

M.L. 2014, Chp. 226, Sec. 2, Subd. 04e-1 **Project Abstract**
For the Period Ending June 30, 2017

PROJECT TITLE: Mountain pine beetle: Invasive threat to Minnesota's pines
PROJECT MANAGER: Brian Aukema
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2014, Chp. 226, Sec. 2, Subd. 04e-1

APPROPRIATION AMOUNT: \$175,000
AMOUNT SPENT: \$175,000
AMOUNT REMAINING: \$0

Overall Project Outcomes and Results

Native to the western United States and Canada, mountain pine beetle is the most devastating forest insect in North America, impacting almost 125 million acres of western mature pine forests to date. Mountain pine beetle reproduces under the bark in the water conducting tissues of many species of pines. During outbreaks, mountain pine beetles *must* kill their trees in order to reproduce and prefer live, vigorous, large-diameter trees. Minnesota is at risk of invasion from mountain pine beetle via two different routes. First, populations reproducing in Alberta, Canada could spread through a corridor of jack pine stretching across Canada's boreal forest into northern Minnesota. Second, green pine logs imported from western states could inadvertently bring this insect to the Midwest.

This project, in partnership with the Minnesota Department of Agriculture, had two objectives. First, pine stands in several areas of the state were surveyed for the presence of this insect. No populations were detected to date (see MDA update). Second, we exposed logs of pine species common in Minnesota, such as red pine, jack pine, white pine, and Scots pine, to the nearest known mountain pine beetle populations in the Black Hills of South Dakota, to gain baseline data on the risk to Minnesota's species of pines.

We found that mountain pine beetles were able to tunnel into cut logs of Minnesota's pines, attract mates, and lay eggs. The eggs were fertile, and insects could complete their development. The insects were cold hardy and the data suggest they could survive Minnesota's winters if established here. Development times in Minnesota's pines were slightly faster than those in historical western pine hosts, which was surprising. These results indicate that we should continue to take the threat of range expansion of mountain pine beetle seriously.

Project Results Use and Dissemination

During the course of this project, the MDA enacted an exterior state quarantine for pine logs with bark on them from western states, and the project manager met with DNR officials to discuss management/silvicultural responses to mountain pine beetle should the insect arrive in the state. This project fostered collaborations with five partner state and federal agencies, three universities, trained a PhD student who received a faculty position, and engaged several dozens of undergraduate university students by incorporating this project into classroom education such as redesigned laboratory practical exercises. In one instance, we hosted an undergraduate student from a different state who flew to Minnesota to conduct her internship on this project (at no cost to the project). If you are a student seeking to help with one of the most serious pending challenges in North America, the state of

Minnesota is a great place to come! This research project has resulted in five peer-reviewed publications to date, with others currently in review, along with several presentations at various scientific conferences.



Environment and Natural Resources Trust Fund (ENRTF) M.L. 2014 Final Report

Date of Final Report: November 13, 2017
Date of Work Plan Approval: June 4, 2014
Project Completion Date: June 30, 2017
Does this submission include an amendment request? Y

PROJECT TITLE: Mountain Pine Beetle Invasive Threat to Minnesota's Pines (UMN Activities 2 & 3)

Project Manager: Brian Aukema
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Location: Statewide (survey Activity 1); with insect work undertaken both in the Quarantine Lab at the University of Minnesota as well as out-of-state in the Black Hills of South Dakota to avoid unintentional introduction of this pest to Minnesota

| | | |
|------------------------------------|-----------------------------|------------------|
| Total ENRTF Project Budget: | ENRTF Appropriation: | \$175,000 |
| | Amount Spent: | \$175,000 |
| | Balance: | \$0 |

Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 04e-1

Appropriation Language:

\$175,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota and \$75,000 the second year is from the trust fund to the commissioner of agriculture to survey for the presence and characterize the potential risk of the invasive mountain pine beetle to Minnesota's pine forests to inform early detection and rapid response. This appropriation is available until June 30, 2017, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Mountain Pine Beetle Invasive Threat to Minnesota's Pines (UMN Activities 2 & 3)

II. PROJECT STATEMENT:

This project focuses on survey and characterization of risk to Minnesota's pines from mountain pine beetle. Native to the western United States and Canada, mountain pine beetle is the most devastating forest insect in North America. In the fall of 2012, mountain pine beetle was found in a shipment of logs to Minnesota. Fortunately, the insect was dead, but live insects may be here already.

Mountain pine beetle reproduces in almost all species of pines. It breeds in the water-conducting tissues of the tree, just underneath the bark, much like emerald ash borer. During outbreaks, mountain pine beetle *must* kill their tree in order to reproduce. The insect can only breed in trees larger than 5" diameter, so prefers healthy, larger diameter trees. US Forest Service data from 2011 indicates that Minnesota has 191,000,000 red, jack, and white pines large enough for mountain pine beetle to attack. Our pine forests create valuable wildlife habitat, regulate water runoff, and promote recreational opportunities. To date, mountain pine beetle has impacted almost 125 million acres of mature pine forests in western North America.

This project is being initiated due to two high-priority routes of entry to Minnesota (see graphic page):

- 1. Through import of green logs into the state from proximate western states with the insect.** Interstate movement of logs is not regulated, so it is challenging to quantify the extent of this risk. The Minnesota Department of Agriculture (MDA) recently formed an expert task force on mountain pine beetle. Early investigation revealed one supplier in Montana who indicated they distribute wood to 900 builders, including "hundreds" in Minnesota and Wisconsin. Minnesota Department of Agriculture attempted contact with 79 business potentially receiving wood from western sources. Seventeen businesses were interviewed and 5 did state importing pine from western areas in the past. One site visit was made to a business as a result and dead mountain pine beetle were found in lodgepole pine logs from Montana. Raw wood imports brought Douglas fir beetle, a kissing cousin of mountain pine beetle, to Grand Rapids, MN, a few years ago. For unknown reasons, those insects died after being established from 2002-2006. The state was very fortunate, and needs to learn from that experience. This project implements critically-needed statewide monitoring and should be continued until evidence suggests the beetle could not establish here.
- 2. From the northwest through a corridor of jack pine stretching across Canada's boreal forest into northern Minnesota.** Currently, an ongoing outbreak of mountain pine beetle in western Canada totals 45 million acres in size, making it the world's largest outbreak of any forest insect. The insect is typically kept in check by cold winter temperatures, but recent warming trends have unleashed the beetle over the Rocky Mountains on a path to Minnesota's pines. In a "good" year, the insects can disperse up to 500 miles (even visible on Doppler radar). Minnesota is 500 miles from the Black Hills of South Dakota, but there is little pine forest in between. We are twice this distance from the approaching front in Canada, but there is contiguous pine in between. Estimating the approaching front is difficult, as monitoring is an imperfect science: much like emerald ash borer, we know where trees have died, not how much closer the beetle is now.

This project uses a collaborative multi-agency team to undertake two objectives. The Minnesota Department of Agriculture will assume Objective 1 (Activity 1), while the University of Minnesota will undertake Objective 2 (Activities 2 & 3).

Objective 1. Survey state locations for presence of mountain pine beetle. If low numbers of insects have been introduced, they may persist for a number of years before exploding (similar to emerald ash borer).

Unlike emerald ash borer, there *is* an effective trap and lure. Management of isolated, endemic populations may not be impossible – *if* we know they are there first.

Objective 2. Characterize the risk to Minnesota’s pine species. Studies by Canadian researchers indicate that jack pine is an excellent food source for the insect. We will characterize development and winter survival in red, white, and Scots pines to inform and direct rapid response management for Minnesota’s pine species.

III. PROJECT STATUS UPDATES:

Amendment Approved by the LCCMR 10-24-2014: We are seeking permission to rebudget \$4,000 from “salary” to “services.” In addition to three University of Minnesota student helpers, we were able to partner with two WCSS students to help with fieldwork in the Black Hills of South Dakota studying colonization of mountain pine beetle on pines brought from Minnesota. Because the WCSS students are not U Minnesota students, we could not engage in traditional undergraduate employment but instead seek to offer honoraria for their help. According to the university, this qualification falls under “compensation for services rendered” not strict “employment.” The budget and scope of work does not change.

Project Status as of November 15, 2014: This project has started well. A highly talented graduate student was recruited and we were able to deploy pines harvested from Minnesota to beetle populations in the Black Hills of South Dakota immediately after appropriation of funds from LCCMR. The timing worked well, as beetle flight occurred in the temporal window expected. Because beetles reproduce once per year, results related to reproduction and winter cold tolerance will not be available until late 2015. Details on the attraction study are provided below. No changes to project direction or scope are anticipated or required at this time.

Project Status as of May 15, 2015: This project continues to progress well. A trip to the Black Hills in April revealed that the beetles are developing in the logs. The logs were placed in emergence tubes for anticipated emergence late this coming summer. We were able to involve several undergraduates in sorting the survey samples from the MDA (detailed below). No changes to project direction or scope are anticipated or required at this time.

Project Status as of November 15, 2015: In June insects started to emerge from material in the Black Hills. We were able to hire three people this summer (in addition to the graduate student) to help with collections of beetles that emerged from the material in the Black Hills. These data form the basis of the first comparisons of insects emerging from different species of pine hosts present in the Great Lakes region. Because of the unprecedented range expansion of this insect, the topic continues to garner international attention. We presented an update on this work at an international forestry conference in Argentina in August (although no project funds were used for travel).

Amendment [Requested November 24, 2015, Approved December 8, 2015]: We are requesting small shifts in allocations of funds among Activities 1 and 2. We would like to add \$800 to supplies for Activity 1 because it was necessary to purchase a small freezer for sample storage, as the science station did not have an available freezer. We also purchased a large number of vials and Eppendorf tubes to store insects for Activity 1, although many of these are being used for Activity 2 following collection. As such, we would like to decrease the supply budget for Activity 2 by \$500. We would like to add \$100 for printing to Activity 1 given the ongoing requests for scientific presentations and decrease travel for Activity 2 by \$400 given less travel than anticipated in the Black Hills (logs for Activity 2 are held at a central location). The net change in budget to the project after reallocations is \$0.

With this amendment we are also correcting a typo in the approved work plan to allocations listed under section IV. Project Activities and Outcomes so the amounts match Appendix 1. Previously, the amounts listed in the workplan summed to \$173,200 instead of the project appropriation of \$175,000.

Project Status as of May 15, 2016: The work continues to go extremely well. In November, work was presented by both the graduate student and an undergraduate student on this project at the national meeting of the Entomological Society of America. Both of them won President's Prizes (first place) in their respective divisions. The likelihood of both students winning is literally 1 in 100, and reflects some superb work on their part and the high profile nature of this potential invasion event that could decimate pines in eastern North America. Moreover, the first scientific publication from this project that was submitted just prior to the last progress report has been accepted for publication at an international peer-reviewed journal.

Project Status as of November 15, 2016: This past six months saw two peer-reviewed publications come out on this work. The first (mentioned above) is now available from the international journal *Entomologia Experimentalis et Applicata* and details a new method to determine the sex of mountain pine beetles. This is important because females attack the trees and attract males; this publication lays a useful methodological framework as we publish the remaining research from this project. The second publication details how we have involved undergraduates from the university in the survey work from the MDA. Many universities continue to look for ways to keep students engaged and motivated in classes; we involved students in Entomology 4251 (Forest & Shade Tree Entomology) to learn how to sort and identify insect predators attracted to the survey traps. Students appreciated the laboratory activity as it is contributing to an important real-world resource management challenge.

Retroactive Amendment Request 9/28/17

In Activity 2, we request permission to move the \$1,000 remaining in "professional contracts" back to "personnel." Previous approved amendment requested reallocation from personnel to contracts. This was strictly to satisfy internal UMN HR classifications when employing students at the Science Station in the Black Hills. In final project year, all students came from UMN so contract was not necessary and this amendment simply reverses the first. We also request permission to reallocate \$787 savings from travel in MN (we were able to find suitable trees at Cloquet in the second year) to \$640 research supplies (cost increase in chemicals used to attract and trap mountain pine beetles for this research) and \$147 personnel. We would like to reallocate \$33 unspent in printing (posters for research result dissemination to scientific community) to \$25 personnel and \$8 communications (tax on \$100 SIM card purchase). Finally, we would like to allocate a remaining \$107 in travel outside of MN to personnel to zero all categories; these small increases in personnel reflect changing benefits rates through the life of the project.

In Activity 3, with some effort we were able to complete cold tolerance work outside of the BSL-2 quarantine lab (but with an abundance of precautions and appropriate permit). We request reallocation of the BSL-2 rental savings (\$4500), \$1000 from supplies (thermocouples), \$300 from printing (oral presentations instead of posters), and \$714 from travel (shared with Activity 2) for total reallocation of \$6686 to personnel, as technicians spent more hours than expected painstakingly extracting these insects from underneath the bark for cold tolerance assays.

Overall Project Outcomes and Results:

Native to the western United States and Canada, mountain pine beetle is the most devastating forest insect in North America, impacting almost 125 million acres of western mature pine forests to date. Mountain pine beetle reproduces under the bark in the water conducting tissues of many species of pines. During outbreaks, mountain pine beetles *must* kill their trees in order to reproduce and prefer live, vigorous, large-diameter trees. Minnesota is at risk of invasion from mountain pine beetle via two different routes. First, populations reproducing in Alberta, Canada could spread through a corridor of jack pine stretching across Canada's boreal forest into northern Minnesota. Second, green pine logs imported from western states could inadvertently bring this insect to the Midwest.

This project, in partnership with the Minnesota Department of Agriculture, had two objectives. First, pine stands in several areas of the state were surveyed for the presence of this insect. No populations were detected to date (see MDA update). Second, we exposed logs of pine species common in Minnesota, such as red pine, jack pine, white pine, and Scots pine, to the nearest known mountain pine beetle populations in the Black Hills of South Dakota, to gain baseline data on the risk to Minnesota's species of pines.

We found that mountain pine beetles were able to tunnel into cut logs of Minnesota's pines, attract mates, and lay eggs. The eggs were fertile, and insects could complete their development. The insects were cold hardy and the data suggest they could survive Minnesota's winters if established here. Development times in Minnesota's pines were slightly faster than those in historical western pine hosts, which was surprising. These results indicate that we should continue to take the threat of range expansion of mountain pine beetle seriously.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1 (MDA): Survey Minnesota pine forests for mountain pine beetle

(Note: This description is copied from the separate workplan for MDA for project coherence. For Budget and Outcomes, please see MDA workplan.)

Description:

MDA will survey pine locations during the timeframe of potential MPB flight period (July – September) throughout Minnesota for three years. Sites will be selected based on known or suspected importation routes of green timber. MDA will identify trap contents for mountain pine beetle, related species and natural enemies. We anticipate that we will be able to maintain a total of approximately 100 traps. These traps will be divided across sites to optimize the number of sites trapped and the trapping coverage at each site. We expect that there will be approximately 25 targeted sites with 4 traps surrounding each site, however the actual number of sites trapped each year may vary based on the discovery of new sites or the determination that previously trapped sites do not justify additional survey.

ACTIVITY 2 (UMN): Determine attractiveness and developmental rate in Minnesota's pines

Description:

Beetles will develop faster, slower, or not at all in "new" tree species. Initial Canadian data suggests that mountain pine beetle can easily kill and reproduce in jack pine. A graduate student will characterize development of mountain pine beetle in logs of red, jack, white, and Scots pine. We will do this by harvesting logs of these species in Minnesota and driving them immediately to the Black Hills of South Dakota where there are active populations of mountain pine beetle. *Note: we will conduct this work outside of the state of Minnesota, as we have no desire to inadvertently introduce this insect to Minnesota.* We will use the Wheaton College Science Station just outside of Rapid City, South Dakota as a home base for summer work. This location is in the middle of several excellent field sites.

Mountain pine beetles will be collected from flight traps in the Black Hills and then introduced to the Minnesota logs in the WCSS laboratory. Mountain pine beetle will readily infest fresh material when they are confined in a small gel capsule over a small nick through the surface of the bark. We will also infest local South Dakota logs harvested from lodgepole and Ponderosa pine. This will allow us to determine attraction/reproduction relative to usual western hosts. Logs of all species will be deployed from the Science Station to the field and the number of flying mountain pine beetles attracted to the infested logs and captured in flight traps will be counted. We will aim for 12 field sites, but that number may be adjusted due to populations of beetles and travel distance from the WCSS.

A second subset of logs will be infested and then screened to prevent escape or additional invasion. The logs will be stored outdoors in South Dakota. Some of these infested logs will be transported to back to Minnesota in the winter directly to the Quarantine Laboratory at the University of Minnesota (see Activity 3,

below). There, cold hardiness in Minnesota’s pines and western hosts will be tested. The remaining logs will be preserved in South Dakota until the following summer to count emerging progeny and determine reproductive rates. These experiments will be repeated twice.

Summary Budget Information for Activity 2:

ENRTF Budget: \$ 124,800
Amount Spent: \$ 124,800
Balance: \$ - 0

Activity Completion Date: June 30, 2017

| Outcome | Completion Date | Budget |
|--|------------------------|---------------|
| 1. Comparison of Minnesota’s pines for attractiveness to flying beetles | 06/30/2016 | \$56,850 |
| 2. Comparison of development times in Minnesota’s pines | 06/30/2016 | \$56,850 |
| 3. Comparison to western pine hosts and final results reported | 06/30/2017 | \$12,000 |

Activity Status as of November 15, 2014:

Logs of red, jack, white, and Scots pine were harvested from the Cloquet Forestry Center and driven to the Black Hills of South Dakota in late July after the project was initiated. Before transport, the ends were sealed with molten wax to reduce dessication. At the same time, logs of Ponderosa and lodgepole pine were harvested and retrieved from South Dakota and Wyoming, respectively. All logs were sectioned into bolts before introduction of female mountain pine beetles using a gel cap method described above. After infestation, the logs were screened to prevent infestation of additional beetles and transported to the field. We were successful in setting up twelve sites for the experiments. Each site had one of the logs above with a funnel trap placed to capture beetles arriving to the logs. Similar to the MDA trapping results, we are now sorting through the trap catches during the flight period of mountain pine beetle and analyzing numbers of mountain pine beetles arriving to each host species.

Activity Status as of May 15, 2015: A trip to our research sites in April indicated that the insects appeared to be surviving the winter. The logs were placed in emergence containers for anticipated emergence of the insects later this summer.

While waiting for the insects to develop (i.e., only one generation per year) we have been sorting the MDA samples. We incorporated this into a laboratory activity that the graduate student Derek Rosenberger led for an Invertebrate Zoology class at Bethel College. Derek and Angie Ambourn from the MDA gave a presentation to the class about the threat of mountain pine beetle to Minnesota’s pines. The students had learned about taxonomic classification of insects and were provided some of the summer survey samples to sort into “bark beetles” and “other.” We feel the exercise went very well, as it gave the class experience in identification of insects and some degree of personal fulfillment knowing that their efforts were helping confront a serious “real-life” challenge. We have been following up identifying the student-sorted specimens, and to date no mountain pine beetles have been detected.

Activity Status as of November 15, 2015:

This summer the insects began emerging from the logs, indicating that mountain pine beetles are able to complete development in cut logs of Minnesota’s pines. Analyses of these data continue. We were surprised to find differences in emergence times between hosts that were a little faster than the western species of pines. If beetles develop too quickly, they risk colonizing trees earlier in the summer and developing to life stages by late fall that are not cold hardy. So, our initial results suggest that some species of pines may be *too good* for the beetles, but more analysis is needed. We are now conducting chemical analyses to understand the chemical differences between the trees, so we can put together a complete picture of how tree chemistry affects the attraction and reproduction of mountain pine beetle in Minnesota’s pines.

One unexpected result from working with these insects this summer was discovering a way to exploit the ‘stress’ response of beetles when they are being handled to determine their sex. With mountain pine beetle (and many tree-killing *Dendroctonus* spp.), the females are the host-selecting sex that bore through the bark. They emit chemicals called pheromones and attract mates, which then overwhelm the host tree they are trying

to colonize. As such, when conducting experiments involving colonization, mate-attraction, and/or reproduction, it is necessary to determine the sex of the beetle. There are two ways to distinguish the sex of the insects: morphological examination of the tergites on the 7th abdominal segment, which is tedious and can injure live insects, and listening to male 'chirps.' These insects will audibly stridulate if you hold them up to your ear; however, it is well known that not all males will stridulate. We discovered that sequential handling of the same cohort of insects will uncover previously silent males in as little as three careful manipulations. We have submitted this observation to a peer-reviewed scientific journal as a note where it is currently under review. We hope this observation will improve future experiments with initial accuracy of sex determination approaching 100%.

Activity Status as of May 15, 2016: We are now wrapping up chemical analyses of the trees. We are finding that our novel eastern pines (red, jack, eastern white, and Scots pine) have very similar amounts of alpha-pinene to the insect's historic hosts in the western part of North America. Alpha-pinene is important to the insects because they use this chemical to make their aggregation pheromone that helps them attract mates and attack and kill trees. Alpha-pinene is only one type of monoterpene chemical. We are also finding that Minnesota's pines, although exhibiting similar levels of alpha-pinene, contain much lower amounts of other monoterpenes. Minnesota's pine exhibit, on average, up to 10X less monoterpenes per gram of tree tissue than western species of pines. These chemicals are often used in tree defense, which suggests that our native pines could have reduced capacity to defend themselves if this insect was introduced to Minnesota. It is important to note however, that these are samples taken immediately upon harvest. All live trees have the capacity to produce more chemical upon "challenge." That said, our data do show thus far that the initial baseline is *not* even.

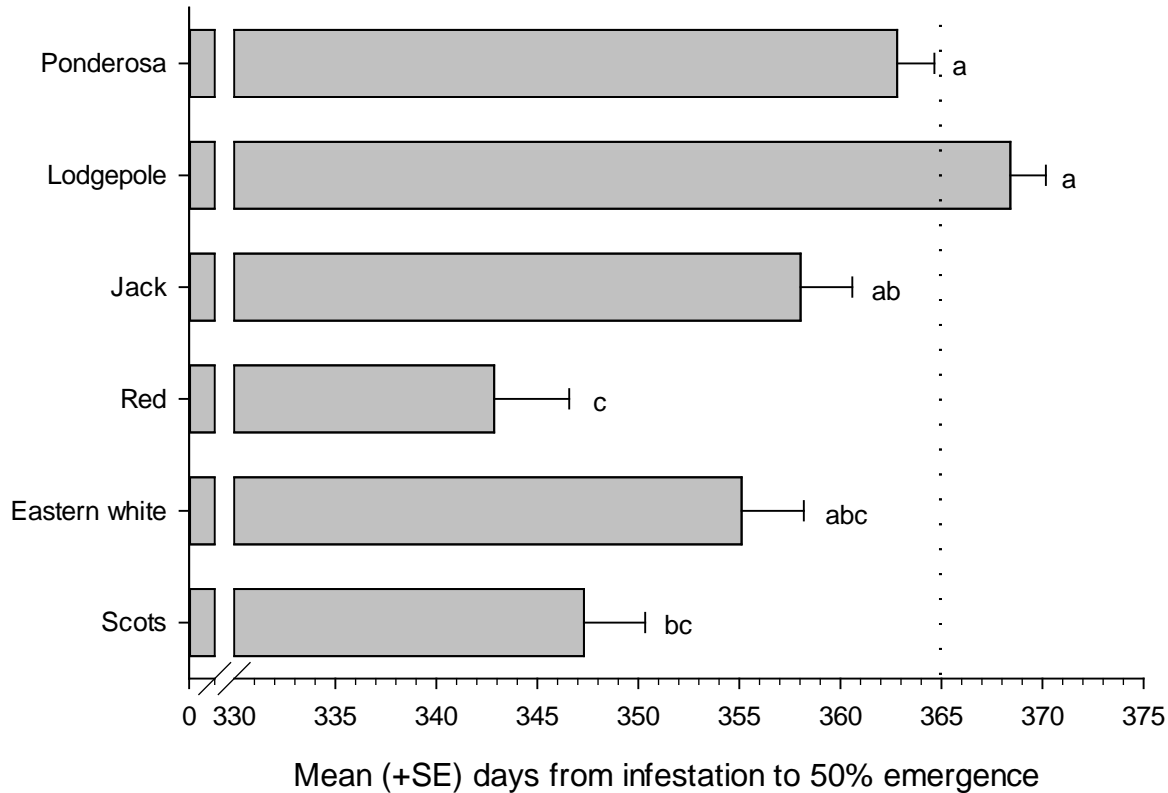
The manuscript on distinguishing sexes of mountain pine beetle has now been accepted for publication in the international peer-reviewed journal, *Entomologia Experimentalis et Applicata*. It will appear in print later this fall.

Activity Status as of November 15, 2016: The manuscript referenced above is now out in print. We are concluding chemical analyses of trees. In the previous status report, we were focusing on alpha-pinene which is a major component of the tree resin. This chemical is an important chemical because that is what the insects use to make their aggregation pheromones that attract mates to the trees. In the past six months, we have also been investigating the trace chemicals found in the resin and attempting to correlate field attraction studies with the chemicals in the phloem tissue in which the beetles tunnel when colonizing a tree. We are finding trace amounts of a phenylpropanoid chemical known as 4-allylanisole, for example, which typically repels flying beetles. Interestingly, we find this compound in the highest amounts in western pines where mountain pine beetle has existed for hundreds of years. Our native pines in Minnesota do not have very much of this compound, likely because trees have never experienced natural selection of these tree-killing bark beetles. Again, this does not bode well for Minnesota's pines if mountain pine beetle were to arrive in the state.

Final Report Summary: The colonization components of this work were published in the peer-reviewed journal PLoS ONE | <https://doi.org/10.1371/journal.pone.0176269>. A portion of the abstract is reproduced below:

We studied how beetle behavior differed among the various stages of colonization on newly cut logs of four novel potential pine host species; jack (*P. banksiana*), red (*P. resinosa* Ait.), eastern white (*P. strobus* L.) and Scots (*P. sylvestris* L.) pines, as well as two historical hosts, ponderosa (*P. ponderosa* Dougl. ex. Laws. var. *scopulorum* Engelm.) and lodgepole (*P. contorta* Dougl. var. *latifolia* Engelm.) pines. Overall, we found that beetle colonization behaviors at each stage in the colonization process differ between pine hosts, likely due to differing chemical and physical bark traits. Pines without co-evolved constitutive defenses against mountain pine beetle exhibited reduced amounts of defensive monoterpene chemicals; however, such patterns also reduced beetle attraction and colonization. Neither chemical nor physical defenses fully defended trees against the various stages of host procurement that can result in tree colonization and death.

The reproductive work is currently in review at a different peer-reviewed journal. Mountain pine beetle could reproduce in cut logs of all of our species of pines – and in many cases developed *faster* than the expected one-year life cycle. Below we reproduce a graph of successful reproduction of mountain pine beetle in Minnesota’s pines:



Caption: Effect of pine species on time to 50% emergence of mountain pine beetles in Black Hills, SD ($F_{5,42} = 8.6$, $P < 0.0001$). Data represent both years. Means followed by the same letter are not significantly different from each other. $n=15-16$ logs per species for each annual replication.

ACTIVITY 3 (UMN): Characterize cold tolerance in Minnesota’s pines

In western pines, cold tolerance differs depending on species of pine. The same graduate student and an undergraduate summer worker will characterize the effects of freezing temperatures on beetle mortality levels in logs of red, jack, white, and Scots pine under quarantine conditions at the U of M to inform risk maps.

Freshly cut pines will be infested with adult mountain pine beetle and screened as per Activity 2. Beetles will be allowed to reproduce and offspring to develop until late December or early January. This approach provides adequate time and conditions for mountain pine beetle to naturally acclimate to cold. Preliminary observations (D. Rosenberger, U of MN, data not shown) indicate that mountain pine beetle from cut logs are as cold hardy as from standing trees of the same host (i.e., ponderosa pine).

We use artificial infestation because the pine species of interest do not occur with the current range of mountain pine beetle. Also, mountain pine beetle prefers to colonize large diameter trees so transplanting eastern species is not a feasible option.

In December/January, infested logs will be secured in triple containment (i.e., three independent means to prevent the escape of the insects) and returned to Minnesota. Under secure conditions, infested logs will be peeled and the distribution of life stages noted. Cold hardiness of recovered insects will be measured in three ways. We will measure the supercooling points of overwintering life stages. The supercooling point is the

temperature at which insects begin to freeze. Mountain pine beetle uses a freeze-avoidant strategy to survive the winter on western pines because individuals will die if they freeze. The insect produces cryoprotectants and is able to prevent freezing until temperatures approach -35°C. However, we recognize that the overwintering strategy might be different on new pine species. So, we will also measure the lower lethal temperature of the insect. For these measurements, insects are cooled to randomly-selected temperatures between 0 and -40°C. Insects are immediately removed upon reaching these temperatures. Survival (as demonstrated by normal behavior of the insect) will be recorded at 24, 48, and 72 hours after cold exposure. These studies will allow us to determine if some individuals might die before or upon freezing or if some fraction of the population can survive freezing. Lastly, if we can obtain enough insects, we will measure lower lethal time. For these measures, insects will be held at sub-zero temperature for up to 160 days. Batches of insects will be removed at regular intervals and survivorship assessed. At the conclusion of experiments, material will be returned to South Dakota.

Summary Budget Information for Activity 1:

ENRTF Budget: \$ 50,200
Amount Spent: \$ 50,200
Balance: \$ 0

Activity Completion Date: June 30, 2017

| Outcome | Completion Date | Budget |
|---|-----------------|--------|
| 1. Determination of lower lethal temperature in Minnesota's pines | 06/30/2017 | 50,200 |

Activity Status as of November 15, 2014: At the same time that logs were infested in Activity 2, a subset of logs was infested and remains in South Dakota. The beetles laid eggs which hatched, and the larvae began tunneling until their activity slowed into the winter. Overwintering beetles will be tested for cold tolerance later this winter.

Activity Status as of May 15, 2015: The graduate student conducted initial cold tolerance testing of developing beetles in each of the six hosts. We have found qualitative differences in the supercooling points of the insects among hosts, which was a bit surprising, and suggests that the insects may find "refuges" in some of our native pines vs. others. Full analysis is ongoing to detect quantitative differences.

Activity Status as of November 15, 2015: We have conducted additional runs to determine supercooling points of insects within different hosts. There seem to be year to year differences that we are trying to tease apart. The rankings among trees appear to be similar, but the magnitude of the responses differs. What is clear is that beetles emerging out of red pine consistently appear to be the most cold tolerant. None of the insects are cold tolerant beyond what they experience in their historic range (-40F).

We are now trying to determine whether there are unique chemistries or fungi that may be responsible for host-specific differences. Mountain pine beetles, like many bark beetles, are not sterile and have cuticular structures that vector fungi into the trees they attack. The fungi serve a variety of functions for the insects, such as serving as nutritional sources for the developing brood and potentially making the insects more (or less) cold hardy. An undergraduate, Jonah Widmer, began exploring whether there are differences in the presence of one fungus, *Ophiostoma montium*, between species of pines in an undergraduate research project mentored by the project lead, the graduate student, and Prof. Robert Blanchette's laboratory in Plant Pathology. Insects collected in Activity one were collected into individual microcentrifuge tubes using sterile technique and frozen until extraction. The beetles were crushed before their DNA was extracted, and then the solution with species-specific primers was run through PCR. The products were observed with gel electrophoresis. Jonah's undergraduate project continues in the data analysis stage, where he is working to determine the proportion of beetles carrying this fungus. We are not observing that the beetles emerging from red pine carry the highest proportions of this fungus, although he has successfully recovered *O. montium* from insects emerging from all of our native pines tested.

Activity Status as of May 15, 2016: Undergraduate Jonah Widmer presented the results of his independent study at the Entomological Society of America, and won first place in the President's Prize competition! To summarize, he has demonstrated that *Ophiostoma montium*, an important fungal associate of mountain pine beetle, can develop in novel eastern pine hosts and be vectored by emerging beetles. This is the first time this has been demonstrated, and could be related to the differences among supercooling points. A copy of his poster is attached to the end of this file.

In addition, we hosted a second undergraduate who worked with us at the Wheaton College Science Station in Rapid City on Activity 2 this past summer. Hannah needed to complete a two-credit research experience course at Wheaton College, so continued working on an aspect of mountain pine beetle ecology this semester. She flew up from Wheaton College to the University of Minnesota twice this past semester (no travel charges to project). She worked to determine whether the staining left in logs colonized by the beetles varied between species of pines tested. We found that white pine had the highest amount of staining overall. Staining can be a problem in recovery of timber for various wood products. We were a little surprised to find that white pine had more staining than red pine, which yields beetles that appear to be the most cold tolerant. We were pleased that Hannah wanted to continue her involvement in this work, even though she is not a student at the University of Minnesota!

Activity Status as of November 15, 2016: We are finished collecting cold tolerance data in the laboratory, and are now working to correlate the number of insects reared out of logs of different species of pine with the field temperatures recorded each winter. We have developed developmental indices for each log based on the distributions of life stages of mountain pine beetle going into each winter. We have found that insects in red pine develop the most rapidly compared to all species – and these insects appeared to be hit the hardest in the cold winter of 2013-2014. So, while red pine might be the best for mountain pine beetles, the insects risk developing to cold sensitive life stages from late summer into fall. Of course, if winters continue to warm, this will not affect the insects at all.

Final Report Summary: This work was published in a peer-reviewed journal as Rosenberger, Aukema, and Venette (2017) Cold tolerance of mountain pine beetle among novel eastern pines: A potential for trade-offs in an invaded range? *Forest Ecology & Management* 400: 28-37. The abstract is reproduced below:

Novel hosts may have unforeseen impacts on herbivore life history traits. The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) is a tree-killing bark beetle native to western North America but constrained by cold temperatures in the northern limits of its distribution. In recent years, this insect has spread north and east of its historical range, and continued expansion, or accidental introduction, could result in the mountain pine beetle becoming invasive in eastern North America. The limiting effect of cold temperatures among novel host pines is unknown, yet crucial for understanding the risk posed to northeastern North American forests. We report the susceptibility of mountain pine beetle to cold temperatures while overwintering in six different pine species. Brood developed in two western pine hosts (*Pinus contorta* Dougl. var. *latifolia* Engelm. and *P. ponderosa* Dougl. ex. Laws. var. *scopulorum* Engelm.) as well as four eastern pines (*P. banksiana* Lamb., *P. resinosa* Ait., *P. strobus* L. and *P. sylvestris* L.) novel to this insect. The cold tolerance and cold tolerance strategy of the most common overwintering stage varied by host and year. Models describing lower lethal temperatures more accurately predicted observed field mortality of overwintering larvae than models based on temperatures at which larvae froze. Rapid development to less cold tolerant pupal and adult stages by brood in novel hosts prior to winter may constitute a trade-off between increased host suitability and winter mortality. We demonstrate that overwintering survival of mountain pine beetles in novel hosts depends on a match between the climate and ecophysiological effects of pine species. These results have implications for risk assessment models and management planning for eastern forests as mountain pine beetle continues to expand its range.

V. DISSEMINATION:

Description:

This work will be shared with relevant stakeholders through meetings and presentations (e.g., Upper Midwest Invasive Species Council, MN Forest Resource/Stewardship Council, North Central Forest Pest Workshop, etc.). Presentations have already been given on this important topic for groups such as the Great Lakes Log Crafters Association. We will be available for media requests, as well. This insect is well known in the western United States and Canada and western media outlets periodically request interviews from personnel in states at risk of introduction or invasion to find out about preparedness levels.

Status as of November 15, 2014:

The potential expansion of mountain pine beetle to pine forests of eastern North America is one of the most serious forest health threats today. As such, we have received a number of invitations to present ongoing work on risk assessment at several venues. This LCCMR project was highlighted at several meetings over the past five months:

| | | |
|---------------|---|--------------------|
| July 22, 2014 | Southern Forest Insect Work Conference | Charleston, SC |
| Sept 8, 2014 | North Central Forest Pest Workshop | Chariton, IA |
| Oct 6, 2014 | International Union of Forestry Research Organizations World Congress | Salt Lake City, UT |
| Oct 22, 2014 | Upper Midwest Invasive Species Meeting | Duluth, MN |
| Nov 11, 2014 | Bethel College Tri-Beta Undergraduate Biology Honors Banquet | Arden Hills, MN |

In addition, we contribute a short article updating the rate of spread through the Canadian boreal forest towards Minnesota:

Aukema, B.H., McKee, F.R., and D.W. Rosenberger. Update on mountain pine beetle, a potentially devastating threat. Pp. 14-15, in *Tree Farming for Better Forests*, Summer 2014

Status as of May 15, 2015:

We were interviewed by National Geographic cartographers for details about how range expansion of mountain pine beetle could affect the forest resource of the Midwest. We received an acknowledgement in the resulting article (see cartography section, <http://ngm.nationalgeographic.com/2015/04/pine-beetles/rosner-text>, "Pine Beetle Epidemic: the Bug that's Eating the Woods" April 2015).

Status as of November 15, 2015: Presentations on this work were given at the North Central Forest Pest Workshop at Mosinee Indian Reservation in Keshena, WI Sept 24-27 and the International Union of Forest Research Organizations joint working party meeting "Population dynamics of bark and wood-boring beetles" and "Invasive insects and international trade" in Bariloche, Argentina, Aug 31 – 3 Sept. Travel funds were not used outside of the state of Minnesota even though we continue to receive such external requests for updates on this high-profile project.

The lead PI also met with officials at the DNR on November 3 to discuss this project and management/silvicultural responses to MPB should the insect arrive in the state.

Status as of May 15, 2016:

Two presentations were given at the national meeting of the Entomological Society of America, November 15-18 at the convention center in Minneapolis. Each student received a First Place President's Prize in their division:

- **Widmer, J.,** Rosenberger, D.W., Blanchette, R., Held, B., Venette, R.C., and B.H. Aukema. The suitability of novel hosts for *Grosmannia clavigera* and *Ophiostoma montium*, two common fungal associates of mountain pine beetle (Coleoptera: Curculionidae)

- **Rosenberger, D.W.**, Aukema, B.H., and R.C. Venette. How climate change and host-specific cold tolerance may mediate invasion potential of mountain pine beetle, *Dendroctonus ponderosae*, in eastern pine forests

Status as of November 15, 2016:

Three presentations were given since the last update. One presentation was given at the North American Forest Insect Work Conference, which is a national gathering of forest health professionals that meets every five years. The 2016 meeting was in Washington, DC, from May 31-June 3. There, we were also able to meet with colleagues from Alberta, Canada involved in spread control of mountain pine beetle as we work to understand factors that may slow potential arrival in the Lake states region. Another presentation was given at the International Congress of Entomology in October, which met in Orlando, Florida. This meeting meets every four years, and provided another opportunity to share lessons with others involved in controlling outbreaks of bark beetles. Finally, Brian Aukema gave an invited department seminar at UW-Madison at the end of September on mountain pine beetle. This provided a valuable opportunity to share research with Wisconsin colleagues, who have also been trapping the state for potential arrival of mountain pine beetle.

Final Report Summary:

During the course of this project, the MDA enacted an exterior state quarantine for pine logs with bark on them from western states, and the project manager met with DNR officials to discuss management/silvicultural responses to mountain pine beetle should the insect arrive in the state. This project fostered collaborations with five partner state and federal agencies, three universities, trained a PhD student who received a faculty position, and engaged several dozens of undergraduate university students by incorporating this project into classroom education such as redesigned laboratory practical exercises. In one instance, we hosted an undergraduate student from a different state who flew to Minnesota to conduct her internship on this project (at no cost to the project). If you are an undergraduate looking to help on one of the most serious pending challenges in North America, the state of Minnesota is a great place to come!

This research project has resulted in five peer-reviewed publications to date, with others currently in review:

Rosenberger, D.W., R.C. Venette, Maddox, M.P., and B.H. Aukema. (2017) Colonization behaviours of mountain pine beetle on novel hosts: implications for range expansion into eastern North America. *PLoS ONE* <https://doi.org/10.1371/journal.pone.0176269>

Rosenberger, D.W., Aukema, B.H., and R.C. Venette. (2017) Cold tolerance of mountain pine beetle among novel eastern pines: A potential for trade-offs in an invaded range? *Forest Ecology & Management* 400: 28-37

Rosenberger, D.W. and B.H. Aukema. (2016) Stimulating curiosity and engagement with insects beyond the college classroom through citizen science. *American Entomologist* 62: 120-122. (*this paper summarized an invited symposium talk at a national scientific conference, where we highlighted how we involved undergraduate students at the University of Minnesota to sort through MDA survey samples in Forest Entomology 4251. Students enjoyed the enhancement of classroom learning with hands-on tackling a serious, real life ecological challenge!).

Rosenberger, D.W., Venette, R.C., and B.H. Aukema. (2016) Sex determination of live mountain pine beetles (Coleoptera: Curculionidae): refinement of a behavioural method for *Dendroctonus* spp. *Entomologia Experimentalis et Applicata*. 160: 195-199.

There are two other scientific papers pending.

Our project results were also shared at several academic and stakeholder venues. Because of the high profile nature of this work and continent-wide implications for this native insect expanding its range, we received several international invitations to share progress reports. Venues included the University of Wisconsin-Madison, IUFRO World Forestry Congress, the Western Forest Insect Work Conference, the Upper Midwest Invasive Species Conference, the International Congress of Entomology, the North Central Forest Pest Workshop, the Ecological Society of America, the Entomological Society of America, and the Walker Northern Silviculture Workshop to list a few. Many of these presentations were undertaken without cost to the project through external travel scholarships to students.

One of the highlights of this project was the receipt of two President's Prizes for student presentations at the national meetings of the Entomological Society of America in the fall of 2015.

We are pleased with the quality and quantity of research that was facilitated, the benefits of student training, and how quickly we were able to disseminate results. We thank the LCCMR commission for their investment in this important natural resource issue.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview (UMN Activities 2 & 3 only):

| Budget Category | \$ Amount | Explanation |
|----------------------------------|-------------------------|---|
| Personnel: | \$ 140,000 \$147,965 | One graduate student at 50%FTE for 2 years, one undergraduate summer student at 30% FTE each of 3 years, 1 summer faculty time at 15% 5% FTE for 3 years Summer faculty pay was voluntarily substituted to recruit and pay postdoc Dr. Kevin Chase who joined this project for six months training in advance of MPB Phase II (MITPPC, 2016-2020). |
| Professional/Technical Contracts | \$4,000 \$3,000 | Two undergraduates research honoraria in lieu of salary as based at Wheaton College Science Station outside of Minnesota for field coordination help. |
| Equipment/Tools/Supplies: | \$4,500 \$4,140 | Lab and field supplies Increase in price of chemical reagents and lures; we erred on side of being able to attract enough insects for research |
| Printing: | \$700 \$367 | Printing/poster charges for dissemination of results |
| Travel Expenses in MN: | \$2000 \$1,213 | \$1,000 each of two years collecting pine material to transport to Black Hills (est. 2 day truck rental plus 750 miles at \$0.39/mile plus one night lodging x 2 trips each year) July & Aug for Activity 2 |
| Other: | \$23,800 \$18,315 | <ul style="list-style-type: none"> Travel to, from, among field sites in Black Hills of South Dakota (\$19,100) (\$18,300) Rental of UMN Quarantine Facility 3 years (\$4,500) (\$0) Cellular air time prepaid 2 years for safety in case of field emergencies (\$200). Proposed as cheaper than ACR |

| | | |
|----------------------------|--|---|
| | | ResQLink+ Personal locator beacon (\$289, REI) or SPOT tracker (\$100+\$100 subscription). We have used satellite phones (Iridium) in past research, but cell reception is acceptable at WCSS so propose this as least expensive route; if unallowable will pursue more expensive option. |
| TOTAL ENRTF BUDGET: | | \$175,000 |

Explanation of Use of Classified Staff:

Explanation of Capital Expenditures Greater Than \$5,000:

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 2.35

B. Other Funds:

| Source of Funds | \$ Amount Proposed | \$ Amount Spent | Use of Other Funds |
|--|--------------------|-----------------|--|
| Non-state | | | |
| Canada: NSERC TRIA-Net \$3M Cdn (in kind) | \$0 | \$ | Because Canadian grant funds cannot be spent outside of Canada, these funds cannot support work in Minnesota. However, we will be in touch with Canadian workers on their projects (invasion pathway through the Canadian boreal forest) and their ongoing spread control work |
| State | | | |
| Waived indirect costs of 52% U of M \$91,000 | \$0 | \$ | |
| TOTAL OTHER FUNDS: | \$0 | \$ | |

VII. PROJECT STRATEGY:

A. Project Team/Partners

Similar to the ongoing EAB projects on biological control, detection, and monitoring, this proposal is a joint partnership with the MDA, USDA Forest Service, and the University of Minnesota.

Receiving funds: The MDA (Abrahamson) will lead the survey efforts (Activity 1). The U of M and the Forest Service (Aukema/Venette) will lead the characterization of risk to Minnesota’s pines through studies of reproduction and cold tolerance (Activities 2/3).

Not receiving funds: The Forest Service will not receive funds. All institutions will provide in-kind equipment, facilities, intellectual input, and GIS/technical support, and we will collaborate with the DNR and other federal agencies, including Canadian. As stated above, a collaborative Canadian research team was recently awarded \$3M from their federal authorities to study the approaching eastward invasion front. Our proposal complements their and does not overlap.

B. Project Impact and Long-term Strategy:

This project has immediate impact for Minnesota by surveying whether the insect has established in the state, given that dead insects were found on imported pine logs in the fall of 2012 with a random inspection.

Mountain pine beetle can exist for years at “endemic” levels where it reproduces in but does not kill trees.

When environmental conditions permit, the insect suddenly erupts and begins killing trees until either 1) it runs out of trees to kill or 2) unfavorable winter temperatures kill a significant portion of the insects.

A longer-term strategy has already begun here and elsewhere. In Minnesota, the threat of mountain pine beetle has prompted convening of an expert task force through the Minnesota Department of Agriculture. Several outreach presentations have been given to relevant stakeholder groups highlighting the necessity to reduce likelihood of transporting the insect – or any of its associates – to the state.

In the event that mountain pine beetle is found or arrives in the near future, the work on risk assessment in various pine species and cold tolerance will inform rapid response strategies. We will know within a few years which tree species produce the most beetles, and what level of cold might be needed to kill populations in the winter.

LCCMR has not spent any funds on the emerging mountain pine beetle problem to date. Over the past 10 years, Canada has spent \$1.5B on spread control and mitigation of ecologic consequences. This figure does not include \$285,000 earmarked this year by provinces such as Ontario that share a border with MN. Wisconsin has already deployed sentinel traps in five locations for early detection.

C. Spending History:

| Funding Source | M.L. 2008 or FY09 | M.L. 2009 or FY10 | M.L. 2010 or FY11 | M.L. 2011 or FY12-13 | M.L. 2013 or FY14 |
|--|----------------------------------|----------------------------------|----------------------------------|-------------------------------------|----------------------------------|
| U of M Graduate School Fellowship for PhD Student to recruit Derek Rosenberger | | | \$42,0000 | | |

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. VISUAL ELEMENT or MAP(S): See shared MDA – UMN graphic

X. ACQUISITION/RESTORATION REQUIREMENTS WORKSHEET: N/A

XI. RESEARCH ADDENDUM: N/A

XII. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted no later than 11/15/2014, 5/15/2015, 11/15/2015, 5/15/2016 and 11/15/2016. A final report and associated products will be submitted between June 30 and August 15, 2017.

Jonah's winning poster: presented at the national meeting of the Entomological Society of America to a national audience of insect research and management experts.

Ophiostoma montium, a fungal associate of mountain pine beetle, can grow in and be transferred from logs of novel eastern pine hosts



Jonah R. Widmer¹, Derek W. Rosenberger¹, Robert A. Blanchette², Benjamin W. Held², Robert C. Venette³, Aubree M. Wilke¹, & Brian H. Aukema¹

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²Dept. of Plant Pathology, University of Minnesota, St. Paul, MN

³USDA Forest Service, Northern Research Station, St. Paul



Ophiostoma montium

Introduction

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is a native insect that can kill lodgepole (*P. contorta*) and ponderosa (*P. ponderosa*) pine when at outbreak levels in western North America.¹

Recent eastward range expansion resulted in interactions with a novel host, jack pine (*P. banksiana*)² and further eastward movement could result in contact with red (*P. resinosa*) eastern white (*P. strobus*) and Scots (*P. sylvestris*) pines.

While mountain pine beetle has attacked these species in off-site plantings the past, it is unknown whether fungal associates of the beetle, vital to nutrition and development of offspring³, are able to colonize these novel hosts.

Here we used a species specific primer⁴ to test for the presence of the phoretic blue stain fungal associate, *Ophiostoma montium*, on beetles emerging from logs of native and novel pine species.

Research Questions

- Can species specific-primers detect *O. montium* on emerged beetles?
- Can *O. montium* grow in novel hosts and be transferred by emerging beetles?

Methods – Beetle Collection

- Trees of each species were cut in Minnesota, Wyoming and South Dakota and infested with mountain pine beetles caught in August, 2013.
- Logs overwintered in natural common garden conditions then were placed in emergence tubes in an unheated laboratory for spring and summer.
- Upon emergence in July and August 2014 beetles were collected into individual microcentrifuge tubes using sterile techniques and frozen until extraction.

Methods - Fungal extraction and detection



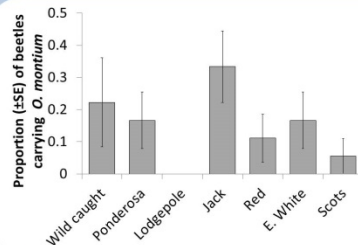
Beetles crushed and DNA extracted



DNA solution with species-specific primers run through PCR



PCR products visualized with gel electrophoresis



Results

- *O. montium* was found on beetles emerging from logs of each novel pine species.
- Proportions of beetles vectoring fungi from ponderosa logs were similar to wild beetles emerging from naturally colonized ponderosa pine in the Black Hills.

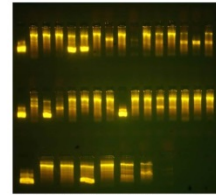


Fig. 1 Gel of 36 beetle samples. Left-most wells are positive controls. Bottom right-most well (bottom row, well 8) is negative control.

Discussion

- Our results indicate that *Ophiostoma montium*, an important fungal associate of mountain pine beetle, can develop in novel eastern pine hosts and be vectored by emerging beetles.
- While *O. montium* was not recovered from beetles emerging from lodgepole pine logs in this study, the fungus has been recovered from this tree species in previous studies.
- One limitation to this study was that while fungi were exposed to constitutive defenses in logs, they were not exposed to induced defenses of the living tree, which can further limit fungal growth.

Acknowledgments

This research was made possible by a University of Minnesota Graduate Fellowship to DWR, the Minnesota Department of Natural Resources and a grant from the Minnesota Environment and Natural Resources Trust Fund.

Contact Information

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References

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3. Berry, B. J., and D. L. Six. 2006. Epibiotic content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae: Scolytinae). *Ann. Entomol. Soc. Am.* 99: 129-134.
4. Shadmehr, L. S., M. Masoumi-Khameneh, R. Hamelink, J. Kuhlmann, and C. Brwell. 2010. Target-specific PCR primers can detect and differentiate ophiostomatoid fungi from microbial communities associated with the mountain pine beetle *Dendroctonus ponderosae*. *Fungal Biol.* 114: 825-33.



Environment and Natural Resources Trust Fund
M.L. 2014 Project Budget - FINAL REPORT



Project Title: Mountain Pine Beetle Invasive Threat to Minnesota's Pines (UMN Activities 2 & 3)
Legal Citation: M.L. 2014, Chp. 226, Sec. 2, Subd. 04e-1
Project Manager: Brian Aukema
Organization: University of Minnesota
M.L. 2014 ENRTF Appropriation: \$175,000
Project Length and Completion Date: 3 year project, to be completed June 30, 2017
Date of Report: September 28, 2017

| ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET | Activity 1 Budget | Amount Spent | Activity 1 Balance | Activity 2 Budget | Revised Activity 2 Budget 09/28/17 | Amount Spent | Activity 2 Balance | Activity 3 Budget | Revised Activity 3 Budget 09/28/17 | Amount Spent | Activity 3 Balance | TOTAL BUDGET | TOTAL REVISED BUDGET 9/28/17 | TOTAL SPENT | TOTAL BALANCE |
|--|-------------------------|--------------|--------------------|---|------------------------------------|--------------|--------------------|--------------------------|------------------------------------|--------------|--------------------|--------------|------------------------------|-------------|---------------|
| BUDGET ITEM | Survey (See MDA budget) | | | Attractiveness and development in Minnesota's pines | | | | Determine cold tolerance | | | | | | | |
| Personnel (Wages and Benefits) | | | | \$98,000 | \$99,279 | \$99,279 | \$0 | \$42,000 | \$48,686 | \$48,686 | \$0 | \$140,000 | \$147,965 | \$147,965 | \$0 |
| 1 Graduate Research Assistant: \$41,500 in year 1, \$42,500 in Year 2, evenly shared between Activities 2 & 3, 50% FTE for 2 years | | | | | | | | | | | | | | | |
| 1 Undergraduate Research Assistant or Technician: \$8,000 each of 3 2 years and \$4,000 one year (92% salary, 8% benefits), approximately 40 hours/week in summers Activity 2 (may be three different students) | | | | | | | | | | | | | | | |
| U of M: One 3 year PTE faculty \$12,000/year (80% salary, 20% fringe) for method development Activity 2 | | | | | | | | | | | | | | | |
| Professional/Technical Contracts | | | | \$4,000 | \$3,000 | \$3,000 | \$0 | | | | | \$4,000 | \$3,000 | \$3,000 | \$0 |
| Two undergraduates research honoraria in lieu of salary if based at WCSS outside of Minnesota for field coordination help | | | | | | | | | | | | | | | |
| Equipment/Tools/Supplies | | | | \$3,500 | \$4,140 | \$4,140 | \$0 | | | | | \$3,500 | \$4,140 | \$4,140 | \$0 |
| Lures, ethanol for storing collected insects, screening, staples, rope, misc. field supplies for Activity 2 | | | | | | | | | | | | | | | |
| Syringes and thermocouples for Activity 3 | | | | | | | | \$1,000 | \$0 | \$0 | \$0 | \$1,000 | \$0 | \$0 | \$0 |
| Printing | | | | \$400 | \$367 | \$367 | \$0 | \$300 | \$0 | \$0 | \$0 | \$700 | \$367 | \$367 | \$0 |
| Scientific posters for dissemination results (\$200/year) split between Activities 2 & 3 | | | | | | | | | | | | | | | |
| Travel expenses in Minnesota | | | | \$2,000 | \$1,213 | \$1,213 | \$0 | | | | | \$2,000 | \$1,213 | \$1,213 | \$0 |
| \$1,000 each of two years collecting pine material to transport to Black Hills (est. 2 day truck rental plus 750 miles at \$0.39/mile plus one night lodging x 2 trips each year) July & Aug for Activity 2 | | | | | | | | | | | | | | | |
| Other | | | | \$16,700 | \$16,593 | \$16,593 | \$0 | \$2,400 | \$1,514 | \$1,514 | \$0 | \$19,100 | \$18,107 | \$18,106 | \$1 |
| Travel expenses outside of Minnesota: Activity 2: \$7,600/year for first two years. Work will be conducted in Black Hills of SD to avoid introduction of MPB to MN. Flight season typically mid-July through August or early Sept, approx. 60 days. Expenses include pickup truck rental 2 x 2 mo rental of 3/4 ton trucks at \$800/mo, plus est. 3600 miles/year gas at \$0.39/mi, total \$4,600 year; includes deploying trap lines to collect MPB, procuring lodgepole pine from Big Horn Mtns, WY, deploying MN and western pines to field sites in Black Hills, checking attraction every 3d from base station. Travel includes \$1,500 lodging for team inclusive of research base space at Wheaton College Science Station in South Dakota (storage of logs from MN, use of upper classrooms to infest material to deploy to field). Budget also allows two overnight trips to fetch material for Activity 3 approx October, Jan two years in trucks for return to UMN Quarantine Facilities (est. \$1,400; 2 trips x 2 trips x \$100 truck rental x 1200 miles at \$0.39 + hotel). \$1.5K each of three years also budgeted for travel to meet with MPB specialists to share findings and stay abreast of management strategies and progress slowing spread from Canada. | | | | | | | | | | | | | | | |
| Cell phone airtime: \$100/year for 2 years pay-as-you-go for safety emergencies in the field on Activity 2. Proposed as cheaper than ACR ResQLink+ Personal locator beacon (\$289, REI) or SPOT tracker (\$100+\$100 subscription). We have used satellite phones (Iridium) in past, but cell reception is acceptable at WCSS so propose this as least expensive route. | | | | \$200 | \$208 | \$208 | \$0 | | | | | \$200 | \$208 | \$208 | \$0 |
| UMN Quarantine Facility rental for Activity 3 | | | | | | | | \$4,500 | \$0 | \$0 | \$0 | \$4,500 | \$0 | \$0 | \$0 |
| COLUMN TOTAL | | | | \$124,800 | \$124,800 | \$124,800 | \$0 | \$50,200 | \$50,200 | \$50,200 | \$0 | \$175,000 | \$175,000 | \$175,000 | \$0 |

Mountain Pine Beetle: Invasive Threat to Minnesota's Pines

Has it reached us yet?

This beetle was imported into Dodge County in fall 2012. Fortunately, this mountain pine beetle was dead. MDA surveyed, but did not detect any new insects.



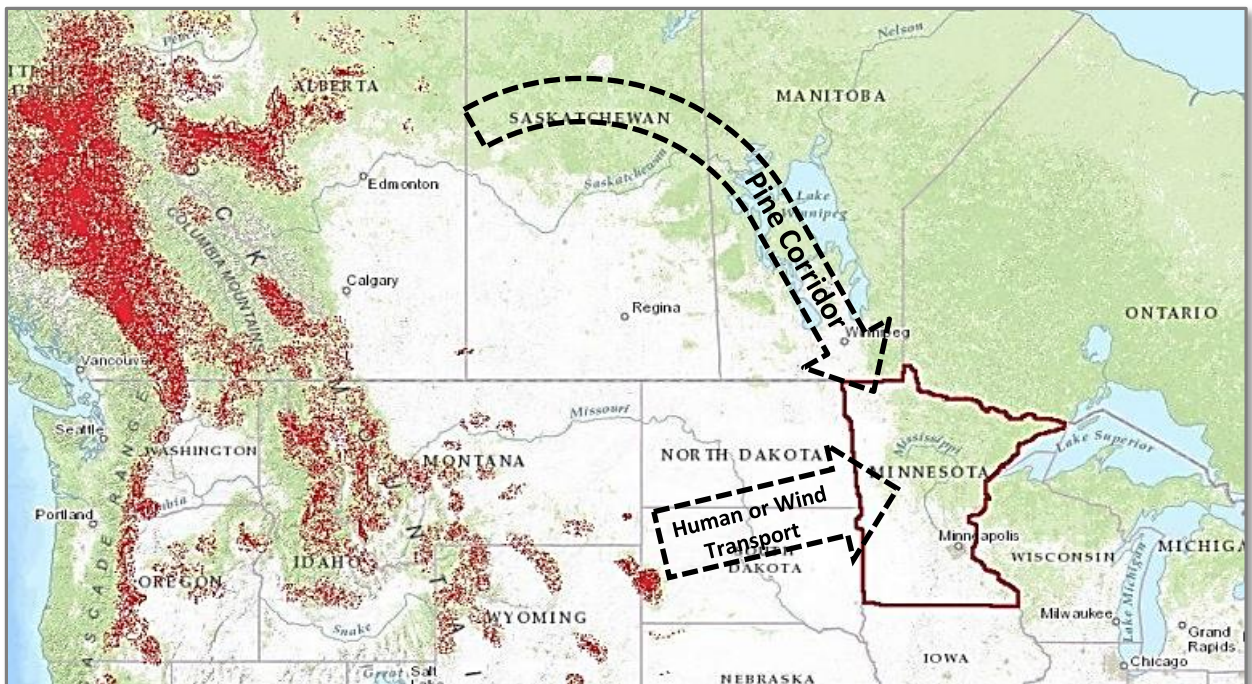
Can it reproduce in our pines?

We found the insect can attract mates, lay eggs, develop, and emerge from cut logs of our native pines – sometimes faster than in their historic western range.



Conclusion

**This insect
remains a serious
invasive threat**



Shaded areas indicate conifer forest. Dark areas on the left indicate the current extent of forests with high mortality due to mountain pine beetle. Routes to Minnesota from current epidemic populations are shown.

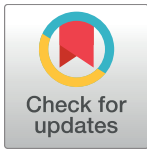
RESEARCH ARTICLE

Colonization behaviors of mountain pine beetle on novel hosts: Implications for range expansion into northeastern North America

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Abstract

As climates change, thermal limits may no longer constrain some native herbivores within their historical ranges. The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is a tree-killing bark beetle native to western North America that is currently expanding its range. Continued eastward expansion through the newly invaded and novel jack pine (*Pinus banksiana* Lamb.) trees of the Canadian boreal forest could result in exposure of several species of novel potential host pines common in northeastern North America to this oligophagous herbivore. Due to the tightly co-evolved relationship between mountain pine beetle and western pine hosts, in which the insect utilizes the defensive chemistry of the host to stimulate mass attacks, we hypothesized that lack of co-evolutionary association would affect the host attraction and acceptance behaviors of this insect among novel hosts, particularly those with little known historical association with an aggressive stem-infesting insect. We studied how beetle behavior differed among the various stages of colonization on newly cut logs of four novel potential pine host species; jack, red (*P. resinosa* Ait.), eastern white (*P. strobus* L.) and Scots (*P. sylvestris* L.) pines, as well as two historical hosts, ponderosa (*P. ponderosa* Dougl. ex. Laws. var. *scopulorum* Engelm.) and lodgepole (*P. contorta* Dougl. var. *latifolia* Engelm.) pines. Overall, we found that beetle colonization behaviors at each stage in the colonization process differ between pine hosts, likely due to differing chemical and physical bark traits. Pines without co-evolved constitutive defenses against mountain pine beetle exhibited reduced amounts of defensive monoterpenoid chemicals; however, such patterns also reduced beetle attraction and colonization. Neither chemical nor physical defenses fully defended trees against the various stages of host procurement that can result in tree colonization and death.

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Introduction

In recent decades, human activity and climate change have contributed to the geographic range expansion of some herbivorous insects [1–4]. A number of forest insects have been highly successful in invading new areas [5] at high cost to the public [6–8]. Host shifts are one important factor that can mediate geographic range expansions [9], providing the invaders access to a new resource pool and/or corridor(s) for expansion. However, the ability to utilize new hosts is dependent upon a match between insect offensive and host defensive traits, or the “ecological fit” between herbivore and novel host [9,10].

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera, Curculionidae), is a bark beetle native to western North America ranging from southern California to British Columbia and east to the western edge of the Great Plains in western South Dakota. The beetle’s predominant hosts are lodgepole (*Pinus contorta* Dougl.) and ponderosa (*P. ponderosa* Dougl. ex. Laws.) pines, although the insect feeds and reproduces on almost all pines within its range [11]. This insect typically undergoes a one-year lifecycle, exhibiting a temperature-mediated synchronized emergence of adults in late summer crucial for host procurement activities [12,13]. Insect densities typically remain at low levels for decades, but populations can erupt when suitable host pools and environmental conditions coincide [4,14]. At outbreak levels, mountain pine beetles exhibit landscape-level effects on western North American pine forests [15], altering forest ecosystem services [16], forest regeneration [17], fire severity [18], carbon budgets [19–21], and even local climate [22].

Spread of mountain pine beetle to northeastern North America and its potential impact on forest and plantation trees such as red (*P. resinosa* Ait.), eastern white (*P. strobus* L.), jack (*P. banksiana* Lamb) and Scots (*P. sylvestris* L.) pines are serious concerns [23,24]. To date, little is known about the ability of this insect to colonize these hosts. Two potential pathways could facilitate the introduction of mountain pine beetle to eastern forests (Fig 1). Anthropogenic movement of infested wood comprises the first pathway [1,2,25,26]. Similar anthropogenic introductions have been reported for other *Dendroctonus* spp. [27–29]. The second pathway reflects continued natural spread through the boreal forest [24]. In 2006, the beetle breached the geoclimatic barrier of the northern Rocky Mountains due to increased climatic suitability, and moved into lodgepole pine forests of western Alberta [30,31]. Over the past decade, populations of the insect expanded east into a lodgepole-jack pine hybrid zone and are now established and expanding through stands of pure jack pine, a “novel” host for this insect [32].

Similar to many bark beetles, the colonization of a susceptible tree by mountain pine beetle is characterized by a series of discrete events [34]. Female pioneers orient to the tree, find a bracing point on the bark, and determine the potential suitability of the tree from gustatory stimulant-deterrent cues in the bark and phloem [35,36]. If the host is accepted and boring commences, female mountain pine beetles produce an aggregation pheromone, *trans*-verbenol, from α -pinene, a monoterpene found in the trees’ phloem tissues [37]. Joining males produce additional aggregation pheromones [38], which enhance the signal of *trans*-verbenol in combination with several critical host volatiles [37,39–42]. This attraction fosters a mass attack that can concentrate low density populations [43] and quickly overwhelms the tree’s defenses [44].

Pines utilize several defensive mechanical and chemical traits such as bark texture, preformed resin ducts, and toxic chemical deterrents to curtail attacks [45], such that select trees with a higher defensive capacity may prevent colonization [46–48]. Various studies have reported differences in susceptibility to colonization among trees of a single species, concurrent with intraspecific variation in particular defensive traits [44,48–50]. However, greater variation in susceptibility can occur between trees of different species, where there are differences

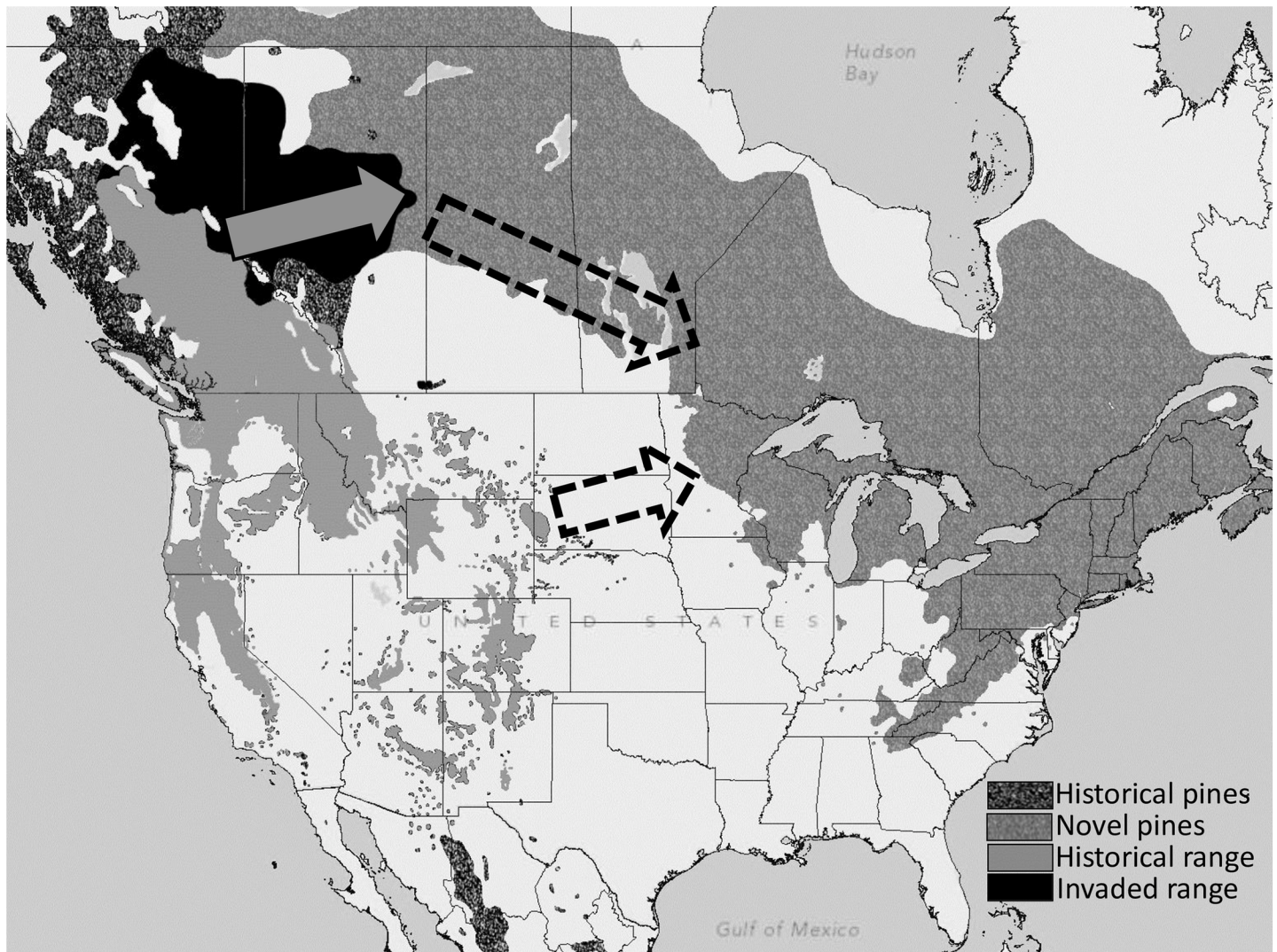


Fig 1. Approximate historical (light grey and mottled grey) and current (light grey and black) extent of mountain pine beetle range in North America. The light arrow represents current range expansion and dashed arrows represent potential pathways to eastern pine forests. Pine regions shown represent those of pine species used in this study from [33]. Historical and range expansion data obtain from data presented in [24] and the approximate geographic limit of beetle presence reported by the Alberta Ministry of Agriculture and Forestry in 2014.

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in both expression of defensive traits and the defensive strategy used (e.g., bark surface texture and internal tree chemistry) [41,46,50–53]. Many host defense studies have been conducted on live trees [51,52], however, constitutive defenses first encountered by the insects are also integral to mediating early colonization behaviors [53]. Interspecific differences in susceptibility to bark beetles have also been observed in harvested logs, for example [54–57]

Mountain pine beetle appears to have a long co-evolutionary history with western hosts, utilizing secondary chemicals produced by the tree to defend against insect and fungal attack [57,58] to instead produce aggregation pheromones and pheromone synergists that result in mass attack and host procurement [39]. Long associations may have resulted in particularly high secondary chemical concentrations in some hosts, which has conferred some level of resistance due to deterrence when concentrations of secondary chemicals are too high [36,59]. Thus, colonization behaviors mediated by defensive traits of a host may differ between

historical hosts and novel host species. Empirically, mountain pine beetle has colonized novel host species on only two known occasions in arboreta, exhibiting varying degrees of success among species and between studies [60,61]. Thus, it is unclear if pine species with which the insect has had no co-evolutionary relationship will be suitable hosts[9].

To determine whether novel eastern pine hosts may be suitable hosts for mountain pine beetle, and/or whether they have traits that may limit colonization, we designed a series of field and laboratory behavioral experiments to assess beetle response to novel pine hosts at each stage of the colonization process (attraction to infested material, bark acceptance, phloem acceptance, and egg gallery establishment). We used two common historical hosts, ponderosa and lodgepole pine, as positive controls to account for potential interspecific variation among historical hosts [54]. We hypothesized that interspecific differences among pine species will mediate host colonization behavior.

Materials and methods

Host material

We tested eastern white pine, jack pine, and red pine, all native to northeastern North America and of uncertain host status for mountain pine beetle; Scots pine, a Eurasian species commonly planted in North America and also of unclear host status, and ponderosa (*P. ponderosa* Dougl. ex. Laws. var. *scopulorum* Engelm.) and lodgepole pine (*P. contorta* var. *latifolia* Dougl.), two species common to western North America that are known hosts of mountain pine beetle. In 2013, we harvested two trees of each species on July 29 and 30 and two more on August 5 and 6. In 2014, the same numbers of trees were cut on August 4 and 5 and August 11 and 12, for a total of 48 pines for both years. All trees were free from any sign of damage or disease and approximately 24 cm DBH (diameter at breast height, approximately 1.4m above ground level) (Table 1).

The eastern pines as well as quaking aspen (*Populus tremuloides* Michx.), which served as a negative control for bark acceptance experiments, were sourced, with permission, from the University of Minnesota Cloquet Forestry Center, Cloquet, MN, USA (latitude, longitude: 46.701735, -92.521798). Ponderosa pines were cut with permission from the USDA-Forest Service Rocky Mountain Region from stands in the Black Hills of South Dakota, USA in 2013 (latitude, longitude: 44.12955, -103.48513) and 2014 (44.12587, -103.56700). Lodgepole pines, with permission from the USDA-Forest Service Rocky Mountain Region were harvested from the Bighorn National Forests in the central Bighorn Mountains, Wyoming, USA in 2013 (44.60337, -107.21505 and 44.62710, -107.16303) and 2014 (44.31865, -106.94633 and

Table 1. Mean (SE) diameter at breast height (DBH) (cm) of trees cut ($n = 4$ per species per year) and mean (SE) phloem thickness (mm) of each species for each of 6 logs cut from the bole of each tree. Means within a column followed by the same letter are not significantly different.

| Species | 2013 | | 2014 | |
|---------------|--------------|----------------|--------------|----------------|
| | DBH (SE) cm | Phloem (SE) mm | DBH (SE) cm | Phloem (SE) mm |
| Ponderosa | 25.76 (0.58) | 2.02 (0.08) | 24.22 (0.21) | 3.10 (0.06)a |
| Lodgepole | 24.28 (0.57) | 2.01 (0.07) | 23.61 (0.48) | 3.18 (0.05)a |
| Jack | 22.63 (0.71) | 1.56 (0.04) | 22.93 (0.55) | 2.00 (0.06)c |
| Red | 24.63 (1.30) | 1.89 (0.04) | 23.10 (0.36) | 2.47 (0.04)b |
| Eastern White | 24.60 (0.78) | 1.86 (0.06) | 24.13 (0.63) | 2.50 (0.10)b |
| Scots | 24.03 (0.90) | 1.61 (0.03) | 23.56 (0.93) | 2.09 (0.07)bc |
| $F_{5,18}$ | 1.46 | 2.61 | 0.83 | 12.51 |
| P | 0.25 | 0.06 | 0.54 | <0.0001 |

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44.22341, -106.93212). From each tree, we cut five logs at 1 m lengths and immediately sealed cut ends with paraffin wax to reduce desiccation. Sealed logs were enclosed immediately in black terrapin body bags (BP medical supplies, Brooklyn, NY, USA) to prevent infestation by other insects. We transported all logs to an experiment station in the central Black Hills, SD within 24 h of harvest, and stored them on their cut ends in a closed building until use.

We utilized cut material instead of live trees for several reasons, including regulatory and biosafety concerns in introducing mountain pine beetle and its associated fungi to live trees located outside of its current range. Freshly cut material has often been used by others to assess colonization dynamics of mountain pine beetle [35,42,54,62–66] and allows an assessment of baseline constitutive effects between species in a common garden environment.

Source of insects

Mountain pine beetles were collected in 12-funnel Lindgren funnel traps [67] with a commercially available pheromone lure (Contech Enterprises Inc, Delta, BC) from 6–8 locations along an approximately 6 km transect during peak flight in the first and second weeks of August in 2013 and 2014 in the central Black Hills National Forest with permission from the USDA-Forest Service Rocky Mountain Region. Collection cups contained clear cellophane shred (Spring-Fill Industries, Northbrook, IL) as refugia to reduce insect damage from crowding. Beetles were collected daily, transferred to Petri dishes lined with a lightly moistened tissue paper (Kimwipe: Kimberly-Clark, Irving, TX), and stored at approximately 5°C. We separated beetles by sex using auditory stridulation within 24 h of trapping [68]. Beetles were stored for 1–5 d before use.

Experiment 1: Bark entry

To test the frequency at which mountain pine beetles enter the bark of each species of pine, female beetles were caged on logs. Log sections 60 cm in length were cut from the lower bole of harvested trees. We measured the phloem thickness at three equidistant locations around the perimeter of the cut surface, and sealed the cut ends with paraffin wax to reduce desiccation. In 2013, ten 144 cm² cells (12 x 12cm) were constructed around the middle of the log using a border of 32 x 4 mm closed-cell vinyl foam tape (W.J. Dennis & Company, Elgin IL). In 2014, two 625 cm² cells (25 x 25cm) were constructed on each log. Care was taken not to disturb bark texture, and gaps in the cell border were filled with additional strips of tape. We secured charcoal-colored aluminum screening (New York Wire, Hanover, PA) to the cells with staples but ensured no bracing point other than the bark itself was available for beetles to begin boring. We introduced 5 beetles per cell in 2013 and 15 per cell in 2014. Two trees of each species were used in 2013 and three trees in 2014. Scots pine was tested in 2014 but not 2013.

Logs were placed upright indoors with natural light (approx.14L: 10D) and variable temperature (19–24°C). Logs were kept indoors to prevent colonization by other insects during the study. The logs were examined at 24, 48 and 72 h. Bark acceptance by beetles was judged by the presence and color of boring dust (i.e., dark = bark; light = phloem) and visual inspection of whether the insects were visible or had begun vertical boring within the log. At 72 h, we debarked the logs to confirm the number of beetles that had bored through the outer bark. Because no beetles bored into aspen, the negative control, it was not included in statistical analyses. To determine if bark acceptance behavior was similar between cut logs and live trees, we repeated the bark acceptance experiment in 2014 with four live ponderosa pine of similar diameter and origin to the logs used in the laboratory assays.

Experiment 2: Phloem entry

We examined the propensity of female beetles to initiate tunneling once the phloem was reached. We cut two 40 cm log sections from three of the 1 m logs of each tree. In 2013, two additional lengths were cut from each tree for a total of 168 logs in 2013 and 144 in 2014. Phloem thickness was measured, and ends were sealed with paraffin wax, as before. We drilled six equidistant holes that were 5 cm from one cut surface in 2013 and seven holes in 2014. Holes were 63 mm in diameter and just scored the phloem. A female beetle was introduced to each hole within 24–48 h of trees being felled. We placed female beetles in microcentrifuge tubes (0.2 ml capacity; Eppendorf, Hamburg, Germany) with tops removed and inserted the open ends into the holes. Tubes were checked after 12 h for acceptance of phloem. Inactive females that had not entered the phloem (i.e., no boring dust in the microcentrifuge tube) were recorded as rejections. Rejecting beetles were replaced with new females for the following experiments.

Experiment 3: Brood establishment

We determined the proportion of adult female beetles that established ovipositional galleries and laid fertile eggs after accepting the phloem from 144 (24 of each pine species) of the 168 logs from 2013 and all 144 logs from 2014 used in Experiment 2. Males were added to three holes approximately 18–24 h after the first female introductions. Vinyl screen was loosely attached over the entrance hole to reduce the chance of beetles falling out while the logs were handled. Logs were wrapped in charcoal-colored aluminum screening (New York Wire, Hanover, PA) and secured at both ends with staples to preclude entry by other wood-boring insects or predators before being stored outdoors for autumn and winter of 2013–14 and 2014–15. For a separate experiment [69], a subset of logs were debarked in January of each year. The remaining logs were returned indoors in April and placed in cardboard emergence tubes. Logs were debarked in mid-August after beetles had emerged [69]. To determine brood establishment, galleries of mated females that had been provided a male and had established ovipositional galleries were inspected for a minimum of one horizontal larval gallery.

Experiment 4: Attraction to tunneling beetles

We assessed differences in attraction of beetles to infested pine substrate in a field study utilizing artificially infested logs in a choice experiment [63]. Twelve sites, no closer than 350 m, and directly adjacent to or within active outbreaks (characterized by pines with fading needles and fresh pitch tubes around the bole) were established in early August of 2013 and 2014 in the Black Hills National Forest with permission from the USDA-Forest Service Rocky Mountain Region. At each location, we arranged seven 12-unit Lindgren funnel traps suspended from iron t-posts spaced every 3 m equidistantly around a ring. The funnel traps were attached to the t-posts with 35.5 cm long aluminum shelving brackets that were secured with wire to the t-posts. Screen logs from experiment two, which contained both paired and unpaired females, were used as bait. One infested log of each of the six pine species was transported to each of twelve field sites within 48–60 h of females being inserted. Logs were arranged at random and fixed with a hook next to each trap with the beetle entry holes midway down the trap length. As a negative control, one trap was left with no log. Traps were checked approximately every 48 h and all insects were removed and counted. Traps remained set for 6 d as beetles are expected to produce relatively consistent amounts of pheromone over this period [70], and were then replaced with a second set of fresh logs that were prepared in the same manner as above. In this manner, approximately 2 wk of data were collected both years, and the signal of tunneling beetles in exposed logs never lasted more than 9 d (i.e., log preparation plus testing

time). In 2013, logs were kept on the same t-post at each site for the length of the week, so total beetle catch for that treatment was summed for each week. In 2014, logs were re-randomized at each site each time a collection occurred. In 2013, twelve sites were used each week. In 2014, twelve sites were used the first week, and six sites the second week.

Chemical analysis of tree material

We collected phloem samples from logs to quantify monoterpene concentrations. A bark sample (approx. 5x5 cm) with the phloem intact was collected from a log of each of the four trees of each species in 2014 and two trees of red, eastern white, jack and ponderosa pine and one tree of lodgepole and Scots pine in 2013. Samples were removed within four days after trees were cut and stored in a freezer at -20°C until processing. A 1.5 cm^2 phloem sample was removed from the bark and phloem sample and cut into approximately 1 mm^2 pieces. Phloem constituents were extracted twice with 0.75 mL (1.5 mL total) high performance liquid chromatography grade hexane for 24 hours in a 2 mL vial at room temperature. Hexane was removed from the sample after each extraction with a 1 mL syringe. The two extractions were combined and passed through a $0.45\text{ }\mu\text{m}$ polyvinylidene fluoride syringe filter (Analytical Sales and Services Inc, Pompton Plains, NJ) in preparation for gas chromatography mass spectrometry (GCMS) analysis. The extracted phloem was placed in a fume hood for 1 wk at room temperature to dry. Once dry, the mass of phloem was recorded and used to normalize the concentrations of organic extracts.

GCMS analysis was carried out by using a Shimadzu QP2010S equipped with a Restek Rxi-5 ms column (30 m x 0.25 mm). Helium was used as the carrier gas at a column flow rate of 0.60 mL/min. Initial oven temperature was 55°C . This temperature was held for 5 min., stepped to 70°C at 1°C per minute, and then stepped to 160°C at 15°C per minute and held for 2 minutes. Finally, the oven was stepped to 250°C at 30°C per min. and held for 4 minutes.

All samples and standards contained heptyl acetate as an internal standard at a final concentration of 0.025 mM. Analytic standards of the phenylpropanoid 4-allylanisole and the most common and biologically important monoterpenes [71] α -pinene, β -pinene, 3-carene, myrcene, limonene, and camphene were used to generate calibration curves and response factors compared to the internal standard. β -phellandrene, also an important monoterpene for which a standard was unavailable, was identified as a 99% match with the NIST08 library. These titration curves and response factors were used to determine final concentrations and ratios for each compound in the phloem extracts. β -phellandrene and limonene co-eluted under the separation conditions. The concentration of β -phellandrene was approximated by subtracting the limonene signal based on unique ions in the mass spectrum and its calibration curve. The remaining peak area was attributed to β -phellandrene and used to approximate its concentration.

Analysis

Statistical analyses were completed in mixed effects frameworks in R (R Core Team, 2014). Separate generalized linear models with binomial distributions (lme4 package in R) were used to model the proportions of beetles of the total exposed to the treatment that entered the bark, accepted the phloem, and established brood, respectively. Fixed effects in the model were tree species, tree origin (historical or novel host), phloem thickness, and total monoterpene concentrations. Random effects in the analysis of data from Experiments 2–4 include tree and log nested within tree. In selecting the most parsimonious variables that could explain each response variable examined, we relied on graphical data analysis, Akaike's Information

Criteria to judge model suitability (AIC), and p-values associated with inferential tests of the significance of the variables ($\alpha = 0.05$).

We examined how phloem thickness, individual monoterpene concentrations and the number of mountain pine beetles captured in funnel traps (Experiment 4) varied with pine species in separate mixed-effect analysis of variance (ANOVA) models. Site and week were included as random effects. To meet model assumptions of homoscedasticity and normality of errors, all trap data and concentrations of 4-allylanisole were square-root transformed, and concentrations of β -pinene, 3-carene, myrcene, limonene, β -phelandrene and camphene were $\log(y+1)$ transformed. Where significant treatment effects existed ($\alpha = 0.05$), protected least significant difference tests were used to separate means in multiple comparisons [72].

We tested outliers by examining whether the presence of suspicious data points statistically changed the magnitude of the effect of interest (e.g., species of pine) on the response variable (e.g., number of insects captured). We did this by including a binomial indicator variable for suspiciously high trap catches as a covariate in the mixed effects model. If the P-value associated with the questionable catch was less than 0.05 divided by the total number of observations (i.e., Bonferroni's correction), it was considered an outlier. No outliers were found in 2013; however, in 2014, three points were removed. Two of these points came from a trap near a newly-attacked tree, which can skew catch numbers [65].

We constructed a test statistic to assess the degree of similarity between the rankings of pine species used as baits to capture flying beetles in Experiment 4 in 2013 and 2014. Pine species both years were ranked from most attractive to least attractive based on mean numbers of insects captured. The test statistic was devised by squaring the differences in ranks per treatment between years and summing those values. This procedure was then repeated 999 times with randomly generated rankings for both years. The placement of the test statistic from the empirical data relative to the 999 randomly generated test statistics reflects the probability of rankings having the same degree of similarity between years.

A Monte Carlo simulation was used to obtain an integrated estimate of the probability of brood production by a female alighting on each pine based on the outcomes of Experiments 1–3. Maximum likelihood estimates of species-specific proportions of beetles that entered the bark, entered the phloem, and produced brood were integrated into one model. Each parameter was assumed to be normally distributed with mean and variances derived from the maximum likelihood estimates of the logit-linked transformed proportions. A random draw was taken from each of the three distributions, then multiplied to obtain an estimate of susceptibility for a given species. The model was run 100,000 times for each pine species to obtain an overall susceptibility distribution. The upper and lower 2.5 percentiles of the distribution were truncated to obtain the middle 95% of the distribution, indicating susceptibility of a given pine species.

Results

Experiment 1: Bark entry

Overall, 532 of the 840 females initiated boring within 72 h of assay initiation, a boring rate of 63.3%. Beetles bored into the bark of all species of pines tested although the cumulative proportion that entered the bark by 24, 48 and 72 h varied among species (Table 2). Approximately 20–25% more beetles entered pines representing their historical hosts than novel eastern hosts by the 2-day and 3-day time points (contrasts; Day 1: $\chi^2 = 1.68$, $df = 1$, $P = 0.20$; Day 2: $\chi^2 = 7.67$, $df = 1$, $P = 0.006$; Day 3: $\chi^2 = 7.80$, $df = 1$, $P = 0.005$). Phloem thickness did not affect propensity of an insect to penetrate the bark (Day 1: $\chi^2 = 0.57$, $df = 1$, $P = 0.45$; Day 2: $\chi^2 = 2.82$, $df = 1$, $P = 0.093$; Day 3: $\chi^2 = 1.60$, $df = 1$, $P = 0.21$).

Table 2. Proportion of female mountain pine beetles that bored through the bark of six species of pine over a three day period. Means within a column followed by the same letter are not significantly different.

| Species | Host | 24 hours | 48 hours | 72 hours |
|---------------|------------|----------------------|----------------------|----------------------|
| | | mean % (±95%CI) | mean % (±95%CI) | mean % (±95%CI) |
| Ponderosa | historical | 44.0 (36.3, 52.0) a | 67.3 (59.4, 74.4) a | 74.8 (66.5, 81.7) a |
| Lodgepole | historical | 28.0 (19.9, 36.9) b | 58.7 (50.6, 66.3) ab | 68.8 (60.0, 76.4) ab |
| Jack | novel | 31.3 (23.0, 40.7) b | 50.7 (42.7, 58.6) b | 56.7 (47.7, 65.3) bc |
| Red | novel | 38.7 (29.5, 48.4) ab | 60.0 (52.0, 67.5) ab | 68.1 (59.3, 75.8) ab |
| Eastern White | novel | 17.3 (11.2, 24.9) c | 28.7 (22.0, 36.4) c | 47.3 (38.5, 56.3) c |
| Scots | novel | 30.0 (19.7, 42.1) b | 57.8 (47.4, 67.5) ab | 65.7 (54.1, 75.7) ab |
| χ^2 | | | 20.68 | 50.36 |
| P | | | 0.00093 | 1.168E-09 |
| | | | | 24.55 |
| | | | | 0.00017 |

No beetles bored into aspen, the negative control, which is therefore excluded from the analysis.

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When we compared our laboratory assays to insects boring in live trees, we found that the proportion of beetles entering the bark of live trees versus cut logs of ponderosa pine were similar after 24 h (Fig 2; $\chi^2 = 0.88$, $df = 1$, $P = 0.35$). However, approximately 10% more insects entered the live trees versus cut logs after 48 h ($\chi^2 = 7.32$, $df = 1$, $P = 0.007$) and 72 h ($\chi^2 = 6.38$, $df = 1$, $P = 0.01$). Overall success rates approached 95% for the live trees, and 80% for the cut logs (Fig 2). No beetles initiated boring before subsequently rejecting the live hosts in the three days of observation.

Experiment 2: Phloem entry

Our second experiment assessed whether beetles that had entered the bark would subsequently tunnel into the phloem. The majority (84.7%) of the 1123 beetles in this experiment actively bored into the phloem within 12 h of being introduced to the phloem, although the proportion that bored varied by species (Fig 3A; $\chi^2 = 19.12$, $df = 5$, $P = 0.002$). Jack pine exhibited the lowest percentage of females entering the phloem, with 15–19 percent fewer beetles entering jack pine phloem than ponderosa, red or eastern white pine phloem (Fig 3A). There was no overall effect of historical association of pine with the mountain pine beetle on phloem entry ($\chi^2 = 0.907$, $df = 1$, $P = 0.34$). Likewise, we found no overall effect across years of phloem thickness on the proportion of females that entered the phloem ($\chi^2 = 0.027$, $df = 1$, $P = 0.87$). However, there was a weak negative effect of phloem thickness on likelihood of phloem entry in 2014 ($\chi^2 = 4.90$, $df = 1$, $P = 0.027$). There was no relationship between total monoterpene concentration and insect’s acceptance of phloem ($\chi^2 = 0.15$, $df = 1$, $P = 0.70$).

Experiment 3: Brood establishment

A total of 840 galleries were established by the paired female and male beetles from the subset of logs used in Experiment 2. Of these galleries, 70.2% established brood, although the likelihood of brood establishment varied among pine species (Fig 3B; $\chi^2 = 27.18$, $df = 5$, $P < 0.0001$). The percentage of females that established brood was 13% greater in historical hosts than in novel hosts ($\chi^2 = 4.70$, $df = 1$, $P = 0.03$). Brood establishment rates appear to be driven, in part, by phloem thickness, with thicker phloem in historical hosts (Table 1). While there was some evidence for a relationship between likelihood of brood establishment and phloem thickness overall ($\chi^2 = 3.74$, $df = 1$, $P = 0.053$), we found that phloem thickness explained more variation in likelihood of successful brood establishment in 2014 than did species of pine. Thinner phloem

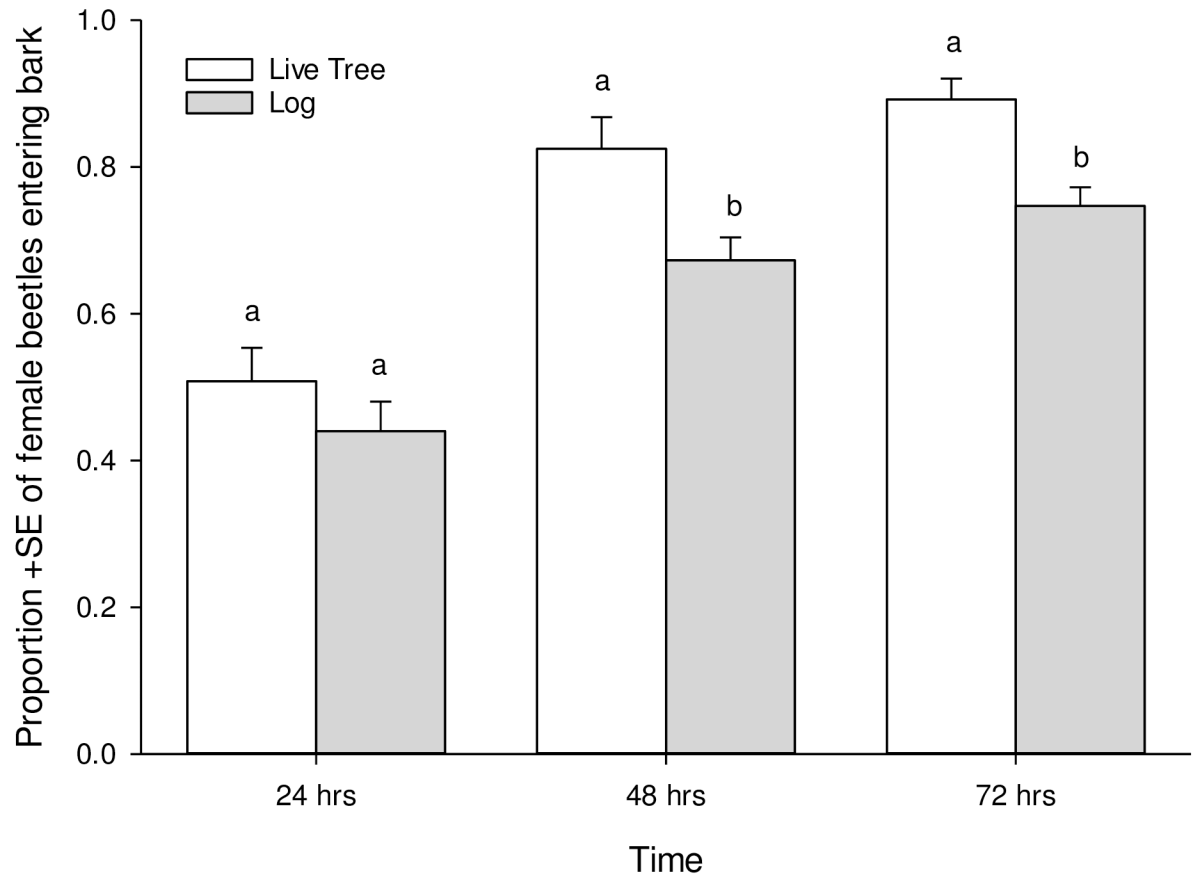


Fig 2. Comparison of the mean (+SE) proportion of female beetles entering four live ponderosa pine trees ($n = 120$ beetles) and logs cut from five ponderosa pine trees ($n = 150$ beetles) over a three day period. Bars within a time period with the same letter are not significantly different.

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($\chi^2 = 22.4$, $df = 1$, $P < 0.0001$) and greater total monoterpene concentrations ($\chi^2 = 6.1$, $df = 1$, $P = 0.01$) resulted in fewer successful galleries.

Modeled susceptibility to colonization

By integrating the results of Experiments 1–3 (i.e., bark is entered, boring is initiated in the phloem, and brood establishment occurs), we examined overall susceptibility to colonization (Fig 4). In general, less than 50% of adult females placed on the bark completed the series of discrete steps in host colonization that would result in live progeny under the bark. There were notable differences between species, however. Ponderosa pine appeared to be twice as susceptible to mountain pine beetle colonization as lodgepole pine. Susceptibility also differed between novel hosts, with red pine being more susceptible than any of the other novel hosts and even lodgepole pine. Eastern white pine was the least susceptible pine, although still similar overall to lodgepole pine (Fig 4).

Host attraction

The number of mountain pine beetles caught in funnel traps associated with infested logs varied among pine species in 2013 ($F_{6,138} = 3.70$, $P = 0.002$) and 2014 ($F_{6,372} = 3.10$, $P = 0.009$) (Fig 5). In general, traps associated with ponderosa and Scots pine logs caught the most beetles,

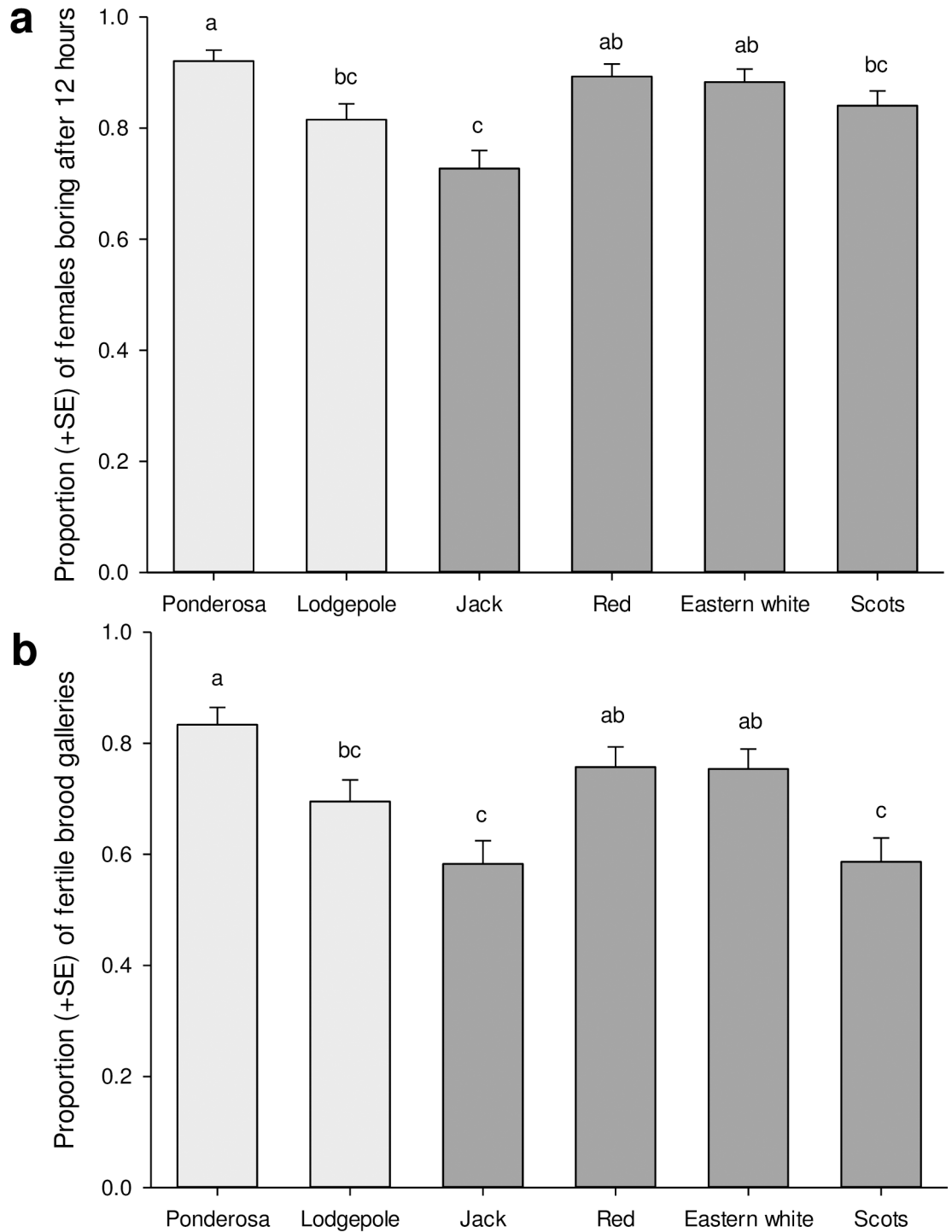


Fig 3. Mean (+SE) phloem acceptance and egg gallery establishment. (A) Mean (+SE) proportion of female beetles accepting phloem after 12 hours when placed into direct contact with phloem through pre-drilled holes. (B) Mean (\pm SE) proportion of galleries from logs infested with mountain pine beetle with larval galleries present after at least five months. Presence of larval galleries indicates mated pairs accepted the log and laid fertile eggs. Light and dark bars represent historical and novel pine hosts respectively. Bars with the same letter are not significantly different.

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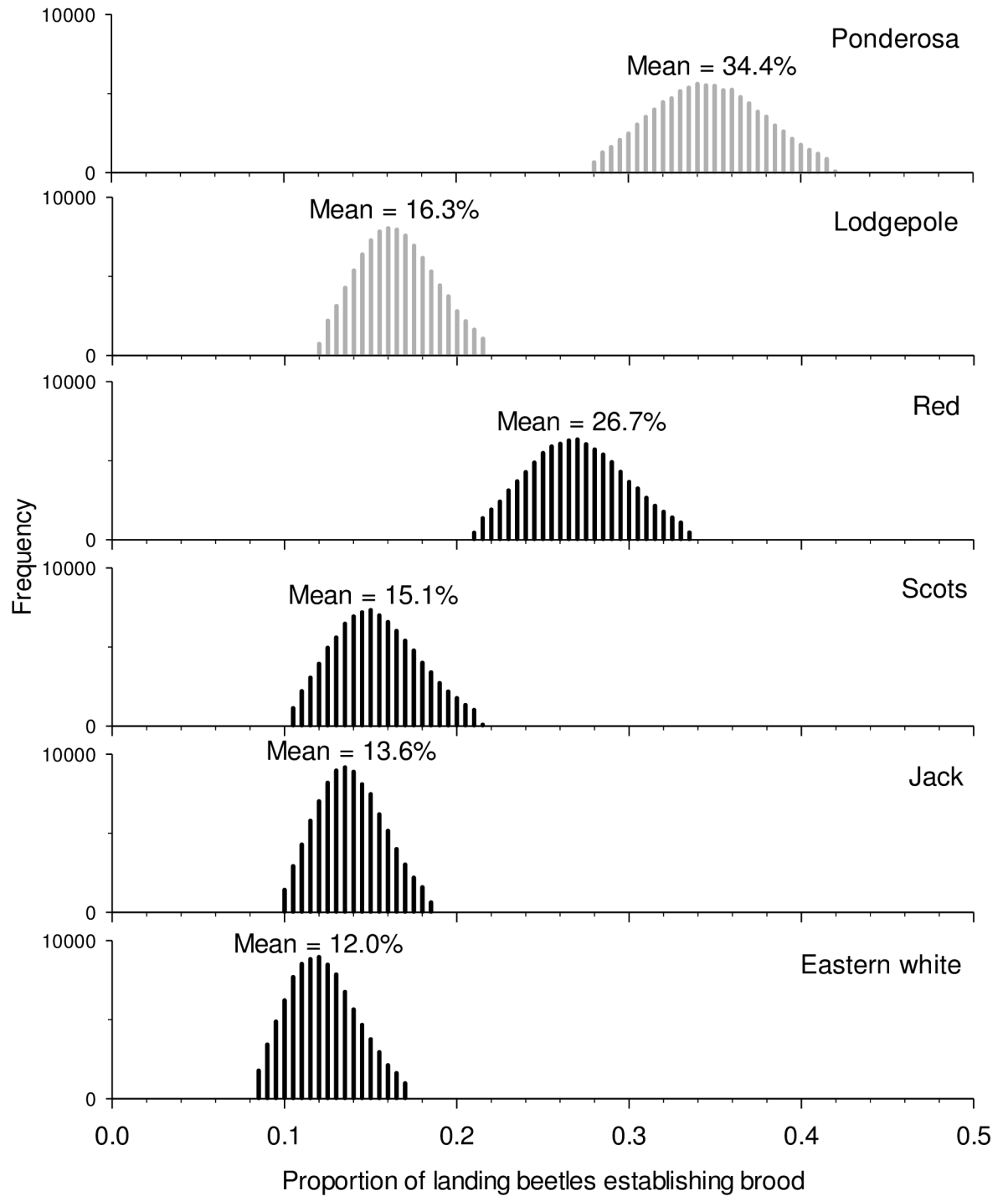


Fig 4. Monte Carlo simulation integrating bark entry, phloem acceptance and egg gallery establishment for cumulative susceptibility. Percentages indicated the likelihood of a landing beetle establishing a fertile egg gallery. Light and dark bars represent historical and novel hosts respectively. Probability distributions show the middle 95% of the distribution, with upper and lower 2.5% of the tails removed.

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while traps associated with logs of eastern white pine captured the least. The rankings of attraction between the seven treatments were consistent between years, with the exception of jack

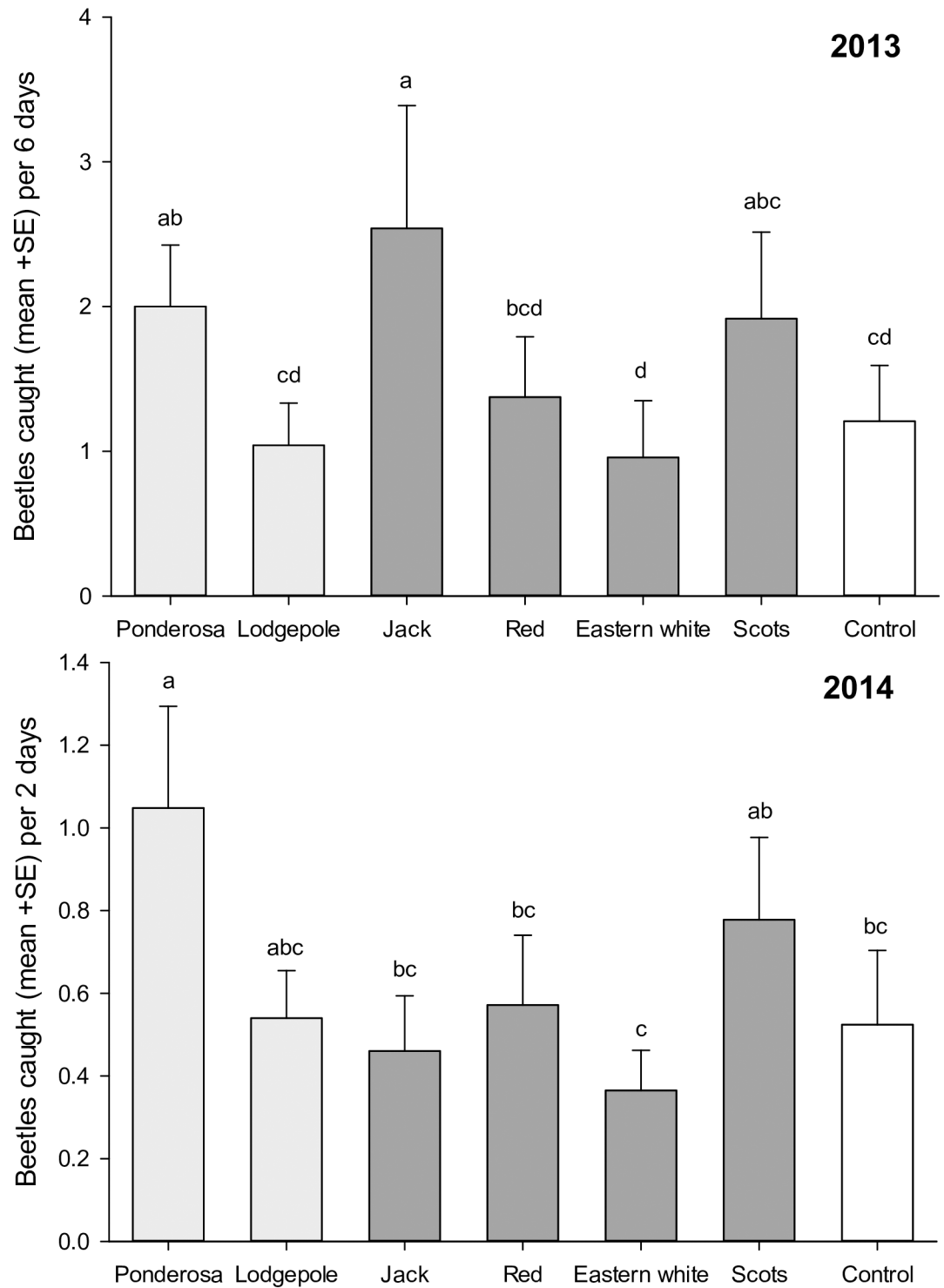


Fig 5. Mean (+SE) number of beetles caught in funnel traps. Traps were adjacent to various species of logs infested with boring beetles ($n = 12$ sites) over 2013 and 2014 flight periods. Light grey and dark grey bars differentiate historical and novel hosts respectively. White bars represent the control. Bars with the same letter are not significantly different.

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pine. Traps baited with infested jack pine captured more beetles than ponderosa pine in 2013, but fewer than all but eastern white pine in 2014. While there was not a significant likelihood ($P = 0.17$) of rankings being consistent between years with jack pine included, removal of the jack pine treatment resulted in a significant likelihood of consistency of the remaining six rankings between years not being due to chance ($P = 0.006$). Despite the consistency of these patterns, overall, very few beetles were captured. Traps associated with infested ponderosa pine logs were the only treatments that caught significantly more beetles in both years than unbaited control traps (Fig 5). More flying beetles were caught in traps associated with historical than novel hosts in 2014 ($F_{2,376} = 4.41, P = 0.013$), although this pattern was not apparent in 2013 ($F_{2,142} = 0.595, P = 0.553$).

Chemical concentrations in pines

The total absolute concentration of key monoterpene of known biological significance, present in the phloem, differed among pine species (Fig 6; $F_{5,28} = 12.91, P < 0.0001$). On average,

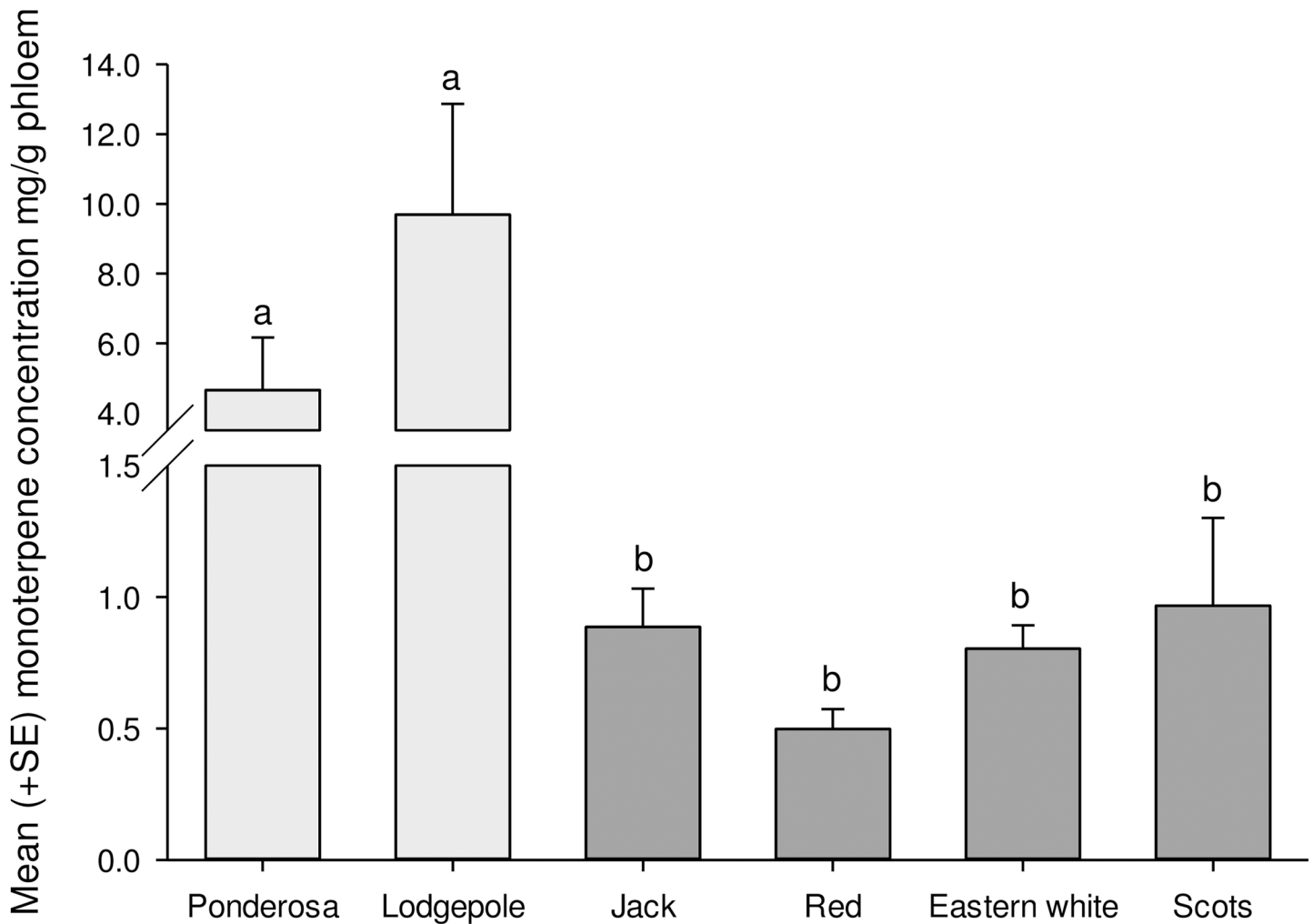


Fig 6. Total mean (+SE) monoterpene concentration (mg/g of phloem) of six species of pines used in this study. Samples were extracted from two uninfested logs of each pine within four days of being cut in 2013 with the exception of Scots and lodgepole from which only one was taken, and each of the four trees of each species in 2014. Light and dark bars represent historical and novel hosts respectively. Bars with the same letter are not significantly different.

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historical hosts (i.e., ponderosa and lodgepole pines) had 6 and 8 times more total monoterpenes than did novel hosts on average, respectively. Notably, Scots pine was the only novel host to have absolute concentrations of a known pheromone synergist, 3-carene, similar to those of historical pines (Fig 7B). We found minimal concentrations of limonene and the phenylpropanoid 4-allylanisole, two known beetle deterrents, in each species of novel hosts, including red pine. The only chemical that did not differ among pines in absolute concentrations was α -pinene (Fig 7; $F_{5,28} = 1.44$, $P < 0.24$), though relative concentrations of α -pinene (i.e., percent α -pinene relative to all other monoterpenes measured) did vary (Fig 8; $F_{5,28} = 42.1$, $P < 0.0001$).

Discussion

Our results fail to provide evidence that constitutive physical or chemical defenses of novel hosts *a priori* protect pine species from an aggressive herbivore by interfering with discrete colonization behaviors. Constitutive monoterpene concentrations can be lethal to other bark beetles in just a few days [58], or deter mountain pine beetle attacks altogether [59,73]. Induced defenses are also critical to tree defense from these insects [45,74], but are only stimulated after the insects and their fungi have breached the host [62,75,76]. Indeed, we found that entry rates of female beetles in our freshly-cut logs and live trees were similar after 24 h (Fig 2). Moreover, our findings that 68.7% of beetles entered the bark of logs of lodgepole pines after three days are similar to boring rates of 61.9% of beetles on live lodgepole pines over three days in Alberta, Canada [77]. Thus, even though inducible defenses would presumably increase upon beetle/fungal challenge in live novel hosts (impossible to test at this time due to quarantine regulations), we expect that our results are comparable to what might occur on live trees in early stages of attack, the focus of this behavioral study.

The consistent pattern of attraction to infested logs of different species between years ($P = 0.006$), and few treatments being more or less attractive than the control (Fig 5), is consistent with certain aspects of their chemical profiles. For example, the concentration of *trans*-verbenol released by boring beetles is correlated with the amount of its monoterpene precursor, α -pinene, present in the phloem [70]. In our study, we note that attractive ponderosa (2013, 2014) and jack pines (2013; Fig 7A) also tended to exhibit higher absolute concentrations of α -pinene than other species, although the mean absolute concentration of α -pinene was statistically similar across species (Fig 7A). Relative, rather than absolute, α -pinene concentration has also been suggested to be important in explaining differential beetle attraction [65]. However, while red, eastern white, and Scots pines exhibited 3.5–4.5 times the relative proportions of α -pinene vs. ponderosa pines (Fig 8), none were more attractive, suggesting that concentrations of other volatile monoterpenes that enhance attraction to mountain pine beetle pheromones may explain observed differences in attraction.

Concentrations of synergists may offer further explanation. Reduced concentrations of synergists would result in low attraction even if high concentrations of *trans*-verbenol were produced [39,78]. Indeed, all four novel host candidates had significantly lower concentrations of myrcene [78,79] than lodgepole and ponderosa pines, and all but Scots pine had lower concentrations of 3-carene [42] than the historical hosts (Fig 7). Terpinolene has also been shown to synergize response of flying beetles to *trans*-verbenol [78]. Terpinolene is present in high concentrations in ponderosa, lodgepole and Scots pine [42,46,80,81] but at low concentrations or absent in jack, red and eastern white pine [66,70,82].

Greater concentrations in lodgepole pine of 4-allylanisole (Fig 7H), a phenylpropanoid deterrent of mountain pine beetle and other bark beetles may explain why our lodgepole pine logs were less attractive than ponderosa pine logs [46,83–85]. Possible deterrence to flying

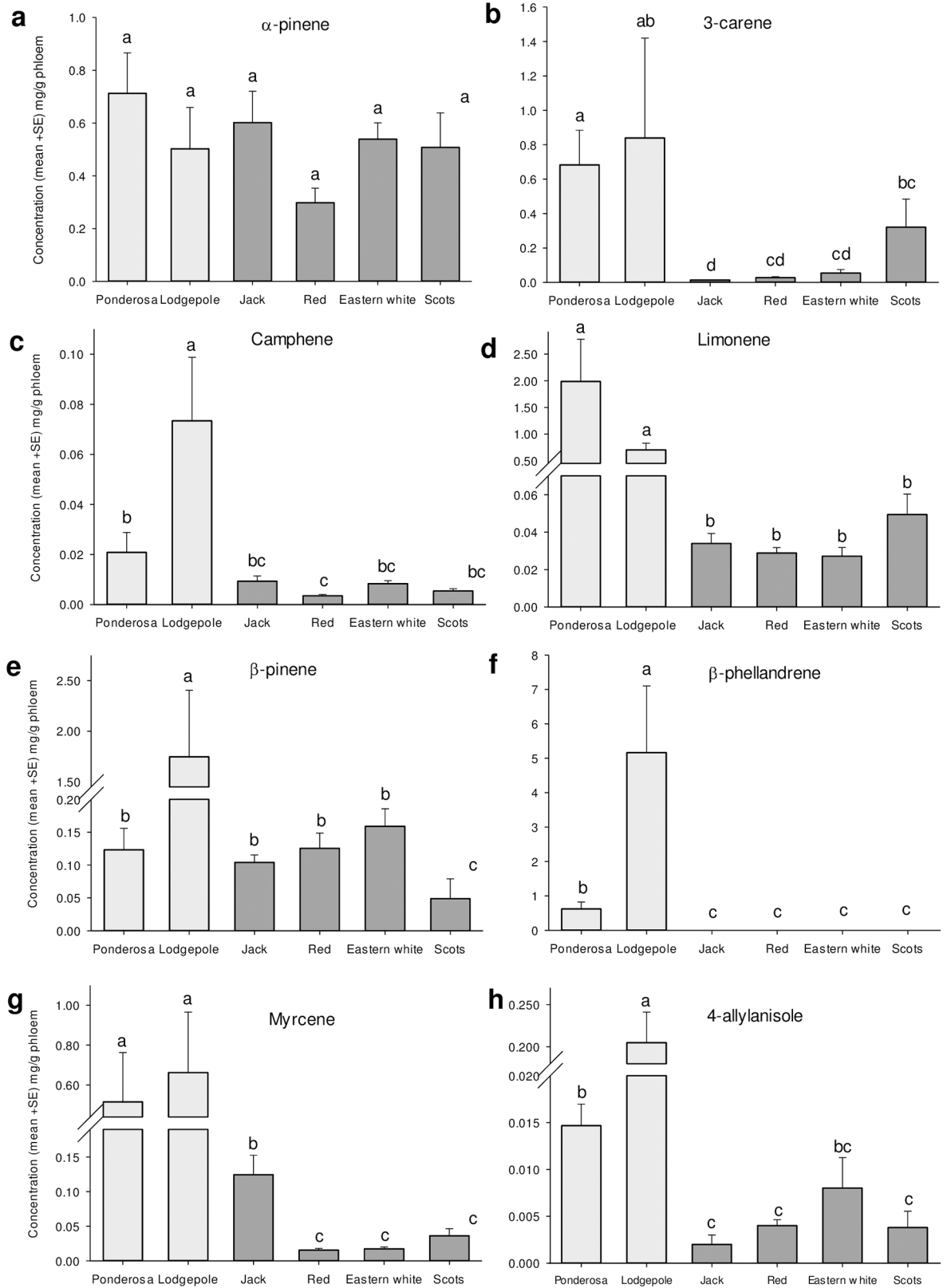


Fig 7. Mean (+SE) absolute chemical composition of logs from the six species of pines used in this study. Samples were extracted from two logs of each pine within four days of being cut in 2013 with the exception of Scots and lodgepole from

which only one was taken, and each of the four trees of each species in 2014. Light and dark bars represent historical and novel hosts respectively. Bars with the same letter are not significantly different. Note that scales of y -axis vary between chemicals.

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beetles by 4-allylanisole suggests a defensive adaptation in lodgepole pine to historical beetle pressure not strongly developed in novel hosts, and deserves further study. The hypothesis that 4-allylanisole has evolved as a deterrent is further supported by the fact that ponderosa and eastern white pine, the species with the second and third highest concentrations of 4-allylanisole respectively (Fig 7H), have also historically faced the two other most aggressive bark beetles in the *Dendroctonus* genus [86,87]; western pine beetle (*D. brevicornis*) and southern pine beetle (*D. frontalis*), respectively, and are also likely deterred by this chemical [83,85].

Colonization behaviors in novel northeastern hosts

Our results suggest that eastern forests are likely susceptible to the mountain pine beetle due to a general fit between historical and novel host traits and beetle behaviors [9]. By integrating the three steps in host acceptance, once attraction has occurred, we were able to estimate overall susceptibility to compare beetle preference in historical and novel hosts (Fig 4). Susceptibility of Scots, jack and eastern white pine was similar to lodgepole pine, and red pine was similar to ponderosa pine. Here, we provide a summary of fit for each “novel” species based on beetle behaviors and chemical traits in our experiments.

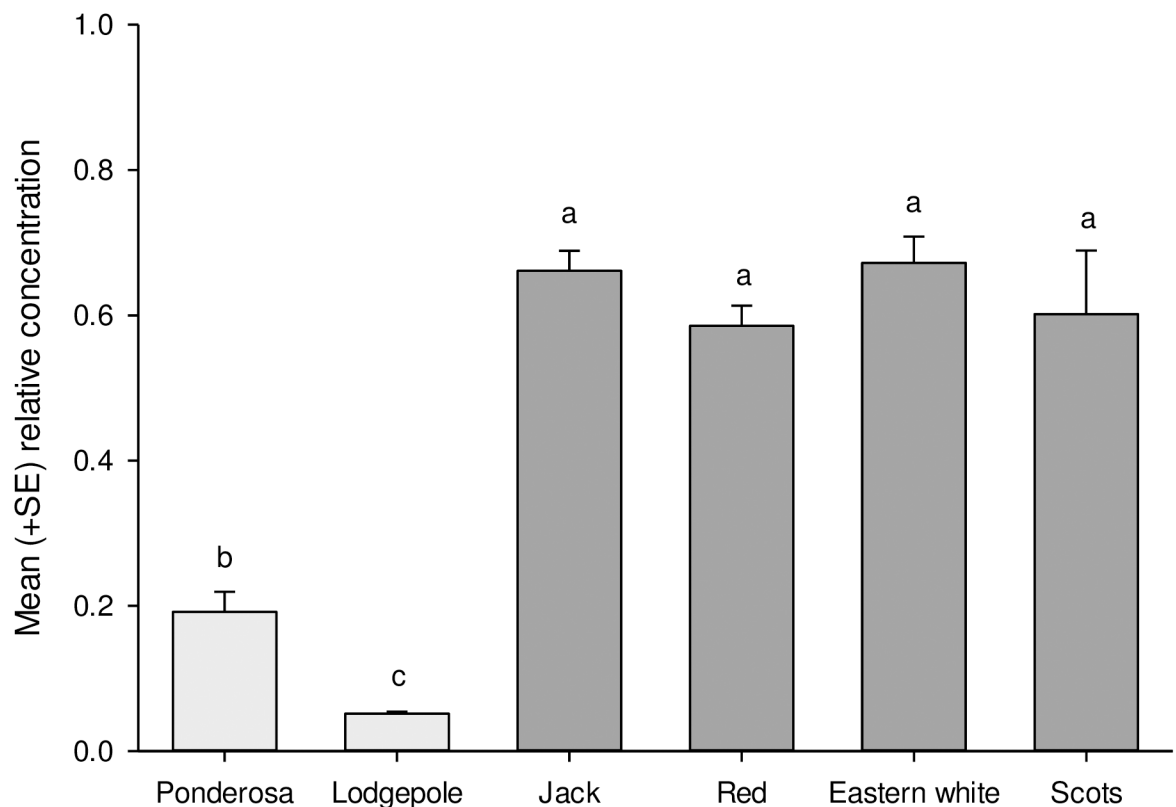


Fig 8. Mean (+SE) relative concentration of α -pinene in pine logs in relation to the seven primary monoterpenes measured. Samples were extracted from two logs of each pine within four days of being cut in 2013 with the exception of Scots and lodgepole from which only one was taken, and each of the four trees of each species in 2014. Light and dark bars represent historical and novel hosts respectively. Bars with the same letter are not significantly different.

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Overall, the least susceptible novel host, eastern white pine, was no more susceptible to mountain pine beetle than the least susceptible historical host, lodgepole pine (Fig 4). Some resistance to mountain pine beetle in lodgepole pine was evident at each stage of colonization. In contrast, the constitutive resistance displayed in eastern white pine is primarily conferred at the bark level as less than 50% of the beetles had entered the phloem of eastern white pine even after three days exposure (Table 2). Bark rejection may be due to physical traits such as bark texture [88,89], high lignin content [90], or repulsive gustatory cues [35,91]. Resistance conferred by the bark of eastern white pine may reduce its susceptibility at low beetle densities, but does not imply that the pine will not be attacked, or that stands will fully resist mountain pine beetle populations. Indeed, a high proportion of the eastern white pines that beetles entered were successfully attacked and killed in an arboreta in Idaho in the 1960s [61] and 2014 [69]. Primary reliance on resistance at only one point in the colonization process suggests that once the bark is breached, mountain pine beetle will demonstrate little further deterrence. This corroborates reports of general resistance by eastern white pine to southern pine beetle at endemic levels, but heightened susceptibility at outbreak levels when few other options were available [92,93]. We do further note that eastern white pine was the least attractive to foraging beetles (Fig 5), likely due to low concentrations of synergists and possibly higher concentrations of 4-allylanisole (Fig 7).

Overall, red pine appears to exhibit the highest suitability for mountain pine beetle of the novel hosts examined (Fig 4). Beetles tunneled into the bark of red pine at rates greater than eastern white pine (Table 2), although infested red pine logs were no more attractive than controls to foraging beetles (Fig 5). Consistent patterns of reduced attraction in our study likely reflects low concentrations of pheromone synergists in red pine phloem (Fig 7), supporting work by others who also found low concentrations of pheromone synergists but yet also demonstrated pheromone production by mountain pine beetle in red pine logs [66]. Induced defenses of live red pines in response to fungi vectored by the beetles could also reduce overall susceptibility. In general, red pine demonstrates rapid and high induced monoterpene responses to pathogenic fungi [58,94]. Induced responses to *Grosmannia clavigera* and *Ophiostoma montium*, the common fungal associates of mountain pine beetle [95], remain unquantified, and induced responses can differ between fungal species [58,75,96]. Regardless, attacks on red pines in an arboreta in Idaho have demonstrated apparent susceptibility [61].

Infested Scots pine, and possibly jack pine, may be more attractive to flying beetles due to the presence of pheromone synergists in their phloem. Jack pine in Alberta have high concentrations of the synergist 3-carene [70,97] and are particularly attractive to mountain pine beetles [42]. However, eastern jack pine populations have little of this monoterpene [70], although it does have the greatest concentration of another synergist, myrcene, among the novel hosts (Fig 7). Increased relative attraction of mountain pine beetle to infested Scots pine may be due to higher concentrations of 3-carene and terpinolene [80] relative to the other novel hosts. This greater attraction may also explain why Scots pines were the only common northeastern pine attacked in an arboretum in California [60] and why they were the first trees to be attacked in the recent attacks at Shattuck Arboretum in Idaho [69].

It is unclear why attraction to traps associated with infested jack pine varied between years while ranking of attraction to other host treatments remained remarkably consistent (Fig 5). Possible reasons for variation in attraction may include phloem thickness, age, abiotic variation between years or variation in chemotypes. Experimental design precludes us from assessing within-species variation in the present work, but variables affecting variation in insect attraction within a host species merits further study, and has been suggested for jack pine previously [70,98].

Reduced susceptibility in Scots and jack pine relative to the highly susceptible red pine (Fig 4) may be correlated with thinner phloem (Table 1) as previous observations in arboreta where nearly half [61] or all [60] attacks on live Scots pines were unsuccessful. Phloem thickness is positively correlated with attack probability [99] and reproductive success in mountain pine beetle [100–103], although its relationship with colonization success has been less well studied. A positive correlation between phloem thickness and colonization success supports the preference-performance hypothesis, which posits that parents choose the most suitable host for offspring fitness [104,105]. Since thin phloem results in fewer offspring [102], preference for pine species with thick phloem once the outer bark has been breached suggests that female assessment of phloem thickness at early stages of colonization drive this preference. Phloem thickness is a plastic trait that may vary between years. Indeed, we observed generally thicker phloem the second year. Phloem thickness is positively related to growth rate and tree diameter [106,107], although overall factors affecting phloem thickness deserve more study. Reduced phloem thickness at older ages is consistent with greater stand susceptibility in over mature stands [12]. Particularly high resin flow in live Scots pine [108], relative to lodgepole pine [109], may also provide additional defensive capabilities in this host that we did not test here.

Conclusions

Our study is the first to quantify how the initial colonization behaviors of mountain pine beetle vary among historical and novel hosts in a common garden environment. We found little evidence that constitutive defenses, critical in early stages of attack, will preclude mountain pine beetle from colonizing eastern pines. Red pine may be most susceptible to landing beetles while eastern white pine, similar to observations with southern pine beetle in the southeastern United States [92,93], may be least susceptible.

We do note that colonization (i.e., susceptibility) is distinct from reproduction (i.e., suitability), which was not the focus of this study. Tree mortality can occur after a colonization event, irrespective of the successful reproduction of the insect progeny however. Mountain pine beetle, like some other bark beetles, vector virulent fungi [110] that extract nutrients from the sapwood [111] and reduce water flow from the roots to the canopy, accelerating tree mortality [112,113].

Our results may be useful and applicable to other systems undergoing dramatic range shifts. While the mountain pine beetle is a future threat to common northeastern pines that have never exhibited association with an aggressive bark beetle, the southern pine beetle has already begun expanding its range north from the southeastern United States [114]. This insect has spread hundreds of miles north in recent years and was found in New England in 2014 [115], where it has successfully attacked red, eastern white and Scots pines (Dodds, K. pers. comm.). Indeed, both the mountain pine beetle and southern pine beetle attack trees in a similar fashion via mass attacks, and respond in similar ways to host monoterpenes during colonization events [71].

Much future work is necessary to more fully understand the impacts mountain pine beetle may have among novel hosts as the insect moves higher in elevation and expands eastward, subjecting new pine populations to attack [24,86]. Future work should investigate foliar volatile organic compounds and their potential role in colonization [73], suitability of eastern pines for fungal and microbial symbionts, differences in physical and induced defenses of these pines to beetle-vectored fungi, novel interactions with other subcortical insects and predators, and reproductive potential. Our finding that novel northeastern pines have little innate defenses that preclude susceptibility to beetle colonization provides further evidence that

accidental introduction or continued range expansion into eastern areas of North America could have serious effects on several species of economically and ecologically important native pines [116].

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Writing – review & editing: DWR BHA RCV MPM.

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TECHNICAL NOTE

Sexing live mountain pine beetles *Dendroctonus ponderosae*: refinement of a behavioral method for *Dendroctonus* spp.

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Introduction

Members of the genus *Dendroctonus* (Coleoptera: Curculionidae, Scolytinae) are some of the most aggressive tree-killing bark beetles in the world. As such, much research on this genus has been undertaken to understand the factors that affect the population dynamics of these insects (Six & Bracewell, 2015; Aukema et al., 2016). Despite biome-level ecological impacts of the most aggressive members of this genus when at outbreak levels, the flight periods of many temperate species are constrained to just a few weeks of peak emergence during which beetles locate and procure hosts via pheromone-mediated mass attacks (Rudinsky, 1962; Raffa, 2001; Bentz et al., 2014). Females initiate boring into a host. Thus, for many manipulative laboratory and field experiments assessing reproduction or host selection, the ability to quickly and accurately determine the sex of live insects is required.

Sexual dimorphism on the frons and pronotum is present in some species of *Dendroctonus*, most prominently in those species closely related to the southern pine beetle (*Dendroctonus frontalis* Zimmermann) (Wood, 1982), and provide varying degrees of accuracy in determination (Osgood & Clark, 1963; Tate & Bedard, 1967). The only consistently 100% accurate method of sex determination via secondary characters for *Dendroctonus* spp. requires examination of adults for the presence of a highly sclerotized plectrum on the seventh abdominal tergite (Lyon, 1958; Safranyik & Carroll, 2006). This plectrum is used for stridulation by males but absent in females. This morphological character is highly accurate (Lyon, 1958; Jantz & Johnsey, 1964; Godbee & Franklin, 1978), but can pose

challenges when working with live insects (Tate & Bedard, 1967). For example, female mountain pine beetles (*Dendroctonus ponderosae* Hopkins) tend to draw their abdomens tight against the elytra when prodded. Squeezing the abdomen or manipulating its position with a metal probe under a dissecting microscope (McCambridge, 1962) can extend handling times and result in harm to the insect (Godbee & Franklin, 1978). Morphological examinations remain a popular technique, however, and work reliably when executed properly.

Several authors have tested the efficacy of stridulatory behavior as a potential method for sex determination of live *Dendroctonus* beetles (Table 1). When disturbed, males will use stridulation to produce predominantly simple ‘stress’ chirps that are characterized by rapid short bursts (McCambridge, 1962; Michael & Rudinsky, 1972; Fleming et al., 2013). Chirps are produced as males move the plectrum against the pars stridens on the underside of the elytra (Hopkins, 1909; Michael & Rudinsky, 1972). Female *Dendroctonus* spp. beetles are also able to stridulate, using a different stridulatory apparatus, but their short, simple chirps, characterized by a low sound pulse rate, are easily differentiated from the rapid chirping and higher sound pulse rates of males (Barr, 1969; Rudinsky & Michael, 1973; Yturralde & Hofstetter, 2015). Sonic emissions of female *Dendroctonus* spp. are typically restricted to courtship behaviors, though a stress response has been detected in the red turpentine beetle (*Dendroctonus valens* LeConte) and the larger Mexican pine beetle (*Dendroctonus approximatus* Dietz) (Ryker & Rudinsky, 1976a; Yturralde & Hofstetter, 2015). The ability to chirp likely confers reproductive advantage to the joining sex, as this trait has been independently gained in both *Dendroctonus* and *Ips* (Barr, 1969; Lewis & Cane, 1990), and is conserved among males across *Dendroctonus* spp. (Ryker, 1988).

Though audible observations of stridulation can be useful for sexing adults of *Dendroctonus* spp., some error is

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Table 1 Results of this and other studies assessing accuracies of sex determination in *Dendroctonus* spp. by a single assessment of audible stridulation

| <i>Dendroctonus</i> species | Mean (\pm SEM) | | Reference |
|-----------------------------|---|-----|--------------------------|
| | % of total beetles correct ¹ | n | |
| <i>D. brevicomis</i> | 87.5 \pm 1.2 | 741 | Tate & Bedard (1967) |
| <i>D. frontalis</i> | 82.0 \pm 3.8 | 100 | Osgood & Clark (1963) |
| <i>D. ponderosae</i> | 97.7 \pm 0.6 | 663 | This study |
| <i>D. pseudotsugae</i> | 97.5 \pm 1.6 | 100 | Jantz & Johnsey (1964) |
| <i>D. terebrans</i> | 94.7 \pm 1.3 | 300 | Godbee & Franklin (1978) |

¹To standardize reporting, we report means of the datasets utilized. For example, if the paper reports results of experienced vs. inexperienced worker, we report the mean of the results.

common, which has reduced its use among researchers. Reported accuracy has varied between species. For example, up to 97.5% accuracy in sex determination has been obtained by listening for male stress chirps in Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) (Jantz & Johnsey, 1964). Up to 92% accuracy has been achieved in identifying female black turpentine beetles [*Dendroctonus terebrans* (Olivier)] based on females being silent (43%) or producing a low-pitch rasping sound (57%), with error occurring due to silent males (Godbee & Franklin, 1978). This 'low-pitch rasping' may be similar to the stress response reported in the closely related female red turpentine beetles (Ryker & Rudinsky, 1976a). For the western pine beetle (*Dendroctonus brevicomis* LeConte), 85–90% of males and <1% of females stridulate (Tate & Bedard, 1967). Only 82% accuracy was reported for *D. frontalis*, although misidentifications may have been exacerbated by experimental design issues acknowledged by the authors (Osgood & Clark, 1963). Inaccuracies most frequently result from some males remaining silent and not due to females chirping (Tate & Bedard, 1967; Godbee &

Franklin, 1978). In summary, although this method can be fairly accurate, improvements to audible examination of insects would likely enhance further employment of this technique.

A related approach to determine the sex of adult bark beetles is to look at, not listen to, stridulatory movements from males under low magnification. Specifically, the abdomen will make rapid stridulatory movements when the insect is disturbed by touching (McCambridge, 1962) or dropping (Lyons, 1982). This method has been used most successfully in the native elm bark beetle, *Hylurgopinus rufipes* (Eichhoff), with accuracies of 99.5% (Lyons, 1982). A similar method has been used with mountain pine beetles, for which observation of these movements has proven to be 95.9% accurate at distinguishing adult males from females (McCambridge, 1962). However, observation of stridulatory movements is less accurate than listening for audible chirps in other *Dendroctonus* spp. (Tate & Bedard, 1967; Godbee & Franklin, 1978). To our knowledge, although many investigators familiar with mountain pine beetle have used the audible method for sexing beetles (Hynum & Berryman, 1980; Raffa & Berryman, 1982; Fleming et al., 2013), no studies have explicitly examined the accuracy of using auditory male stress chirps for sexing mountain pine beetles.

Materials and methods

We have found in our work with mountain pine beetle that auditory examination can identify males and females with >99% accuracy, especially when subjecting initial cohorts to a second or third assessment that reveals males which previously remained silent. This repetition can be integrated easily into previously designed protocols to reduce the time needed for sexing relative to morphological examinations (Lyon, 1958). For example, beetles can be sexed initially during collections and placement in storage containers. A second assessment of purported females can be quickly completed during handlings for experiments. Alternatively, beetles can be sexed in a production line scenario with multiple workers sexing the same beetles.

Table 2 Classification accuracy of the sex of adult mountain pine beetles according to the number of times beetles were assessed for stridulatory chirping

| Source | n | First assessment | | Second assessment | | Improvement |
|-------------------|-----|---------------------|-----------------------|---------------------|-----------------------|----------------------------|
| | | % correct males (n) | % correct females (n) | % correct males (n) | % correct females (n) | % reclassified females (n) |
| Field-captured | 292 | 100 (139) | 95.4 (153) | 100 (142) | 97.3 (150) | 2.0 (3) |
| Laboratory-reared | 371 | 100 (117) | 96.9 (254) | 100 (121) | 98.4 (250) | 1.6 (4) |

To determine the sex of mountain pine beetles using audible chirping signals, a beetle is held gently by the margins of the pronotum between thumb and forefinger, with hindwings fully folded, the abdomen facing up, and the ventral side facing out. Holding them in this way will elicit stress chirps from males (Fleming et al., 2013). These chirps can be detected by holding the insect within 10 cm of the worker's ear (Fleming et al., 2013) especially when tapping the side of the thumb holding the beetle with the forefinger of the other hand 5–10×. This additional stimulation will often elicit chirps from initially silent beetles. We have found that sex can be determined at a rate of ca. seven beetles per minute, or 400–500 beetles per h, though additional time to allow for delayed response may increase accuracy.

To investigate the accuracy of audible sexing, mountain pine beetles were captured in the Black Hills of South Dakota, USA (44°7'N, 103°34'W). Lindgren funnel traps were baited with mountain pine beetle lure (Contech Enterprises, Delta, BC, Canada) in ponderosa pine (*Pinus ponderosa* Douglas ex C Lawson) forests during peak flight in mid-August 2013. Beetles were also sourced from infested logs in 2015. Beetles emerged naturally or were removed from the logs once peak emergence had passed. If beetles had been stored in a refrigerator, they were allowed to warm at room temperature before being processed. Sexing was accomplished by listening 2–3× for stridulatory chirps in all beetles tested, as described above. Males and females were placed in separate containers each time they were assessed. A minimum of 3 min was allotted between each assessment of the same beetle. After 2–3 consecutive assessments, beetles were moved to a freezer to be killed for later sex verification using the morphology of the seventh abdominal tergite (Lyon, 1958). To determine whether our method improves upon previous reports on stridulation in mountain pine beetles, we compared our audible stridulatory results with the visual stridulatory movement results reported by McCambridge (1962). For this analysis, we used a generalized linear logistic model in R v. 3.1.0 (R Development Core Team, Vienna, Austria) with the 'lme4' package for binomial data. The bimodal response variable was the accurate or inaccurate identification of sex based on the presence or absence of chirps (our data) or abdominal movements (data from McCambridge, 1962).

Results and discussion

In total, 1 095 male and female mountain pine beetles were used to evaluate the accuracy of audible sexing with repeated assessments. All beetles that produced rapid chirps were accurately classified as males during the initial

assessment with no false positives (Table 2). Initial accuracy of sex classification was 97–98% for both field and laboratory-sourced individuals, and was not significantly different from results reported by McCambridge (1962), who used a visual stridulatory technique (field-captured: $\chi^2 = 1.82$, $P = 0.18$; laboratory-reared: $\chi^2 = 2.87$, $P = 0.09$, both d.f. = 1). The proportion of beetles accurately identified as females improved with an additional assessment as previously silent males were identified. The accuracy of sexing after two assessments using an auditory technique was significantly greater than the 95.9% accuracy of a single visual identification reported by McCambridge (1962) for both our field-captured (98.6%; $\chi^2 = 5.46$, d.f. = 1, $P = 0.019$) and laboratory-emerged (98.9%; $\chi^2 = 8.43$, d.f. = 1, $P = 0.0037$) beetles.

Using a separate cohort of 432 field-captured beetles, we found that accuracy of female identification was 99.1% after three assessments. As we did not measure results after one or two assessments for this group, we exclude this cohort from Table 2.

We suspect the initial or total silence of some males may have been due to a combination of obstructions to the stridulatory apparatus, e.g., incomplete folding of the hindwings, malformed stridulatory structures, or other physical damage. Indeed, a damaged or malformed male, unable to stridulate, would unlikely be accepted by a female, as stridulation is required for mate acceptance (Ryker & Rudinsky, 1976b). Thus, female rejection of silent males may serve as a means of ensuring male physical or genetic fitness and ability to assist in gallery formation. This study suggests that such reduced fitness may be present in as many as 2–3% of males.

Further research should examine whether repetitive assessments can increase accuracies in sex determination among other *Dendroctonus* spp. (Table 1), and whether male production of rapid stress chirps can be used for sex differentiation in other groups of bark beetles. The morphology of the pars stridens and plectrum are similar across the tribe Hylurgini, to which the genus *Dendroctonus* belongs, and stress signals have been observed in members of other groups within the tribe (Rudinsky & Vallo, 1979; Oester et al., 1981; Lyons, 1982; Swedenborg et al., 1989). The male stress chirp may be a basal characteristic across *Dendroctonus* spp., as this behavior has been observed in all *Dendroctonus* spp. tested to date (Table 1; Ryker, 1988; Yturralde & Hofstetter, 2015), spanning most of the clades within the genus (Reeve et al., 2012).

Although researchers have been aware of the audible approach to sexing adult mountain pine beetles for some time, our study is the first to experimentally demonstrate the reliability of this method and demonstrate how audible

sexing can be improved. Our results indicate that beetles sorted as males can be 100% accurate after just one assessment, and female accuracy can approach 100% after 2–3 assessments. We show that inaccuracy in sexing by listening to stridulatory chirping is due to males that remain silent and thus are wrongfully identified as females. However, assuming they are in good condition, silent males will not necessarily remain silent in additional assessments.

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Cold tolerance of mountain pine beetle among novel eastern pines: A potential for trade-offs in an invaded range?



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ABSTRACT

Novel hosts may have unforeseen impacts on herbivore life history traits. The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) is a tree-killing bark beetle native to western North America but constrained by cold temperatures in the northern limits of its distribution. In recent years, this insect has spread north and east of its historical range, and continued expansion, or accidental introduction, could result in the mountain pine beetle becoming invasive in eastern North America. The limiting effect of cold temperatures among novel host pines is unknown, yet crucial for understanding the risk posed to north-eastern North American forests. We report the susceptibility of mountain pine beetle to cold temperatures while overwintering in six different pine species. Brood developed in two western pine hosts (*Pinus contorta* Dougl. var. *latifolia* Engelm. and *P. ponderosa* Dougl. ex. Laws. var. *scopulorum* Engelm.) as well as four eastern pines (*P. banksiana* Lamb., *P. resinosa* Ait., *P. strobus* L. and *P. sylvestris* L.) novel to this insect. The cold tolerance and cold tolerance strategy of the most common overwintering stage varied by host and year. Models describing lower lethal temperatures more accurately predicted observed field mortality of overwintering larvae than models based on temperatures at which larvae froze. Rapid development to less cold tolerant pupal and adult stages by brood in novel hosts prior to winter may constitute a trade-off between increased host suitability and winter mortality. We demonstrate that overwintering survival of mountain pine beetles in novel hosts depends on a match between the climate and ecophysiological effects of pine species. These results have implications for risk assessment models and management planning for eastern forests as mountain pine beetle continues to expand its range.

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1. Introduction

Climate change is having significant impacts on many insect populations, resulting in the shifting of historical ranges and modifications to activity and development (e.g., Bentz et al., 2010; Weed et al., 2013; Bebbler et al., 2013). Among phytophagous forest insects, the effects of climate change can be particularly important due to the ecological and economic significance of many of these species (Gandhi and Herms, 2010). Mitigating the effects of climate change will require effective management of these insects in new ranges and among novel hosts (Raffa et al., 2015; Tobin et al., 2014). However, novel hosts may influence the life history traits of these insects in unexpected ways (Awmack and Leather,

2002), complicating assessments of forest vulnerability (Fuentealba et al., 2013).

The mountain pine beetle (*Dendroctonus ponderosa* Hopkins), a tree-killing bark beetle native to western North America, is expanding its range due to climatic warming (Carroll et al., 2004; Raffa et al., 2015; Safranyik et al., 2010). Recent outbreaks have killed multiple pine species (*Pinus* spp.) across millions of hectares (Meddens et al., 2012) and contributed to a depletion of the forest carbon stock similar to that of all forest fires in the same region combined (Hicke et al., 2013). Winter temperatures have historically limited the northern range of this insect to southern British Columbia, Canada (Safranyik et al., 1975). In recent years, mountain pine beetle has been spreading north and east due to increasingly suitable climate (Carroll et al., 2004; Safranyik et al., 2010). Spread into western Alberta, Canada (de la Giroday et al., 2012) has coincided with expansion through the lodgepole-jack pine hybrid zone and recently into jack pine (*P. banksiana* Lamb.) of

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the boreal forest, a pine species and ecosystem historically naïve to this insect (Cullingham et al., 2011; Erbilgin et al., 2014). Continued eastward spread through the boreal forest, or anthropogenic movement of infested wood, could introduce this insect to other pine species that have no coevolutionary history with it (Raffa et al., 2015). Some assessments suggest that with continued climate change, most North American pine forests will have winters that are moderately to highly suitable for mountain pine beetle survival (Bentz et al., 2010).

Under historical climatic conditions and among western hosts, mountain pine beetle generally exhibits one generation per year. Broods initiated in late summer typically advance through four instars prior to winter, although developmental rate may differ among hosts (Cerezke, 1995). Development at low temperatures is more rapid for early instars than late instars (Bentz et al., 1991; Régnière et al., 2012), which enables early instars to “catch up” with siblings oviposited earlier, synchronizing much of the population as late instars prior to winter (Powell et al., 2000). This strategy supports mass attack behavior by adult beetles on the following summer that can overwhelm the defenses of trees when insects are at outbreak levels.

Overwintering success is critical for sustained mountain pine beetle outbreaks (Cole, 1981; Langor, 1989; Safranyik and Linton, 1991) and can be affected by the cold tolerance of overwintering life stages and the severity and duration of cold exposure. Eggs and early instars are less cold tolerant than late instars (Reid and Gates, 1970; Safranyik and Linton, 1998), and pupae and teneral adults rarely survive the winter (Amman, 1973; Reid, 1963). Supercooling points (i.e., the temperatures at which insect bodily fluids begin to freeze) for late instars vary, but average between -26.5 (Bentz and Mullins, 1999) and -36.7 °C (Cooke, 2009) in midwinter. Much research on mountain pine beetle cold tolerance and modeling climatic suitability has focused on supercooling point (Bentz and Mullins, 1999; Cooke, 2009; Somme, 1964; Strongman, 1982) although freezing is not coincidental with mortality in all insects (Sinclair, 1999). For mountain pine beetle, early instars (Somme, 1964) and late instars in lodgepole pine (*P. contorta* Dougl. var. *latifolia* Engelm.) (Bentz and Mullins, 1999) are reported to die upon freezing, but survive exposure to low, non-freezing temperatures (Bentz and Mullins, 1999). Larvae can adjust cryoprotectant concentrations (Somme, 1964) and cold tolerance (Yuill, 1941) in response to winter temperature fluctuations. However, pine hosts may also affect cold tolerance of larvae as evidenced from laboratory experiments (Wygant, 1940; Yuill, 1941), and field observations (Langor and Spence, 1991) among hosts in western North America. Indeed, increasing evidence from several other insect-host systems suggests that host may play a role in cold tolerance and winter survival (Feng et al., 2016; Gash and Bale, 1985; Morey et al., 2016; Trudeau et al., 2010). Thus host species may be an important factor to consider when assessing cold tolerance among novel hosts.

The stage distribution and cold tolerance of mountain pine beetle overwintering in novel hosts from eastern North America have not yet been assessed, and knowledge of how hosts may mediate life-history traits may improve our understanding of climatic suitability (Bentz et al., 2010; Régnière and Bentz, 2007). Here, we report the results of experiments designed to assess host effects on mountain pine beetle overwintering success. We measured supercooling points, lower lethal temperatures and development rates of overwintering brood in six pine species. Two species, lodgepole and ponderosa pine (*P. ponderosa* P. & C. Lawson) were historical hosts, and four pine species, jack, red (*P. resinosa* Ait.), eastern white (*P. strobus* L.) and Scots pine (*P. sylvestris* L.) had no historical association with mountain pine beetle. We hypothesized that the insects would be freeze-intolerant and that cold tolerance would differ among host species.

2. Methods

2.1. Material preparation

These experiments required us to work with cut logs because (i) many of the *Pinus* spp. of interest do not grow within the current range of mountain pine beetle; (ii) adult beetles will only colonize large diameter trees; and (iii) we cannot introduce mountain pine beetle into the environs of eastern North America. A total of eight trees of each species were harvested, four in 2013 and four in 2014. Each year uninfested trees were cut on two occasions, with two trees of each species being harvested each time, one week apart in early August, spanning peak flight time. Lodgepole pine were harvested in the eastern Big Horn mountains of Wyoming west of Big Horn, WY the first year (latitude, longitude: 44.60337, -107.21505 and 44.62710, -107.16303) and west of Buffalo, WY the second year (44.31865, -106.94633 and 44.22341, -106.93212). Ponderosa pine were harvested in the Black Hills near Nemo, SD the first year (44.12955, -103.48513) and near Silver City, SD the second year (44.12587, -103.56700). Jack, red, eastern white and Scots pines were harvested at the Cloquet Forest Research Center, MN (46.701735, -92.521798). All pines were 23–30 cm diameter at breast height (DBH, approximately 1.3 m above ground) and had a full crown with no signs of bark beetle attack and no visible mistletoe infection. The main stem of each tree was cut into 1 m sections, immediately waxed to slow desiccation, and placed into zippered tarapuan body bags (BP medical supplies, Brooklyn, NY, USA) for transport to the Black Hills, where they were further cut into 40 cm bolts. The ends of the bolts were waxed, and material was stored indoors.

Beetles used to infest bolts were caught in 12-funnel Lindgren funnel traps baited with pheromone and kairomone lures (trans-verbenol, exo-brevicomin, and myrcene) (Contech Enterprises Inc, Delta, BC) in an ongoing outbreak in ponderosa pine forests in the Black Hills of South Dakota, U.S.A. near Silver City. Beetles were immediately sexed by stridulation (Rosenberger et al., 2016) and stored in Petri dishes on moist KimWipes (Kimberly-Clark, Irving, TX) at approximately 5 °C for ≤ 3 days before use. Within 36–48 h of being harvested, six holes in 2013 and seven in 2014, each 5 cm from one cut end, were drilled on each bolt to the phloem with a 0.63 cm-diameter drill bit. A female beetle was placed in a microcentrifuge tube that was gently inserted into the hole to allow the beetle to enter the phloem. Bolts were stored upright with beetles near the base. Beetles were checked after 12 h for boring dust, evidence of initial host acceptance. Dead or inactive beetles were replaced. Males were added to three of the holes within 24 h of female introduction and replaced after 12 h if they did not enter the gallery. Bolts were covered in aluminum screening to keep other insects from infesting them. The bolts were hung for 5 days in the Black Hills National Forest as part of a separate experiment on colonization dynamics on novel hosts (Rosenberger et al., 2017) before moving them to the USDA Forest Service Mystic Ranger Station outside of Rapid City, SD to overwinter. In mid-October 2013, temperature probe data-loggers (HOBO, Onset Computer Corporation, Bourne, Mass.) were inserted under the bark on the north and south sides of two bolts of each species. In 2014, probes were inserted immediately after being moved to the overwintering location. Air temperatures were also recorded 30 cm above the ground.

2.2. Beetle extraction and storage

A subsample of the total infested bolts representing two bolts of each of the four trees of each species were moved from Rapid City, SD to St. Paul, MN in late December 2013 and early January 2015 in

their aluminum screening in construction-grade plastic bags inside zippered tarpaulin bags. The bolts remained in bags and were stored in a secured but unheated building (temp. $<0^{\circ}\text{C}$). Individual insects were carefully extracted by removing the bark with a chisel as needed over the course of the experiments in January and early February. All life stages except eggs (i.e., early instar, late instar, pupae, teneral adult, and parent) of the mountain pine beetle were collected and tallied to determine proportion of brood at each stage of development prior to winter. Larvae with head capsules less than approximately 0.75 mm wide were likely first or second instars, while larvae with head capsules greater than approximately 0.75 mm were likely third and fourth instars (Rosenberger, 2016). Fourth instars used in cold tolerance tests were further differentiated with 98% accuracy from third instars by visual estimation of head capsule width and body size (Rosenberger, 2016). Individuals to be used for cold tolerance tests were placed immediately into individual 1.5 ml microcentrifuge tubes, and all brood were stored at -5°C . All tests were completed within four days of individuals being removed from a bolt.

Discolored (i.e., darkened) larvae were considered dead and not used in cold tolerance tests (Amman, 1973; Wygant, 1940). Discolored larvae were prevalent in 2015, likely due to an early November 2014 cold snap and subsequent warming that would have allowed for decomposition to begin.

2.3. Cold tolerance tests

Tests to determine supercooling points and lower lethal temperatures of fourth instar mountain pine beetles (i.e., the most commonly found stage) from each pine species were conducted in January and early February 2014 and in January 2015. Insects were extracted from eight trees of each species across the two years of the experiment. Microcentrifuge tubes containing insects were removed from storage at -5°C and placed in contact with a copper-constantan thermocouple (Stephens et al., 2015). Temperatures starting from a room temperature of 21°C were recorded once per second via an analog data acquisition unit (USB-TC, Measurement Computing Corporation, Norton, MA) and TracerDAQ Pro software (Measurement Computing Corporation, Norton, MA). In 2014, individual microcentrifuge tubes with the insects and thermocouples were cooled at $\sim 1^{\circ}\text{C}$ per minute (Carrillo et al., 2004). In 2015, a refrigerated bath circulator (A40, Thermo Fisher Scientific, Newington, NH) with SIL-180 silicon oil was used to cool the insects at $\sim 1^{\circ}\text{C}$ per minute. The microcentrifuge tubes containing the thermocouple and beetle were placed in glass test tubes and lowered into the coolant bath.

2.4. Supercooling point tests

To determine the effect of host on larval supercooling points, we used a randomized complete block design in which larvae from each pine species were tested in the same run (i.e., block). Supercooling points were determined by recording the lowest temperature before the exotherm as the latent heat of fusion was released. Larvae were removed after approximately 2–3 min, once the exotherm returned to the supercooling point. Larval mass was measured following treatment.

While most overwintering mountain pine beetles were larvae, in 2014, some pupae and teneral adults were also available for testing. The supercooling points of pupae and male and female teneral adults from each of the pine species were measured. Individuals from the different pine species were pooled for statistical analysis.

2.5. Lower lethal temperature tests

We used a randomized complete block design for lower lethal temperature tests, similar to above. Fourth instar larvae extracted from bolts into microcentrifuge tube and stored at -5°C were placed in contact with a thermocouple, as above, and cooled from 21°C to a randomly chosen temperature between -20 and -42°C , inclusive, in one degree increments in 2014 and between -21 and -38°C in 2015. In 2015, half the batches were cooled to a randomly assigned temperature and half were removed directly after the exotherm when the temperature of the insect had returned to the supercooling point. This approach allowed us to obtain a large number of insects from the same populations for which we had both supercooling point measurements and lower lethal temperature measurements. After the target temperature was achieved, larvae were removed from the cooling unit, weighed, and warmed to room temperature while remaining in microcentrifuge tubes. For every two lower lethal temperature treatment batches, control larvae from each pine species were set aside and remained at room temperature to estimate mortality not due to cold exposure. Supercooling point measurements obtained from lower lethal temperature experiments were combined with the supercooling point data for analysis. We tested 40 blocks (one larvae from each pine species in each block) in 2014 and 63 blocks in 2015.

2.6. Mortality evaluation

We modified a method from Wygant (1940) to assess survival after cold exposure: larvae were held in the microcentrifuge tubes that were stuffed with 2.5×2.5 cm pieces of KimWipe to simulate a pupal cell. The KimWipe was dampened with 2 mL of deionized water, and a small hole was made in the cap for ventilation. Tubes were stored on their sides to allow larvae to move and kept in a dark box at room temperature. Individuals that bored into the moist Kim Wipe or molted to the next developmental stage were considered alive. Survival was assessed after 1, 2, 4, and 7 days after cold exposure and then twice a week until the insect reached adulthood or died. Thus in our experiments, we assessed effects that were not immediately lethal, yet still result in mortality. Approximately 10% of control and 15% of chilled larvae did not move when warmed to room temperatures, indicating that some healthy-appearing larvae were dead or mortally injured during extraction from bolts (Wygant, 1940), which could affect lower lethal temperature curves. Thus, insects that never resumed movement in lower lethal temperature tests were removed from further analysis allowing us to account for prior unobservable mortality. Because beetles can rapidly de-acclimate at room temperature (Cooke, 2009) (Supplementary Material), assessment of mortal injury before cold exposure was not possible.

2.7. Statistical analysis

All data were analyzed using R v. 3.1.0 (R Development Core Team, Vienna, Austria).

2.7.1. Development stage

To test whether proportions of brood at each stage differed between pine host species we used a generalized linear logistic model for binomial data (“lme4” package) in R. The binomial response variable was the presence of a specific stage “1” (early instar, late instar, pupae or adult), or that of another stage “0”. Values for each tree species were separated using a Tukey HSD post-hoc test with the “multcomp” package in R (Hothorn et al., 2008).

2.7.2. Survival of early winter cold snap

To determine the effect of host on larval mortality or developmental stage on mortality in bolts exposed to sub-zero temperatures in November 2014, proportions of discolored (dead) vs. creamy (likely alive) larvae were analyzed by using a generalized linear mixed effects model with a binomial distribution in the lme4 package (Bates et al., 2015). A term for host species was included as a fixed effect. To account for multiple individuals assessed from the same bolt and multiple bolts from the same tree, terms for bolt and tree were included as random effects. Chi square tests were used to test for differences in survival between larval stages and between species.

2.7.3. Overwintering supercooling points between stages

Mixed effects ANOVA models (“nlme” package in R) (Pinheiro et al., 2013) were used to evaluate the effect of natal host on the supercooling points of larvae and to compare supercooling points of pupae and adults. Model assumptions of homoscedasticity and normality of errors were assessed via inspections of residual plots. For all models, terms for tree and bolt within tree were included as random effects. *F*-tests were used to obtain a global estimate of the treatment effect, and means were separated between species using a Tukey HSD post-hoc test with the “multcomp” package in R.

2.7.4. Comparison of lower lethal temperature data between species and between years

Lower lethal temperature data were analyzed by using logistic regression. Survival curves were modeled by using logistic regression (binomial distribution and logit link function) and compared between years to determine if data could be pooled. Within year, modeled survival curves were compared between species to determine whether mortality rates at 0 °C (intercept) and/or the change in mortality with each degree change in temperature (slope) differed between species. For this analysis a positive event (i.e., survival) was the dependent variable and temperature, data group (i.e., year one or two for comparison of years, and species for comparison between species curves) and their interaction were included as independent variables. Wald tests, using a marginal fit, were used to compare these models.

2.7.5. Comparison of lower lethal temperature and supercooling point

Likelihood of larvae freezing as temperature declined was modeled using logistic regression to obtain a modeled cumulative supercooling point curve. At a population level, the cumulative supercooling point curve described the entire proportion of individuals that were expected to have started freezing if exposed to a specified temperature, x , with some individuals beginning to freeze at a temperature $\geq x$ ($-45\text{ °C} \leq x \leq 0\text{ °C}$, in our application). Modeled cumulative supercooling point curves modeled lower lethal temperature survival curves were compared using logistic regression as above. Comparisons of these curves allow us to determine whether the extent of freezing and mortality are equivalent at a specified temperature for this insect (i.e., evidence of freeze-intolerance), or whether mortality occurs before or after freezing, which would indicate chill intolerance or freeze-tolerance, respectively (Cira et al., 2016; Stephens et al., 2015). To determine overall similarity between freezing and mortality for each year, the dependent variable was a positive event (i.e., survival for lower lethal temperature data and freezing for the cumulative supercooling point curve). Independent variables were temperature, data group (lower lethal temperature or supercooling point), and an interaction between the two variables. The same analysis was then conducted to compare freezing and mortality at the natal pine species level by constructing separate models for each species in each year. Estimated LT_{50} and LT_{90} values and associated variances were obtained from the lower lethal temperature curves with the

“MASS” package (Venables and Ripley, 2002) in R by using the dose.p function.

2.7.6. Field validation

To determine whether our models were able to predict survival at a specified temperature, we used the predict.glm function in R to obtain predicted mortality for fourth instar larvae at the minimum temperature (i.e., the average of minimum temperatures recorded from underbark probes on the north and south side of two bolts of each species) recorded in each pine species prior to debarking in January 2014. Only 2014 models were used as the early cold snap in November 2014 likely killed beetles prior to winter acclimation (Somme, 1964) and our models are constructed for winter acclimated insects. Observed survival was obtained by tallying the proportion of dark (dead) versus creamy (alive) larvae from eight bolts from four trees of each pine species in January and February 2014. We considered models in which observed survival fell within 95% confidence limits to be superior.

3. Results

3.1. Development and early winter field mortality

In both years of these experiments, we observed significant effects of host pine species on the proportion of brood at each stage of development by January (Table 1). In the first year we found predominantly pupae and teneral adults in the novel hosts and larvae in the historical hosts (Table 1). We observed little natural mortality (8.2%) as indicated by dark larvae (Wygant, 1940) among brood overall the first year, despite under-bark temperatures reaching around -21 °C in early to mid-December (Fig. A.1). In the second year, most individuals were larvae (Table 1) and higher mortality (25%) was observed. An early winter cold snap occurred the second year when daily low air temperature rapidly dropped from 2 °C on 09-Nov-2014 to -22 °C on 13-Nov-2014. Daily low average under-bark temperatures (averaged for the north and south faces of the cut bolts) reached -13 °C (Fig. A.1). Host had a modest effect on survival of early instar larvae the second year, with a greater proportion of larvae surviving in ponderosa, jack and red pines than in lodgepole, eastern white or Scots pines (Fig. 1, $\chi^2 = 10.04$, $df = 5$, $P = 0.07$). There was no clear effect of pine species on survival of late instar larvae (Fig. 1, $\chi^2 = 5.83$, $df = 5$, $P = 0.32$). Larval stage did affect survival ($\chi^2 = 145.6$, $df = 1$, $P < 0.001$), with early instar larvae in all but jack pine exhibiting lower survival than late instars (Fig. 1).

3.2. Life-stage and host effects on supercooling points

In the first year, supercooling points differed among mountain pine beetle life stages tested (i.e., fourth instar, pupa, and teneral adult) ($F_{3,295} = 149.0$; $P < 0.0001$). Fourth instar larvae had significantly lower supercooling points than pupae or male or female teneral adults (Fig. 2) or all teneral adults combined (mean \pm SE: $-16.6\text{ °C} \pm 1.3$). The mean supercooling points of pupae and male teneral adults were not significantly different but were both significantly warmer than those of female teneral adults. The second year, the mean fourth instar supercooling point ($-30.7\text{ °C} \pm 0.22$) was similar to the first year ($F_{1,46} = 0.021$; $P = 0.89$). The minimum fourth instar supercooling point observed over both years in our experiments was -38.4 °C and the maximum was -19 °C . Natal pine species affected supercooling points of larvae in the first ($F_{5,18} = 9.07$; $P = 0.0002$), but not the second ($F_{5,18} = 1.14$; $P = 0.38$) year (Table 2). Fourth instars from lodgepole and Scots pine had the lowest and highest mean supercooling points respectively, each year although the average supercooling point from lodgepole

Table 1
Stage distribution of mountain pine beetles in the winters of 2013–14 and 2014–15 from evolutionarily novel and historical host species reared in eight logs of each pine species each year in Black Hills, SD. Stage distribution was determined in January of each year. Data were analyzed for differences between hosts using a generalized linear model. Proportions with the same letter within a column and year are not significantly different from each other.

| Year | Host species | Host Type | n | Proportion | | | |
|---------|---------------|------------|------|--------------|-------------|---------|----------|
| | | | | Early Instar | Late Instar | Pupae | Adults |
| 2013–14 | Ponderosa | Historical | 1085 | 0.08b | 0.45ab | 0.19a | 0.28d |
| | Lodgepole | Historical | 694 | 0.09b | 0.51a | 0.15ab | 0.24d |
| | Jack | Novel | 856 | 0.06b | 0.37c | 0.14b | 0.43bc |
| | Red | Novel | 985 | 0.01c | 0.22e | 0.13b | 0.64a |
| | Eastern white | Novel | 1399 | 0.07b | 0.41bc | 0.15ab | 0.38c |
| | Scots | Novel | 686 | 0.15a | 0.28d | 0.12b | 0.44b |
| | | | | χ^2_{5} | 139.6 | 227.3 | 21.7 |
| | | | P | <0.0001 | <0.0001 | <0.001 | <0.0001 |
| 2014–15 | Ponderosa | Historical | 1393 | 0.04c | 0.94a | 0.004b | 0.002c |
| | Lodgepole | Historical | 747 | 0.17a | 0.82c | 0.000ab | 0.003bc |
| | Jack | Novel | 701 | 0.08b | 0.90b | 0.001ab | 0.016ab |
| | Red | Novel | 878 | 0.09b | 0.86bc | 0.019a | 0.035a |
| | Eastern white | Novel | 1213 | 0.03c | 0.96a | 0.001b | 0.002c |
| | Scots | Novel | 534 | 0.11b | 0.89b | 0.004ab | 0.000abc |
| | | | | χ^2_{5} | 149.9 | 156.4 | 38.3 |
| | | | P | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

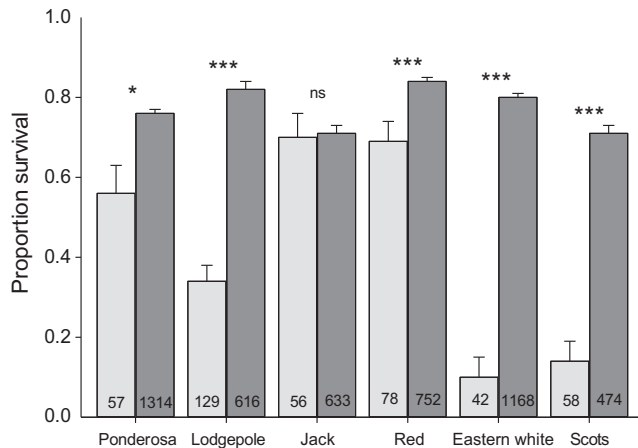


Fig. 1. Proportion of early (light bars) and late (dark bars) instars in overwintering pine bolts infested in August 2014, that appeared to survive through January 2015 after experiencing a cold snap in early November 2014. Discolored larvae were classified as dead, and cream colored larvae were classified as alive. Numbers on bars indicate the total number of early or late instar larvae for that pine. Late instars were more likely to survive than early instars ($\chi^2 = 145.6$, $df = 1$, $P < 0.001$). Stars indicate a significant difference between survival of small and large larvae (ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

was not significantly different from jack and eastern white, and Scots was not different from ponderosa and red (Table 2). An interaction between pine species and year precluded us from combining larval supercooling point data from both years. Live mass of larvae did not affect supercooling point the first ($F_{1,267} = 0.003$; $P = 0.96$) or second year ($F_{1,223} = 0.665$; $P = 0.42$).

3.3. Lower lethal temperatures

Lower lethal temperature curves described survival as a function of exposure to sub-zero temperatures. Comparisons of these curves among the six pine species indicated that projected mortality at 0 °C (i.e., the intercept) was not affected by host in the first ($\chi^2 = 4.34$, $df = 5$, $P = 0.50$) or second year ($\chi^2 = 8.0$, $df = 5$, $P = 0.16$). The change in survivorship with each degree of cooling (i.e., the slope) was also not affected by host in either year (Year 1: $\chi^2 = 4.22$, $df = 5$, $P = 0.52$; Year 2: slope: $\chi^2 = 6.26$, $df = 5$,

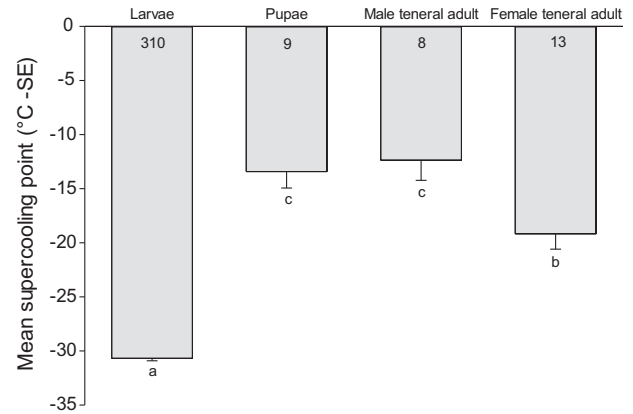


Fig. 2. Mean (-SE) supercooling points of fourth instar ($-30.7\text{ }^\circ\text{C} \pm 0.23\text{SE}$), pupae ($-13.4\text{ }^\circ\text{C} \pm 1.5\text{SE}$), adult male ($-12.4\text{ }^\circ\text{C} \pm 1.9\text{SE}$) and adult female ($-19.2\text{ }^\circ\text{C} \pm 1.4\text{SE}$) mountain pine beetle in 2014. Insects were extracted from all six pine species. Numbers on bars represent sample sizes. Bars with the same letter are not significantly different from each other.

$P = 0.28$). However, host may have affected the temperature at which mortality occurred as we observed a 4 °C spread in LT_{50S} between the coolest and warmest values the first year (comparison of red pine to Scots pine: $t = 1.97$, $df = 62$, $P = 0.053$) and 5.5 °C the second year (comparison of red pine to jack pine: $t = 2.18$, $df = 63$, $P = 0.033$) (Table 2). Survival among controls was 84.4% overall, but no effect of species on control mortality was observed ($\chi^2 = 2.7$, $df = 5$, $P = 0.75$).

3.4. Comparison of modeled supercooling point and lower lethal temperature

We found that fourth instars demonstrated similar freezing and mortality relationships with temperatures in some pines, but not in others (Fig. 3). In 2014, the proportion of larvae that froze or died at 0 °C (i.e., intercepts) was similar for the individuals from ponderosa, jack, red and Scots pines, as was the rate of change in the proportion of individuals that died or began to freeze as temperatures declined below 0 °C. In contrast, freezing and mortality appeared to be unrelated among larvae from lodgepole and eastern

Table 2

Mean supercooling point and temperatures required for 50 % and 90 % mortality of fourth instar mountain pine beetles from different pine species. Mean supercooling points with the same letter are not significantly different from each other.

| | 2014 | | | | 2015 | | | | | |
|---------------|----------------|----|----------------|----------------|------|---------------|----|----------------|----------------|----|
| | SCP (°C ± SE) | n | LT50 (°C ± SE) | LT90 (°C ± SE) | n | SCP (°C ± SE) | n | LT50 (°C ± SE) | LT90 (°C ± SE) | n |
| Ponderosa | −30.3 ± 0.46bc | 56 | −32.1 ± 1.5 | −38.2 ± 2.7 | 30 | −30.0 ± 0.68 | 44 | −28.3 ± 1.3 | −36.5 ± 2.8 | 50 |
| Lodgepole | −33.2 ± 0.43a | 49 | −30.6 ± 1.8 | −37.6 ± 3.8 | 30 | −31.6 ± 0.48 | 39 | −29.2 ± 1.0 | −34.4 ± 1.4 | 53 |
| Jack | −31.2 ± 0.51ab | 53 | −31.1 ± 1.0 | −34.6 ± 1.6 | 35 | −30.5 ± 0.51 | 46 | −25.9 ± 2.4 | −37.2 ± 3.5 | 51 |
| Red | −28.8 ± 0.63c | 51 | −33.3 ± 1.5 | −38.6 ± 2.6 | 29 | −31.0 ± 0.48 | 45 | −31.4 ± 0.9 | −36.8 ± 1.7 | 55 |
| Eastern white | −33.1 ± 0.47ab | 45 | −31.6 ± 1.6 | −39.3 ± 3.1 | 34 | −31.6 ± 0.48 | 42 | −27.7 ± 1.5 | −36.4 ± 2.4 | 56 |
| Scots | −28.2 ± 0.47c | 57 | −29.4 ± 1.4 | −35.5 ± 2.4 | 34 | −29.6 ± 0.61 | 38 | −27.5 ± 1.8 | −39.6 ± 4.2 | 58 |

white pines because mortality commenced before freezing began (Fig. 3). Factors other than freezing contributed to mortality at low temperatures among these hosts. In 2015, freezing was only associated with mortality in ponderosa and red pines, while other factors appeared to be involved in mortality among larvae from lodgepole, jack, eastern white and Scots pines in which a high proportion of mortality occurred in the population before freezing (Fig. 3, Table A.1). For larvae from ponderosa, jack, red, and Scots pines (i.e., hosts from which the rates of change in the proportion of individuals that began to freeze and that began to die were the same), the mean supercooling points and LT₅₀s were not different except in red pine ($t = 2.88$, $df = 39$, $P = 0.0064$), which had a warmer mean supercooling point than LT₅₀ in 2014. Larvae from red pine demonstrate some degree of freeze-tolerance.

3.5. Survival of supercooling

In addition to observing mortality before and at the point of freezing, we also observed that 13.5% of fourth instars survived after an exotherm was detected. Natal host did not significantly affect the proportion of individuals that survived the onset of freezing ($\chi^2 = 8.79$, $df = 5$, $P = 0.12$), although four times as many larvae reared in red pine survived ice formation, compared with larvae from lodgepole or eastern white pine (Fig. 4).

3.6. Field validation of models

We used under-bark temperatures and the supercooling point and lower lethal temperature models from 2014 (Fig. 3) to determine expected mortality of late instar larvae in overwintering bolts given a certain exposure temperature. Cumulative supercooling point models consistently overestimated the proportion of live late instar larvae found in bolts (Table 3). However lower lethal temperature models provided generally more reliable estimates of mortality, and all observed values fell within 95% confidence intervals around the predicted value (Table 3).

4. Discussion

Our work empirically demonstrates that the ecophysiological effects of host species on mountain pine beetle can affect cold tolerance and pre-winter development rate, two life history traits that may mediate the population dynamics of this insect (Bentz and Powell, 2014; Régnière and Bentz, 2007). These findings have critical implications for the suitability of novel hosts for this insect, and thus the potential for management of outbreaks among novel hosts in eastern forests.

4.1. Effects of life stage and sex on cold tolerance

Previous work has suggested that different life stages of the mountain pine beetle may have different tolerances to cold temperatures (Cooke, 2009; Lester and Irwin, 2012; Safranyik and

Linton, 1998; Strongman, 1982; Wygant, 1940). This study shows, for the first time in a common garden environment, that fourth instars are significantly more cold tolerant than pupae or teneral adults (Fig. 2). Indeed, supercooling points of fourth instars were 12–18 °C lower than later life stages. Our results also suggest a sex-related difference in cold tolerance among overwintering adults (Fig. 2). This bimodality has previously received little attention (Renault et al., 2002; Salin et al., 2000), yet may have important implications for sex ratios and colonization dynamics of mountain pine beetle throughout its range as males may be less likely to survive winter, possibly contributing to previously observed female bias in this species (James et al., 2016). The mean supercooling point of teneral adults in our study (−16.6 °C) was substantially warmer than reported for diapausing adults (−28.7 °C) that were overwintering after depositing brood (Lester and Irwin, 2012). The reason for this difference is unclear but may be related to exposure to different temperature regimes (Bentz and Mullins, 1999) or to the significant shifts in adult physiology that occur during and post host colonization (Pitt et al., 2014).

We observed higher mortality in early than in late instars (Fig. 1), similar to others (Reid and Gates, 1970; Safranyik and Linton, 1998), despite reports of little difference in supercooling points among instars (Bentz and Mullins, 1999; Somme, 1964). Similar supercooling points, yet higher mortality in early instars (Fig. 1) may suggest potential chill intolerance in these early stages. While we investigated the relationship between cold and mortality (cold tolerance strategy) of fourth instars, further work is needed to determine the cold tolerance strategy of other life stages.

4.2. Host mediated trade-offs

The combination of rapid pre-winter development, and differences in cold tolerance among life stages, may constitute a trade-off in novel hosts. More rapid development is often positively correlated with other performance traits (Amman, 1982; Bentz, 1999; Cerezke, 1995; Langor, 1989) and is likely an indicator of greater nutrition (Goodsman et al., 2012) and a higher quality host (Amman, 1982; Safranyik and Linton, 1983). However, a trade-off may occur as the likelihood of cold induced mortality increases in advanced life stages. Overwintering in pupal and adult stages is uncommon in the beetle's historical range (Reid, 1963). This may be due to host specific selection pressure for appropriate development rates in lodgepole and ponderosa pines (Bentz et al., 2014). Indeed, we observed a low proportion of brood in these late stages in historical hosts the first year (Table 1). In novel hosts this interaction between development rates and host species may be disrupted. Brood in red pine in particular developed at faster rates both years (Table 1), resulting in brood in these hosts generally becoming less cold tolerant as a population once winter arrived (due to some insects reaching less cold tolerant stages). Faster development prior to winter and variable rates of develop-

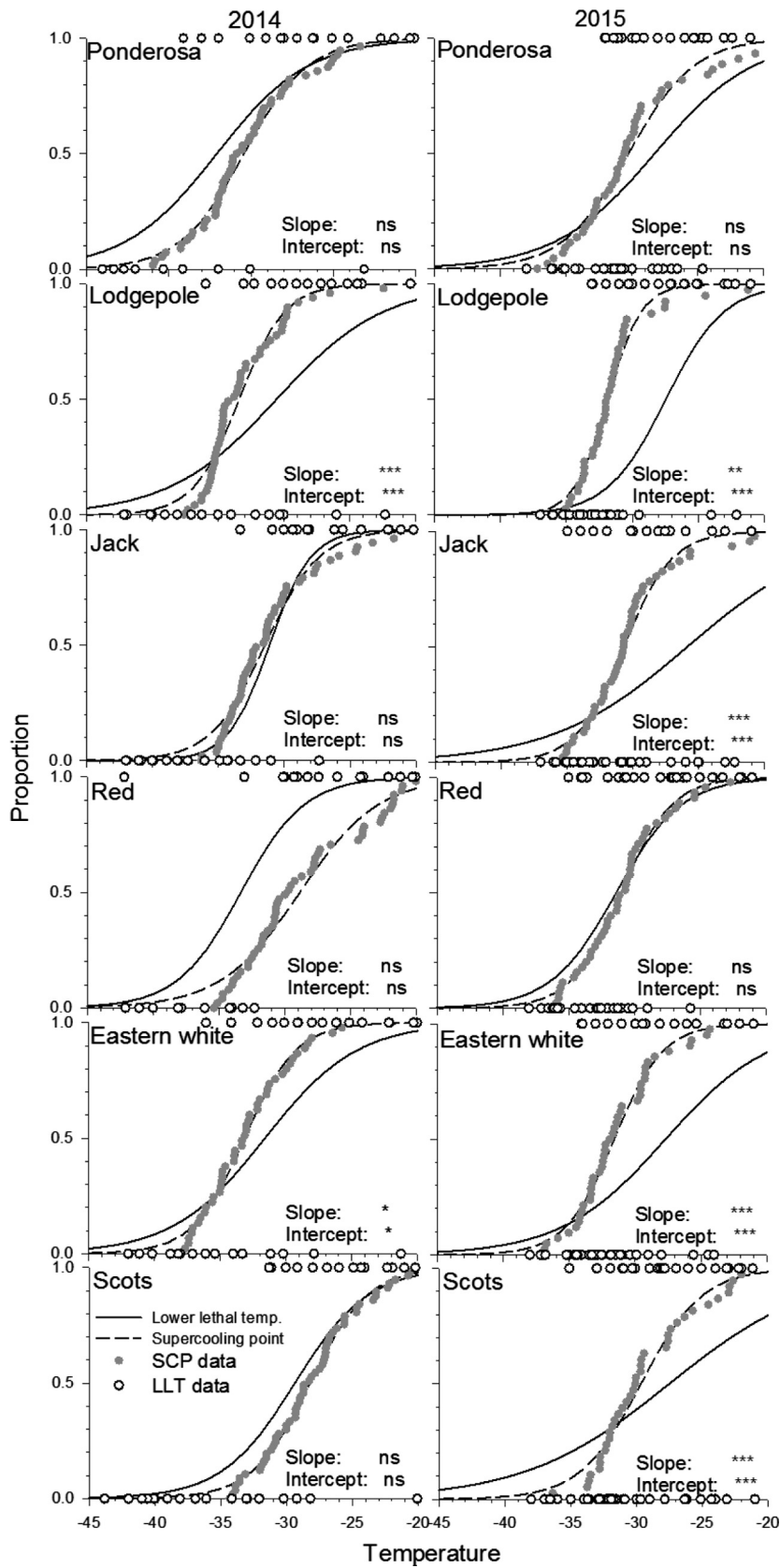


Fig. 3. Proportion of fourth instar mountain pine beetles from different hosts that had survived (i.e., lower lethal temperature) or had not yet started to freeze (i.e., reached the supercooling point) when exposed to various temperatures. Filled circles indicate individual supercooling points. Dashed lines describe the modelled proportions of individuals that had not given an exotherm when cooled to the specified temperature. Open circles and solid lines describe observed and modelled survival of individuals cooled to the specified temperature respectively. Models were based on logistic regressions. In each panel, tests compare slopes and intercepts of the two models: ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

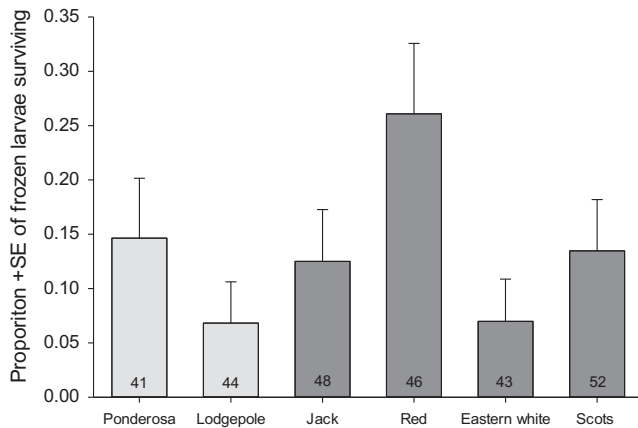


Fig. 4. Proportion of fourth instar mountain pine beetles within each pine species that survived freezing in lower lethal temperature experiments. Survival was determined by evidence of feeding or molting. Numbers on bars are the total number of larvae tested. Light bars are historical hosts and dark bars are novel, potential hosts. The proportion of larvae that survived freezing was not affected by host ($\chi^2 = 8.79$, $df = 5$, $P = 0.12$).

ment among pine species within a stand may result in earlier and desynchronized emergence the following year. Establishment of brood earlier in the summer could result in an even larger proportion of the population developing to pupal or adult stages prior to winter. Thus, differences in rates of development could have cascading impacts on the population dynamics of the mountain pine beetle among novel hosts and in eastern forests.

4.3. Host impact on cold tolerance

Contrary to our expectations, mortality was not strictly associated with freezing in all hosts, with many larvae in hosts such as lodgepole and eastern white pine, for example, dying before freezing (Fig. 3), thus demonstrating chill-intolerance. Mortality prior to freezing in an otherwise freeze-intolerant insect has been observed by others (Renault et al., 2002), but the possible role of host in mediating survival of chilling is intriguing.

Our results showing little fourth instar survival of the onset of ice formation in lodgepole pine are consistent with other studies (Bentz and Mullins, 1999; Somme, 1964). In contrast, survival of the onset of freezing was more prevalent in red pines, where over 25% of the larvae survived (Fig. 4). We did not quantify the extent of ice formation or assess survival after holding individuals in a semi-frozen state for an extended period (e.g., >24 h), two metrics often used to determine freeze-tolerance (Wygant, 1940). Thus, we are reluctant to suggest that mountain pine beetle larvae can be fully freeze-tolerant, but posit that these insects could be consid-

ered partially freeze-tolerant. This finding is ecologically significant because it indicates that the supercooling points may be a less reliable measure of overwintering capacity than lower lethal temperatures for overwintering fourth instar larvae of mountain pine beetle.

In addition to how larvae respond to freezing, we found that the effects of cold temperatures on larval mortality differed among pine species (Table 2). Previous studies of lower lethal temperatures conducted in common garden environments (Wygant, 1940; Yuill, 1941), as well as field observations of mountain pine beetle in mixed stands (Langor and Spence, 1991), have suggested that larvae experience different lower lethal temperatures among different western hosts. Host effects on cold tolerance of insects are becoming more widely documented in recent years (e.g., Liu et al., 2007; Trudeau et al., 2010; Feng et al., 2016; Morey et al., 2016). In fourth instars, we observed a spread in LT_{50} s of 4 °C the first year and 5.5 °C the second year (Table 2), similar to previously reported spreads of 3 °C (Yuill, 1941) and 5 °C among historical hosts (Wygant, 1940). Similarly, differences in supercooling points among hosts varied by as much as 5 °C (Table 2). Thus a greater proportion of larvae may be killed in Scots, jack and eastern white than in red pine in a mixed stand exposed to the same temperatures.

4.4. Parameters for climatic suitability models

By utilizing under-bark temperatures in the different pine hosts, we were able to compare observed and predicted mortality (Table 3). In this field validation of cumulative supercooling point and lower lethal temperature models (Fig. 3), supercooling point models were consistently conservative in their estimate of mortality, while our lower lethal temperature models more accurately predicted observed mortality in most cases (Table 3). Host-mediated differences in cold tolerance may reduce the accuracy of models, parameterized on a single host, to accurately project winter mortality. Our results for supercooling points for lodgepole pine (-33.2 °C \pm 0.43SE in Year 1, and -31.6 °C \pm 0.48SE in Year 2) are consistent with the global value (-32.3 °C \pm 0.06SE) for full cold tolerance used in the model developed by Régnière and Bentz (2007), parameterized from a sample of larvae from primarily lodgepole pine (Bentz and Mullins, 1999). However, mean supercooling point values from larvae from other pines in our study deviated from that value by as much as 4 °C (Table 2). Deviation from the global value further increased by 6 °C when LT_{50} s were used. However while host may exact varying effects, we observed LT_{90} s among all hosts to be warmer than a threshold of -40 °C (Safranyik et al., 1975), an absolute lower limit for survival (Carroll et al., 2004; Safranyik et al., 2010, 1975).

Table 3

Predicted and observed survival of late instar mountain pine beetles overwintering in the Black Hills, SD in December 2013 in eight bolts of each of six pine species. Predicted survival is based on the minimum observed temperature (averaged for the north and south sides of each log) and January-February 2014 laboratory measures and subsequent models of the supercooling point (SCP) and lower lethal temperature (LLT). The supercooling model assumes that each individual dies as it starts to freeze.

| Species | n | Observed Temp °C | Observed Survival | 2014 SCP model | | | 2014 LLT model | | |
|---------------|------|------------------|-------------------|----------------|--------------------|--------------|----------------|--------------------|--------------|
| | | | | Lower 95% CI | Predicted Survival | Upper 95% CI | Lower 95% CI | Predicted Survival | Upper 95% CI |
| Ponderosa | 489 | -20.3 | 91.2% | 99.2% | 99.5% | 99.7% | 77.9% | 98.6% | 99.9% |
| Lodgepole | 356 | -21.7 | 92.7% | 99.7% | 99.8% | 99.9% | 54.0% | 88.4% | 98.0% |
| Jack | 316 | -21.1 | 97.2% | 98.7% | 99.2% | 99.5% | 83.8% | 99.8% | 100.0% |
| Red | 213 | -21.2 | 80.8% | 91.1% | 92.8% | 94.2% | 80.8% | 99.4% | 100.0% |
| Eastern white | 569 | -21.8 | 95.3% | 99.5% | 99.7% | 99.8% | 68.5% | 94.3% | 99.2% |
| Scots | 194 | -21.4 | 90.2% | 94.8% | 95.9% | 96.7% | 65.4% | 94.7% | 99.4% |
| All samples | 2137 | -21.4 | 92.3% | 98.1% | 98.2% | 98.3% | 91.7% | 96.8% | 98.8% |

4.5. Significance to eastern forests

Our results have implications for mountain pine beetle winter survival should it reach forests of eastern North America. In the beetle's native range, natural selection acts to match development rate and cold tolerance to the climate of the region (Bentz et al., 2014), facilitating outbreak potential (Sambaraju et al., 2012; Stahl et al., 2006; Weed et al., 2015). In areas of eastern North America with winter temperatures similar to western forests (e.g., central Great Lakes region and New England), more rapid development in certain novel hosts may result in high mortality among pupae and adults. In areas with winters typically colder than the native range of the insect (e.g., Northwestern Great Lakes region and areas north of New England), brood developing in red pine may be particularly successful due to generally greater cold tolerance and reduced likelihood of reaching less cold tolerant life stages prior to winter (Table 1, Fig. 2). However, the risk of sudden drops in temperature could still limit success. Further research is needed to more fully integrate climate, development, and cold tolerance throughout the range most at risk for invasion.

4.6. Conclusions

This research was inspired by a simple, pragmatic question: could mountain pine beetle survive winters in northeastern North America? The question cannot be answered without considering the effects of novel hosts. Indeed, the effects of novel host plants on an insect herbivore's capacity to successfully overwinter are likely important to consider in assessments of climatic suitability for other insects that are experiencing range expansions, or introduced to new regions (Morey et al., 2016). Our study revealed host mediated trade-offs between development and cold tolerance. If future, extreme-low temperatures are similar to historical norms, winter mortality of mountain pine beetle may be an important driver of population dynamics among novel hosts due to rapid development to less cold tolerant stages, and serve to limit range expansion and outbreak potential (Bentz et al., 2010). But, if low-winter temperatures rise, that rate of range expansion may accelerate due to faster development on novel hosts than historical hosts.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foreco.2017.05.031>.

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have merit. However, an examination of the course topics, laboratory exercises, and assessment tools reveals a much different story (Table 1). A forensic entomology course can serve as an excellent vehicle for exploring key concepts in biology, ecology, and entomology by engaging student interests. The course I have developed explores many of the same concepts that a traditional entomology course includes (e.g., succession, competition, parasitism/predation, growth and development, environmental influences, seasonal adaptations, morphology, and taxonomy), as well as a small insect collection with identifications that are much more specific to the topic. For example, all Dipteran and Coleopteran specimens are required to be identified to genus and species, while any other insects should be identified to family. Identification specificity is due to the fact that collections are used in the context of crime scene processing and expert witness reports, and thus the insects represent physical evidence obtained from mock crime scenes set up around campus. The latter is the key to motivating students in tedious taxonomic work; they enjoy the application to mock crime scenes, a feature that seems to be universal to high school students and undergraduates (Schoenly et al. 2006). Involvement in real-world projects or authentic enterprise is also an important experience for students to develop their own identity as scientists and making informed decisions about career paths (Thiry et al. 2011).

Conclusions

The primary goal of using forensic entomology to teach undergraduates is not to convert all enrollees to entomology. No, the world can only stomach so many of us at a time! Rather, the discipline serves as a thematic framework for engaging otherwise disinterested students into topics and concepts essential for broadly trained biologists. Does the forensic entomology curriculum work? If the desired outcomes are increased student interest, motivation, and learning, the answer is unequivocally yes. Subsequent student enrollments in my general entomology course and involvement in research have also increased, an indication of sustained student interest in insect biology. Many other conceptual themes could also be well suited for this type of curricular design, and should be based on the interests and backgrounds of students and instructors. Hearing students comment to their peers that they cannot wait to take forensic entomology is not only gratifying, but also bodes well for the future of our discipline.

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INSTANT SYMPOSIUM 8

Stimulating Curiosity and Engagement with Insects Beyond the College Classroom Through Citizen Science

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Many professional entomologists were first introduced to the study of insects through a college biology course, yet little work has been published demonstrating effective means of stimulating curiosity and engagement with insects beyond such first exposures. At many small liberal arts colleges, a single introductory course in entomology or invertebrate biology may be all that is offered. This limitation increases a need to understand effective pedagogies that stimulate further engagement of students.

Undergraduate research experiences are particularly effective at stimulating the pursuit of graduate programs in science-related fields post-graduation (Lopato 2007). While we often perceive research experiences to be grounded behind a lab bench, course-based undergraduate research experiences can also promote continued engagement with science (reviewed in Corwin et al. 2015). However, instructors of introductory courses are often limited in time and resources,

Table 1. Example content from the lecture and laboratory components of an undergraduate forensic entomology course taught at Loyola University Maryland.

| Lecture | Laboratory |
|---|---|
| Insect use in legal investigations | Examination of general external and internal morphology |
| Short introduction to morphology, growth, and development | Morphological characters of forensically important flies and beetles |
| Necrophagous fly reproduction | Rearing forensically important species |
| Chemical communication and signaling in carrion communities | Collection and preservation techniques |
| Natural and artificial influences on succession | Group case analyses |
| Applied topics of insects and death, abuse/neglect, terrorism | Examination of physical decomposition under a range of conditions |
| Modeling insect growth, degree days, and estimating the postmortem interval | Field trip to morgue |
| Specialized topics on forensic archaeoentomology and deadly insects | Crime scene investigation: A multi-week capstone project using mock crime scenes for processing, analyses, and presentation |

making facilitation of such experiences difficult. Thus, engagement in authentic research—one of the more effective tools for developing future scientists—is often precluded from a venue where future entomologists are likely to be birthed: the introductory college classroom.

Integrating citizen science into introductory science courses may be one means of addressing this issue (Vitone et al. 2016), and can be a win-win for students, instructors, and researchers. Citizen science can be defined as “the active engagement of the public in scientific research projects to address real-world problems” (Wiggins and Crowston 2011). A slight modification of this definition is well-suited for student learning outcomes within a course syllabus: “Students in this course will actively engage in scientific research projects to address real-world problems.”

In practice, researchers have long collaborated with volunteers to collect data. In recent years, many researchers have turned to formalized citizen-science projects to recruit help. Some excellent examples of such projects, within the field of entomology, can be found on Scistarter.com and the Xerces Society. Projects cover a range of areas within entomology and are often focused on conservation and species monitoring (Johansen and Auger 2013). While such projects have great potential for supplying valuable data to researchers, recruitment of large numbers of participants can be difficult (Vitone et al. 2016).

Here, we share our success in integrating two citizen-science projects into three different courses at different institutions: Invertebrate Biology (BIO 308) at Bethel University (MN), Forest and Shade Tree Entomology (ENT 4251) at the University of Minnesota (MN), and Issues in Environmental Science (BIO 314) at Wheaton College (IL). Each investigation-type project (Wiggins and Crowston 2011) has taken one of two forms: a partnership with a state agency, brought into the classroom (Case Study 1), or the utilization of an established citizen-science program (Case Study 2). Partnerships with state agencies in non-formal citizen-science projects can be particularly rewarding for students, and provide networking and résumé-building opportunities. However, such projects are likely limited to the course, and require significant management and quality control on the part of

Table 1. Example project outlines of how the two projects were integrated into laboratory sessions. Other bycatch or monitoring projects could utilize similar frameworks.

| Case Study 1: Bycatch Assessment Project | |
|---|--|
| Laboratory session 1 | |
| An entomologist from the Minnesota Department of Agriculture introduces invasive insect monitoring in Minnesota. | |
| The instructor introduces the project, forms groups based on trap regions, and discusses project purposes and research questions. | |
| The instructor shows examples of target insects and provides data sheets and materials. | |
| Students sort each collection into target and non-target insects. Sorted bags are labeled and stored. | |
| The instructor works closely with the students to identify target insect groups. | |
| Laboratory session 2 | |
| Students finish sorting insects. | |
| Students submit results to the instructor. | |
| Groups begin working on the lab write-up (introduction, methods, results, implications). | |
| Laboratory session 3 | |
| Each group presents results from the region they assessed. | |
| The instructor presents consolidated results and implications are discussed by the class. | |
| Results are shared with the Minnesota Department of Agriculture. | |
| Case Study 2: Campus Bark Beetle Monitoring Project | |
| Laboratory session 1 | |
| The instructor introduces the project, forms groups, and discusses project research questions. | |
| Groups construct, distribute, and bait traps in transects around campus. | |
| Students check traps daily for one or two weeks and store samples in a freezer. | |
| Laboratory session 2 | |
| Students assess trap catch and determine presence and quantity of bark beetles. | |
| Students submit collection results to the instructor. | |
| Groups begin working on the lab write-up (introduction, methods, results, implications). | |
| All bark beetles are sent to Backyard Bark Beetles to be identified. | |
| Laboratory session 3 | |
| Each group presents results to the class. | |
| The instructor presents consolidated results. Implications of findings are discussed by the class. | |

the instructor. Conversely, student participation in formalized citizen-science programs, often with user-friendly websites and established protocols, can continue following completion of the course. We present two case studies as examples of ways citizen-science projects can be integrated into science courses.

Case Study 1

The Minnesota Department of Agriculture (MDA) is responsible for the monitoring of invasive species in Minnesota. One program deploys traps baited with aggregation pheromones throughout the pine regions of the state to monitor for potential introductions of mountain pine beetle (*Dendroctonus ponderosae*), a bark beetle native in western North America.

Resource managers and regulating officials are also interested in whether potential predators or competitors of existing native bark beetles would respond to aggregation pheromones produced by mountain pine beetle, if the insect were to arrive in the region. However, MDA time and resources were not allocated beyond monitoring for the mountain pine beetle. Thus, in BIO 308 and ENT 4251, we partnered with the MDA to process and analyze the bycatch from traps for potential predators and competitors of the mountain pine beetle (Table 1).

There were three principal learning outcomes of this project. First, the students experienced authentic scientific discovery as they collected and analyzed data to address a real-world question with

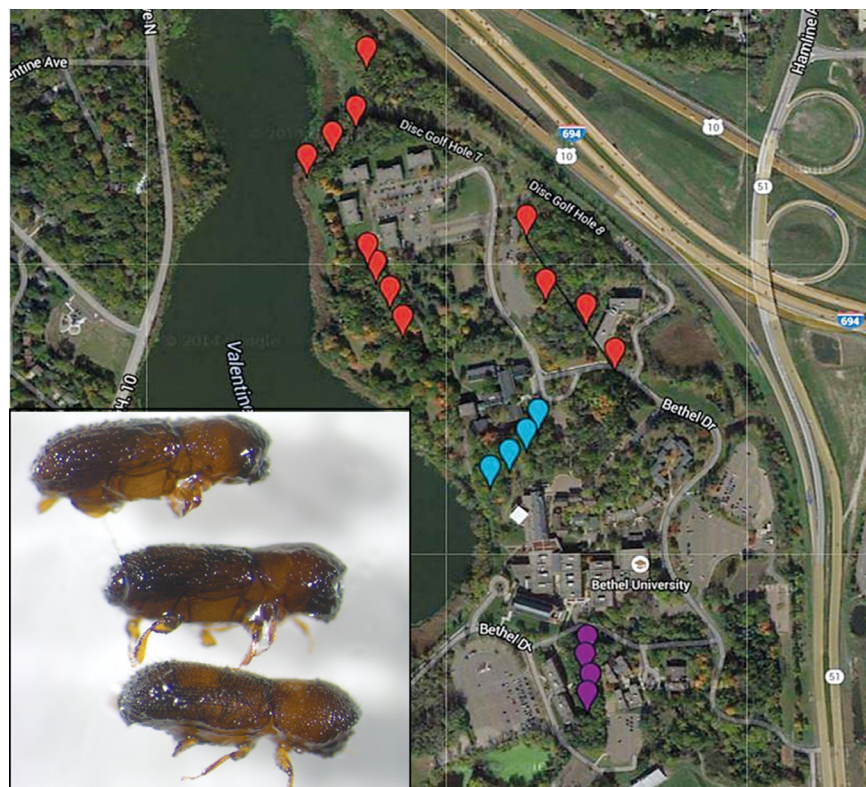


Fig. 1. Five transects of four traps (colored points) established by groups of students on the Bethel University (MN) campus to monitor for bark beetles during the bark beetle monitoring project (Case Study 2). Students used Google Maps to record trap locations. The inset image shows fruit tree pinhole borers (*Xyleborinus saxesenii*) caught in student traps. These captures represented the first record of this insect in the state of Minnesota.

an unknown answer. Second, students gained a familiarity with insect diversity by spending two lab periods sorting through the diversity of insects found in bycatch and identifying different groups. Students handled insects from many different orders and families, including many species that few people encounter. Third, students gained presentation and communication skills by writing laboratory reports and orally presenting their work to classmates. Students responded positively to this project and the chance to interact with a professional entomologist from the MDA. Several students inquired about further opportunities for involvement, including potential summer positions with the MDA.

Case Study 2

Backyard Bark Beetles (www.backyard-barkbeetles.org) is a formalized citizen-science project coordinated by the Hulcr lab at the University of Florida. This project is designed to monitor potentially invasive bark beetles and assess distributions. Citizen scientists engage in this project by monitoring a homemade window trap made from an upside-down two-liter

soft-drink bottle with one side removed. Hand sanitizer, which is typically 70% ethanol, is placed in the bottom of the trap, where it serves both as an attractant (emulating a stressed tree) and preservative. Traps are checked for bark beetles and refilled daily. Samples are sent to Backyard Bark Beetles to be identified.

This project was effectively integrated into all three courses during modules focused on insect diversity or invasive species (Table 1). Students were motivated by the recognition that the abundance and identity of the various species of bark beetles on campus was unknown to science. As such, if students did not monitor for the presence of invasive species, potential new introductions could go undetected. Indeed, the efforts of the students in BIO 308 resulted in the first detection of the fruit-tree pinhole borer, *Xyleborinus saxesenii* (Ratzeburg) (Coleoptera: Curculionidae), in Minnesota in April 2015 (Fig. 1). This ambrosia beetle was previously unknown to state agencies such as the MDA and the Department of Natural Resources, and we could not find previous records in the literature or the University of Minnesota Insect Museum.

Students completed evaluations that asked about interests prior to and following involvement in the campus bark beetle monitoring project. A majority of students expressed that they were interested in engaging in additional citizen-science projects in the future after having been involved in the project. In addition, a majority of the science majors responded that they would be interested in engaging in insect-related research in the future after having participated in this project (D. Rosenberger, unpublished data).

These case studies offer examples of how the use of citizen-science projects as authentic course-based undergraduate research experiences can be valuable for researchers, instructors, and students. Course-based citizen science offers researchers and instructors opportunities to engage students in authentic research experiences, achieving both scientific and educational goals. The accessibility of formalized projects provides students the opportunity to continue their engagement in entomological research beyond the classroom. Thus, this initial engagement could be the first step towards a life-long interest or career in entomology. Future work should seek to quantify such outcomes and investigate incorporation of course-based citizen science into disciplines beyond entomology.

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