to be observed (see section 6.2.1).

Literature Cited

Abbasi, R., Heschuk, D., Kim, B., Whyard, S. 2020. A novel paperclip double-stranded RNA structure demonstrates clathrin-independent uptake in the mosquito *Aedes aegypti*. Insect Biochem. Mol. Biol. 127:103492. doi: 10.1016/j.ibmb.2020.103492.

Ainslie, B., and Jackson, P.L. 2011. Investigation into mountain pine beetle above-canopy dispersion using weather radar and an atmospheric dispersion model. Aerobiologia **27**(1): 51–65. doi:10.1007/s10453-010-9176-9.

Andersson, M.N., Grosse-Wilde, E., Keeling, C.I., Bengtsson, J.M., Yuen, M.M., Li, M., Hillbur, Y., Bohlmann, J., Hansson, B.S., and Schlyter, F. 2013. Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles*, Ips typographus* and *Dendroctonus ponderosae* (Coleoptera: Curculionidae: Scolytinae). BMC Genomics **14**(1): 198. doi:10.1186/1471-2164-14-198.

Andrus, R.A., Hart, S.J., and Veblen, T.T. 2020. Forest recovery following synchronous outbreaks of spruce and western balsam bark beetle is slowed by ungulate browsing. Ecology **101**(5): e02998. doi:10.1002/ecy.2998.

Anjum, A., Jaggi, S., Varghese, E., Lall, S., Bhowmik, A., and Rai, A. 2016. Identification of Differentially Expressed Genes in RNA-seq Data of *Arabidopsis thaliana*: A Compound Distribution Approach. J. Comput. Biol. **23**(4): 239–247. doi:10.1089/cmb.2015.0205.

Audley, J.P., Fettig, C.J., Steven Munson, A., Runyon, J.B., Mortenson, L.A., Steed, B.E., Gibson, K.E., Jørgensen, C.L., McKelvey, S.R., McMillin, J.D., and Negrón, J.F. 2020. Impacts of mountain pine beetle outbreaks on lodgepole pine forests in the Intermountain West, U.S., 2004–2019. For. Ecol. Manag. **475**: 118403. doi:10.1016/j.foreco.2020.118403.

Aw, T., Schlauch, K., Keeling, C.I., Young, S., Bearfield, J.C., Blomquist, G.J., Tittiger, C. 2010. Functional genomics of mountain pine beetle (*Dendroctonus ponderosae*) midguts and fat bodies. Genomics **11**:215. https://doi.org/10.1186/1471-2164-11-215

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., and Roberts, J. 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. **25**(11): 1322–1326. Nature Publishing Group. doi:10.1038/nbt1359.

Bentz, B.J., and Hansen, E.M. 2018. Evidence for a Prepupal Diapause in the Mountain Pine Beetle (*Dendroctonus ponderosae*). Environ. Entomol. **47**(1): 175–183. doi:10.1093/ee/nvx192.

Bentz, B.J., and Powell, J.A. 2014. Mountain Pine Beetle Seasonal Timing and Constraints to Bivoltinism: (A Comment on Mitton and Ferrenberg, "Mountain Pine Beetle Develops an Unprecedented Summer Generation in Response to Climate Warming"). Am. Nat. **184**(6): 787–796. doi:10.1086/678405.

Beyenbach, K.W., Schöne, F., Breitsprecher, L.F., Tiburcy, F., Furuse, M., Izumi, Y., Meyer, H., Jonusaite, S., Rodan, A.R., and Paululat, A. 2020. The septate junction protein Tetraspanin 2A is critical to the structure and function of Malpighian tubules in *Drosophila melanogaster*. Am. J. Physiol.-Cell Physiol. **318**(6): C1107–C1122. doi:10.1152/ajpcell.00061.2020.

Bingsohn, L., Knorr, E., Billion, A., Narva, K.E., and Vilcinskas, A. 2017. Knockdown of genes in the Toll pathway reveals new lethal RNA interference targets for insect pest control. Insect Mol. Biol. **26**(1): 92–102. doi:10.1111/imb.12273.

Bleiker, K. P. (Ed.). (2019). Risk assessment of the threat of mountain pine beetle to Canada's Boreal and Eastern pine forests. Canadian Council of Forest Ministers, Forest Pest Working Group

Bleiker, K.P., and Van Hezewijk, B.H. 2016. Flight Period of Mountain Pine Beetle (Coleoptera: Curculionidae) in its Recently Expanded Range. Environ. Entomol. **45**(6): 1561–1567. doi:10.1093/ee/nvw121.

Bracewell, R.R., Pfrender, M.E., Mock, K.E., and Bentz, B.J. 2011. Cryptic postzygotic isolation in an eruptive species of bark beetle (*Dendroctonus ponderosae*). Evolution **65**(4): 961–975. doi:10.1111/j.1558-5646.2010.01201.x.

Bracewell, R.R., Pfrender, M.E., Mock, K.E., and Bentz, B.J. 2013. Contrasting Geographic Patterns of Genetic Differentiation in Body Size and Development Time with Reproductive Isolation in *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae). Ann. Entomol. Soc. Am. **106**(3): 385–391. doi:10.1603/AN12133.

Bragg, Z., and Rieske, L.K. 2022a. Feasibility of Systemically Applied dsRNAs for Pest-Specific RNAi-Induced Gene Silencing in White Oak. Front. Plant Sci. **13**: 830226. doi:10.3389/fpls.2022.830226.

Bragg, Z., and Rieske, L.K. 2022b. Spatial Distribution and Retention in Loblolly Pine Seedlings of Exogenous dsRNAs Applied through Roots. Int. J. Mol. Sci. **23**(16): 9167-N.PAG. MDPI. doi:10.3390/ijms23169167.

British Columbia. (n.d.). B.C.'s Bark Beetles: What does the science say? Available from https://www2.gov.bc.ca/gov/content/industry/forestry/managing-our-forest-resources/forest-health/forest-pests/bark-beetles [accessed 30 August 2024].

Bulus, M. 2022. pwrss: Statistical Power and Sample Size Calculation Tools. doi:10.32614/CRAN.package.pwrss.

Burton, P.J. 2006. Restoration of forests attacked by mountain pine beetle: Misnomer, misdirected, or must-do? J. Ecosyst. Manag. **7**(2). doi:10.22230/jem.2006v7n2a537.

CABI Digital Library. 2020. *Dendroctonus ponderosae* (black hills beetle), https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.18354 [accessed March 2023].

Cappelle K, de Oliveira CF, Van Eynde B, Christiaens O, Smagghe G. 2016. The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. Insect Mol Biol. **25**(3):315-23. doi:10.1111/imb.12222.

Chaudhari, B.Y., Pradhan, A.G., and Joshi, R.S. 2025. Metabolic gatekeepers: Dynamic roles of sugar transporters in insect metabolism and physiology. Insect Mol. Biol. **34**(1): 1–18. doi:10.1111/imb.12963.

Chiu, C.C., and Bohlmann, J. 2022. Mountain Pine Beetle Epidemic: An Interplay of Terpenoids in Host Defense and Insect Pheromones. Annu. Rev. Plant Biol. **73**(1): 475–494. doi:10.1146/annurev-arplant-070921-103617.

Christiaens, O., Whyard, S., Vélez, A.M., and Smagghe, G. 2020. Double-Stranded RNA Technology to Control Insect Pests: Current Status and Challenges. Front. Plant Sci. **11**: 1-10. doi: 10.3389/fpls.2020.00451.

Coggins, S.B., Coops, N.C., Wulder, M.A., Bater, C.W., and Ortlepp, S.M. 2011. Comparing the impacts of mitigation and non-mitigation on mountain pine beetle populations. J. Environ. Manage. **92**(1): 112–120. doi:10.1016/j.jenvman.2010.08.016.

Cooper, A.M., Silver, K., Zhang, J., Park, Y., and Zhu, K.Y. 2019. Molecular mechanisms influencing efficiency of RNA interference in insects. Pest Manag. Sci. **75**(1): 18–28. doi:10.1002/ps.5126.

Corbett, L.J., Withey, P., Lantz, V.A., and Ochuodho, T.O. 2016. The economic impact of the mountain pine beetle infestation in British Columbia: provincial estimates from a CGE analysis. For. Int. J. For. Res. **89**(1): 100–105. doi:10.1093/forestry/cpv042.

Costa, A., and Reeve, J.D. 2012. The effect of larval predators Thanasimus dubius (Coleoptera: Cleridae), produced by an improved system of rearing, against the southern pine beetle *Dendroctonus frontalis* (Coleoptera: Curculionidae). Biol. Control **60**(1): 1–6. doi:10.1016/j.biocontrol.2011.08.006.

Coulter, S.J. 2018. Mitigation of the effect of variability in digital PCR assays through use of duplexed reference assays for normalization. BioTechniques **65**(2): 86–91. doi:10.2144/btn-2018-0058.

Cullingham, C.I., Cooke, J.E.K., Dang, S., Davis, C.S., Cooke, B.J., Coltman, D.W. 2011. Mountain pine beetle host-range expansion threatens the boreal forest. Mol. Ecol. **20**: 2157-2171. doi: 10.1111/j.1365-294X.2011.05086.x

Cullingham, C.I., Janes, J.K., Hamelin, R.C., James, P.M.A., Murray, B.W., and Sperling, F.A.H. 2019. The contribution of genetics and genomics to understanding the ecology of the mountain pine beetle system. Can. J. For. Res. **49**(7): 721–730. doi:10.1139/cjfr-2018-0303.

Cullingham, C.I., Roe, A.D., Sperling, F.A.H., and Coltman, D.W. 2012. Phylogeographic insights into an irruptive pest outbreak. Ecol. Evol. **2**(5): 908–919. doi:10.1002/ece3.102.

Deem, K.D., and Brisson, J.A. 2024. Problems with Paralogs: The Promise and Challenges of Gene Duplicates in Evo-Devo Research. Integr. Comp. Biol. **64**(2): 556–564. doi:10.1093/icb/icae009.

Dhar, A., Parrott, L., and Hawkins, C. 2016a. Aftermath of Mountain Pine Beetle Outbreak in British Columbia: Stand Dynamics, Management Response and Ecosystem Resilience. Forests **7**(8): 171. doi:10.3390/f7080171.

Dhar, A., Parrott, L., and Heckbert, S. 2016b. Consequences of mountain pine beetle outbreak on forest ecosystem services in western Canada. Can. J. For. Res. **46**(8): 987–999. NRC Research Press. doi:10.1139/cjfr-2016-0137.

Dias, R.O., Cardoso, C., Leal, C.S., Ribeiro, A.F., Ferreira, C., and Terra, W.R. 2019. Domain structure and expression along the midgut and carcass of peritrophins and cuticle proteins analogous to peritrophins in insects with and without peritrophic membrane. J. Insect Physiol. **114**: 1–9. doi:10.1016/j.jinsphys.2019.02.002.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics **29**(1): 15–21. doi:10.1093/bioinformatics/bts635.

Dowling, D., Pauli, T., Donath, A., Meusemann, K., Podsiadlowski, L., Petersen, M., Peters, R.S., Mayer, C., Liu, S., Zhou, X., Misof, B., and Niehuis, O. 2016. Phylogenetic Origin and Diversification of RNAi Pathway Genes in Insects. Genome Biol. Evol. **8**(12): 3784–3793. doi:10.1093/gbe/evw281.

Duman-Scheel, M. 2019. Saccharomyces cerevisiae (Baker's Yeast) as an Interfering RNA Expression and Delivery System. Curr. Drug Targets **20**(9): 942–952. doi:10.2174/1389450120666181126123538.

Dunivant, T.S., Singh, V., Livingston, K.E., Ross, J.D., and Hileman, L.C. 2024. *CYCLOIDEA* paralogs function partially redundantly to specify dorsal flower development in *Mimulus lewisii*. Am. J. Bot. **111**(2): e16271. doi:10.1002/ajb2.16271.

Evenden, M.L., Whitehouse, C.M., and Sykes, J. 2014. Factors Influencing Flight Capacity of the Mountain Pine Beetle (Coleoptera: Curculionidae: Scolytinae). Environ. Entomol. **43**(1): 187–196. doi:10.1603/EN13244.

Fabrick, J.A., and Hull, J.J. 2017. Assessing Integrity of Insect RNA. USDA-ARS Research Notes. 5991-7903EN: 1-12. Available from

https://www.ars.usda.gov/ARSUserFiles/20200500/Pubs%202018/FabrickHull%20AgilentNot4es2017.pdf [Accessed May 24, 2023].

Fettig, C.J., Gibson, K.E., Munson, A.S., and Negrón, J.F. 2014. Cultural Practices for Prevention and Mitigation of Mountain Pine Beetle Infestations. For. Sci. **60**(3): 450–463. doi:10.5849/forsci.13-032.

Finetti, L., Benetti, L., Leyria, J., Civolani, S., and Bernacchia, G. 2023. Topical delivery of dsRNA in two hemipteran species: Evaluation of RNAi specificity and non-target effects. Pestic. Biochem. Physiol. **189**: 105295. doi:10.1016/j.pestbp.2022.105295.

Fraser, J.D., Bonnett, T.R., Keeling, C.I., and Huber, D.P.W. 2017. Seasonal shifts in accumulation of glycerol biosynthetic gene transcripts in mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), larvae. PeerJ **5**: e3284. doi:10.7717/peerj.3284.

Ghosh, S.K.B., Hunter, W.B., Park, A.L., and Gundersen-Rindal, D.E. 2018. Double-stranded RNA Oral Delivery Methods to Induce RNA Interference in Phloem and Plant-sap-feeding Hemipteran Insects. J. Vis. Exp. JoVE (135): 57390. doi:10.3791/57390.

Gilbert, S.F. 2000. Differential Gene Expression. *In* Developmental Biology. 6th edition. Sinauer Associates. Available from https://www.ncbi.nlm.nih.gov/books/NBK10061/ [accessed 10 September 2024].

Girard, M., Berthaud, V., Martin, E., Vallon, L., Rebollo, R., Vallier, A., Vigneron, A., Hay, A.-E., Valiente Moro, C., and Minard, G. 2025. Evaluation of non-invasive dsRNA delivery methods for the development of RNA interference in the Asian tiger mosquito *Aedes albopictus*. J. Pest Sci. **98**(1): 581–596. doi:10.1007/s10340-024-01779-w.

González-Caballero, N., Rodríguez-Vega, A., Dias-Lopes, G., Valenzuela, J.G., Ribeiro, J.M.C., Carvalho, P.C., Valente, R.H., Brazil, R.P., and Cuervo, P. 2014. Expression of the mevalonate pathway enzymes in the *Lutzomyia longipalpis* (Diptera: Psychodidae) sex pheromone gland demonstrated by an integrated proteomic approach. J. Proteomics **96**: 117–132. doi:10.1016/j.jprot.2013.10.028.

Griffin, J., and Turner, M. 2012. Changes to the N cycle following bark beetle outbreaks in two contrasting conifer forest types. Oecologia **170**(2): 551–565. doi:10.1007/s00442-012-2323-y.

Gupta, K., Attri, J., Singh, A., Kaur, H., and Kaur, G. 2016. Basic concepts for sample size calculation: Critical step for any clinical trials! Saudi J. Anaesth. **10**(3): 328. doi:10.4103/1658-354X.174918.

Haller, S., Widmer, F., Siegfried, B.D., Zhuo, X., and Romeis, J. 2019. Responses of two ladybird beetle species (Coleoptera: Coccinellidae) to dietary RNAi. Pest Manag. Sci. **75**(10): 2652–2662. doi:10.1002/ps.5370.

Hashiro, S., and Yasueda, H. 2022. RNA Interference-Based Pesticides and Antiviral Agents: Microbial Overproduction Systems for Double-Stranded RNA for Applications in Agriculture and Aquaculture. Appl. Sci. **12**(6): 2954. Multidisciplinary Digital Publishing Institute. doi:10.3390/app12062954.

Hicke, J.A., Meddens, A.J.H., Allen, C.D., and Kolden, C.A. 2013. Carbon stocks of trees killed by bark beetles and wildfire in the western United States. Environ. Res. Lett. **8**(3): 035032. IOP Publishing. doi:10.1088/1748-9326/8/3/035032.

Hoback, W.W., and Stanley, D.W. 2001. Insects in hypoxia. J. Insect Physiol. **47**(6): 533–542. doi:10.1016/S0022-1910(00)00153-0.

Hollowell, H., Wallace, M., and Rieske, L.K. 2022. The trigger for RNA interference to silence essential genes in southern pine beetle, *Dendroctonus frontalis*, demonstrates no lethal effects on pine-associated nontarget insects. Agric. For. Entomol. doi:10.1111/afe.12550.

Horianopoulos, L.C., Boone, C.K., Samarasekera, G.D.N.G., Kandola, G.K., and Murray, B.W. 2018. Selection of the sex-linked *inhibitor of apoptosis* in mountain pine beetle (*Dendroctonus ponderosae*) driven by enhanced expression during early overwintering. Ecol. Evol. **8**(12): 6253–6264. doi:10.1002/ece3.4164.

Howe, M., Carroll, A., Gratton, C., and Raffa, K.F. 2021. Climate-induced outbreaks in high-elevation pines are driven primarily by immigration of bark beetles from historical hosts. Glob. Change Biol. **27**(22): 5786–5805. doi:10.1111/gcb.15861.

Hu, X., Richtman, N.M., Zhao, J.-Z., Duncan, K.E., Niu, X., Procyk, L.A., Oneal, M.A., Kernodle, B.M., Steimel, J.P., Crane, V.C., Sandahl, G., Ritland, J.L., Howard, R.J., Presnail, J.K., Lu, A.L., and Wu, G. 2016. Discovery of midgut genes for the RNA interference control of corn rootworm. Sci. Rep. **6**(1): 30542. doi:10.1038/srep30542.

Hu, X., Steimel, J.P., Kapka-Kitzman, D.M., Davis-Vogel, C., Richtman, N.M., Mathis, J.P., Nelson, M.E., Lu, A.L., and Wu, G. 2019. Molecular characterization of the insecticidal activity of double-stranded RNA targeting the smooth septate junction of western corn rootworm (*Diabrotica virgifera virgifera*). PLoS ONE **14**(01): 1–18. Public Library of Science. doi:10.1371/journal.pone.0210491.

Huang, C., Lü, J., Bai, C., Guo, Y., Guo, C., Song, J., and Xi, J. 2025. Transcriptome reveals patterns of response by *Lasioderma serricorne* (Coleoptera: Ptinidae) larvae to s-methoprene exposure. J. Stored Prod. Res. **111**: 102521. doi:10.1016/j.jspr.2024.102521.

Huang, X., Whitman, D.W., Ma, J., McNeill, M.R., and Zhang, Z. 2017. Diet alters performance and transcription patterns in *Oedaleus asiaticus* (Orthoptera: Acrididae) grasshoppers. PLOS ONE **12**(10): e0186397. doi:10.1371/journal.pone.0186397.

Huho, B.J., Ng'habi, K.R., Killeen, G.F., Nkwengulila, G., Knols, B.G.J., and Ferguson, H.M. 2007. Nature beats nurture: a case study of the physiological fitness of free-living and laboratory-reared male *Anopheles gambiae s.l.* J. Exp. Biol. **210**(16): 2939–2947. doi:10.1242/jeb.005033.

Izumi, Y., Motoishi, M., Furuse, K., and Furuse, M. 2016. A tetraspanin regulates septate junction formation in *Drosophila* midgut. J. Cell Sci.: jcs.180448. doi:10.1242/jcs.180448.

Izumi, Y., Yanagihashi, Y., and Furuse, M. 2012. A novel protein complex, mesh-ssk, is required for septate junction formation in *Drosophila* midgut. J. Cell Sci.: jcs.112243. doi:10.1242/jcs.112243.

Janes, J.K., Li, Y., Keeling, C.I., Yuen, M.M.S., Boone, C.K., Cooke, J.E.K., Bohlmann, J., Huber, D.P.W., Murray, B.W., Coltman, D.W., and Sperling, F.A.H. 2014. How the Mountain Pine Beetle (*Dendroctonus ponderosae*) Breached the Canadian Rocky Mountains. Mol. Biol. Evol. **31**(7): 1803–1815. doi:10.1093/molbev/msu135.

Jantz, O.K., and Johnsey, R.L. 1964. Determination of Sex of the Douglas-fir Beetle *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Scolytidae). Can. Entomol. **96**(10): 1327–1329. doi:10.4039/Ent961327-10.

Jenkins, M.J., Hebertson, E., Page, W., and Jorgensen, C.A. 2008. Bark beetles, fuels, fires and implications for forest management in the Intermountain West. For. Ecol. Manag. **254**(1): 16–34. doi:10.1016/j.foreco.2007.09.045.

Jiang, H., Zhang, N., Chen, M., Meng, X., Ji, C., Ge, H., Dong, F., Miao, L., Yang, X., Xu, X., Qian, K., and Wang, J. 2019a. Transcriptional and post-translational activation of AMPKα by oxidative, heat, and cold stresses in the red flour beetle, *Tribolium castaneum*. Cell Stress Chaperones **24**(6): 1079–1089. doi:10.1007/s12192-019-01030-x.

Jiang, T., Ma, L., Liu, X.-Y., Xiao, H.-J., and Zhang, W.-N. 2019b. Effects of starvation on respiratory metabolism and energy metabolism in the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). J. Insect Physiol. **119**: 103951. doi:10.1016/j.jinsphys.2019.103951.

Joga, M.R., Zotti, M.J., Smagghe, G., and Christiaens, O. 2016. RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. Front. Physiol. **7**. Available from https://www.frontiersin.org/articles/10.3389/fphys.2016.00553 [accessed 21 November 2022].

Johnson, E.C. 2024. An assessment of Alberta's strategy for controlling mountain pine beetle outbreaks. arXiv. doi:10.48550/arXiv.2412.08786.

Jonusaite, S., Beyenbach, K.W., Meyer, H., Paululat, A., Izumi, Y., Furuse, M., and Rodan, A.R. 2020. The septate junction protein Mesh is required for epithelial morphogenesis, ion transport, and paracellular permeability in the *Drosophila* Malpighian tubule. Am. J. Physiol.-Cell Physiol. **318**(3): C675–C694. American Physiological Society. doi:10.1152/ajpcell.00492.2019.

Jurado, A.R., Tan, D., Jiao, X., Kiledjian, M., and Tong, L. 2014. Structure and Function of Pre-mRNA 5′-End Capping Quality Control and 3′-End Processing. Biochemistry **53**(12): 1882. American Chemical Society. doi:10.1021/bi401715v.

Keeling, CI., Campbell, E.O., Batista, P.D., Shegelski, V.A., Trevoy, S.A.L., Huber, D.P.W., Janes, J.K., Sperling, F.A.H. 2021. Chromosome-level genome assembly reveals genomic architecture of northern range expansion in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae). Mol Ecol Resour. 22:1149–1167. doi:10.1111/1755-0998.13528.

Keeling, C.I., Chiu, C.C., Aw, T., Li, M., Henderson, H., Tittiger, C., Weng, H.-B., Blomquist, G.J., and Bohlmann, J. 2013a. Frontalin pheromone biosynthesis in the mountain pine beetle, *Dendroctonus ponderosae* , and the role of isoprenyl diphosphate synthases. Proc. Natl. Acad. Sci. **110**(47): 18838–18843. doi:10.1073/pnas.1316498110.

Keeling, C.I., Li, M., Sandhu, H.K., Henderson, H., Yuen, M.M.S., and Bohlmann, J. 2016. Quantitative metabolome, proteome and transcriptome analysis of midgut and fat body tissues in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and insights into pheromone biosynthesis. Insect Biochem. Mol. Biol. **70**: 170–183. doi:10.1016/j.ibmb.2016.01.002.

Kirsch, R., Heckel, D.G., and Pauchet, Y. 2016. How the rice weevil breaks down the pectin network: Enzymatic synergism and sub-functionalization. Insect Biochem. Mol. Biol. **71**: 72–82. doi:10.1016/j.ibmb.2016.02.007.

Klepzigl, K.D., Moser, J.C., Lombardero, F.J., Hofstetter, R.W., and Ayres, M.P. 2001. Symbiosis and Competition: Complex Interactions Among Beetles, Fungi and Mites. Symbiosis. **30**: 83-96.

Klutsch, J.G., Cale, J.A., Whitehouse, C., Kanekar, S.S., and Erbilgin, N. 2017. Trap trees: an effective method for monitoring mountain pine beetle activities in novel habitats. Can. J. For. Res. **47**(10): 1432–1437. doi:10.1139/cjfr-2017-0189.

Koch, D., Lewis, M.A., and Lele, S. 2021. The Signature of Endemic Populations in the Spread of Mountain Pine Beetle Outbreaks. Bull. Math. Biol. **83**(6): 65. doi:10.1007/s11538-021-00899-z.

Krueger, F., James, F., Ewels, P., Afyounian, E. & Schuster-Boeckler, B. 2021. FelixKrueger/TrimGalore: v0.6.7 - doi via Zenodo. doi:10.5281/zenodo.5127899.

Kyre, B.R., Bentz, B.J., and Rieske, L.K. 2020. Susceptibility of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) to gene silencing through RNAi provides potential as a novel management tool. For. Ecol. Manag. **473**: 118322. doi:10.1016/j.foreco.2020.118322.

Kyre, B.R., Dupuis, J.R., Zúñiga, G., and Rieske, L.K. 2024. Variation in RNA interference sensitivity in the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Curculionidae). Biol. J. Linn. Soc. **142**(2): 147–154. doi:10.1093/biolinnean/blad126.

Kyre, B.R., and Rieske, L.K. 2022. Using RNAi to silence heat shock protein has congeneric effects in North America's *Dendroctonus* bark beetles. For. Ecol. Manag. **520**: 120367. doi:10.1016/j.foreco.2022.120367.

Kyre, B.R., Rodrigues, T.B., and Rieske, L.K. 2019. RNA interference and validation of reference genes for gene expression analyses using qPCR in southern pine beetle, *Dendroctonus frontalis*. Sci. Rep. **9**: 5640. doi:10.1038/s41598-019-42072-6.

Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., and Garrido, C. 2008. Heat shock proteins: essential proteins for apoptosis regulation. J. Cell. Mol. Med. **12**(3): 743–761. doi:10.1111/j.1582-4934.2008.00273.x.

Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. 2014. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. **15**(2): R29. doi:10.1186/gb-2014-15-2-r29.

Leelesh, R.S., and Rieske, L.K. 2020. Oral Ingestion of Bacterially Expressed dsRNA Can Silence Genes and Cause Mortality in a Highly Invasive, Tree-Killing Pest, the Emerald Ash Borer. Insects **11**(7): 440. Multidisciplinary Digital Publishing Institute. doi:10.3390/insects11070440.

Li, K., Chen, T., Li, Y., Sun, K., Pang, K., Yu, X., and Hao, P. 2025. Risk Assessment of RNAi-Based Potential Pesticide dsNlAtg3 and Its Homologues for *Nilaparvata lugens* and Non-Target Organisms. Insects **16**(2): 225. doi:10.3390/insects16020225.

Linz, D.M., Clark-Hachtel, C.M., Borràs-Castells, F., and Tomoyasu, Y. 2014. Larval RNA Interference in the Red Flour Beetle, *Tribolium castaneum*. J. Vis. Exp. (92): 52059. doi:10.3791/52059.

List, F., Tarone, A.M., Zhu-Salzman, K., and Vargo, E.L. 2022. RNA meets toxicology: efficacy indicators from the experimental design of RNAi studies for insect pest management. Pest Manag. Sci. **78**(8): 3215–3225. doi:10.1002/ps.6884.

Liu, B., and Chen, H. 2022. Disruption of CYP6DF1 and CYP6DJ2 increases the susceptibility of *Dendroctonus armandi* to (+)-α-pinene. Pestic. Biochem. Physiol. **188**: 105270. doi:10.1016/j.pestbp.2022.105270.

Liu, B., Fu, D., Gao, H., Ning, H., Sun, Y., Chen, H., and Tang, M. 2021. Cloning and Expression of the Neuropeptide F and Neuropeptide F Receptor Genes and Their Regulation of Food Intake in the Chinese White Pine Beetle *Dendroctonus armandi*. Front. Physiol. **12**: 662651. doi:10.3389/fphys.2021.662651.

Liu, B., Fu, D., Ning, H., Tang, M., and Chen, H. 2022. Knockdown of CYP6CR2 and CYP6DE5 reduces tolerance to host plant allelochemicals in the Chinese white pine

beetle *Dendroctonus armandi*. Pestic. Biochem. Physiol. **187**: 105180. doi:10.1016/j.pestbp.2022.105180.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods **25**(4): 402–408. doi:10.1006/meth.2001.1262.

Lodhi, L., Ahi, J.D., Gupta, N., Pradhan, B.L., Sen, P., Ghosh, M., and Dey, K.K. 2024. Exploring the structure and dynamics of soft and hard cuticle of Bombyx mori using solid-state NMR techniques. Sci. Rep. **14**(1): 22378. doi:10.1038/s41598-024-69984-2.

Lohman, B.K., Weber, J.N., and Bolnick, D.I. 2016. Evaluation of TagSeq, a reliable low-cost alternative for RNAseq. Mol. Ecol. Resour. **16**(6): 1315–1321. doi:10.1111/1755-0998.12529.

Lü, J., Nanda, S., Chen, S., Mei, Y., He, K., Qiu, B., Zhang, Y., Li, F., and Pan, H. 2022. A survey on the off-target effects of insecticidal double-stranded RNA targeting the Hvβ′COPI gene in the crop pest *Henosepilachna vigintioctopunctata* through RNA-seq. J. Integr. Agric. **21**(9): 2665–2674. doi:10.1016/j.jia.2022.07.015.

Lubawy, J., Chowański, S., Adamski, Z., and Słocińska, M. 2022. Mitochondria as a target and central hub of energy division during cold stress in insects. Front. Zool. **19**(1): 1. doi:10.1186/s12983-021-00448-3.

McCambridge, W.F., Morris, M.J., and Edminster, C.B. 1982. Herbage Production Under Ponderosa Pine Killed by the Mountain Pine Beetle in Colorado. USDA Forest Service, Rocky Mountain Forest and Range Experiment Station.

McEwan, D.L., Weisman, A.S., and Hunter, C.P. 2012. Uptake of Extracellular Double-Stranded RNA by SID-2. Mol. Cell **47**(5): 746–754. doi:10.1016/j.molcel.2012.07.014.

Mock, K.E., Bentz, B.J., O'neill, E.M., Chong, J.P., Orwin, J., and Pfrender, M.E. 2007. Landscape-scale genetic variation in a forest outbreak species, the mountain pine beetle (*Dendroctonus ponderosae*). Mol. Ecol. **16**(3): 553–568. doi:10.1111/j.1365-294X.2006.03158.x.

Morris, J.L., Cottrell, S., Fettig, C.J., Hansen, W.D., Sherriff, R.L., Carter, V.A., Clear, J.L., Clement, J., DeRose, R.J., Hicke, J.A., Higuera, P.E., Mattor, K.M., Seddon, A.W.R., Seppä, H.T., Stednick, J.D., and Seybold, S.J. 2017. Managing bark beetle impacts on ecosystems and society: priority questions to motivate future research. J. Appl. Ecol. **54**(3): 750–760. doi:10.1111/1365-2664.12782.

Morris, J.L., Courtney Mustaphi, C.J., Carter, V.A., Watt, J., Derr, K., Pisaric, M.F.J., Anderson, R.S., and Brunelle, A.R. 2015. Do bark beetle remains in lake sediments correspond to severe outbreaks? A review of published and ongoing research. Quat. Int. **387**: 72–86. doi:10.1016/j.quaint.2014.03.022.

Murphy, K.A., Tabuloc, C.A., Cervantes, K.R., and Chiu, J.C. 2016. Ingestion of genetically modified yeast symbiont reduces fitness of an insect pest via RNA interference. Sci. Rep. **6**(1): 22587. doi:10.1038/srep22587.

Niu, X., Kassa, A., Hu, X., Robeson, J., McMahon, M., Richtman, N.M., Steimel, J.P., Kernodle, B.M., Crane, V.C., Sandahl, G., Ritland, J.L., Presnail, J.K., Lu, A.L., and Wu, G. 2017. Control of Western Corn Rootworm (*Diabrotica virgifera virgifera*) Reproduction through Plant-Mediated RNA Interference. Sci. Rep. **7**: 12591. doi:10.1038/s41598-017-12638-3.

Paine, T.D., Raffa, K.F., and Harrington, T.C. 1997. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. Annu. Rev. Entomol. **42**(1): 179–206. doi:10.1146/annurev.ento.42.1.179.

Pampolini, F., and Rieske, L.K. 2023. Foliar Application of dsRNA to Induce Gene Silencing in Emerald Ash Borer: Systemic Distribution, Persistence, and Bioactivity. Forests **14**(9): 1853. Multidisciplinary Digital Publishing Institute. doi:10.3390/f14091853.

Petek, M., Coll, A., Ferenc, R., Razinger, J., and Gruden, K. 2020. Validating the Potential of Double-Stranded RNA Targeting Colorado Potato Beetle Mesh Gene in Laboratory and Field Trials. Front. Plant Sci. **11**: 1250. doi:10.3389/fpls.2020.01250.

Pettit, J.M., Voelker, S.L., DeRose, R.J., and Burton, J.I. 2020. Spruce beetle outbreak was not driven by drought stress: Evidence from a tree-ring iso-demographic approach indicates temperatures were more important. Glob. Change Biol. **26**(10): 5829–5843. doi:10.1111/gcb.15274.

Phatnani, H.P., and Greenleaf, A.L. 2006. Phosphorylation and functions of the RNA polymerase II CTD. Genes Dev. **20**: 2922-2936. doi:10.1101/gad.1477006.

Poreddy, S., Li, J., and Baldwin, I.T. 2017. Plant-mediated RNAi silences midgut-expressed genes in congeneric Lepidopteran insects in nature. BMC Plant Biol. **17**(1): 199. doi:10.1186/s12870-017-1149-5.

Raffa, K.F., Aukema, B.H., Bentz, B.J., Carroll, A.L., Hicke, J.A., Turner, M.G., and Romme, W.H. 2008. Cross-scale Drivers of Natural Disturbances Prone to Anthropogenic Amplification: The Dynamics of Bark Beetle Eruptions. BioScience **58**(6): 501–517. doi:10.1641/B580607.

Rangasamy, M., and Siegfried, B.D. 2012. Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) adults. Pest Manag. Sci. **68**(4): 587–591. doi:10.1002/ps.2301.

Reaume, C.J., and Sokolowski, M.B. 2011. Conservation of gene function in behaviour. Philos. Trans. R. Soc. B Biol. Sci. **366**(1574): 2100–2110. doi:10.1098/rstb.2011.0028.

Reed, D.E., Ewers, B.E., Pendall, E., Frank, J., and Kelly, R. 2018. Bark beetle-induced tree mortality alters stand energy budgets due to water budget changes. Theor. Appl. Climatol. **131**(1–2): 153–165. Springer Nature. doi:10.1007/s00704-016-1965-9.

Reinders, J.D., Moar, W.J., Head, G.P., Hassan, S., and Meinke, L.J. 2023. Effects of SmartStax® and SmartStax® PRO maize on western corn rootworm (*Diabrotica virgifera virgifera* LeConte) larval feeding injury and adult life history parameters. PLOS ONE **18**(7): e0288372. doi:10.1371/journal.pone.0288372.

Rieu, I., and Powers, S.J. 2009. Real-Time Quantitative RT-PCR: Design, Calculations, and Statistics. Plant Cell **21**(4): 1031–1033. doi:10.1105/tpc.109.066001.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. **43**(7): e47. doi:10.1093/nar/gkv007.

Robert, J.A., Bonnett, T., Pitt, C., Spooner, L.J., Fraser, J., Yuen, M.M.S., Keeling, C.I., Bohlmann, J., and Huber, D.P.W. 2016. Gene expression analysis of overwintering mountain pine beetle larvae suggests multiple systems involved in overwintering stress, cold hardiness, and preparation for spring development. PeerJ **4**: e2109. doi:10.7717/peerj.2109.

Robert, J.A., Pitt, C., Bonnett, T.R., Yuen, M.M.S., Keeling, C.I., Bohlmann, J., and Huber, D.P.W. 2013. Disentangling Detoxification: Gene Expression Analysis of Feeding Mountain Pine Beetle Illuminates Molecular-Level Host Chemical Defense Detoxification Mechanisms. PLOS ONE **8**(11): e77777. Public Library of Science. doi:10.1371/journal.pone.0077777.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. 2010. edgeR : a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**(1): 139–140. doi:10.1093/bioinformatics/btp616.

Rodrigues, T.B., Duan, J.J., Palli, S.R., and Rieske, L.K. 2018. Identification of highly effective target genes for RNAi-mediated control of emerald ash borer, *Agrilus planipennis*. Sci. Rep. **8**(1): 5020. doi:10.1038/s41598-018-23216-6.

Rodrigues, T.B., Mishra, S.K., Sridharan, K., Barnes, E.R., Alyokhin, A., Tuttle, R., Kokulapalan, W., Garby, D., Skizim, N.J., Tang, Y., Manley, B., Aulisa, L., Flannagan, R.D., Cobb, C., and Narva, K.E. 2021. First Sprayable Double-Stranded RNA-Based Biopesticide Product Targets Proteasome Subunit Beta Type-5 in Colorado Potato Beetle (*Leptinotarsa decemlineata*). Front. Plant Sci. **12**: 728652. doi:10.3389/fpls.2021.728652.

Rosenberger, D.W., Venette, R.C., and Aukema, B.H. 2016. Sexing live mountain pine beetles Dendroctonus ponderosae: refinement of a behavioral method for *Dendroctonus* spp. Entomol. Exp. Appl. **160**(2): 195–199. doi:10.1111/eea.12463.

Safranyik, L. 1998. Mortality of mountain pine beetle larvae, *Dendroctonus ponderosae* (Coleoptera: Scolytidae) in logs of lodgepole pine (*Pinus contorta* var. latifolia) at constant low temperatures. J. Entomol. Soc. Br. Columbia **95**: 81–88.

Saleh, M.-C., Van Rij, R.P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P.H., and Andino, R. 2006. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat. Cell Biol. **8**(8): 793–802. doi:10.1038/ncb1439.

Samarasekera, G.D.N., Bartell, N.V., Lindgren, B.S., Cooke, J.E.K., Davis, C.S., James, P.M.A., Coltman, D.W., Mock, K.E., and Murray, B.W. 2012. Spatial genetic structure of the mountain pine beetle (*Dendroctonus ponderosae*) outbreak in western Canada: historical patterns and contemporary dispersal. Mol. Ecol. **21**(12): 2931–2948. doi:10.1111/j.1365-294X.2012.05587.x.

Schüller, R. 2013. Insights into the RNA Polymerase II CTD code. Helmholtz Center Munich German Research Center for Environment and Health (GmbH) Institute for Clinical Molecular Biology and Tumor Genetics Department of Molecular Epigenetics. 135. Available from https://edoc.ub.uni-muenchen.de/16565/1/Schueller_Roland.pdf [accessed February 27 2025].

Shegelski, V.A., Campbell, E.O., Thompson, K.M., Whitehouse, C.M., and Sperling, F.A.H. 2021. Source and spread dynamics of mountain pine beetle in central Alberta, Canada. Can. Entomol. **153**(3): 314–326. doi:10.4039/tce.2020.83.

Silva, G.M., and Vogel, C. 2016. Quantifying gene expression: the importance of being subtle. Mol. Syst. Biol. **12**(10): 885. doi:10.15252/msb.20167325.

Simard, M., Romme, W.H., Griffin, J.M., and Turner, M.G. 2011. Do mountain pine beetle outbreaks change the probability of active crown fire in lodgepole pine forests? Ecol. Monogr. **81**(1): 3–24. doi:10.1890/10-1176.1.

Singh, I.K., Singh, S., Mogilicherla, K., Shukla, J.N., and Palli, S.R. 2017. Comparative analysis of double-stranded RNA degradation and processing in insects. Sci. Rep. **7**(1): 17059. Nature Publishing Group. doi:10.1038/s41598-017-17134-2.

Sørensen, J.G., Addison, M.F., and Terblanche, J.S. 2012. Mass-rearing of insects for pest management: Challenges, synergies and advances from evolutionary physiology. Crop Prot. **38**: 87–94. doi:10.1016/j.cropro.2012.03.023.

Stewart, A.T.M., Mysore, K., Njoroge, T.M., Winter, N., Feng, R.S., Singh, S., James, L.D., Singkhaimuk, P., Sun, L., Mohammed, A., Oxley, J.D., Duckham, C., Ponlawat, A., Severson, D.W., and Duman-Scheel, M. 2023. Demonstration of RNAi Yeast Insecticide Activity in Semi-Field Larvicide and Attractive Targeted Sugar Bait Trials Conducted on *Aedes* and *Culex* Mosquitoes. Insects **14**(12): 950. doi:10.3390/insects14120950.

Sun, Y., Fu, D., Kang, X., Liu, B., Ning, H., and Chen, H. 2021. Function of mevalonate pathway genes in the synthesis of frontalin in Chinese white pine beetle*, Dendroctonus armandi*

(curculionidae: Scolytinae). Arch. Insect Biochem. Physiol. **107**(4): e21828. doi:10.1002/arch.21828.

Tamaki, S., Takemoto, S., Uryu, O., Kamae, Y., and Tomioka, K. 2013. Opsins are involved in nymphal photoperiodic responses in the cricket *Modicogryllus siamensis*. Physiol. Entomol. **38**(2): 163–172. doi:10.1111/phen.12015.

Taylor, S.C., Laperriere, G., and Germain, H. 2017. Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. Sci. Rep. **7**(1): 2409. Nature Publishing Group. doi:10.1038/s41598-017-02217-x.

Taylor, S.P., Wilson, I.M., and White, K.J. 1997. Topical application of carbon dioxide and liquid nitrogen against the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae).

Tehrani, S.D., Shojaei, S.R., Hosseini, S.R., and Shayan, P. 2024. Investigating Differential Expression Genes Profile in Hct-8 Cell Line Infected with Cryptosporidium Parvum in Host-Parasite Interactions. Bulg. J. Vet. Med. **27**(3): 475–488. Trakia University. doi:10.15547/bjvm.2022-0089.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J.-L., Barthel, A., Bebas, P., Bitra, K., Bravo, A., Chevalier, F., Collinge, D.P., Crava, C.M., De Maagd, R.A., Duvic, B., Erlandson, M., Faye, I., Felföldi, G., Fujiwara, H., Futahashi, R., Gandhe, A.S., Gatehouse, H.S., Gatehouse, L.N., Giebultowicz, J.M., Gómez, I., Grimmelikhuijzen, C.J.P., Groot, A.T., Hauser, F., Heckel, D.G., Hegedus, D.D., Hrycaj, S., Huang, L., Hull, J.J., Iatrou, K., Iga, M., Kanost, M.R., Kotwica, J., Li, C., Li, J., Liu, J., Lundmark, M., Matsumoto, S., Meyering-Vos, M., Millichap, P.J., Monteiro, A., Mrinal, N., Niimi, T., Nowara, D., Ohnishi, A., Oostra, V., Ozaki, K., Papakonstantinou, M., Popadic, A., Rajam, M.V., Saenko, S., Simpson, R.M., Soberón, M., Strand, M.R., Tomita, S., Toprak, U., Wang, P., Wee, C.W., Whyard, S., Zhang, W., Nagaraju, J., ffrench-Constant, R.H., Herrero, S., Gordon, K., Swevers, L., and Smagghe, G. 2011. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. J. Insect Physiol. **57**(2): 231–245. doi:10.1016/j.jinsphys.2010.11.006.

Thompson, K.M., Huber, D.P.W., and Murray, B.W. 2020. Autumn shifts in cold tolerance metabolites in overwintering adult mountain pine beetles. PLOS ONE **15**(1): e0227203. doi:10.1371/journal.pone.0227203.

Tittiger, C., Keeling, C.I., Blomquist, G.J. 2005. Some insights into the remarkable metabolism of the bark beetle midgut. *In* Chemical Ecology and Phytochemistry of Forest Ecosystems. Romeo, J.T., editor c2005, **38.** Oxford, UK**:** Elsevier. p. 57-78.

Trevoy, S.A.L., Janes, J.K., Muirhead, K., and Sperling, F.A.H. 2019. Repurposing population genetics data to discern genomic architecture: A case study of linkage cohort detection in

mountain pine beetle (*Dendroctonus ponderosae*). Ecol. Evol. **9**(3): 1147–1159. doi:10.1002/ece3.4803.

Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P., and Newcomb, R.D. 2006. RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. Insect Mol. Biol. **15**(3): 383–391. doi:10.1111/j.1365-2583.2006.00656.x.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3**(7): research0034.1-research0034.11.

Vélez, A.M., Khajuria, C., Wang, H., Narva, K.E., and Siegfried, B.D. 2016. Knockdown of RNA Interference Pathway Genes in Western Corn Rootworms (*Diabrotica virgifera virgifera* Le Conte) Demonstrates a Possible Mechanism of Resistance to Lethal dsRNA. PLOS ONE **11**(6): e0157520. doi:10.1371/journal.pone.0157520.

Verhagen, A.M., Coulson, E.J., and Vaux, D.L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome Biology **2**(7): 1-10. doi:10.1186/gb-2001-2-7-reviews3009.

Wallace, M., and Rieske, L.K. 2023. Ingestion of Species-Specific dsRNA Alters Gene Expression and Can Cause Mortality in the Forest Pest, *Ips calligraphus*. Forests **14**(2): 422. doi:10.3390/f14020422.

Wang, J., Zhang, R.-R., Gao, G.-Q., Ma, M.-Y., and Chen, H. 2016. Cold tolerance and silencing of three cold-tolerance genes of overwintering Chinese white pine larvae. Sci. Rep. **6**(1): 34698. doi:10.1038/srep34698.

Wangen, C.E., Powell, J.A., and Bentz, B.J. 2024. A mountain pine beetle (Coleoptera: Curculionidae) adult development rate model confirms evolved geographic differences. J. Insect Sci. **24**(4): 16. doi:10.1093/jisesa/ieae074.

Wei, Q., Khan, I.K., Ding, Z., Yerneni, S., and Kihara, D. 2017. NaviGO: interactive tool for visualization and functional similarity and coherence analysis with gene ontology. BMC Bioinformatics **18**(1): 177. doi:10.1186/s12859-017-1600-5.

Whitney, H.S., and Spanier, O.J. 1982. An improved method for rearing axenic mountain pine beetles, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Can. Entomol. **114**(11): 1095–1100. doi:10.4039/Ent1141095-11.

Whyard, S., Singh, A.D., and Wong, S. 2009. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem. Mol. Biol. **39**(11): 824–832. doi:10.1016/j.ibmb.2009.09.007.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H., and Hunter, C.P. 2007. *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. Proc. Natl. Acad. Sci. **104**(25): 10565–10570. doi:10.1073/pnas.0611282104.

Wittische, J., Janes, J.K., and James, P.M.A. 2019. Modelling landscape genetic connectivity of the mountain pine beetle in western Canada. Can. J. For. Res. **49**(11): 1339–1348. doi:10.1139/cjfr-2018-0417.

Wood, S.L. 1963. A Revision of the Bark Beetle Genus *Dendroctonus* Erichson (Coleoptera: Scolytidae). Gt. Basin Nat. **23**(1/2): 1–117. Monte L. Bean Life Science Museum, Brigham Young University.

Xin, Z., Cai, Y., Dang, L.T., Burke, H.M.S., Revote, J., Charitakis, N., Bienroth, D., Nim, H.T., Li, Y.-F., and Ramialison, M. 2022. MonaGO: a novel gene ontology enrichment analysis visualisation system. BMC Bioinformatics **23**(1): 1–16. BioMed Central. doi:10.1186/s12859-022-04594-1.

Xu, C., Tang, H.-W., Hung, R.-J., Hu, Y., Ni, X., Housden, B.E., and Perrimon, N. 2019. The Septate Junction Protein Tsp2A Restricts Intestinal Stem Cell Activity via Endocytic Regulation of aPKC and Hippo Signaling. Cell Rep. **26**(3): 670-688.e6. doi:10.1016/j.celrep.2018.12.079.

Yoon, J.-S., Mogilicherla, K., Gurusamy, D., Chen, X., Chereddy, S.C.R.R., and Palli, S.R. 2018. Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in Coleopteran insects. Proc. Natl. Acad. Sci. **115**(33): 8334–8339. doi:10.1073/pnas.1809381115.

Yoon, J.-S., Shukla, J.N., Gong, Z.J., Mogilicherla, K., and Palli, S.R. 2016. RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors. Insect Biochem. Mol. Biol. **78**: 78–88. doi:10.1016/j.ibmb.2016.09.002.

Zawadzki, J.L., Kotze, A.C., Fritz, J.-A., Johnson, N.M., Hemsworth, J.E., Hines, B.M., and Behm, C.A. 2012. Silencing of essential genes by RNA interference in *Haemonchus contortus*. Parasitology **139**(5): 613–629. doi:10.1017/S0031182012000121.

Zhang, R., Gao, G., and Chen, H. 2016. Silencing of the olfactory co-receptor gene in *Dendroctonus armandi* leads to EAG response declining to major host volatiles. Sci. Rep. **6**(1): 23136. doi:10.1038/srep23136.

Zhao, C., Alvarez Gonzales, M.A., Poland, T.M., and Mittapalli, O. 2015. Core RNAi machinery and gene knockdown in the emerald ash borer (*Agrilus planipennis*). J. Insect Physiol. **72**: 70–78. doi:10.1016/j.jinsphys.2014.12.002.

Zhao, S., Liu, H., Wu, Y., Wu, P., Fu, J., Yang, H., James, A.A., Chen, X. 2024. The odorant-binding protein genes *obp67* and *obp56d-like* encode products that guide oviposition site selection in the Asian tiger mosquito, *Aedes albopictus*. Insect Sci. 0: 1-13. doi:10.1111/1744-7917.13430.

Zhu, F., Xu, J., Palli, R., Ferguson, J., and Palli, S.R. 2011. Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. Pest Manag. Sci. **67**(2): 175–182. doi:10.1002/ps.2048.

Zhu, K.Y., and Palli, S.R. 2020. Mechanisms, Applications, and Challenges of Insect RNA Interference. Annu. Rev. Entomol. **65**(1): 293–311. doi:10.1146/annurev-ento-011019-025224.

Zimmermann, G. 2007. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontrol Sci. Technol. **17**(6): 553–596. doi:10.1080/09583150701309006.

Zmienko, A., Samelak-Czajka, A., Goralski, M., Sobieszczuk-Nowicka, E., Kozlowski, P., and Figlerowicz, M. 2015. Selection of Reference Genes for qPCR- and ddPCR-Based Analyses of Gene Expression in Senescing Barley Leaves. PLOS ONE **10**(2): e0118226. doi:10.1371/journal.pone.0118226.

Appendix

Chapter 2

Table S1 Protein-coding sequences and accession numbers of gene targets in mountain pine beetle (Dendroctonus ponderosae). Long dsRNA sequence is highlighted in yellow. Digital droplet PCR (ddPCR) primers (highlighted in green) were designed outside of the dsRNA region to avoid inclusion of the dsRNA in the gene quantification.

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***vha19* - XM_019903780.2**

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***ssk* - XM_048663913.1**

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dsRS

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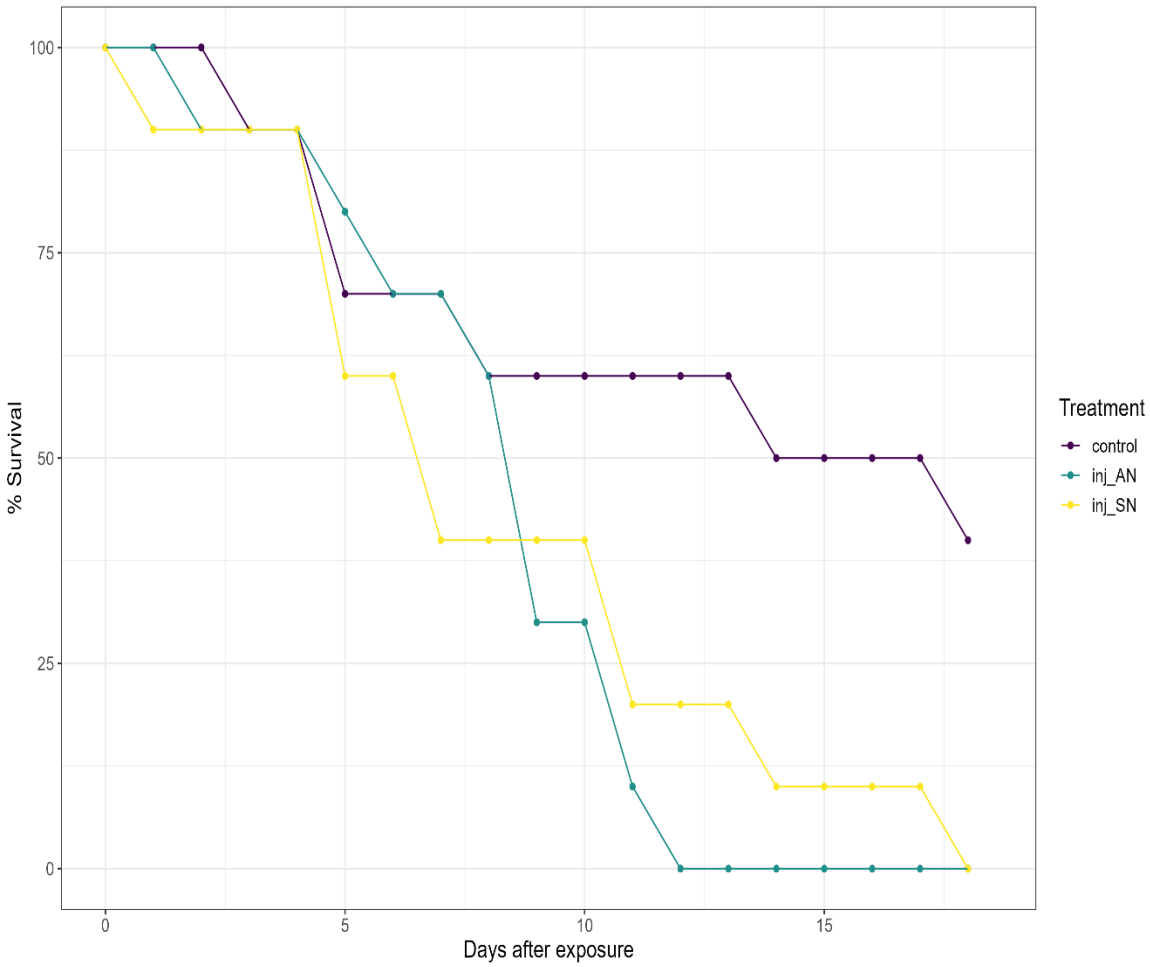

Figure S1 Mortality of adult mountain pine beetle (*Dendroctonus ponderosae*, MPB) in the microinjection trial. Survival of the beetles injected with angled (inj_AN) and side-delivery (inj_SN) needles (Hamilton, Canada) were compared to the survival of non-injected (control) beetles. The initial survival pattern along with the injection process with each needle, were considered in the selection of t he selection of the best needle.

Chapter 3

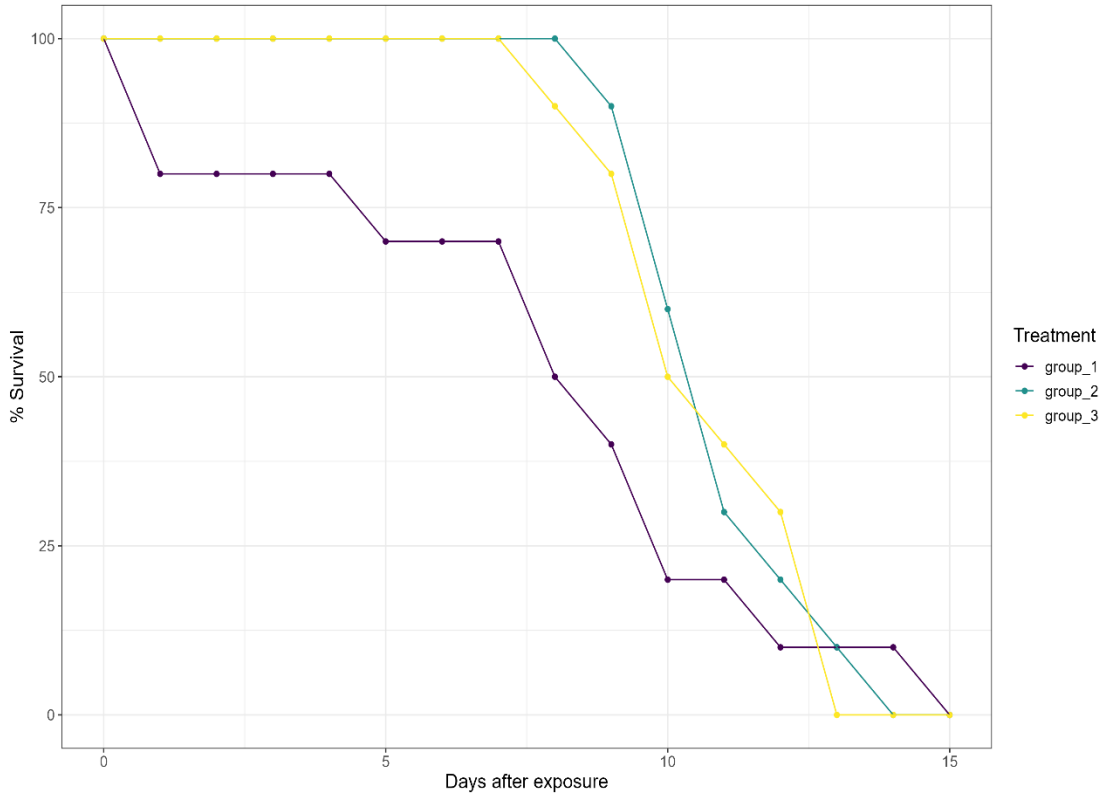

Figure S2 Adult MPB percent survival in three different sucrose feeding treatment groups: no treatment (group_1), submerged in a 1% sucrose solution (group_2), and force fed a vertically suspended droplet (group_3). Mortality was recorded for 15 days.

Table S2 A tally of the presence or absence of blue dye in MPB adult guts after exposure to a blue-dyed 1% sucrose solution.

	Blue Dye Present	Blue Dye Absent
Vertical Droplet Suspension	0	10
Beetle Submergence	2	8

Table S3 Observation of the presence or absence of blue dye in MPB adult guts after exposure to blue-dyed 1% sucrose as proof of ingestion.

	Blue Dye Present	Blue Dye Absent
Vertical Droplet Suspension	0	10
Beetle Submergence	4	6

Table S4 Presence or absence of blue dye in the guts of adult MPB after being submerged in blue-dyed 1% sucrose for 12h.

Blue Dye Present	Blue Dye Absent
6	4

Table S5 Final tally of surviving "starved" and "fed" adult MPB 16 days post-submergence in the three treatment groups: water, 0.05mg/ml and 0.1mg/ml hpRS-expressing yeast solutions.

	Water	0.05mg/ml hpRS	0.1mg/ml hpRS
Starved	2 of 3	2 of 3	3 of 3
Fed	3 of 3	2 of 3	2 of 3

Chapter 4

Table S6 Quantification of hpRS translocated in phloem at the top (phT#), middle (phM#), and bottom (phB#) of pine rounds placed in yeast solutions (see section 4.2.3.4.2). Included are no-reverse transcriptase controls (-RTC_#), a no-template control (NTC), and a positive control (E gBlock). For further definitions of controls, refer to Chapter 2, section 2.2.8.1 and 2.2.8.4.

Well	Sample	Copies/µl	Copies
A10	Top1	18.2	364
A11	phT1	19.7	394
B10	phT2	4.96	99.2
B11	phT2	6.35	127
C10	phT3	5.71	114.2
C11	phT3	6.26	125.2
D10	phM1	6.48	129.6
D11	phM1	4.21	84.2
E10	phM2	2.51	50.2
E11	phM2	3.62	72.4
F10	phM3i	9.24	184.8
F11	phM3i	8.43	168.6
G10	phB1	2.4	48
G11	phB1	1.9	38
H10	phB2	4.31	86.2
H11	phB2	3.49	69.8
A12	phB3	2.07	41.4
B12	phB3	2.22	44.4
C12	-RTCM1	0.298	5.96
D12	-RTCM1	0.513	10.26
E12	-RTCT2	0.116	2.32
F12	-RTCB1	0.243	4.86
G12	E gBlock	26.3	526
H12	NTC	0	0

Chapter 5

Table S7 Quality check results for total RNA eluted from adult MPB in previous RNAi assays meeting requirements for 3'TagSeq library prep and sequencing. Concentration measured with the Qubit *4* Fluorometer Broad Range RNA assay (Thermo Fisher Scientific Inc., 2015) and the sample integrity (RIN) was determined using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). The ND-1000 Nanodrop (v.3.8.1, NanoDrop Technologies 2024) was used to measure the 260/280 and 260/230 ratios. The 3'Tag-Seq analysis requirements are provided in red. * Though the RIN assigned to the samples is not reliable for insect RNA as there is an endogenous break in the 28S rRNA, the RNA quality was visually assessed using the electropherogram for each sample, showing little to no degredation and the two peaks close together (Fabrick and Hull 2017).

Sample	Biological Rep	Delivery Method	Amount sent (ng)	RIN score (>6)*	260/280 ratio (1.8-2.1)	260/230 ratio (>1.5)
C22_1	A	No treatment 2022	1500	N/A	2.1	1.8
C22_3	B	No treatment 2022	1500	6.4	2.1	1.5
C22_4	C	No treatment 2022	1500	6.8	2.1	1.5
C23_1	A	No treatment 2023	1500	6.2	2.1	1.7
C23_4	B	No treatment 2023	1500	7.1	2.1	2
C23_5	C	No treatment 2023	1500	6.9	2.1	2.1
J22S_D3	A	Submergence	1500	5.6	2.1	1.6
J22S_D4	B	Submergence	1500	5.6	2.1	1.9
J22S_D5	C	Submergence	1500	N/A	2.1	1.6
J22S_I1	A	Submergence	1500	6.8	2.1	2.1
J22S_I4	B	Submergence	1500	7.1	2.1	1.7
J22S_I5	C	Submergence	1500	6.9	2.1	2
A16M_W1	A	Microinjection	1500	5.9	2.1	2
A16M_W3	B	Microinjection	1500	6.8	2.1	1.5
A16M_W5	C	Microinjection	1500	6.3	2.1	1.5
A16M_D2	A	Microinjection	1500	6.3	2.1	1.9
A16M_D3	B	Microinjection	1500	6.3	2.1	1.8
A16M_D5	C	Microinjection	1500	6.7	2.1	1.2
PHPF_W1	A	Phloem-fed	1500	6.5	2.1	2
PHPF_W4	B	Phloem-fed	1500	6.6	2.1	2
PHPF_W5	C	Phloem-fed	1500	7.0	2.1	2
PHPF_D2	A	Phloem-fed	1500	6.1	2.1	1.5
PHPF_D3	B	Phloem-fed	1500	7.6	2	1.5
PHPF_D5	C	Phloem-fed	1500	6.6	2.1	1.6

Table S8 Read count after quality control (QC) and mapping rate summary for each total MPB RNA sample in the differential gene expression analysis.

Sample	Reads after QC	Reads uniquely mapped	Uniquely mapped rates (%)
A16M_D2	6014032	3522181	58.57
A16M_D3	6089371	3964016	65.10
A16M_D5	6547129	4187597	63.96
A16M_W1	6841191	3968350	58.01
A16M_W3	6259254	3526978	56.35
A16M_W5	6613472	3880999	58.68
C22_1	7243905	3872899	53.46
C22_3	6917986	4085141	59.05
C22_4	7691051	4093132	53.22
C23_1	7570401	3874023	51.17
C23_4	7067160	3593754	50.85
C23_5	7043497	3299466	46.84
J22S_D3	7628782	3778522	49.53
J22S_D4	6732600	3466626	51.49
J22S_D5	6459394	3571064	55.28
J22S_I1	7299763	3628493	49.71
J22S_I4	6902227	3787147	54.87
J22S_I5	7465229	4417637	59.18
PHPF_D2	6728546	4047544	60.15
PHPF_D3	6777632	4264421	62.92
PHPF_D5	6137461	3317399	54.05
PHPF_W1	7163238	4279602	59.74
PHPF_W4	6702677	3949065	58.92
PHPF_W5	6575207	3915640	59.55

Table S9 List of annotated GO terms associated with up-regulated genes in adult MPB submerged in dsIAP (J22S_I) compared to adult MPB not submerged at all (C22) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0005044	0.000597	2	MF	scavenger receptor activity
GO:0140662	0.001312	2	MF	ATP-dependent protein folding chaperone
GO:0004564	0.001345	1	MF	beta-fructofuranosidase activity
GO:0008010	0.001347	1	MF	structural constituent of chitin-based larval cuticle
GO:0042302	0.001347	1	MF	structural constituent of cuticle
GO:0062129	0.001347	1	CC	chitin-based extracellular matrix
GO:0003998	0.002685	1	MF	acylphosphatase activity
GO:0003994	0.004025	1	MF	aconitate hydratase activity
GO:0042853	0.005363	1	BP	L-alanine catabolic process
GO:0004017	0.010699	1	MF	adenylate kinase activity
GO:0035312	0.012028	1	MF	5'-3' exodeoxyribonuclease activity

GO:0003995	0.016007	1	MF	acyl-CoA dehydrogenase activity
GO:0098599	0.01865	1	MF	palmitoyl hydrolase activity
GO:0098734	0.01865	1	BP	macromolecule depalmitoylation
GO:0004089	0.01997	1	MF	carbonate dehydratase activity
GO:0046940	0.026541	1	BP	nucleoside monophosphate phosphorylation
GO:0016829	0.029891	2	MF	lyase activity
GO:0005739	0.030417	6	CC	mitochondrion
GO:0042026	0.033069	1	BP	protein refolding
GO:0008483	0.033069	1	MF	transaminase activity
GO:0004364	0.035669	1	MF	glutathione transferase activity
GO:0006457	0.036711	2	BP	protein folding
GO:0006099	0.044715	1	BP	tricarboxylic acid cycle
GO:0031672	0.049847	1	CC	A band

Table S10 List of annotated GO terms associated with down-regulated genes in adult MPB submerged in dsIAP (J22S_I) compared to adult MPB not submerged at all (C22) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:1990439	0.000724	1	MF	MAP kinase serine/threonine phosphatase activity
GO:0140791	0.000724	1	MF	histone H2AXS140 phosphatase activity
GO:0180004	0.000724	1	MF	RNA polymerase II CTD heptapeptide repeat Y1 phosphatase activity
GO:0180005	0.000724	1	MF	RNA polymerase II CTD heptapeptide repeat T4 phosphatase activity
GO:0180006	0.000724	1	MF	RNA polymerase II CTD heptapeptide repeat S2 phosphatase activity
GO:0180007	0.000724	1	MF	RNA polymerase II CTD heptapeptide repeat S5 phosphatase activity
GO:0180008	0.000724	1	NA	RNA polymerase II CTD heptapeptide repeat S7 phosphatase activity
GO:0010349	0.000724	1	MF	L-galactose dehydrogenase activity
GO:0033192	0.003612	1	MF	calmodulin-dependent protein phosphatase activity
GO:0008422	0.004333	1	MF	beta-glucosidase activity
GO:0090385	0.010083	1	BP	phagosome-lysosome fusion
GO:0005385	0.017939	1	MF	zinc ion transmembrane transporter activity
GO:0016042	0.019635	2	BP	lipid catabolic process
GO:0051119	0.020072	1	MF	sugar transmembrane transporter activity
GO:0071577	0.020781	1	BP	zinc II ion transmembrane transport
GO:0006829	0.0222	1	BP	zinc II ion transport
GO:0016592	0.026443	1	CC	mediator complex
GO:0034219	0.043247	1	BP	carbohydrate transmembrane transport
GO:0008643	0.046021	1	BP	carbohydrate transport

Table S11 List of annotated GO terms associated with up-regulated genes in adult MPB submerged in dsRS (J22S_D) compared to adult MPB not submerged at all (C22) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0005739	3.49E-05	12	CC	mitochondrion
GO:0003995	0.000196	2	MF	acyl-CoA dehydrogenase activity
GO:0008299	0.001023	2	BP	isoprenoid biosynthetic process
GO:0006099	0.001622	2	BP	tricarboxylic acid cycle
GO:0004163	0.00176	1	MF	diphosphomevalonate decarboxylase activity
GO:0008010	0.001763	1	MF	structural constituent of chitin-based larval cuticle
GO:0042302	0.001763	1	MF	structural constituent of cuticle
GO:0062129	0.001763	1	CC	chitin-based extracellular matrix
GO:0006097	0.00351	1	BP	glyoxylate cycle
GO:0050992	0.00351	1	BP	dimethylallyl diphosphate biosynthetic process
GO:0004450	0.00351	1	MF	isocitrate dehydrogenase (NADP+) activity
GO:0004452	0.00351	1	MF	isopentenyl-diphosphate delta-isomerase activity
GO:0019287	0.005261	1	BP	isopentenyl diphosphate biosynthetic process, mevalonate pathway
GO:0034462	0.005261	1	BP	small-subunit processome assembly
GO:0003994	0.005261	1	MF	aconitate hydratase activity
GO:0017099	0.005261	1	MF	very-long-chain-acyl-CoA dehydrogenase activity
GO:0046592	0.005261	1	MF	polyamine oxidase activity
GO:0140300	0.005261	1	BP	serine import into mitochondrion
GO:0016829	0.005283	3	MF	lyase activity
GO:0042853	0.007009	1	BP	L-alanine catabolic process
GO:0004035	0.007009	1	MF	alkaline phosphatase activity
GO:0016491	0.007721	5	MF	oxidoreductase activity
GO:0000472	0.008754	1	BP	endonucleolytic cleavage generating mature 5'-end of SSU-rRNA
GO:0009240	0.008754	1	BP	isopentenyl diphosphate biosynthetic process
GO:0120170	0.008754	1	MF	intraciliary transport particle B binding
GO:0050660	0.010207	2	MF	flavin adenine dinucleotide binding
GO:0006102	0.010496	1	BP	isocitrate metabolic process
GO:0003985	0.010496	1	MF	acetyl-CoA C-acetyltransferase activity
GO:0000480	0.012234	1	BP	endonucleolytic cleavage in 5'-ETS of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
GO:0000447	0.015703	1	BP	endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA

				from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
GO:0033539	0.01916	1	BP	fatty acid beta-oxidation using acyl-CoA dehydrogenase
GO:0004089	0.026039	1	MF	carbonate dehydratase activity
GO:0000062	0.027751	1	MF	fatty-acyl-CoA binding
GO:0030992	0.029461	1	CC	intraciliary transport particle B
GO:0042026	0.043031	1	BP	protein refolding
GO:0008483	0.043031	1	MF	transaminase activity
GO:0005044	0.046395	1	MF	scavenger receptor activity
GO:0004364	0.046395	1	MF	glutathione transferase activity

Table S12 List of annotated GO terms associated with down-regulated genes in adult MPB submerged in dsRS (J22S_D) compared to adult MPB not submerged at all (C22) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008061	4.11E-05	2	MF	chitin binding
GO:0005549	0.000449	3	MF	odorant binding
GO:0102210	0.001243	1	MF	rhamnogalacturonan endolyase activity
GO:0010349	0.001243	1	MF	L-galactose dehydrogenase activity
GO:0030599	0.001243	1	MF	pectinesterase activity
GO:0042545	0.001243	1	BP	cell wall modification
GO:0045490	0.001243	1	BP	pectin catabolic process
GO:0005415	0.004951	1	MF	nucleoside:sodium symporter activity
GO:0008422	0.007418	1	MF	beta-glucosidase activity
GO:0009922	0.008649	1	MF	fatty acid elongase activity
GO:0034625	0.008649	1	BP	fatty acid elongation, monounsaturated fatty acid
GO:0034626	0.008649	1	BP	fatty acid elongation, polyunsaturated fatty acid
GO:0019367	0.009879	1	BP	fatty acid elongation, saturated fatty acid
GO:0005337	0.012334	1	MF	nucleoside transmembrane transporter activity
GO:1901642	0.012334	1	BP	nucleoside transmembrane transport
GO:0020037	0.013273	2	MF	heme binding
GO:0005344	0.016006	1	MF	oxygen transporter activity
GO:0042761	0.016006	1	BP	very long-chain fatty acid biosynthetic process
GO:0015671	0.017227	1	BP	oxygen transport
GO:0007608	0.018424	3	BP	sensory perception of smell
GO:0016460	0.030563	1	CC	myosin II complex
GO:0051119	0.03417	1	MF	sugar transmembrane transporter activity

GO:0019825	0.037764	1	MF	oxygen binding

Table S13 List of annotated GO terms associated with up-regulated genes in adult MPB fed water-associated with phloem (PHPF_W) compared to adult MPB not fed at all (C23) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008810	2.24E-14	5	MF	cellulase activity
GO:0030245	2.24E-14	5	BP	cellulose catabolic process
GO:0016798	2.91E-12	9	MF	hydrolase activity, acting on glycosyl bonds
GO:0045490	3.77E-06	2	BP	pectin catabolic process
GO:0004553	0.000753	3	MF	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0004497	0.001204	3	MF	monooxygenase activity
GO:0005975	0.001671	5	BP	carbohydrate metabolic process
GO:0000246	0.001967	1	MF	delta24(24-1) sterol reductase activity
GO:0050614	0.001967	1	MF	delta24-sterol reductase activity
GO:0030599	0.001971	1	MF	pectinesterase activity
GO:0042545	0.001971	1	BP	cell wall modification
GO:0008218	0.001971	1	BP	bioluminescence
GO:0010349	0.001971	1	MF	L-galactose dehydrogenase activity
GO:0004650	0.001971	1	MF	polygalacturonase activity
GO:0071555	0.001971	1	BP	cell wall organization
GO:0005777	0.002812	3	CC	peroxisome
GO:0005506	0.003276	3	MF	iron ion binding
GO:0080019	0.003923	1	MF	fatty-acyl-CoA reductase (alcohol-forming) activity
GO:0102965	0.003923	1	MF	alcohol-forming long-chain fatty acyl-CoA reductase activity
GO:0004768	0.005879	1	MF	stearoyl-CoA 9-desaturase activity
GO:0004505	0.005879	1	MF	phenylalanine 4-monooxygenase activity
GO:0006571	0.005879	1	BP	tyrosine biosynthetic process
GO:0019293	0.005879	1	BP	tyrosine biosynthetic process, by oxidation of phenylalanine
GO:0052689	0.006649	3	MF	carboxylic ester hydrolase activity
GO:0006196	0.00978	1	BP	AMP catabolic process
GO:0008422	0.011724	1	MF	beta-glucosidase activity
GO:0009922	0.013665	1	MF	fatty acid elongase activity
GO:0034625	0.013665	1	BP	fatty acid elongation, monounsaturated fatty acid
GO:0034626	0.013665	1	BP	fatty acid elongation, polyunsaturated fatty acid
GO:0008061	0.015603	1	MF	chitin binding

GO:0019367	0.015603	1	BP	fatty acid elongation, saturated fatty acid
GO:0006559	0.015603	1	BP	L-phenylalanine catabolic process
GO:0016791	0.016119	3	MF	phosphatase activity
GO:0015187	0.019466	1	MF	glycine transmembrane transporter activity
GO:0016717	0.021392	1	MF	oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water
GO:0006633	0.022107	2	BP	fatty acid biosynthetic process
GO:0005549	0.023898	2	MF	odorant binding
GO:0008253	0.025234	1	MF	5'-nucleotidase activity
GO:0015816	0.025234	1	BP	glycine transport
GO:0042761	0.025234	1	BP	very long-chain fatty acid biosynthetic process
GO:0008252	0.02906	1	MF	nucleotidase activity
GO:0005283	0.044221	1	MF	sodium:amino acid symporter activity
GO:0035336	0.047975	1	BP	long-chain fatty-acyl-CoA metabolic process

Table S14 List of annotated GO terms associated with down-regulated genes in adult MPB fed water-associated with phloem (PHPF_W) compared to adult MPB not fed at all (C23) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0006635	4.56E-05	3	BP	fatty acid beta-oxidation
GO:0004108	0.00119	1	MF	citrate (Si)-synthase activity
GO:0004163	0.00119	1	MF	diphosphomevalonate decarboxylase activity
GO:0008775	0.00119	1	MF	acetate CoA-transferase activity
GO:0042132	0.002375	1	MF	fructose 1,6-bisphosphate 1-phosphatase activity
GO:0005986	0.002375	1	BP	sucrose biosynthetic process
GO:0019287	0.003561	1	BP	isopentenyl diphosphate biosynthetic process, mevalonate pathway
GO:0006083	0.003561	1	BP	acetate metabolic process
GO:0140300	0.003561	1	BP	serine import into mitochondrion
GO:0005739	0.004052	7	CC	mitochondrion
GO:0003986	0.004745	1	MF	acetyl-CoA hydrolase activity
GO:0006101	0.005928	1	BP	citrate metabolic process
GO:0003985	0.00711	1	MF	acetyl-CoA C-acetyltransferase activity
GO:0003857	0.00711	1	MF	3-hydroxyacyl-CoA dehydrogenase activity

GO:0030388	0.009469	1	BP	fructose 1,6-bisphosphate metabolic process
GO:0032222	0.009469	1	BP	regulation of synaptic transmission, cholinergic
GO:0030431	0.010647	1	BP	sleep
GO:0003995	0.014172	1	MF	acyl-CoA dehydrogenase activity
GO:0006002	0.015344	1	BP	fructose 6-phosphate metabolic process
GO:0006000	0.016515	1	BP	fructose metabolic process
GO:0004467	0.017685	1	MF	long-chain fatty acid-CoA ligase activity
GO:0097428	0.018853	1	BP	protein maturation by iron-sulfur cluster transfer
GO:0070403	0.02002	1	MF	NAD+ binding
GO:0016747	0.025524	2	MF	transferase activity, transferring acyl groups other than amino-acyl groups
GO:0042026	0.029308	1	BP	protein refolding
GO:0008299	0.031616	1	BP	isoprenoid biosynthetic process
GO:0051537	0.031616	1	MF	2 iron, 2 sulfur cluster binding
GO:0046949	0.032769	1	BP	fatty-acyl-CoA biosynthetic process
GO:0016226	0.032769	1	BP	iron-sulfur cluster assembly
GO:0006099	0.039656	1	BP	tricarboxylic acid cycle
GO:0006695	0.043082	1	BP	cholesterol biosynthetic process
GO:0035025	0.045359	1	BP	positive regulation of Rho protein signal transduction

Table S15 List of annotated GO terms associated with up-regulated genes in adult MPB fed hpRS-associated with phloem (PHPF_D) compared to adult MPB not fed at all (C23) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008810	4.63E-06	2	MF	cellulase activity
GO:0030245	4.63E-06	2	BP	cellulose catabolic process
GO:0004768	1.38E-05	2	MF	stearoyl-CoA 9-desaturase activity
GO:0140575	2.75E-05	2	MF	transmembrane monodehydroascorbate reductase activity
GO:0008061	0.000128	2	MF	chitin binding
GO:0016717	0.00025	2	MF	oxidoreductase activity
GO:0005506	0.000325	4	MF	iron ion binding
GO:0006633	0.002082	3	BP	fatty acid biosynthetic process
GO:0033961	0.002175	1	MF	cis-stilbene-oxide hydrolase activity
GO:0010349	0.002179	1	MF	L-galactose dehydrogenase activity
GO:0008010	0.002179	1	MF	structural constituent of chitin-based larval cuticle

GO:0042302	0.002179	1	MF	structural constituent of cuticle
GO:0062129	0.002179	1	CC	chitin-based extracellular matrix
GO:0016798	0.002808	3	MF	hydrolase activity, acting on glycosyl bonds
GO:0006636	0.003238	2	BP	unsaturated fatty acid biosynthetic process
GO:0016491	0.004172	6	MF	oxidoreductase activity
GO:0080019	0.004336	1	MF	fatty-acyl-CoA reductase (alcohol-forming) activity
GO:0102965	0.004336	1	MF	alcohol-forming long-chain fatty acyl-CoA reductase activity
GO:0008107	0.004336	1	MF	galactoside 2-alpha-L-fucosyltransferase activity
GO:0004180	0.004672	2	MF	carboxypeptidase activity
GO:0004477	0.006496	1	MF	methenyltetrahydrofolate cyclohydrolase activity
GO:0004487	0.006496	1	MF	methylenetetrahydrofolate dehydrogenase (NAD+) activity
GO:0102158	0.008653	1	MF	very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase activity
GO:0004488	0.008653	1	MF	methylenetetrahydrofolate dehydrogenase (NADP+) activity
GO:0006196	0.010805	1	BP	AMP catabolic process
GO:0004185	0.010805	1	MF	serine-type carboxypeptidase activity
GO:0097176	0.010805	1	BP	epoxide metabolic process
GO:0008422	0.012952	1	MF	beta-glucosidase activity
GO:0004301	0.017233	1	MF	epoxide hydrolase activity
GO:0018812	0.017233	1	MF	3-hydroxyacyl-CoA dehydratase activity
GO:0035999	0.019366	1	BP	tetrahydrofolate interconversion
GO:0016803	0.021495	1	MF	ether hydrolase activity
GO:0004497	0.022581	2	MF	monooxygenase activity
GO:0008253	0.027855	1	MF	5'-nucleotidase activity
GO:0042761	0.027855	1	BP	very long-chain fatty acid biosynthetic process
GO:0030497	0.029966	1	BP	fatty acid elongation
GO:0016298	0.031907	2	MF	lipase activity
GO:0008252	0.032073	1	MF	nucleotidase activity
GO:0004089	0.032073	1	MF	carbonate dehydratase activity
GO:0036065	0.036273	1	BP	fucosylation
GO:0020037	0.038015	2	MF	heme binding

Table S16 List of annotated GO terms associated with down-regulated genes in adult MPB fed hpRS-associated with phloem compared (PHPF_D) to adult MPB not fed at all (C23) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008010	0.001139	1	MF	structural constituent of chitin-based larval cuticle
GO:0042302	0.001139	1	MF	structural constituent of cuticle
GO:0062129	0.001139	1	CC	chitin-based extracellular matrix
GO:0004170	0.002272	1	MF	dUTP diphosphatase activity
GO:0006226	0.002272	1	BP	dUMP biosynthetic process
GO:0046081	0.002272	1	BP	dUTP catabolic process
GO:0017108	0.006802	1	MF	5'-flap endonuclease activity
GO:0000808	0.009059	1	CC	origin recognition complex
GO:0005664	0.009059	1	CC	nuclear origin of replication recognition complex
GO:0032222	0.009059	1	BP	regulation of synaptic transmission, cholinergic
GO:0030431	0.010186	1	BP	sleep
GO:0000400	0.012436	1	MF	four-way junction DNA binding
GO:0003688	0.015802	1	MF	DNA replication origin binding
GO:0004467	0.016922	1	MF	long-chain fatty acid-CoA ligase activity
GO:0097428	0.01804	1	BP	protein maturation by iron-sulfur cluster transfer
GO:0000900	0.019157	1	MF	translation repressor activity, nucleic acid binding
GO:0045947	0.026943	1	BP	negative regulation of translational initiation
GO:0051537	0.030262	1	MF	2 iron, 2 sulfur cluster binding
GO:0016226	0.031366	1	BP	iron-sulfur cluster assembly
GO:0046949	0.031366	1	BP	fatty-acyl-CoA biosynthetic process
GO:0030371	0.037964	1	MF	translation repressor activity
GO:0035025	0.043429	1	BP	positive regulation of Rho protein signal transduction

Table S17 List of annotated GO terms associated with up-regulated genes in adult MPB fed hpRS-associated with phloem (PHPF_D) compared to adult MPB fed water-associated with phloem (PHPF_W) obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008010	1.50E-07	2	MF	structural constituent of chitin-based larval cuticle
GO:0042302	1.50E-07	2	MF	structural constituent of cuticle
GO:0062129	1.50E-07	2	CC	chitin-based extracellular matrix

GO:0015020	0.01397	1	MF	glucuronosyltransferase activity
GO:0008080	0.022919	1	MF	N-acetyltransferase activity
GO:0042246	0.028576	1	BP	tissue regeneration
GO:0004497	0.04339	1	MF	monooxygenase activity

Table S18 List of annotated GO terms associated with down-regulated genes in adult MPB fed hpRS-associated with phloem compared to adult MPB fed water-associated with phloem obtained from NaviGO. Categories of GO terms include: cellular component (CC), biological processes (BP), and molecular function (MF). Enrichment is provided with calculated p-value, significance level is $p<0.05$.

GO term	P-value	Count	Category	Annotation
GO:0008810	0.000362	1	MF	cellulase activity
GO:0030245	0.000362	1	BP	cellulose catabolic process
GO:0004170	0.000723	1	MF	dUTP diphosphatase activity
GO:0006226	0.000723	1	BP	dUMP biosynthetic process
GO:0046081	0.000723	1	BP	dUTP catabolic process
GO:0004505	0.001085	1	MF	phenylalanine 4-monooxygenase activity
GO:0006571	0.001085	1	BP	tyrosine biosynthetic process
GO:0019293	0.001085	1	BP	tyrosine biosynthetic process, by oxidation of phenylalanine
GO:0017108	0.002168	1	MF	5'-flap endonuclease activity
GO:0006559	0.00289	1	BP	L-phenylalanine catabolic process
GO:0000400	0.003972	1	MF	four-way junction DNA binding
GO:0007094	0.010802	1	BP	mitotic spindle assembly checkpoint
GO:0004867	0.036669	1	MF	serine-type endopeptidase inhibitor activity
GO:0004497	0.03807	1	MF	monooxygenase activity
GO:0016798	0.046089	1	MF	hydrolase activity, acting on glycosyl bonds