Susceptibility of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) to gene silencing through RNAi provides potential as a novel management tool

Bethany R. Kyre a, Barbara J. Bentz b, Lynne K. Rieske a,⁎

a Department of Entomology, University of Kentucky S225 Ag North, Lexington, KY 40546-0091, USA
b USDA Forest Service Rocky Mountain Research Station, Logan, UT 84321, USA

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**ABSTRACT**

The mountain pine beetle (MPB), *Dendroctonus ponderosae*, is an eruptive endemic forest pest that is undergoing substantial range expansion in response to recent climatic changes, breaching geographic barriers, exploiting novel hosts, and affecting millions of hectares of conifer forests in western North America. Current management approaches have been unable to keep pace with MPB population outbreaks, and novel and aggressive management responses are required as MPB’s range expansion progresses. Gene silencing through RNA interference (RNAi) is an emerging pest management approach that is being developed for agricultural pests, and has also been shown to be effective against some xylophagous forest pests, including the southern pine beetle (SPB), *D. frontalis*. When essential genes are targeted, RNAi can cause rapid insect mortality; here we focus on evaluating its effectiveness in MPB. We identified reference genes for quantitative real-time PCR (qPCR) and validated RNAi responses in MPB by analyzing gene expression and beetle survival. Using an adult bioassay that combined oral ingestion and dermal absorption of dsRNA targeting three genes (*hsp, iap, and shi*), we measure gene expression and demonstrate silencing, as well as insect mortality, following dsRNA exposure. All three genes were silenced and all treatment beetles died within 7 d. This validates reference genes for expression analyses and demonstrates that MPB, similar to the congeneric SPB, has a highly sensitive RNAi response. Additionally, we document sex-specific differences in gene expression for one of the three target genes, *hsp*; any differences in gene expression and subsequent mortality based on sex must be considered as this technology progresses as a pest management tool. RNAi causes rapid insect mortality when essential genes are targeted, is highly specific to the target pest, and has no environmental contamination risks, making it an attractive approach for further development in forest pest suppression.

1. Introduction

In addition to globalization, increasingly erratic temperatures and precipitation are enabling forest pests to expand beyond their historical geographic ranges (Liebold and Williams, 2002, Ziska et al., 2011, Ramsfield et al., 2016); an expansion facilitated by exploitation of naïve hosts (Cudmore et al., 2010, Cipollini and Rigsby, 2015) and lack of natural enemies (Keane and Crawley, 2002, Wolfe, 2002, Olson and Rieske, 2019). Forest invaders may be exotic, such as the emerald ash borer, *Agrilus planipennis* (Li et al., 2019) and the red bay ambrosia beetle, *Xyleborus glabratus* (Lira-Noriega et al., 2018), or they may be native but undergoing substantial range expansion, such as the gold spotted oak borer, *Agrilus auroguttatus* (Coleman and Seybold, 2011), the southern pine beetle, *Dendroctonus frontalis* (Lesk et al., 2017), and the mountain pine beetle, *D. ponderosae* (Carroll et al., 2003). The impacts of these invading forests pests are economic (Corbett et al., 2016, Bachert et al., 2017), with devastating losses in timber (Pye et al., 2011) and tourism revenue (Cahyanto et al., 2018, Arnerger et al., 2018), and ecological, as extensive tree mortality leads to significant disturbances that influence ecosystem function (Jenkins et al., 1999, Clarke et al., 2000, Kenis et al., 2009, Lovett et al., 2013, Dhar et al., 2016a, 2016b).

In forests of western North America, native bark beetles are among the most important sources of tree mortality, in some years exceeding that of tree mortality due to wildfire (Hicke et al., 2016, Berner et al., 2017). Although the majority of native bark beetles are integral components of forest ecosystems, contributing to decomposition and affecting successional trajectories (Matson and Addy, 1975, Axelson et al., 2018), a few species can cause extensive tree mortality during population outbreaks, interfering with land management objectives and...
disrupting ecosystem services (Gregoire et al., 2015). Because multiple insect life history traits that influence population success are temperature dependent (Bentz and Jönsson, 2015), warming temperatures associated with climate change have resulted in altered outbreak frequencies of many species within their historical ranges (Weed et al., 2015, Buote et al., 2016). Moreover, for species with historical distributions limited by climate rather than host availability, migration to new, thermally suitable environments is also occurring as temperatures warm (Chen and Godwin, 2011). As these native invaders expand into new regions they are encountering novel forest types and naive hosts, raising the potential for increasing outbreak frequencies and catastrophic tree losses. Novel and aggressive management responses are required.

Mountain pine beetle (Dendroctonus ponderosae Hopkins, Coleoptera: Curculionidae, Scolytinae) (MPB) is an eruptive endemic forest pest that is responding to recent climatic changes through substantial range expansion (Carroll et al., 2003, Safranyik et al., 2010). MPB is native to western North America, inhabiting pine (Pinus spp.) forests from northern Baja California, Mexico to western Canada (Wood, 1982, Dowlle et al., 2017), and has caused tree mortality on millions of hectares of forest in recent decades (Bentz et al., 2010, Meddens et al. 2012, Hickie et al., 2016). Suitable pine hosts occur both to the north and south of the historical MPB distribution, suggesting a role for climate in influencing its current range (Safranyik et al., 2010). The breach of geoclimatic barriers as a result of warmer annual temperatures has led to rapid northward and eastward expansion of MPB in western Canada (Cudmore et al., 2010, De la Giordana et al., 2012), with continued northward expansion projected for the near future (Bentz et al., 2019). MPB is known to attack and reproduce in the majority of pine species within its historical distribution, with few exceptions (Eidson et al., 2018). Additionally, MPB is capable of utilizing several novel pine species which span North America (Furniss and Schenk, 1969), including jack pine, P. banksiana, and the non-native but naturalized Scots pine, P. sylvestris, (Fries, 2017). When populations are low, MPB is found in pines with weakened host defenses that have been colonized by other less aggressive bark beetles (Smith et al., 2011), and it acts to maintain a functionally healthy forest landscape (Dordel et al., 2008, Axelson et al., 2009, Klutch et al., 2009, Raffa et al., 2009) as an important player in forest succession. During outbreaks, however, MPB will attack larger, healthier trees with more robust defenses (Boone et al., 2011), and has the potential to move beyond Pinus spp. into other members of the Pinaceae (Furniss and Schenk, 1969, Huber et al., 2009, Rosenberger et al., 2017).

The large-scale tree mortality associated with recent MPB activity has multiple ecological and economic impacts, including a reversal of the essential role of forests from carbon sinks to carbon sources, at least in the short term prior to regrowth (Hansen et al., 2015, Arora et al., 2016). Outbreaks can affect forest regeneration (Karst et al., 2015), influence ecosystem services (Logan et al., 2010), and affect ignition and fire behavior in complex ways (Hicke et al., 2012, Harvey et al., 2014, Hart and Preston, 2020).

Large ongoing infestations impact forests on a landscape level and are capable of altering local climates (Maness et al., 2012). Habitats that have coevolved with MPB are resilient to MPB related deforestation, however, naïve ecosystems are less capable of rebounding from bark beetle related damage. The ability of MPB to alter meteorological conditions, coupled with its current range expansion, make MPB an ecosystem engineer on a global scale (Bunnell et al., 2011).

Traditional management strategies for MPB include one or a combination of sanitation, insecticides, and semiochemicals (Fettig et al., 2014). Insecticide and semiochemical treatment of individual trees works best on small-scale management areas such as campgrounds and urban settings, focusing on prize trees or high value stands (Fettig et al., 2018). Semiochemical lures can be used to create trap trees and monitor populations, although again these strategies are limited to small and accessible areas (Seybold et al., 2018). At the forest level the use of insecticides and semiochemical baited traps are not cost effective (Fettig et al., 2007) and most common large-scale management falls to strategies that include preventative thinning and controlled burns that are conducted prior to outbreak initiation (Fettig et al., 2010, Fettig et al., 2014, Hood et al., 2016). In particular, salvage operations following an outbreak can negatively impact forest resilience and regeneration (Dobor et al., 2020), and the removal of dead standing trees may be detrimental to wildlife habitat and ecosystem readjustment (Dhar et al., 2016b, Saab et al., 2014). For these reasons, management strategies focusing on MPB and other forest pests must embrace innovative approaches that utilize current advancements in molecular biology and biotechnology.

An emerging approach to pest management uses gene silencing to cause insect mortality by manipulating the cellular RNA interference (RNAi) pathway. RNAi is a naturally occurring immune response in which the translation of protein from RNA is disrupted (Mack, 2007, Dietrich et al., 2017). This process can be induced artificially by introducing exogenous double stranded RNA (dsRNA) into an insect’s midgut either by injection or through oral ingestion (Huvenne and Schmagge, 2010, Yu et al., 2013). The dsRNA contacts Dicer, an endonuclease that cleaves long double-stranded RNA in to short-stranded RNA, or small interfering RNA (siRNA) (Song and Rossi, 2017). These siRNAs are then unwound by a multi-protein complex known as the RNA-induced silencing complex (RISC). One strand is degraded (Qi and Hannon, 2005) while the other acts as a template by which RISC recognizes messenger RNA (mRNA) (Siomi and Siomi, 2009), which is then cleaved by Argonaunt, a protein within the RISC complex (Pratt and Macrae, 2009). Subsequently, the protein which the targeted mRNA coded for is not produced and the function of the gene is silenced. The introduction of carefully targeted dsRNAs can impact any aspect of insect physiology, and because the activation of the RNAi pathway requires a precise match of at least 19 nucleotides (Whyard et al., 2009), non-target effects are rare, occurring primarily in congeneric species, if at all (Poredy et al., 2017). With its success in agriculture, RNAi has recently begun to pique interest in forest pest management research, with successes in gene silencing being demonstrated in emerald ash borer (Zhao et al., 2015, Rodrigues et al., 2017a, 2018), and Asian longhorned beetle, Anoplophora glabripennis (Rodrigues et al., 2017b).

Induction of the RNAi pathway via dsRNA ingestion has also been demonstrated in the southern pine beetle, D. frontalis (SPB) (Kyre et al., 2019), a congeneric of MPB causing widespread tree losses in southern forests. Three genes that code for proteins necessary for basic cellular function and stress responses, heat shock protein (hsp), shibire (shi), and inhibitor of apoptosis (iap), were evaluated. Both hsp and shi, but not iap, are significantly silenced in SPB after introduction of in vitro synthesized dsRNA; this is corroborated with near 100% mortality for the same two genes. Our current focus is to demonstrate the RNAi pathway in MPB, evaluate its efficacy, and investigate its utility as a tool in pest suppression in individual trees, and potentially, whole populations. We evaluated the same three genes in MPB, hsp, shi, and iap, for silencing and subsequent beetle mortality.

2. Materials and methods

2.1. Insects

An MPB-infested limber pine, P. flexilis James, was harvested from the Wasatch-Cache National Forest near Logan, UT, and cut into bolts. Bolts were placed in growth chambers set at 21 °C and adult emergence monitored daily. Upon emergence, beetles were separated by sex (Lyon, 1958) and shipped on ice in standard petri dishes layered with moistened filter paper to the University of Kentucky Forest Entomology Lab to analyze the RNAi response. Once received, beetles were kept on ice until use, and all assays were performed within 72 h of arrival.
2.2. RNA extraction, cDNA synthesis, and qPCR

Total RNA was isolated from adult MPB using TRIzol® Reagent (Life Technologies, Carlsbad, CA), precipitated out using isopropanol, washed twice with 75% EtOH, and resuspended in nuclease free water (autoclaved Mill-Q water, pH 6.998). RNA integrity was verified using gel electrophoresis and absorbances measured at 260/280 and 230/280. Only RNA with absorbances of 1.8 – 2.2 at 260/280 and > 1.7 at 260/230 were used for cDNA synthesis. cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions at a concentration of 1000 ng/µL. A standard curve for qPCR was constructed using a 5-fold dilution that began with a 1/25 dilution; the final dilution was then chosen for gene expression analysis. Each qPCR sample contained 1 µL of 1000 ng/µL synthesized cDNA (diluted 6.4E-5), 0.2 µL of each primer (forward and backward), 3.6 µL of nuclease free ddH2O, and 5 µL of SYBR Green PCR Master Mix (Applied Biosystems, USA); totaling 10 µL. All reactions were performed using SYBR Green Master Mix and amplified under the following cycling conditions using a QuantStudio3 qPCR machine (Applied Biosystems, Beverly, MA): beginning cycle at 95 °C, 40 cycles at 95 °C for denaturation, followed with 30 s at 60 °C for annealing and extension, and ending with generation of a melting curve consisting of a single peak to rule out non-specific product and primer dimer formations. Dissociation curve conditions were 1.6 °C/s at 95 °C for 15 s, followed by 1.6 °C/s at 60 °C for 1 min, and finished with 0.15 °C/s at 95 °C for 15 s.

2.3. Gene expression analysis

For gene expression analysis, three technical replicates were run for each of the six biological samples per treatment and measured using the mean Cq value. Data was normalized using tubulin (Keeling et al., 2012) and ubiquitin (Aw et al., 2010) as reference genes. Relative gene expression was analyzed using the 2−ΔΔCt method (Livak et al., 2001) using the mean expression value of the two reference genes. For statistical analysis of both gene expression and gene silencing, normality was evaluated using a Shapiro-Wilk Test. A Student’s two-tailed T-test was used to evaluate significance (P < 0.05) of target gene expression between males and females. Stacked table of gene silencing was measured using a Student’s one-tailed T-test (P < 0.05) as there was no upregulation of target genes compared to the gfp control. qPCR primers for the three target genes were designed using IDT primer building tools and validated using correlation coefficients (R2) and amplification efficiencies (Eff) (Table 1). A desired R2 is > 0.99 and acceptable amplification efficiencies fell between 90% and 110%. Primers that did not meet R2 and amplification efficiencies were redesigned until appropriate numbers were attained.

2.4. dsRNA synthesis from cDNA

Mountain pine beetle specific primers designed to target heat shock protein (hsp), shibire (shi), and inhibitor of apoptosis (iap) genes containing a T7 promoter sequence were used to amplify dsRNA templates (Table 2) under the following PCR conditions: 94 °C for 4 min then held for 35 cycles of 30 sec, 60°C for 30 sec, then 72°C for 45 sec. Finally, extension incubation took place at 72 °C for 10 min. Resulting PCR templates were purified using a Qiagen PCR purification kit (Qiagen, Germantown, MD) and purified PCR was synthesized into dsRNA using T7 reverse transcriptase kit (Thermo Scientific, Waltham, MA). The reaction mix was incubated 16 h at 37 °C followed by 30 min of DNase treatment at 37 °C. After DNase treatment, dsRNA was recovered using 0.1 x volume of sodium acetate and 2.5 x volume of 100% EtOH. dsRNA was then washed twice with 75% EtOH and dried at 37 °C to be resuspended in nuclease free H2O. dsRNA quality was checked using electrophoresis and quantified using a spectrophotometer (NanoDrop, Wilmington, DE) at absorbances of 260/280 and 260/230.

2.5. Assays

To evaluate the effects of RNAi, adult MPB were exposed to dsRNAs targeting either hsp, shi, and iap, or green florescent protein (gfp) as a control; gene expression was evaluated at a single time interval (24 h), whereas survival was evaluated over 8 days. Beetles were placed anteriorly in microcentrifuge tubes containing 2.5 µg/µL of either MPB specific dsRNA or dsGFP. Enough solution was used to fully submerge the beetle to the posterior end of the pronotum. In addition to oral ingestion, this allowed for dsRNA absorption through sutures and any exposed membranes, without disrupting oxygen uptake. A compressed kimwipe was then placed in the top of the tube to prevent beetle movement (Fig. 1). Tubes with treated beetles were placed in a dark

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**Table 1**

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<th>Gene</th>
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<th>R²</th>
<th>%Eff</th>
<th>Amplicon (bp)</th>
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**Table 2**

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incubator and held at 23 °C. After 12 h, beetles (N = 10) were removed from the RNA-containing tubes and placed into 100 mm petri dishes lined with moistened, autoclaved filter paper and returned to the incubator. Filter paper was replaced at 48 h intervals as needed. Mortality was recorded and dead beetles removed at 24 h intervals for the duration of the week-long assay. A one-way ANOVA was used to analyze beetle mortality, and Tukey's test was used to evaluate differences between individual treatments. Abbott’s formula was used to correct for mortality in the control treatment.

Beetles evaluated for gene silencing (a subsample of N = 6 per dsRNA treatment) were collected 24 h after initial dsRNA exposure and processed immediately upon collection. For statistical analysis of gene silencing, normality was evaluated using a Shapiro-Wilk Test, and significance was measured using a Student’s one-tailed T-test (P < 0.05).

3. Results

3.1. Insect mortality

No mortality was observed in any of the three dsRNA treatments after the initial 24 h. After 4 days all MPB-specific dsRNA treatments experienced ~50% mortality relative to the gfp control. Beetles in the gfp control showed signs of a pathogenic fungal infection on day 3, and notable mortality on day 5, likely due to the excessive moisture in the assay chamber. Despite the fungal-induced mortality in the controls, there were significant differences in mortality between the control and all dsRNA treatments (hsp, shi, iap) (F3, 2 = 25.25, P < 0.0005) (Fig. 2). Seven days post-treatment, there was nearly 100% mortality in all dsRNA treatments.

3.2. Gene expression

Female beetles demonstrated significantly higher expression of hsp than males before dsRNA treatment (Fig. 3). Similarly, expression of iap and shi suggest slight elevation in expression in female beetles relative to males, but those differences were not significant.

3.3. Gene silencing

When female and male beetles were analyzed together, all target genes (hsp, shi, iap) were significantly silenced 24 h after exposure to corresponding dsRNA treatments when compared to the dsGFP control (Fig. 4).

When analyzed by sex after dsRNA exposure, both females and males demonstrated significant silencing in shi and iap relative to the control. However, after exposure to dsHSP, only females demonstrated significant silencing (Fig. 5).

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**Fig. 1.** Adult beetle in MPB-specific dsRNA solution submerged to posterior end of pronotum.

**Fig. 2.** Adult MPB survival after 12 h exposure to 2.5 µg/µL of dsRNA. Significant mortality was observed after day 4 for all three treatments relative to the dsGFP control (ANOVA, F3, 2 = 25.25, P < 0.0005).

**Fig. 3.** Relative expression of hsp, shi, and iap in female (F) and male (M) adult MPB before dsRNA exposure. Relative expression of hsp by female beetles is significantly higher than that of males (t-test, two tailed, P < 0.05), but the relative expression of shi and iap does not differ between sexes (t-test, two tailed, P value: shi P = 0.12, iap P = 0.59).
4. Discussion

Bark beetles pose a significant threat to the sustainability and function of conifer forests globally, causing widespread tree mortality and timber loss, altering susceptibility to wildfire and wildlife habitat, and raising concerns associated with their role as global carbon sinks mitigating the effects of climate change. Increasing temperatures (Raffa et al., 2009), altered precipitation regimes (Kolb et al., 2016), and forest fragmentation exacerbate these issues, making it essential that we develop innovative approaches for bark beetle management (Dobor et al., 2018).

Utilizing novel molecular techniques may expand our management toolbox. Here we show that exogenous dsRNA targeting MPB housekeeping genes to activate the RNAi pathway silences target genes and causes 100% beetle mortality, demonstrating tremendous potential for its use in managing bark beetle populations. Our gene expression results show significant silencing of hsp, shi, and iap, corroborating results of the mortality assays. This high mortality rate demonstrates that, similar to SPB (Kyre et al., 2019), MPB is especially sensitive to the effects of exogenous dsRNA, and implies that the delivery method used here, a combination of ingestion and absorption, is highly efficient. Our findings suggest that hard-bodied scolytinae may uptake exogenous dsRNA through membranous sutures. To date, uptake via topical application has not been evaluated in adult coleopterans, in part because of their chitinious exoskeleton, but also presumably because dsRNA molecules are taken into the gut cells from the gut lumen (Huvenne and Schmagge, 2010) which is more likely to come into contact with dsRNA introduced through ingestion (Yu et al., 2013). Uptake of dsRNA through means other than ingestion creates tremendous potential for deployment (Baum et al., 2007, Burand and Hunter, 2013, Baum and Roberts, 2014, Cagliari et al., 2019).

Ours is the first report of elevated expression of endogenous heat shock proteins in unstressed female insects, though higher expression of heat shock proteins in females has been reported in stressed insects (Shu et al., 2011, Wang et al., 2014), and in animals (Voss et al., 2003, Takahashi et al., 2010). Heat shock proteins are generally recognized for their roles in environmental stress responses (Cai et al., 2017, King and Macrae, 2015), but they may also play important roles in reproduction (Neuer et al., 2000). This would explain the elevated expression we observed, as our experimental insects were not experiencing stress due to abiotic factors imposed after the dsRNA treatment. Our findings are corroborated by the discovery of differential expression of a sex-linked inhibitor of apoptosis gene between male and female MPB in the absence of the common northern neo-x allele (Horianopoulos et al., 2018). Clearly additional insect species should be assessed, and moving forward, variation in gene expression and dsRNA sensitivity between males and females should be fully evaluated and considered when selecting target genes for forest pest management. An understanding of sex-specific differences in gene expression and gene silencing is essential to the success of potential deployment of RNAi technology in this system.

Future efforts will involve the development of mass deployment strategies effective for forest level protection. Exploiting plant-insect and/or plant-microbe interactions could provide an avenue for self-sustainable and/or less labor-intensive deployment, potentially increasing efficacy and reducing the costs of large scale, landscape level management. For example, by exploiting the symbiotic relationships between bark beetles and their fungal symbionts, fungi could be used to vector beetle-targeting dsRNAs. Similarly, phoretic mites could play a role in vectoring dsRNAs. A self-perpetuating system would enable this technology to be driven into a pest complex to maintain populations at endemic levels (Cagliari et al., 2019). If used proactively to inhibit outbreaks, RNAi technology could reduce the labor needed for reactive responses to infestations, which in turn would reduce management costs and free up resources for other aspects of integrated forest management (Hunter and Sinisterra-Hunter, 2018, Cagliari et al., 2019, Fletcher et al., 2020, Rodrigues and Petrick, 2020; Christiaens et al., 2020).

Fig. 4. Following 12 h exposure of adult MPB to 2.5 µg/µL of dsRNA, there is significant silencing of hsp (t-test, one tailed, \( P = 0.0001 \)), shi (\( P = 0.019 \)), and iap (\( P = 0.02 \)) relative to the dsGFP control.

Fig. 5. Female MPB demonstrate significant silencing of hsp 24 h after dsRNA exposure (t-test, one tailed, \( P < 0.05 \)); male beetles do not (t-test, one tailed, \( P = 0.31 \)).
Future research will also investigate the efficacy of alternate target genes. Optimal target genes need not lead to outright mortality, as RNAi can affect multiple aspects of insect physiology, providing various avenues for insect suppression that could include reducing fecundity, altering metabolism, or disrupting chemical communication. Altering the beetles’ ability to detoxify monoterpenes could maintain populations at more manageable levels by limiting their ability to colonize new areas or exploit additional hosts. Similarly, silencing genes associated with pheromone reception could reduce mate finding or aggregation success. Acceptance and implementation by land managers of emerging biotechnologies such as RNAi could help protect trees threatened by eruptive, invasive and native insect species, contributing to great resiliency of our forests in the face of a rapidly changing climate.

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Author Contributions
B.R.K. conducted the experiments. B.R.K. and L.K.R. conceived the experiments, analyzed the results. B.R.K., L.K.R. and B.J.B. interpreted the results and prepared the manuscript. All authors approve of the manuscript.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2020.118322.

References


