“Live as if you were to die tomorrow. Learn as if you were to live forever.”
― Mahatma Gandhi

University of Alberta

Interactions Between Host Trees, Bacteria, and Fungi: Impacts on Mountain Pine Beetle (*Dendroctonus ponderosae*) Reproduction

by

Janet F.C. Therrien

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Abstract

Warming winter trends due to climate change have allowed for a range expansion of the mountain pine beetle, and the beetle now threatens Canada's economically and ecologically important jack pine forests. The beetle's success in jack pine trees will depend upon successful colonization of the host by the beetle and its bacterial and fungal associates. The objectives of this thesis were to determine how tree-bacteria-fungi interactions impact mountain pine beetle reproduction and whether these interactions hinder beetle invasion of jack pines. Results from monitoring beetle reproduction in the presence of various bacterium-fungus combinations in lodgepole, jack, and hybrid pines show that the roles of the bacteria and fungi are mediated by the host tree, and the importance of these microorganisms is dependent upon the biological activities of the beetles under the bark. Further, interactions between the beetle and these microbes are not limiting factors in the invasion of jack pine.

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Contents

List of Tables

List of Figures

Chapter 1

Introduction

Climate change has been a topic of study for over one hundred years (Parmesan 2006), and has the potential to impact species' distributions on a global scale (Parmesan and Yohe 2003). Since the beginning of the twentieth century, average global temperatures have increased by approximately 0.74°C (IPCC 2007), and as a result, several bird, vegetation, and insect species have expanded their ranges northward (Parmesan and Yohe 2003). Included among these species is the mountain pine beetle (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae, Scolytinae]), one of North America's most destructive forest insect species (Carroll et al. 2006, Safranyik and Wilson 2006, Safranyik et al. 2010).

The mountain pine beetle is native to western North American pine forests (Wood 1982), feeding primarily on lodgepole pine (*Pinus contortus* var. *latifolia*), but also on ponderosa pine (*P. ponderosa*), western white pine (*P. monticola*), whitebark pine (*P. albicaulis*), and limber pine (*P. flexilis*). Throughout most of its natural range, the beetle is univoltine, completing one generation per year (Safranyik and Wilson 2006). Dispersal and host selection occurs during the summer months (July-August), during which time beetles leave their overwintering (or parental) hosts in search of un-colonized hosts suitable for mating and reproduction. Beetles require mass attacks in order to overcome the resistance of healthy trees (Pitman et al. 1968, Raffa and Berryman 1983a, Raffa et al. 2005, 2008). Once a suitable host is selected, female beetles release *trans*-verbenol, an aggregation pheromone that attracts both sexes. Arriving female and male beetles release additional aggregation pheromones securing successful host colonization. Following successful colonization, beetles mate and females excavate maternal galleries, in which eggs are laid. Generally, larger female beetles and longer maternal galleries, both of which are positively

influenced by host phloem quality, allow for a greater number of eggs to be laid (McGehey 1971). Egg hatch is followed by larval feeding, which has been shown to be preferential toward phloem colonized by beetle symbiotic fungi (Bleiker and Six 2007). As larvae develop through four instars under the bark, they continue to feed on phloem and fungi (Adams and Six 2007), eventually overwintering as late instar larvae (Safranyik and Wilson 2006). Pupation occurs in the spring, followed by development into teneral adults (also called callow adults).

In general, bark beetles play a vital ecological role in the maintenance of forest health by attacking and killing weakened or stressed trees thus allowing for vegetative succession (Safranyik and Wilson 2006). Historically, the mountain pine beetle has persisted in western North American pine stands in an endemic state, experiencing only periodic outbreaks following unusually warm winters or in pine stands comprised of even-aged, susceptible trees (Safranyik and Wilson 2006, Bleiker and Six 2007). Since the 1970s, however, areas in which temperatures are suitable for beetle survival and reproduction have increased by 75% in western Canada (Carroll et al. 2006), creating the foundation for the current mountain pine beetle epidemic that started in the late 1990s. In western Canada, warming winter trends coupled with recent large beetle populations have enabled the mountain pine beetle to expand its range northward and eastward to include areas of British Columbia and Alberta that have not been part of its historic range (Carroll et al. 2006). One such area is in north central Alberta, where the range of lodgepole pine overlaps with that of jack pine (*P. banksiana*), creating an area of hybridization between the two species (Rice et al. 2007a; Fig. 1-1). In 2006, after significant in-flights of beetles from British Columbia to Alberta, the beetle was able to expand its host range to include these hybrid trees (Rice et al. 2007a, b).

The ability of the mountain pine beetle to thrive in lodgepole-jack pine hybrids, the likelihood that the beetle's range expansion will persist with continued

warming winter trends (Parmesan and Yohe 2003), and the fact that mountain pine beetle epidemics are usually widespread (Safranyik et al. 2010), raised concerns about its potential to expand its host inventory to include jack pine. The range of jack pine in Canada extends from Alberta to the east coast, eventually overlapping with that of eastern white pine, whose range extends south into the eastern United States (Fig. 1-1). Thus, a mountain pine beetle range expansion eastward into jack pine would lead to the destruction of millions of hectares of pine, causing major ecological and economic impacts (Cerezke 1995, Logan et al. 2003, Carroll et al. 2006, Colgan and Erbilgin 2010).

The mountain pine beetle is commonly associated with a number of microbial organisms, including fungi, bacteria, and yeasts. Among these, interactions between beetles and fungi have been studied more extensively than the other microbial associates. Symbiotic and opportunistic fungi carried on the beetle's mycangia and in their exoskeleton are introduced to the new host during initial host colonization, when beetles bore through the bark and into the phloem of the host (Whitney and Farris 1970, Six 2003, Klepzig and Six 2004, Bleiker and Six 2007, DiGuistini et al. 2007). The symbiotic fungi colonize tree phloem and xylem, cutting off water and nutrient flow within the tree, thus diminishing the tree's defensive ability and facilitating colonization of that tree by the beetle (Raffa and Berryman 1983b, DiGuistini et al. 2007). The symbiotic fungi also play an important role in beetle fitness by concentrating nitrogen in the phloem on which beetles feed (Bleiker and Six 2007, Goodsman et al. In press). Beetle size is positively correlated with survival, fecundity, and dispersal ability (Atkins 1967, McGhehey 1971, Safranyik 1976), and since phloem is a relatively nutrient-poor diet (Bleiker and Six 2007), acquiring additional nitrogen from fungal associates within the phloem can be important to improve beetle survival and reproductive success. In contrast, opportunistic fungi may act as antagonists to the beetle, competing with beetle symbiotic fungi for un-colonized phloem, thus limiting the growth of symbiotic fungi, which in turn reduces the positive

effects of those fungi on beetle fitness (Klepzig and Wilkens 1997, Cardoza et al. 2006, Bleiker and Six 2007).

Various species of bacterium are commonly associated with the mountain pine beetle (Adams et al. 2008, 2009, Cardoza et al. 2009), either in direct association with the beetle, by their presence within beetles (Cardoza et al. 2009) or beetle galleries (Adams et al. 2008), or in direct association with beetle hosts, by their presence within beetle host trees (Adams et al. 2008), but their roles in the mountain pine beetle biology and ecology are less understood. The bacteria associated directly with the beetles may increase beetle success by selectively encouraging or inhibiting growth of the beetle's fungal symbionts or antagonists, respectively (Adams et al. 2008, 2009; Cardoza et al. 2006), while bacteria associated with beetle hosts might reduce the overall colonization success of the beetle by inhibiting growth of the beetle's symbiotic fungi (Adams et al. 2008).

While various bacterium and fungus species may differentially affect the colonization and reproductive success of the mountain pine beetle, the beetle's hosts vary in their qualities, such as defensive chemicals, and therefore might differentially impact the composition of microbial communities. Coniferous trees utilize several strategies to defend themselves against attack by bark beetles and their associated microorganisms, including the excretion of resin; a sticky, toxic substance that can entrap and even kill the attacking bark beetles (Franchesi et al. 2005, Raffa et al. 2008). Monoterpenes are chemical compounds that are important constituents of resin (Franchesi et al. 2005), but whose composition can vary greatly between tree species (Adams et al. 2011, Lusebrink et al. 2011). The predominant monoterpene in lodgepole pine phloem is β-phellandrene, while the predominant monoterpenes present in jack pine phloem are α-pinene and β-pinene (Raffa and Berryman 1983a, Adams et al. 2011, Lusebrink et al. 2011, Erbilgin and Colgan In-press), although these monoterpenes can be present in smaller quantities in lodgepole pine as well. Since the mountain pine beetle has

co-evolved with lodgepole pine, it is not surprising that its bacterial associates are more tolerant of β-phellandrene than they are of α-pinene and β-pinene (Adams et al. 2011).

Considering the importance of microbial associates in mountain pine beetle biology and ecology (Bleiker and Six 2007, Rice et al. 2007a, Zilber-Rosenberg and Rosenberg 2008, Adams et al. 2011), understanding how jack pine will influence the symbiotic interactions between the mountain pine beetle and its microbial associates will be critical to assess whether beetle invasion of jack pine boreal forests will be successful. Our incomplete understanding of the invasion biology of the mountain pine beetle is an important knowledge gap hindering our ability to eventually develop appropriate management techniques, so evaluating the role of the mountain pine beetle's microbial associates, and the potential constraints present in the jack pine system, potentially due to host chemical composition, is a crucial step in improving our understanding of this complex system.

In an effort to better understand the complex interactions between the mountain pine beetle, its microbial associates, and its historic and potential host trees, a three-part research project was initiated with the following objectives:

(1) Identify the bacteria present in the mountain pine beetle and in colonized and un-colonized phloem of lodgepole and jack pines, and their hybrids;
(2) Determine how the bacteria identified in the first objective influence the mountain pine beetle's predominantly associated fungi, and how monoterpenes from three potential host trees may influence these interactions;
(3) Determine whether mountain pine beetle reproduction is affected by interactions between bacteria, fungi, and host tree species.

I hypothesized that mountain pine beetle reproduction would be affected by interactions between bacteria, fungi, and host tree species. Specifically, I suspected that beetles would be more successful in their historic host, lodgepole pine, than in jack pine or hybrids, but beetle-associated bacteria and fungi generally accepted to be symbiotic to the beetle would increase beetle reproductive success in jack pine, while tree-associated bacteria and fungi thought to be opportunistic would cause poor reproduction in all host trees. I also hypothesized that differences in reproductive success of the beetle would be explained mainly by the differences in the bacterial communities present in lodgepole pine, jack pine, and their hybrids.

The outcome of this experiment will improve our understanding of the complex interactions that occur between coniferous trees, bark beetles, and microorganisms, and provide evidence of how the small-scale interactions between insects and microbes can impact large-scale insect outbreaks capable of damaging millions of hectares of forests. By manipulating the microbial composition within the assays, we will obtain biological indicators of the roles of these microbes, and how these roles change in different host tree species. We will gain an understanding of the components that are necessary for the expansion of forest insects into a new host and range, and, more specifically, identify the factors that mediate mountain pine beetle adaptation to novel hosts, particularly jack pine.

This thesis is comprised of three chapters, including the introductory chapter, a data chapter summarizing beetle reproductive success in lodgepole pine and jack pine, a discussion chapter, and an appendix. The data chapter is a composition of all of the data collected for beetle reproduction in lodgepole pine and jack pine, and describes the results and conclusions that can be drawn from these data. Since hybrids fall on a continuum between lodgepole pine and jack pine and therefore were not considered a separate category, all of the data collected on

mountain pine beetle reproduction in lodgepole-jack pine hybrids is summarized in Table 4-1 in the appendix. The final chapter of discussion brings together the objectives and outcomes of this portion of the experiment, and discusses the implications of the results.

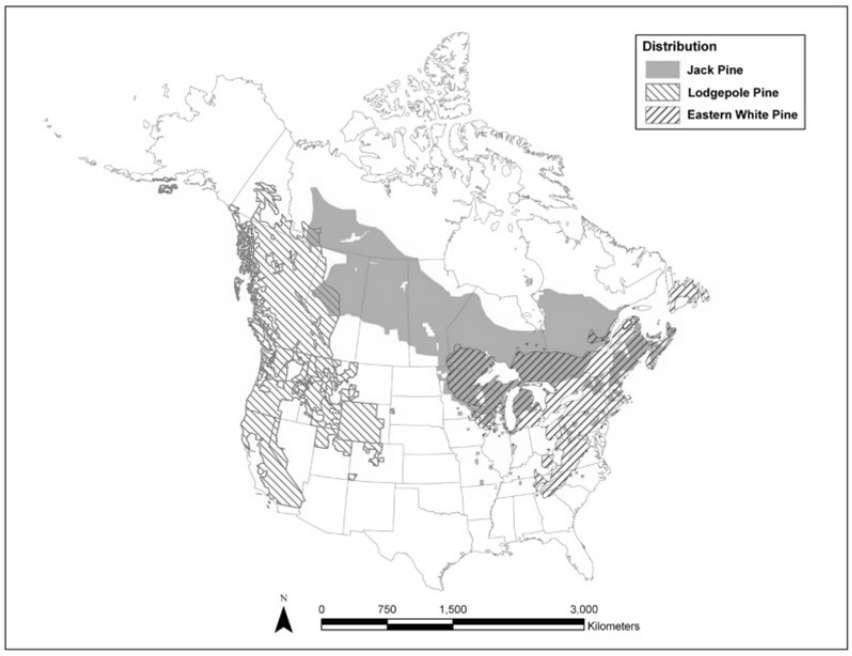

Figure 1-1. Distribution of lodgepole pine, jack pine, and eastern white pine in North America. The lodgepole-jack pine hybrid zone is illustrated by the overlap of lodgepole and jack pine ranges. The overlap of jack pine and eastern white pine ranges indicates an area where both species occur. Map created by Pei-yu Chen.

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Chapter 2: Bacteria-fungi interactions impact mountain pine beetle reproduction in lodgepole pine and jack pine

Introduction

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae, Scolytinae]) is one of the most destructive forest insects in western North America, and due to warming winter trends associated with climate change, has recently expanded its range northward and eastward in Canada (Carroll et al. 2006, Safranyik and Wilson 2006, Rice et al. 2007a). In its endemic state, the mountain pine beetle maintains forest health by attacking and killing stressed trees (Safranyik and Wilson 2006), thus allowing for natural vegetative succession. However, under certain conditions, including unnaturally warm winters and expansive stands of even-aged pine, populations of the mountain pine beetle may reach epidemic levels (Safranyik and Wilson 2006, Bleiker and Six 2007, Safranyik et al. 2010), in which beetles can overcome the resistance of healthy trees through mass attacks, coordinated by the release of aggregation pheromones (Pitman et al. 1968, Raffa and Berryman 1983a, Raffa et al. 2005, 2008).

Although the mountain pine beetle is native to western North American pine forests (Wood 1982), regions in western Canada in which temperatures are suitable for beetle survival and reproduction have increased by 75% since the 1970s, and the beetle is now found in areas of Alberta and British Columbia, Canada, that have not previously been part of its natural range (Carroll et al. 2006, Lusebrink et al. 2011). In 2006, after significant in-flights from British Columbia, the mountain pine beetle was found to be thriving in an area in north-central Alberta where the range of its historic host, lodgepole pine (*Pinus contortus var. latifolia*) overlaps with that of jack pine (*P. banksiana*), creating hybrids of the two species (Fig. 1-1). The presence of the beetle in these hybrid trees spurred studies to determine whether the beetle could thrive in jack pine. These studies

have suggested that the mountain pine beetle and its microbial symbionts are capable of colonizing jack pines (Cerezke 1995, Rice et al. 2007a, b), and that there is a possibility of a host expansion by the mountain pine beetle into jack pines, whose range extends from Alberta to the east coast of Canada (Fig. 1-1). A host and range expansion of this degree would lead to the destruction of millions of hectares of pine, causing major ecological and economic impacts (Cerezke 1995, Logan et al. 2003, Carroll et al. 2006, Colgan and Erbilgin 2010).

Throughout most of its natural range, the mountain pine beetle is univoltine, completing one generation per year (Safranyik and Wilson 2006). During initial tree colonization beetles inoculate the host tree with fungi carried on their exoskeleton and mycangia (Whitney and Farris 1970, Six 2003, Bleiker and Six 2007, DiGuistini et al. 2007). These fungi benefit from dissemination by the beetle (Bleiker and Six 2007), and may be antagonistic or symbiotic to the beetle (Klepzig and Six 2004). The antagonistic fungi are often opportunists (fungi that are transported on beetles and therefore benefit from dispersal, but that do not benefit the beetle) and may compete with symbiotic fungi for un-colonized phloem, reducing the positive effects of symbiotic fungi (Klepzig and Wilkens 1997, Bleiker and Six 2007) and hindering beetle growth. The fungal symbionts colonize tree phloem and xylem, cutting off the flow of water and nutrients within the tree, thus diminishing the tree's ability to defend itself against mountain pine beetle attack, and increasing the likelihood of successful colonization by the beetle (Raffa and Berryman 1983b, DiGuistini et al. 2007). Fungal symbionts also indirectly increase beetle size, which is positively correlated with survival (Safranyik 1976), by concentrating nitrogen in the relatively nutrient-poor phloem on which beetles feed (Bleiker and Six 2007, Goodsman et al. In-press).

Once successful colonization has been established, beetles mate, and female beetles lay eggs in the phloem (Safranyik and Wilson 2006). Generally, the number of eggs laid increases with the length of the maternal gallery and the size

of the female beetle, both of which are positively correlated with host phloem quality (McGehey 1971). When the eggs hatch, the larvae begin feeding on phloem, some of which is colonized by fungi introduced to the tree by parent beetles during initial attack (Adams and Six 2007). Larvae continue to feed throughout four instar periods, eventually overwintering as third or fourth instar larvae (Safranyik and Wilson 2006). Once spring arrives, larvae pupate, and then develop into young adult beetles, called callow or teneral adults (Safranyik and Wilson 2006). As the summer progresses, teneral adults sclerotize, and by the middle of the summer, are ready to bore through the bark and disperse to find new suitable host trees (Safranyik and Wilson 2006).

In addition to its fungal associates, the mountain pine beetle is also associated with various species of bacterium (Adams et al. 2008, 2009, Cardoza et al. 2009). These bacteria may be present in galleries within colonized trees (Adams et al. 2008) or within the beetles themselves (Cardoza et al. 2006, 2009), and thus be directly associated with the beetle. These bacteria may also be present within un-colonized host trees (Adams et al. 2008), and thus be associated with host trees rather than directly with the beetle. Although the roles of these bacteria are not yet well understood, a limited number of studies have suggested that beetle-associated bacteria may encourage growth of the beetle's fungal symbionts (Adams et al. 2008, 2009) and discourage growth of beetle antagonistic fungi (Cardoza et al. 2006). Conversely, tree-associated bacteria may have the ability to reduce colonization success of the mountain pine beetle by inhibiting the growth of symbiotic fungi (Adams et al. 2008).

The mountain pine beetle's historic host, lodgepole pine, is phytochemically different from the beetle's potential host, jack pine (Raffa and Berryman 1983a, Lusebrink et al. 2011, Colgan and Erbilgin 2010, Erbilgin and Colgan In-press). The predominant monoterpene of lodgepole pine phloem is β-phellandrene (Raffa and Berryman 1983a, Adams et al. 2011, Lusebrink et al. 2011), while jack pine

phloem is dominated by α-pinene and β-pinene (Adams et al. 2011, Lusebrink et al. 2011, Erbilgin and Colgan In-press). Mountain pine beetle-associated bacteria are more tolerant of β-phellandrene than they are of α-pinene and β-pinene (Adams et al. 2011), suggesting that microbial communities might differ with varying quantities and compositions of host monoterpenes. Although it is unknown how differences in tree chemistry and bacterial composition between lodgepole and jack pine will impact the colonization and range expansion of the mountain pine beetle into jack pine, knowledge of the role of the beetle's microbial associates and the potential constraints present in the jack pine system will provide a solid foundation for furthering our understanding of this system. This study was the first to examine the interactions between bacteria and fungi, and evaluate the extent of their impacts on mountain pine beetle reproduction. Thus, the objectives of this chapter were laid out as follows, to prove (or disprove) the concept that interactions between bacteria and fungi impact mountain pine beetle reproduction:

(1) To evaluate the role of various combinations of beetle- and tree-associated bacteria and symbiotic and opportunistic fungi in mountain pine beetle reproduction;
(2) To determine whether mountain pine beetle reproduction differs between lodgepole pine and jack pine;
(3) To determine how the roles of fungi and bacteria in beetle reproduction change in jack pine as compared to lodgepole pine.

I suspected that interactions between the bacteria and fungi predominantly associated with the mountain pine beetle and its host trees would impact mountain pine beetle reproduction. I hypothesized that the bacteria and fungi associated with the beetle would have positive impacts on beetle reproduction regardless of host tree species. Conversely, bacteria isolated from trees, and fungi thought to be antagonistic to the mountain pine beetle would likely reduce reproductive success of the beetle. I also hypothesized that differences in beetle reproduction

between host tree species (lodgepole pine and jack pine) could be attributed to varying effects of insect and host microbial associates.

Materials and Methods

Preceding Experiments

The project was in collaboration with colleagues (Drs. Aaron Adams, Cameron Curry, and Kenneth Raffa) at the University of Wisconsin (UW), Madison and Dr. Brian Aukema at the University of Minnesota. Bacterial diversity associated with the mountain pine beetle and its hosts (lodgepole pine, jack pine, and their hybrids) was determined by DNA extractions of beetles and phloem from attacked and un-attacked lodgepole pine collected in British Columbia and Alberta, as well as phloem from attacked and un-attacked jack pine and lodgepole-jack pine hybrids collected in Alberta. Since there were no naturally attacked jack pine trees at the time of sampling, jack pine bolts were inoculated with the mountain pine beetle and emerging beetles were analyzed. The DNA extractions were excised and sequenced out of the gels in order to obtain phylogenetic information, and pure cultures of each bacterium were sequenced for identification using universal eubacterial primers. Detailed methods are described in Adams et al. (submitted).

Pure cultures of the known fungal symbionts of the mountain pine beetle, *Grosmannia clavigera* and *Ophiostoma montium* were obtained from adult beetles by extracting hyphal tips from growing cultures and identifying them as close to species as possible, using morphological characters and molecular methods (as described in Adams et al. submitted). Opportunistic fungi, including *Aspergillus* and *Trichoderma* (Yellow) species, were collected from phloem within larval galleries and from adult beetles, and identified as close to species as possible by sequencing the internally transcribed spacer (ITS) region of the ribosomal DNA (as described in Adams et al. submitted).

To evaluate the effect of host monoterpenes on bacteria and fungi, phloem monoterpene content of each tree species was determined and treatments of monoterpenes (β-phellandrene, α-pinene, and β-pinene) were then added to cultures of the bacteria that were consistently associated with the beetle (AbA1 and D4-22) and its hosts (Hy3TC5 and Hy4T4/1), and cultures of each fungus (*G. clavigera*, *O. montium*, *Aspergillus*, Yellow) identified during the first part of the experiment.

To determine the inhibitive activity of the bacteria towards symbiotic and opportunistic fungi, each bacterium was point-inoculated onto malt extract agar (MEA). Ten days later, fungal spore suspensions were point-inoculated on the same agar, and fungal performance was measured by quantifying linear growth, conidia production, and conidiophore production. Bacteria that exhibited antifungal activity were then introduced to assays containing bacteria, fungi, and monoterpenes representing tree species, to determine how the effects of bacteria on fungi may change under the influence of tree monoterpenes. Bacteria were ranked based on their overall frequency of association with the mountain pine beetle and its hosts, their variation among the beetle's colonized and un-colonized hosts, their ability to selectively inhibit fungal growth, and the significance of their impacts on tree terpenes that inhibit fungal growth. The results indicated that four species of bacterium (two associated directly with beetles, AbA1 and D4-22, and two associated directly with host trees, Hy3TC5 and Hy4T4/1) selectively inhibited fungal growth, showed high variation among tree species, and significantly affected tree monoterpenes that inhibit fungal growth. These four bacteria, as well as the four fungi (*G. clavigera* and *O. montium*, isolated from beetles and expected to be symbiotic to the beetle, and *Aspergillus* and Yellow, isolated from beetles and expected to be opportunistic or antagonistic to the beetle) identified during the first part of the experiment were used to complete the third objective of this project (Table 2-1).

Current Project

Collection

Ten asymptomatic trees each of lodgepole pine, jack pine, and hybrid pine were felled and bucked into 1 metre bolts. Lodgepole pines were collected near Nojack, Alberta (LSD 053-11 W5M). Jack pines were collected from an area north of Lac La Biche, Alberta (LSD 070-13 W4M). Hybrids were collected near Whitecourt, Alberta (LSD 059-09 W5M). Selected trees had a diameter at breast height of approximately 30-35 cm, and showed no signs or symptoms of disease or insect infestation at the time of selection. Three bolts from the basal 3 m of the bole of each tree were collected and stored in a refrigerator at 4 °C to maintain phloem moisture. Although the phloem was still fresh when beetles were introduced to the assays, as the trees were dead, the host resistance was minimal or non-existent (Raffa and Berryman 1983b). Phloem thickness was measured to ensure that differences observed in beetle performance were not due to a difference in phloem thickness between tree species. No significant differences in phloem thickness were detected between tree species (lodgepole pine mean phloem thickness was 1.7 mm, jack pine mean phloem thickness was 1.2 mm), and as a result, phloem thickness was not used as a random factor in subsequent statistical analyses.

Lindgren funnel traps baited with the mountain pine beetle aggregation pheromones, *trans*-verbenol and *exo*-brevicomin, and a pine monoterpene, myrcene, were used to collect live beetles from lodgepole pine stands in the Grande Prairie, Alberta area during peak flight season (July and August) of 2010 and 2011. Beetles were collected and stored on ice, in glass jars with moist paper towels, fresh phloem, and vented plastic lids for a maximum of 8 hours until being stored in a laboratory refrigerator at 5 °C.

Microbial Cultures

All of the microorganisms used in this experiment were isolated from beetles and trees collected within British Columbia and Alberta, Canada. Four bacteria (AbA1, D4-22, Hy3TC5, Hy4T4/1) and four fungi (*G. clavigera*, *O. montium*, *Aspergillus*, Yellow) that are commonly associated with the mountain pine beetle were used in this experiment, based on the findings of our collaborators at the UW, Madison. The fungi were isolated from beetles, and the bacteria were isolated from beetles and from phloem of attacked and un-attacked trees. Each isolate was then identified as close to species as possible. A list of the isolates used, their origins, the groupings into which they were separated for the first part of the data analysis, their closest matched species (to the best of our knowledge), and a brief description of each isolate is listed in Table 2-1. The groups into which bacteria and fungi were separated were based in part on the findings of our collaborators (Adams et al. submitted), and in part due to evidence in published papers as to the roles of various species of fungus that are associated with the mountain pine beetle, particularly *G. clavigera* and *O. montium* (Bleiker and Six 2007, DiGuistini et al. 2007, Goodsman et al. In-press).

Bacteria

Four bacterial suspensions were prepared from cultures obtained from our collaborators. Beetle bacterial inoculation suspensions were prepared by first growing bacteria on sterile tryptic soy agar (3 g of trypticase soy broth, 15 g of bacto agar, and 1 L of distilled water). One agar plate was left open in the lab to determine the presence of airborne spores within the lab. From the bacterial culture plates, the suspensions were made by removing a 1x1 mm square of culture and placing it in a 250 mL solution of sterile tryptic soy broth (3 g of trypticase soy broth and 1 L of distilled water). After being inoculated, the broth was shaken regularly and stored at 23 °C. In order to standardize the inoculum density between *Pseudomonas* bacteria and between *Actinomyces* bacteria for use in beetle inoculations, *Pseudomonas* bacteria (AbA1 and Hy3TC5) were shaken

regularly for 48 hours, and *Actinomyces* bacteria (D4-22 and Hy4T4/1) were shaken regularly for one week, until hyphal spheres were present throughout the suspensions.

Fungi

Four fungi obtained from our collaborators were grown on sterile malt extract agar (15 g of malt extract, 15 g of bacto agar, and 1 L of distilled water). Once fungal growth covered at least half of the surface of the agar, fungal spore suspensions were made by flooding the Petri dish with 100 mL of autoclaved distilled water, and scrubbing the surface of the agar with a sterilized inoculation loop to release fungal spores and aerial hyphae into the solution. The suspension was then collected using a sterilized eye dropper and transferred to a small flask. Fungal spore suspensions were used for up to seven days, at which time new spore suspensions were made.

Phloem Sandwich Assays

The goal of this experiment was to observe the life cycle of the mountain pine beetle from maternal gallery initiation to development of brood larvae, and since phloem sandwich assays accommodate this objective and allow for a large number of assays to be observed within the limited space available in the laboratory, phloem sandwich assays were deemed adequate to accomplish the objectives of this experiment.

Using sterile techniques, beetles were prepared for bacterial inoculation by being surface washed in a solution of 10% bleach, 2% ethanol, and 88% distilled water, then rinsed in autoclaved distilled water twice. Rinsed beetles were submerged in a bacterium suspension for 30 seconds, and then allowed to walk on filter paper saturated with the same bacterium for one hour.

Wild-type beetles treated as controls were also surface washed and rinsed, but autoclaved distilled water was used instead of a bacterium suspension. To determine surface washing success, surface washed and unwashed beetles were rubbed across two different agar plates. These plates were left for one week, then observed to see whether bacteria grew on the plate of the surface washed beetle as compared to that of the non-surface washed beetle. Both plates were colonized by microbes after one week, although we did not attempt to isolate the bacteria from either plate. The surface washing technique does not sterilize the beetles, as bacteria may still be present in beetle guts and fungi may be present on beetle mycangia, but removes particulate matter and some bacteria and fungi from beetle exoskeletons.

Phloem samples were inoculated with a fungal spore suspension by using a bacteria cell spreader to spread the suspension across the surface of the phloem. For wild-type phloem samples a bacteria cell spreader was used to coat the surface of the phloem with autoclaved distilled water.

All instruments and materials were sterilized in a 10% bleach solution. To remove particulate matter and bacteria from the surface of the bark, each bolt was immersed in a 10% bleach solution for 15 minutes prior to use, then allowed to drip dry on the lab bench for two hours. Phloem pieces measuring 15 cm wide and 30 cm long were cut and peeled off of the bolts using a hammer and chisel. Each phloem piece was placed bark-side down onto a 15x30 cm clear Plexiglas plate. A niche measuring approximately 1x1 cm and as deep as the phloem was cut using a chisel and forceps, approximately 3 cm from the bottom edge of the phloem. The phloem was inoculated with the fungal spore suspension, and one male-female bacterium-inoculated beetle pair (to avoid potential contamination caused by reopening the sandwich after 24 hours to introduce the male) was placed in the niche. A second Plexiglas plate was placed over top of the phloem and the phloem sandwich was sealed shut using masking tape and 5/8” binder

clips around the edges. Phloem sandwich assays were stored at 23-25 °C, standing upright, in a dark cupboard.

There were 16 possible bacterium-fungus combinations, and each of these treatment combinations was replicated ten times per tree species, so that a total of 320 treated phloem sandwich assays were completed. In addition, ten replicates were completed for each of the wild-type treatments, so that ten wild-type assays were completed in lodgepole pine phloem, ten wild-type assays were completed in jack pine phloem, and ten wild-type assays were completed in hybrid phloem. Treatments of bacterium-fungus combinations as well as the wild-type "controls" were randomly assigned to phloem samples from randomly selected bolts of each tree species.

Each phloem sandwich assay was observed until teneral adults developed, or until adult beetles were dead and no evidence of offspring was observed. The lengths of the maternal galleries were recorded every week for 10 weeks, using a digital calliper, to monitor the rate of gallery construction and final gallery length. The number of eggs present in each maternal gallery could not be reliably counted due to the large number of samples and extensive fungal staining, so the number of larval galleries originating from the maternal galleries was recorded. Final larval gallery lengths were measured by scanning each assay and then using the measurement function of ARC Map (ESRI 2010). Teneral adult pronotum widths were measured as an indicator of beetle size. Teneral adults were then dried in an oven at 60 °C for 24 hours, removed, and weighed. These data are presented in the appendix in Table 4-2, since a small number of replicates were available for teneral adult dry weights.

Reisolation of Inoculum

Once all observations were made, sandwiches were cut open and samples of phloem and the original adult beetles were tested to ensure that the targeted

bacterium-fungus combinations were still present in the assays. For *G. clavigera* and *O. montium*, strands of phloem adjacent to the maternal gallery were removed using sterilized forceps and placed on malt extract agar amended with 0.05 g of cycloheximide, which is selective media for these genera (Harrington 1981). The locations from which these phloem samples were taken were standardized for all samples. The Petri dish was sealed with Parafilm and inverted the following day. When fungal growth covered at least half of the media, a 1x1 mm square of the fungus believed to be the target fungus was removed and plated on fresh media. The targeted antagonistic/opportunistic fungi (*Aspergillus* and Yellow) were handled the same way as were the symbionts, but the media used was malt extract agar without cycloheximide.

In order to reisolate D4-22 and Hy4T4/1 from the parent beetles, beetles were removed from the phloem sandwich and shaken vigorously in 1 mL of salt buffer (9 g of NaCl in 1 L of autoclaved distilled water). The solution (100 µL) was then spread over chitin agar (750 mL of distilled water, 15 g of bacto agar, 3 g of chitin) amended with 0.0375 g of cycloheximide and 0.0375 g of nystatin. The Petri dish was closed but not sealed until the liquid was dry, at which point the plate was also inverted. Likewise, AbA1 and Hy3TC5 were reisolated from beetles. The parent beetles were removed from the phloem sandwich and shaken vigorously in 1 mL of salt buffer. The solution (50 µL) was plated on tryptic soy agar, then 10 µL were removed and added to 990 µL of distilled water. This process was repeated so that three dilutions were completed and plated. The liquid was always allowed to dry on the media before the plate was sealed and inverted. Reisolated microbes were sent on their respective media in sealed Petri dishes to our collaborators at the UW, Madison for confirmation.

Data Analysis

All data analyses and graphs were completed using the R program (R Development Core Team 2011). The general linear model function (glm) was

used for binary data and the linear model function (lm for models with fixed effects and lme for models with mixed effects) was used for continuous and count data. Data were analysed using "year" as a random effect to determine whether there were differences in the results between the assays completed in 2010 and those completed in 2011. Once it was determined that the results followed similar trends in 2010 and 2011, the random effect of "year" was removed from the models and data were analysed using only fixed effects, including "bacterium", "fungus", and "tree species". All data met the assumptions of normality and homogeneity of variance or were double square-root transformed to meet these assumptions. *Post-hoc* t-tests were performed on select comparisons of interest.

Lodgepole-jack pine hybrids were not used for analysis in this chapter because ANOVA assumes categorical variables, and hybrid trees fall on a continuum between lodgepole and jack pine. Data for hybrid trees is included in the appendix in Tables 4-1 and 4-2.

A number of possible errors could not be controlled during this experiment, and are likely contributing factors to experiment-wise error. These factors include our inability to completely sterilize the beetles, tree bark, and tree phloem; variation within and between tree species, including phloem moisture content and phytochemistry; and variation between beetles.

To reduce the total number of comparisons between treatments, initial data analyses were conducted using groupings of bacteria and fungi. Bacteria and fungi were each split into two groups based on their original supposed role in the mountain pine beetle biology (Table 2-1). The first bacterium group included AbA1 and D4-22 that were directly associated with the beetle whether they were present within beetles or within beetle galleries in host trees (beetle-associated bacteria), and the second group included Hy3TC5 and Hy4T4/1 that were directly associated with the host trees by being present in un-colonized hosts

(tree-associated bacteria). Fungi were split into two groups based on their supposed role as beetle symbionts (*G. clavigera* and *O. montium*) or potential opportunists or antagonists (*Aspergillus* and Yellow).

The success of the phloem sandwich assays was determined by recording whether maternal galleries were initiated. These data were binary, so differences in assay success rates between treatments and wild-types were modeled using the glm function in R and analysed using an ANOVA with a Chi-squared test. The success of the initiated galleries was determined by recording whether larvae were present or absent in those samples. These data were also binary and were analyzed using an ANOVA with a Chi-squared test.

Differences between bacterial and fungal groups in terms of maternal gallery length, time taken to reach maximum maternal gallery length, rate at which maternal beetles constructed galleries, number of larvae, larval density, larval gallery length, and teneral adult pronotum widths were determined by modeling data as linear models, and then analyzing them using an ANOVA to determine statistically significant differences between treatments.

An ANOVA was run on the raw data for lodgepole pine and for jack pine to determine whether significant differences exist between each bacterium-fungus combination for maternal gallery length, time taken to reach maximum maternal gallery length, rate of maternal gallery construction, number of larvae, larval gallery length, and teneral adult pronotum widths within each tree species. The raw data containing means and standard errors for these variables under each bacterium-fungus combination were also ranked based on maternal gallery length, since we were most confident in this variable because the phloem sandwich assays were freshest when we measured this, and began to dry out as the study proceeded. T-tests were then performed on selected tree-bacterium-fungus combinations to determine whether significant differences exist between

treatments within each tree species, and to evaluate differences in treatments between lodgepole pine and jack pine.

Results

Maternal Gallery Initiation

Beetles in the wild-type groups were equally likely to initiate a maternal gallery in lodgepole (100% success) and jack pine (90% success; Table 2-2, Figure 2-1). Beetles in the treatment groups were more likely to initiate a maternal gallery in jack pine (91.3% success) than in lodgepole pine (79.4% success). There was no difference in maternal gallery initiation among the remaining bacterial or fungal groups or their various combinations with different tree species.

Larval Presence/Absence

Wild-type beetles were equally likely to have larvae in both lodgepole pine (90% larval presence) and jack pine (88.9% larval presence; Table 2-3). Treated beetles were also equally likely to have larvae in both lodgepole pine (28.4% larval presence) and jack pine (26.9% larval presence). Larvae were more likely present in the wild-type group (89.5% larval presence) than they were in the assays containing beetle- (29.5% larval presence) or tree-associated (25.8% larval presence) bacteria.

Maternal Gallery Length

When bacterium groups were compared to the wild-types, regardless of tree species, galleries were longer in assays that contained beetle- or tree-associated bacteria (Table 2-4). There were no differences between tree species in the wild-type groups, nor were there differences between groups of bacteria or fungi.

Number of Days Taken to Reach Maximum Maternal Gallery Length

There were no significant differences in the time required to reach maximum maternal gallery length between tree species or between any of the treatments or treatment combinations (Table 2-5).

Rate of Maternal Gallery Construction

Beetles created maternal galleries at a faster rate in assays amended with bacteria and fungi than in wild-type assays (Table 2-6). There were no significant differences in maternal gallery construction rate between tree species or between bacterial or fungal groups.

Number of Larvae Present

Across the treatment and wild-type groups, the number of larvae that were present in the assays ranged from 1 to 24. There were no significant differences in the number of larvae present between any of the treatment groups or the wild-types (Table 2-7).

Larval Density

Larval density was calculated by dividing the number of larvae by the final maternal gallery length (Table 2-8). The density of larvae within the wild-type assays was greater than in those assays containing beetle- or tree-associated bacteria, but the density was not different between assays containing beetle-associated bacteria and tree-associated bacteria. Larval density was also greater in the wild-type groups when compared to those amended with symbiotic or opportunistic fungi. Further, larval density was greater in assays containing opportunistic fungi than in those containing symbiotic fungi.

Larval Gallery Length

Larval galleries among the wild-type groups were longer in lodgepole pine than in jack pine (Table 2-9). Larval galleries in the treatment groups were also longer in

lodgepole pine than in jack pine. Various combinations of tree species and fungus group also differed, with galleries in lodgepole pine being consistently longer than in jack pine, regardless of which fungus group was present. There were no other differences between treatment combinations.

Teneral Adult Pronotum Widths

Pronotum widths of teneral adults were greater in beetles that emerged from assays containing opportunistic fungi than in beetles that emerged from assays amended with symbiotic fungi, but this difference was only moderately significant in the *post-hoc* testing (Table 2-10). No differences in pronotum widths were observed between beetles that emerged from lodgepole pine versus jack pine, or between bacterium groups or any other combinations of bacteria and fungi.

Lodgepole Pine

Comparisons between specific combinations of bacterium and fungus were based upon the rankings of the raw data for lodgepole pine (Table 2-11) and jack pine (Table 2-12). An ANOVA was also performed on each of the ranked tables to determine differences between treatments within each tree species. In lodgepole pine, beetles in assays amended with AbA1 paired with Yellow and Hy4T4/1 paired with *O. montium* had longer maternal galleries than beetles in assays amended with Hy4T4/1 and *Aspergillus* (Table 2-11). Maternal galleries were also longer when beetles were subjected to *O. montium* or Yellow than *Aspergillus* (Table 2-13). Larvae in assays amended with D4-22 constructed the longest galleries, while larvae in assays amended with AbA1 constructed the shortest galleries (Table 2-11). Larval galleries were significantly longer in the wild-type assays, as well as in assays amended with D4-22 or Hy4T4/1 than in assays containing AbA1, and larval galleries were longer in assays containing D4-22 than in assays amended with Hy4T4/1 (Table 2-13). No significant differences were detected in the time taken for beetles to reach their maximum maternal gallery length or in the number of larvae present.

Jack Pine

An ANOVA performed on the ranked raw data for jack pine (Table 2-12) showed that larval galleries were shorter in assays amended with D4-22 and *G. clavigera* or Hy3TC5 and Yellow than assays amended with Hy3TC5 and *Aspergillus*. Specific comparisons within jack pine revealed that beetles in jack pine assays that were amended with either of the tree-associated bacteria (Hy3TC5 or Hy4T4/1) constructed longer maternal galleries than did beetles in the wild-type group (Table 2-14). Larval galleries in assays amended with D4-22 were significantly shorter than galleries in assays amended with AbA1, and larval galleries were also shorter in assays amended with Hy3TC5 and Yellow than Hy3TC5 and *Aspergillus*, and AbA1 and *O. montium* or Hy3TC5 and Yellow than AbA1 and Yellow (Table 2-14). There were no significant differences in the time it took beetles to reach their maximum maternal gallery length or in the number of larvae present in each treatment.

Lodgepole Pine versus Jack Pine

Based on the above results and on previous knowledge of mountain pine beetle fungal symbionts, specific combinations of bacterium and fungus were compared between lodgepole pine and jack pine to determine which combinations were most conducive or disruptive to beetle reproduction (Table 2-15). In the presence of the beetle-associated bacterium, D4-22, paired with the symbiotic fungus, *G. clavigera*, beetles constructed longer larval galleries in lodgepole pine than they did in jack pine. The same beetle-associated bacterium (D4-22) was also paired with a potentially opportunistic or antagonistic fungus, *Aspergillus*. Larval galleries were longer in lodgepole pine than they were in jack pine in assays amended with this treatment. Larval galleries were also longer in lodgepole pine than in jack pine when beetles in lodgepole pine were subjected to AbA1 and Yellow, and beetles in jack pine were subjected to Hy3TC5 and Yellow. The tree-associated bacterium, Hy4T4/1, when paired with either a known fungal symbiont, *O. montium*, or a potential fungal antagonist, *Aspergillus*, caused

beetles to construct longer maternal galleries and shorter larval galleries in jack pine than in lodgepole pine. Beetles took a longer time to reach their maximum maternal gallery length in lodgepole pine assays amended with Hy4T4/1 and *O. montium* than in jack pine assays amended with the same treatment, but took less time in lodgepole pine assays amended with Hy4T4/1 and *Aspergillus* than in jack pine assays amended with that treatment. No significant differences in maternal gallery length, time taken to reach maximum maternal gallery length, or number of larvae were detected between lodgepole pine and jack pine when beetles were subjected to treatments of D4-22 with *G. clavigera* or with *Aspergillus*. Likewise, no significant differences in the number of larvae between the two tree species were observed when assays were amended with combinations of Hy4T4/1 with *O. montium* or with *Aspergillus*, nor were there significant differences in the number of larvae in lodgepole pine assays amended with AbA1 and Yellow compared to jack pine assays amended with Hy3TC5 and Yellow.

Reisolation of Inoculum

Successful reisolations from phloem sandwich assays were completed for Hy3TC5 and Hy4T4/1 bacteria. Attempted reisolations of AbA1 and D4-22 bacteria, as well as the four fungal species, were overgrown with contaminants before successful identification of the species could be completed. Although reisolations were desired, we are confident that the treatment effects observed are due to the presence of the target isolates, since these microorganisms were given a competitive advantage by being introduced in higher concentrations in each assay.

Discussion

The current study was initiated to prove (or disprove) the concept that the mountain pine beetle is affected by its associated bacteria and fungi, and to evaluate the role of various combinations of these bacteria and fungi in mountain pine beetle reproduction. We have proven that mountain pine beetle reproduction

is affected by interactions between the beetle's associated bacteria and fungi and its host trees. More specifically, our results suggest that (1) the roles of bacteria (beetle- or tree-associated), fungi (symbiotic or opportunistic), and their interactions in mountain pine beetle reproduction were mediated by host tree species (lodgepole pine and jack pine). Although bacterial communities were similar in jack pine and lodgepole pine, differences in the roles of bacteria and fungi might be explained by differences in host chemistry. (2) The functions of bacteria, fungi, and their interactions varied with the colonization and life stages of the beetles under the bark, and some of these microorganisms alone or in combinations had a more prevalent role in different aspects of beetle biology and ecology. These results suggest that the bacteria associated with either the mountain pine beetle or its host trees can potentially affect symbiotic or opportunistic fungi associated with beetles. (3) Despite the impacts of host quality on the functions of bacteria and fungi, the interactions between the mountain pine beetle and its microbial associates do not seem to constrain the invasion of jack pine forests by the mountain pine beetle.

The fungi used in this study were isolated from beetles and beetle galleries by our collaborators at the UW, Madison, and were the four predominant fungi found in the samples. Other studies have included another fungus (*Leptographim longiclavatum*) that is often associated with the mountain pine beetle, particularly in the jack pine system. Although this fungus was not one of the predominant fungi noted in our samples, it may have important impacts in the mountain pine beetle system at other geographical locations. This fungus has been found to have similar invasion and colonization abilities as *G. clavigera* in lodgepole pine, jack pine, and their hybrids (Goodsman et al. In-press; Rice et al. 2007a), and to behave similarly to *G. clavigera* in its ability to concentrate nitrogen in tree phloem, thus ameliorating beetle diet (Goodsman et al. In-press).

The phloem sandwich assay unit is not designed to observe the complete mountain pine beetle life cycle, because the phloem tends to dry out and become contaminated by other bacteria and fungi as the experiment progresses, so variables measured after egg hatch and larval development should be interpreted with caution, while our confidence in variables measured during initiation and construction of maternal galleries is high.

In lodgepole pine, *O. montium* and Yellow fungi positively influenced the construction of maternal galleries, while *Aspergillus* seemed to have a negative effect (Table 2-13). In jack pine, Hy3TC5 and Hy4T4/1 bacteria had a positive influence on the construction of maternal galleries, while the lack of a bacterial amendment seemed to have a negative impact (Table 2-14). Maternal gallery length is positively correlated with the number of eggs that are laid (McGhehey 1971). Further, larval gallery construction was influenced by bacteria in lodgepole pine, with AbA1 being present in assays with the shortest larval galleries and D4-22 being present in assays with the longest galleries (Table 2-11 and Table 2-13). In jack pine, on the other hand, D4-22 and Hy3TC5 combined with Yellow promoted short larval galleries, while *Aspergillus* and Hy3TC5, and AbA1 alone or in combination with Yellow promoted long larval galleries (Table 2-12 and Table 2-14). Short larval galleries can indicate subcortical environments that are favourable for larval development, since short larval galleries often terminate in pupal chambers (Six and Paine 1998), and can lead to earlier emergence of mountain pine beetle offspring from parental trees (Smith et al. 2011). Our results suggest that the mountain pine beetle was generally negatively affected by *Aspergillus* in both tree species, but benefited from the presence of Yellow in lodgepole pine and in jack pine when it was paired with Hy3TC5. In contrast, the beetle-associated bacteria, AbA1 and D4-22, were mediated by tree species. In lodgepole pine, AbA1 had a strong positive influence on larvae, while D4-22 had a negative influence (Table 2-11 and Table 2-13), but in jack pine, these effects were reversed (Table 2-12 and Table 2-14). Although

the influence of tree terpenes on the mountain pine beetle and its microbial associates may be altered in phloem sandwich assays due to cutting, the differential effects of AbA1 and D4-22 in lodgepole and jack pines were evident, and could be due, in part, to residual terpenes in the host phloem (Erbilgin et al. 2006). Bacteria have been shown to be differentially tolerant of host monoterpenes (Adams et al. 2011), indicating that perhaps AbA1 is more tolerant of β-phellandrene, the predominant monoterpene present in lodgepole pine (Adams et al. 2011), while D4-22 is more tolerant of α- and β-pinene, the predominant monoterpenes present in jack pine (Adams et al. 2011). Regardless of the mechanisms that facilitate it, despite the negative impacts of *Aspergillus*, the generally positive role of Yellow and the host-mediated role of the bacteria allowed for reproductive success of the mountain pine beetle in both tree species.

The importance of bacteria, fungi, and their interactions in mountain pine beetle reproductive success is dependent upon the biological activities of the beetles under the bark. In particular, the presence of bacteria influenced the construction of long maternal galleries and the development and hatching of eggs in the galleries, while the presence of fungi affected egg development, but also the size of the emerging teneral adults. Further, the presence of both bacteria and fungi caused beetles to create maternal galleries at a faster rate than when the assays were not amended with bacteria or fungi. In this way, the interactions between bacteria and fungi can impact the density of the larvae and adult beetle emergence.

Maternal galleries were significantly longer and were constructed at a faster rate in the assays containing beetle- or tree-associated bacteria than in the wild-type assays (Table 2-4 and Table 2-6). Maternal galleries were also constructed at a faster rate in assays containing fungi than in the wild-type assays (Table 2-6). The presence of a bacterium, regardless of whether that bacterium is directly associated with the beetles or with the host tree species, improved beetle

reproduction by stimulating increased feeding by female beetles, resulting in the construction of longer maternal galleries at a faster rate, which generally results in an increase in the number of eggs that are deposited in the maternal gallery (McGhehey 1971), and likely increased survival since eggs then have a longer time to develop, hatch, and reach the required cold-hardiness for overwintering before the winter season begins. The presence of fungi had a similar effect in that it stimulated faster feeding by female beetles, which, as above, likely results in increased brood survival. Thus, bacteria and fungi might have a positive influence on adult female beetles during feeding, maternal gallery excavation, and oviposition, and therefore indirectly influence overall survival of brood, but this influence depends on beetle activity, and may not directly benefit the development and hatching of eggs.

Larvae were present more often in wild-type assays than in assays containing beetle- or tree-associated bacteria (Table 2-3). This was likely a result of competition between bacteria and fungi, and demonstrates the impact of bacteria and fungi on egg development. Although the number of eggs laid could not be counted directly in the current study, larval presence depends, at least in part, upon oviposition and egg hatching, so the presence of emerged larvae was used as an indicator of oviposition (Table 2-7). *O. montium* is often present in phloem adjacent to mountain pine beetle eggs and early instar larvae, and is therefore likely important for egg hatching and early larval development (Adams and Six 2007). Bacteria present in trees, however, have the ability to inhibit the growth of the beetle's symbiotic fungi, including *O. montium* (Adams et al. 2008), which would decrease the positive influence of these fungi on eggs and larvae. Since species of bacterium closely related to those used in this study were isolated from both tree species, it is likely that these bacteria competed with beetle symbiotic fungi during oviposition and egg development, and therefore decreased the number of eggs laid and the hatch rate of the eggs, subsequently decreasing the presence of larvae in assays containing bacteria.

The use of the nutritionally-limited phloem upon which larvae feed becomes less efficient with increased larval density, which results in increased intraspecific competition between larvae (Raffa and Berryman 1983b). Thus, lower larval density minimizes intraspecific competition and therefore increases larval survival (Berryman and Pienaar 1973). Perhaps as a result of increased feeding behaviour by female beetles in the presence of bacteria, which resulted in longer maternal galleries, in the current study, the density of larvae was less in assays containing beetle- or tree-associated bacteria than in assays not amended with bacteria or fungi (Table 2-8). Larval density was also lowest in the presence of symbiotic fungi, suggesting that symbiotic fungi can influence intraspecific competition and thus larval survival. Increased larval survival has important consequences for sustaining beetle populations, particularly at endemic levels, and perhaps for its epidemic behaviour.

Although the number of emerged teneral adults was low and the phloem sandwich assay is not designed to measure brood adults, we observed that those that emerged from assays amended with opportunistic fungi were significantly larger than those that emerged from assays amended with symbiotic fungi (Table 2-10). Similarly, Bleiker and Six (2007) showed that during development, teneral adults that fed on fungi grew larger than those that did not have any fungi present during development. Beetle size is important for survival, dispersal, fat content, and fecundity (Atkins 1967, McGhehey 1971, Safranyik 1976), so these results show that fungi are important during larval development, and that the opportunistic fungi used in this study had stronger positive impacts on beetle development than did the symbiotic fungi.

As a precursor for invasion success of the mountain pine beetle in jack pine, we investigated the difference in reproductive capacity of the beetle in the two tree species, and found evidence to suggest greater reproductive success of the beetle in jack pine as compared to lodgepole pine. Initiation of maternal galleries

occurred more often in jack pine than in lodgepole pine when beetles were subjected to treatments of bacteria and fungi (Table 2-2). Our results suggest that microoganisms associated with the mountain pine beetle or with tree species have a stronger influence on gallery initiation by beetles in jack pine than they do in lodgepole pine, favoring the new host over the historical host. Also, the relatively negative role of *Aspergillus* seemed to have a weaker impact in jack pine than in lodgepole pine, evidenced in the fact that, despite the *Aspergillus* amendment, maternal galleries were longer in jack pine than in lodgepole pine, and larval galleries were shorter in jack pine than in lodgepole pine. Further evidence that interactions between the mountain pine beetle and its associated microbes may increase, rather than hinder, beetle success in jack pine, lies in the fact that larval galleries were consistently shorter in jack pine than they were in lodgepole pine (Table 2-9, 2-15). This was true for assays containing various combinations of bacteria and fungi, and for assays in the wild-type group. This suggests that the subcortical environment in jack pine is more favourable to beetle larvae than is the subcortical environment in lodgepole pine, regardless of whether it has been amended with bacteria and fungi. Thus, interactions between the mountain pine beetle and its microbial associates do not appear to be a constraint for the beetle during the initiation of galleries in jack pine, and may in fact positively influence the ability of the beetle to establish itself in this host tree species.

Conclusions

These results suggest that the reproductive capacity of the mountain pine beetle in jack pine is comparable to or greater than that in its historic host, lodgepole pine. The ability of the beetle to succeed in this novel host depends on its microbial associates, but these microorganisms do not act as a constraint for beetle host and range expansion because their roles are mediated by host tree species. This study has proven that the mountain pine beetle is affected by interactions between its associated microorganisms. Further, it has improved our understanding of the complex invasion biology of the mountain pine beetle, and has filled an important

knowledge gap regarding how the interactions between bacteria and fungi affect mountain pine beetle reproduction in its historic host and in its potential new host.

Table 2-1. A summary of the bacteria and fungi used to study the impacts of these microorganisms on *Dendroctonus ponderosae* (mountain pine beetle) reproduction. The origin, or source, of each isolate is listed, as well as what groups they were separated into for the first part of the data analysis (Beetle-asssociated bacteria, Tree-associated bacteria, Symbiotic fungi, and Opportunistic fungi), their closest matched species, and a short description, to the best of our knowledge, of the isolate. The closest matched species are educated estimates, since bacteria and fungi could not all be identified to species with the methods used.

Isolate	Origin	Grouping	Closest Species	Description
Bacteria				
AbA1	Beetles, All tree species	Beetle-associated	*Pseudomonas migulae*	Originally presumed to be *Pseudomonas*
D4-22	Beetles, All tree species	Beetle-associated	*Pseudomonas breneri*	Originally presumed to be *Actinomyces*
Hy4T4/1	All tree species	Tree-associated	*Pantoea agglomerans*	Originally presumed to be *Actinomyces*
Hy3TC5AA	All tree species	Tree-associated	*Rahnella aquatilis*	Originally presumed to be *Pseudomonas*
Fungi				
Grosmannia clavigera	Beetles	Symbiotic	---	Blue-stain fungus, beetle symbiont
Ophiostoma montium	Beetles	Symbiotic	---	Blue-stain fungus, beetle symbiont
Aspergillus	Beetles	Opportunistic	Unknown	Possibly opportunistic
Yellow	Beetles	Opportunistic	Unknown but likely a *Trichoderma* sp.	Possibly opportunistic

Table 2-2. Comparison of initiation of maternal galleries by *Dendroctonus ponderosae* (mountain pine beetle) in *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that did not have bacteria or fungi added during the experiment. Beetle-associated bacteria include AbA1 and D4-22, and tree-associated bacteria include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle include *G. clavigera* and *O. montium*, and fungi presumed to be opportunistic or antagonistic to the beetle include *Aspergillus* and Yellow. These data were binary, and were therefore analyzed using a Chi-squared test.

Treatment	Maternal Gallery Initiation	Significance[a]
Wild-type (LP wild-type vs JP wild-type)	NS[b]	
Tree (LP vs JP)	LP < JP	*
Bacterium Group	NS	
Fungus Group	NS	
Tree*Bacterium Group	NS	
Tree*Fungus Group	NS	
Bacterium Group*Fungus Group	NS	
Tree*Bacterium Group*Fungus Group	NS	

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-3. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) larval presence in *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that did not have bacteria or fungi added during the experiment. Beetle-associated bacteria (Beetle) include AbA1 and D4-22, and tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*, and fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were binary and were therefore analyzed using a Chi-squared test.

Treatment	Outcome	Significance[a]
Wild-type (LP wild-type vs JP wild-type)	NS[b]	
Tree (LP vs JP)	NS	
Bacterium Group	Wild-type > Beetle	***
	Wild-type > Tree	***
	Beetle = Tree	
Fungus Group	NS	
Tree*Bacterium Group	NS	
Tree*Fungus Group	NS	
Bacterium Group*Fungus Group	NS	
Tree*Bacterium Group*Fungus Group	NS	

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-4. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery lengths, for assays with larvae, between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were continuous and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (mm) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type JP Wild-type	105.6±28.9 60.5±16.8	NS[b]	
Tree	LP JP	159.4±17.1 124.2±12.4	NS	
Bacterium Group	Wild-type Beetle Tree	84.3±17.6 158.9±16.5 157.1±18.3	Wild-type < Beetle Wild-type < Tree Beetle = Tree	** *
Fungus Group	Wild-type Symbiotic Opportunistic	84.3±17.6 168.5±16.5 150.3±17.7	NS	
Tree*Bacterium Group	LP-Beetle LP-Tree JP-Beetle JP-Tree	182.2±23.4 164.5±31.4 130.7±21.5 148.9±17.5	NS	
Tree*Fungus Group	LP-Symbiotic LP-Opportunistic JP-Symbiotic JP-Opportunistic	192.1±26.9 159.1±27.9 141.3±15.1 140.2±21.0	NS	
Bacterium Group *Fungus Group	Beetle-Symbiotic Beetle-Opportunistic Tree-Symbiotic Tree-Opportunistic	161.9±23.0 157.0±23.1 173.5±23.7 144.0±27.1	NS	
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic LP-Beetle-Opportunistic LP-Tree-Symbiotic LP-Tree-Opportunistic JP-Beetle-Symbiotic JP-Beetle-Opportunistic JP-Tree-Symbiotic JP-Tree-Opportunistic	170.0±38.3 188.8±30.6 206.7±37.9 126.4±47.5 153.7±28.8 113.4±30.9 130.7±14.5 161.5±28.0	NS	

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-5. Comparisons between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group in the number of days required for *Dendroctonus ponderosae* (mountain pine beetle) to excavate their maternal galleries, for assays with larvae. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These were count data and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (days) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type	38.9±6.9	NS[b]	
	JP Wild-type	30.6±4.0		
Tree	LP	31.3±2.4	NS	
	JP	28.2±1.9		
Bacterium Group	Wild-type	35.0±4.1	NS	
	Beetle	31.8±2.5		
	Tree	25.7±2.0		
Fungus Group	Wild-type	35.0±4.1	NS	
	Symbiotic	25.0±2.1		
	Opportunistic	31.1±2.3		
Tree*Bacterium Group	LP-Beetle	31.7±3.6	NS	
	LP-Tree	27.3±3.1		
	JP-Beetle	32.0±3.6		
	JP-Tree	23.9±2.5		
Tree*Fungus Group	LP-Symbiotic	26.6±3.3	NS	
	LP-Opportunistic	31.3±3.2		
	JP-Symbiotic	23.2±2.3		
	JP-Opportunistic	30.7±3.3		
Bacterium Group *Fungus Group	Beetle-Symbiotic	26.8±2.8	NS	
	Beetle-Opportunistic	35.0±3.6		
	Tree-Symbiotic	23.6±2.9		
	Tree-Opportunistic	27.3±2.7		
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic	25.7±4.7	NS	
	LP-Beetle-Opportunistic	35.0±4.7		
	LP-Tree-Symbiotic	27.2±4.7		
	LP-Tree-Opportunistic	27.3±4.2		
	JP-Beetle-Symbiotic	28.0±3.6		
	JP-Beetle-Opportunistic	35.0±5.8		
	JP-Tree-Symbiotic	19.0±2.0		
	JP-Tree-Opportunistic	27.3±3.7		

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-6. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery construction rate, for assays with larvae, between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were continuous and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (mm/day) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type	2.5±0.7	NS[b]	
	JP Wild-type	1.9±0.5		
Tree	LP	5.3±0.5	NS	
	JP	4.9±0.5		
Bacterium Group	Wild-type	2.2±0.4	Wild-type < Beetle	***
	Beetle	5.3±0.6	Wild-type < Tree	***
	Tree	6.3±0.6	Beetle = Tree	
Fungus Group	Wild-type	2.2±0.4	Wild-type < Symbiotic	***
	Symbiotic	6.8±0.4	Wild-type < Opportunistic	***
	Opportunistic	5.1±0.6	Symbiotic = Opportunstic	
Tree*Bacterium Group	LP-Beetle	6.4±0.9	JP-Wild-type < JP-Tree	***
	LP-Tree	5.6±0.6	LP-Beetle > JP-Wild-type	***
	JP-Beetle	4.0±0.5	LP-Wild-type < JP-Tree	***
	JP-Tree	7.0±0.8	LP-Wild-type < LP-Beetle	**
Tree*Fungus Group	LP-Symbiotic	7.2±0.6	NS	
	LP-Opportunistic	5.0±0.9		
	JP-Symbiotic	6.3±0.5		
	JP-Opportunistic	5.2±0.9		
Bacterium Group *Fungus Group	Beetle-Symbiotic	6.2±0.7	NS	
	Beetle-Opportunistic	4.7±0.8		
	Tree-Symbiotic	7.2±0.4		
	Tree-Opportunistic	5.5±1.0		
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic	7.0±1.2	NS	
	LP-Beetle-Opportunistic	6.0±1.2		
	LP-Tree-Symbiotic	7.4±0.6		
	LP-Tree-Opportunistic	4.0±1.2		
	JP-Beetle-Symbiotic	5.4±0.8		
	JP-Beetle-Opportunistic	2.9±0.3		
	JP-Tree-Symbiotic	7.0±0.5		
	JP-Tree-Opportunistic	7.1±1.4		

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-7. Ranges for mean number of *Dendroctonus ponderosae* (mountain pine beetle) larvae present in each phloem sandwich assay. The ranges of number of larvae in each group are shown, as well as the means ± standard errors (there were no significant differences between treatments or wild-types at an α-value of 0.05). Wild-types include all assays that did not have bacteria or fungi added during the experiment. Beetle-associated bacteria (Beetle) include AbA1 and D4-22, and tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*, and fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow.

Combination	**Range (# larvae)**	**Mean (larvae) ± SE**
LP Wild-type	6-10	7.9±0.6
LP-Beetle-Opportunistic	2-24	11.0±1.8
LP-Beetle-Symbiotic	2-10	6.7±1.2
LP-Tree-Opportunistic	1-22	9.3±2.0
LP-Tree-Symbiotic	1-23	8.0±2.7
JP Wild-type	6-10	8.1±0.6
JP-Beetle-Opportunistic	1-11	5.5±1.2
JP-Beetle-Symbiotic	2-19	9.2±3.0
JP-Tree-Opportunstic	1-16	7.4±1.5
JP-Tree-Symbiotic	1-20	4.9±2.6

Table 2-8. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) larval density between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were continuous and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (larvae/mm) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type	0.27±0.16	NS[b]	
	JP Wild-type	0.29±0.10		
Tree	LP	0.18±0.06	NS	
	JP	0.11±0.03		
Bacterium Group	Wild-type	0.28±0.09	Wild-type > Beetle	**
	Beetle	0.11±0.05	Wild-type > Tree	**
	Tree	0.12±0.05	Beetle = Tree	
Fungus Group	Wild-type	0.28±0.09	Wild-type > Symbiotic	***
	Symbiotic	0.05±0.01	Wild-type > Opportunistic	*
	Opportunistic	0.17±0.06	Opportunistic > Symbiotic	**
Tree*Bacterium Group	LP-Beetle	0.14±0.09	NS	
	LP-Tree	0.18±0.10		
	JP-Beetle	0.08±0.02		
	JP-Tree	0.05±0.01		
Tree*Fungus Group	LP-Symbiotic	0.04±0.01	NS	
	LP-Opportunistic	0.25±0.11		
	JP-Symbiotic	0.05±0.02		
	JP-Opportunistic	0.07±0.02		
Bacterium Group *Fungus Group	Beetle-Symbiotic	0.05±0.01	NS	
	Beetle-Opportunistic	0.15±0.01		
	Tree-Symbiotic	0.04±0.01		
	Tree-Opportunistic	0.18±0.01		
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic	0.05±0.02	NS	
	LP-Beetle-Opportunistic	0.19±0.13		
	LP-Tree-Symbiotic	0.04±0.01		
	LP-Tree-Opportunistic	0.31±0.18		
	JP-Beetle-Symbiotic	0.06±0.01		
	JP-Beetle-Opportunistic	0.09±0.03		
	JP-Tree-Symbiotic	0.05±0.03		
	JP-Tree-Opportunistic	0.06±0.01		

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-9. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) larval gallery length between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were continuous and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (mm) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type	39.9±2.7	LP > JP	*
	JP Wild-type	27.8±4.5		
Tree	LP	37.7±1.3	LP > JP	***
	JP	26.9±2.2		
Bacterium Group	Wild-type	37.6±2.4	NS[b]	
	Beetle	37.6±2.2		
	Tree	30.7±1.4		
Fungus Group	Wild-type	37.6±2.4	NS	
	Symbiotic	32.3±1.9		
	Opportunistic	34.8±1.7		
Tree*Bacterium Group	LP-Beetle	39.7±2.3	NS	
	LP-Tree	34.3±1.7		
	JP-Beetle	30.0±5.4		
	JP-Tree	25.3±2.4		
Tree*Fungus Group			LP-Symbiotic > JP-Opportunistic	**
	LP-Symbiotic	38.3±2.2	LP-Symbiotic > JP-Symbiotic	***
	LP-Opportunistic	36.8±1.9	LP-Opportunistic > JP-Opportunistic	**
	JP-Symbiotic	22.3±2.9	LP-Opportunistic > JP-Symbiotic	***
	JP-Opportunistic	29.9±3.6	LP-Wild-type > JP-Opportunistic	***
			LP-Wild-type > JP-Symbiotic	***
Bacterium Group *Fungus Group	Beetle-Symbiotic	34.0±3.2	NS	
	Beetle-Opportunistic	38.9±2.7		
	Tree-Symbiotic	31.2±2.3		
	Tree-Opportunistic	30.4±1.8		
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic	42.5±3.6	NS	
	LP-Beetle-Opportunistic	38.9±2.7		
	LP-Tree-Symbiotic	35.6±2.8		
	LP-Tree-Opportunistic	33.4±2.1		
	JP-Beetle-Symbiotic	19.6±4.5		
	JP-Beetle-Opportunistic	38.7±9.0		
	JP-Tree-Symbiotic	24.0±3.8		
	JP-Tree-Opportunistic	26.0±3.2		

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-10. Comparison of *Dendroctonus ponderosae* (mountain pine beetle) teneral adult pronotum width between *Pinus contorta* (lodgepole pine; LP) and *P. banksiana* (jack pine; JP) and between various combinations of tree, bacterium group, and fungus group. Wild-types include all assays that were not amended with bacteria or fungi. Beetle-associated bacteria (Beetle) include AbA1 and D4-22. Tree-associated bacteria (Tree) include Hy3TC5 and Hy4T4/1. Fungi presumed to be symbiotic to the beetle (Symbiotic) include *G. clavigera* and *O. montium*. Fungi presumed to be opportunistic or antagonistic to the beetle (Opportunistic) include *Aspergillus* and Yellow. These data were continuous and were analyzed using an analysis of variance. The means ± standard errors are presented.

Treatment Groups	Groups Compared	Mean (mm) ± SE	Outcome	Significance[a]
Wild-type	LP Wild-type	1.75±0.08	NS[b]	
	JP Wild-type	None emerged		
Tree	LP	1.72±0.05	NS	
	JP	1.81±0.04		
Bacterium Group	Wild-type	1.75±0.08	NS	
	Beetle	1.79±0.05		
	Tree	1.75±0.05		
Fungus Group	Wild-type	1.75±0.08	Symbiotic < Opportunistic	*
	Symbiotic	1.67±0.04	Wild-type = Symbiotic	
	Opportunistic	1.83±0.05	Wild-type = Opportunistic	
Tree*Bacterium Group	LP-Beetle	1.71±0.08	NS	
	LP-Tree	1.71±0.09		
	JP-Beetle	1.83±0.06		
	JP-Tree	1.79±0.06		
Tree*Fungus Group	LP-Symbiotic	1.67±0.05	NS	
	LP-Opportunistic	1.76±0.13		
	JP-Symbiotic	1.68±0.07		
	JP-Opportunistic	1.87±0.04		
Bacterium Group *Fungus Group	Beetle-Symbiotic	1.71±0.06	NS	
	Beetle-Opportunistic	1.89±0.05		
	Tree-Symbiotic	1.64±0.04		
	Tree-Opportunistic	1.81±0.07		
Tree *Bacterium Group *Fungus Group	LP-Beetle-Symbiotic	1.71±0.08	NS	
	LP-Beetle-Opportunistic	None emerged		
	LP-Tree-Symbiotic	1.62±0.05		
	LP-Tree-Opportunistic	1.76±0.13		
	JP-Beetle-Symbiotic	1.69±0.17		
	JP-Beetle-Opportunistic	1.89±0.05		
	JP-Tree-Symbiotic	1.67±0.07		
	JP-Tree-Opportunistic	1.85±0.06		

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-11. *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, time required to reach maximum maternal gallery length, construction rate of maternal gallery, number of larvae, and larval gallery length within *Pinus contorta* (lodgepole pine) under various bacterium-fungus combinations. Means (± standard errors) were ranked from lowest to highest for each treatment for maternal gallery length.

Bacteria	Fungus	Maternal Gallery Length (mm; mean ± se)	Time to Maximum Gallery Length (days; mean± se)	Maternal Gallery Length Per Day (mm/day; mean± se)	Number of Larvae (larvae; mean ± se)	Larval Gallery Length (mm; mean ± se)
AbA1	*G.clavigera*	No data[a]	No data	No data	No data	No data
AbA1	*O.montium*	No data	No data	No data	No data	No data
AbA1	*Aspergillus*	No data	No data	No data	No data	No data
Hy3TC5	*G.clavigera*	No data	No data	No data	No data	No data
Hy3TC5	Yellow	No data	No data	No data	No data	No data
Hy4T4/1	*G.clavigera*	No data	No data	No data	No data	No data
Hy4T4/1	*Aspergillus*	10.3±2.4	17.5±3.5	0.6±0.0	10.0±5.0	36.6±3.3
Hy3TC5	*Aspergillus*	31.7±14.2	21.0±0.0	1.5±0.7	3.0±2.0	26.7±11.9
Wild-type	**Wild-type**	**105.6±28.9**	**38.9±6.9**	**2.5±0.7**	**7.9±0.6**	**39.9±2.7**
D4-22	*O.montium*	139.4±32.2	24.5±4.5	6.2±1.7	6.3±1.8	34.0±3.5
D4-22	Yellow	171.4±35.4	38.5±7.6	5.0±1.0	11.2±3.3	47.3±5.3
Hy4T4/1	Yellow	202.6±79.8	35.0±7.0	5.5±1.8	12.2±3.0	32.7±2.9
Hy3TC5	*O.montium*	213.7±85.5	29.8±10.5	6.4±1.0	8.9±4.8	33.3±3.6
Hy4T4/1	*O.montium*	231.3±32.9	30.3±2.3	7.6±0.8	7.0±5.5	37.7±4.1
D4-22	*G.clavigera*	231.4±100.9	28.0±14.0	8.6±0.7	7.5±1.5	55.3±6.0
D4-22	*Aspergillus*	237.4±57.1	38.5±10.5	7.1±3.4	13.5±1.5	44.3±3.7
AbA1	Yellow	284.7±2.2	28.0±7.0	10.8±2.6	10.5±1.5	25.9±3.8
ANOVA Results[b]		Ab-Yellow > Hy4-*Asp* Hy4-*O.mon* > Hy4-*Asp*	NS	NS	NS	D4 > Ab

[a] No data indicates that there were no assays from the specified treatment group that were successful

[b] An ANOVA was used to analyse differences between specific treatments within lodgepole pine. NS indicates no significant differences at α=0.05

Table 2-12. *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, time required to reach maximum maternal gallery length, construction rate of maternal gallery, number of larvae, and larval gallery length within *Pinus banksiana* (jack pine) under various bacterium-fungus combinations. Means (± standard errors) were ranked from lowest to highest for each treatment for maternal gallery length.

Bacteria	Fungus	Maternal Gallery Length (mm; mean ± se)	Time to Maximum Gallery Length (days; mean± se)	Maternal Gallery Length Per Day (mm/day; mean± se)	Number of Larvae (larvae; mean ± se)	Larval Gallery Length (mm; mean ± se)
AbA1	*O.montium*	No data[a]	No data	No data	No data	No data
AbA1	*Aspergillus*	No data	No data	No data	No data	No data
D4-22	*O.montium*	No data	No data	No data	No data	No data
Wild-type	**Wild-type**	**60.5±16.8**	**30.6±4.0**	**1.9±0.5**	**8.1±0.5**	**27.8±4.5**
D4-22	Yellow	72.9±43.5	24.5±10.5	2.7±0.6	5.5±1.5	18.9±1.4
Hy4T4/1	*O.montium*	104.1±11.0	14.0±0.0	7.4±0.8	10.5±9.5	24.7±4.5
Hy4T4/1	Yellow	119.0±27.4	21.0±14.0	8.6±4.5	9.0±7.0	0
AbA1	*G.clavigera*	124.6±53.9	24.5±3.5	4.9±1.5	3.0±1.0	37.4±3.9
Hy3TC5	*O.montium*	127.2±34.9	21.0±7.0	6.2±0.4	3.5±2.5	20.9±3.0
D4-22	*Aspergillus*	131.9±18.9	42.0±4.0	3.1±0.2	5.7±2.9	14.8±4.3
Hy3TC5	*G.clavigera*	149.1±42.1	21.0±0.0	7.1±2.0	2.5±0.5	20.6±10.2
Hy4T4/1	*Aspergillus*	153.3±39.5	35.0±4.0	4.2±0.6	8.0±2.6	24.1±3.6
D4-22	*G.clavigera*	155.5±88.0	28.0±7.0	5.1±1.9	10.5±8.5	9.0±1.5
Hy3TC5	*Aspergillus*	162.6±24.5	17.5±3.5	10.0±3.4	4.5±3.5	46.4±7.7
AbA1	Yellow	167.8±123.5	42.0±21.0	3.4±1.3	3.5±1.5	88.7±14.4
AbA1	*O.montium*	181.0±15.4	31.5±10.5	6.3±1.6	14.0±1.0	26.2±10.2
Hy3TC5	Yellow	197.5±92.0	30.3±6.2	7.0±3.4	7.7±0.7	12.1±2.6
ANOVA Results[b]		NS	NS	NS	NS	D4-*G.clav* < Hy3-*Asp* Hy3-Yell < Hy3-*Asp*

[a] No data indicates that there were no assays from the specified treatment group that were successful
[b] An ANOVA was used to analyse differences between specific treatments within lodgepole pine. NS indicates no significant differences at α=0.05

Table 2-13. Differences in *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, days required to reach maximum maternal gallery length, number of emerged larvae, and larval gallery length between specific bacterium-fungus combinations in *Pinus contorta* (lodgepole pine). These data were anlyzed using an analysis of variance. Only those with significant differences are listed.

Maternal Gallery Length[a]	**Time to Maximum Gallery Length**	**Number of Larvae**	**Larval Gallery Length**[a]
O.montium (191.5±33.6) > *Aspergillus* (80.4±42.7)* Yellow (198.4±31.9) > *Aspergillus* (80.4±42.7)*	NS[b]	NS	Wild-type (39.9±2.7) > AbA1 (26.1±3.5) *** D4-22 (45.0±2.7) > AbA1 (26.1±3.5) *** Hy4T4/1 (34.9±2.0) > AbA1 (26.1±3.5) ** D4-22 (45.0±2.7) > Hy4T4/1 (34.9±2.0) **

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-14. Differences in *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, days required to reach maximum maternal gallery length, number of emerged larvae, and larval gallery length between specific bacterium-fungus combinations in *Pinus banksiana* (jack pine). These data were anlyzed using an analysis of variance. Only those with significant differences are listed.

Maternal Gallery Length[a]	**Time to Maximum Gallery Length**	**Number of Larvae**	**Larval Gallery Length**[a]
Hy3TC5 (163.3±29.9) > Wild-type (60.5±16.8) ** Hy4T4/1 (132.6±16.3) > Wild-type (60.5±16.8) *	NS[b]	NS	AbA1 (45.4±9.3) > D4-22 (13.1±1.7) ** Hy3TC5-*Aspergillus* (46.4±7.7) > Hy3TC5-Yellow (12.1±2.6) *** AbA1-Yellow (88.7±14.4) > AbA1-*O.montium* (26.2±10.2) ** AbA1-Yellow (88.7±14.4) > Hy3TC5-Yellow (12.1±2.6) ***

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

Table 2-15. Differences in *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, days required to reach maximum maternal gallery length, number of emerged larvae, and larval gallery length between specific bacterium-fungus combinations in *Pinus contorta* (lodgepole pine; LP) compared to *Pinus banksiana* (jack pine; JP). These data were anlyzed using an analysis of variance. Only those with significant differences are listed. Means and standard errors for all treatments are reported in tables 2-11 and 2-12.

Comparisons	**Maternal Gallery Length**[a]	**Time to Maximum Gallery Length**	**Number of Larvae**	**Larval Gallery Length**[a]
LP-D422-*G.clavigera* vs JP-D422-*G.clavigera*	NS[b]	NS	NS	LP-D422-*G.clavigera* > JP-D422-*G.clavigera* ***
LP-D422-*Aspergillus* vs JP-D422-*Aspergillus*	NS	NS	NS	LP-D422-*Aspergillus* > JP-D422-*Aspergillus* **
LP-Hy4T4/1-*O.montium* vs JP-Hy4T4/1-*O.montium*	LP-Hy4T4/1-*O.montium* < JP-Hy4T4/1-*O.montium* *	LP-Hy4T4/1-*O.montium* > JP-Hy4T4/1-*O.montium* *	NS	LP-Hy4T4/1-*O.montium* > JP-Hy4T4/1-*O.montium* *
LP-Hy4T4/1-*Aspergillus* vs JP-Hy4T4/1-*Aspergillus*	LP-Hy4T4/1-*Aspergillus* < JP-Hy4T4/1-*Aspergillus* **	LP-Hy4T4/1-*Aspergillus* < JP-Hy4T4/1-*Aspergillus* *	NS	LP-Hy4T4/1-*Aspergillus* > JP-Hy4T4/1-*Aspergillus* **
LP-AbA1-Yellow vs JP-Hy3TC5-Yellow	NS	NS	NS	LP-AbA1-Yellow > JP-Hy3TC5-Yellow *

[a] * indicates that the p-value is significant at α=0.05, ** at α=0.005, *** at α=0.0005

[b] No statistical difference at α=0.05

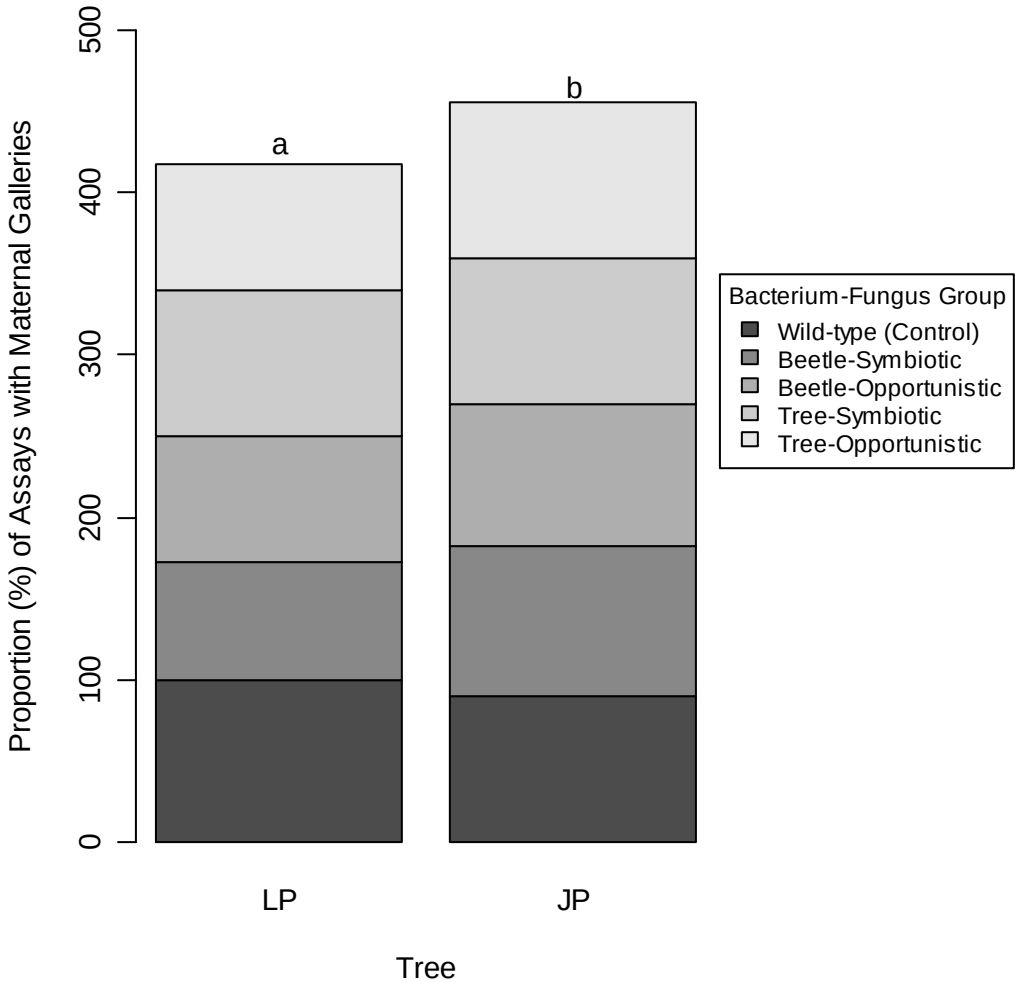

Figure 2-1. Success rate of phloem sandwich assays. This graph shows the proportion of phloem sandwich assays in each treatment group in each tree species that were successful (beetles initiated a maternal gallery). The treatment groups include the wild-type (control), beetle-associated bacteria with symbiotic fungi, beetle-associated bacteria with opportunistic fungi, tree-associated bacteria with symbiotic fungi, and tree-associated bacteria with opportunistic fungi.

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Chapter 3

Discussion

This study was the final part of a collaborative research project between the University of Alberta, the University of Wisconsin (UW), Madison, and the University of Minnesota. The overall objectives of this project were to:

(1) Identify the bacteria present in the mountain pine beetle and in lodgepole, jack, and hybrid pine phloem, both colonized and un-colonized by the beetle;

(2) Determine how the bacteria identified in the first objective influence the mountain pine beetle's predominantly associated fungi, and how monoterpenes from three potential host trees may influence these interactions;

(3) Determine how mountain pine beetle reproduction is affected by interactions between bacteria, fungi, and host tree species.

The first two objectives were accomplished by our collaborators at the UW, Madison, and based on their results, were used to accomplish the final portion of the project, which is summarized in this thesis. My project focused on whether mountain pine beetle reproduction is affected by interactions between bacteria (beetle- or tree-associated), fungi (symbiotic or opportunistic), and host tree species (lodgepole pine, jack pine, and their hybrids). I found that the beetle is affected by interactions between these microorganisms, and that the role of the bacteria and fungi (1) is mediated by host tree species and (2) is dependent upon the biological activities of the beetles under the bark. Based on these findings, I conclude that the interactions between the mountain pine beetle and its associated microbes do not limit the invasion of jack pine forests by the mountain pine beetle.

The role of bacteria and fungi in mountain pine beetle reproduction was mediated by host tree species. Comparisons of microbial roles between lodgepole and jack pine revealed that a symbiotic fungus and a beetle-associated bacterium in lodgepole pine were important for maternal and larval gallery constructions respectively, while a tree-associated bacterium and a tree-associated bacterium paired with an opportunistic fungus played an important role for maternal and larval gallery constructions in jack pine, respectively. Specifically, in lodgepole pine, amending with *Ophiostoma montium* promoted the longest maternal galleries, while AbA1 promoted the shortest larval galleries. In jack pine, Hy3TC5 promoted long maternal galleries, and paired with Yellow, promoted short larval galleries. These results indicate possible switching functions of these microorganisms depending on the tree species. I suspect that differences in host tree chemistry mediated the functions of these microorganisms and their interactions with beetles (Adams et al. 2011).

Further, the importance of bacteria, fungi, and their interactions depended on the subcortical activities of the mountain pine beetle. Assays amended with beetle- or tree-associated bacteria had larvae present less often than did assays in the wild-type group. Reduced larval presence in bacterium-amended assays was probably a result of competition between fungi and bacteria because tree-associated bacteria have been shown to be capable of selectively inhibiting the growth of certain fungi, such as *O. montium*, that are beneficial to mountain pine beetle eggs and larvae (Adams and Six 2007, Adams et al. 2008). Alternatively, the amendment of assays with bacteria led to the construction of longer maternal galleries, which can potentially allow for a greater number of eggs being laid by female beetles (McGhehey 1971), and the presence of bacteria and fungi led to a greater rate of construction of maternal galleries. I suspect that the presence of bacteria promoted host tree entering and the presence of bacteria and fungi promoted host tree establishment, but, during egg development, hindered beetle reproductive success.

Larval density was lower when bacteria or fungi were present, as compared to control assays, but density was also lower in assays containing symbiotic fungi than in assays containing opportunistic fungi, suggesting that both bacteria and fungi can potentially mediate larval density. I am currently unaware of any other studies demonstrating similar results, but low larval density allows for efficient use of phloem nutrients, decreases intraspecific competition (Raffa and Berryman 1983), and ultimately increases larval survival (Berryman and Pienaar 1973). Based on my results and the results of earlier studies cited above, I hypothesize that during maternal gallery construction, which ultimately impacts larval density, female beetles benefit from fungi, particularly symbiotic fungi, and bacteria.

Teneral adults that emerged from assays amended with opportunistic fungi (*Aspergillus* and Yellow) were larger (had wider pronotums) than teneral adults that emerged from assays amended with symbiotic fungi (Ophiostomatoid fungi). Although earlier studies, such as Bleiker and Six (2007), reported that teneral adults were larger when they developed in the presence of *Grosmannia clavigera*, *O. montium*, or both, compared to beetles that developed without these fungi, the positive role of opportunistic fungi in beetle size has not previously been reported. Due to small sample size, I caution about the interpretation of this result, however the consistent impact of opportunistic fungi on different life stages of the mountain pine beetle suggests that these effects, as well as the possibility that similar effects might occur in other tree-killing bark beetle species, deserve further investigation. Nevertheless, regardless of the mechanism, beetle size has been shown to be positively correlated with beetle survival, dispersal ability, fat content, and fecundity (Atkins 1967, McGhehey 1971, Safranyik 1976).

Interactions between the mountain pine beetle and its associated microbes assisted rather than hindered the beetle's ability to colonize jack pine. In fact, in jack pine, the mountain pine beetle was able to utilize microbes to achieve better, or comparable, reproduction to that in its historic host, lodgepole pine: Mountain

pine beetle had a greater probability of entering, established longer maternal galleries, which generally leads to a greater number of eggs being laid, and had shorter larval galleries in jack pine than in lodgepole pine, even in assays containing *Aspergillus*, which generally seemed to inhibit beetle success. Based on these results, I hypothesize that the microbes associated with the beetle and its hosts can potentially help create a favourable subcortical environment for beetles in jack pine.

Management Implications

For the past decade a number of studies have investigated the host and range expansion of the invasive mountain pine beetle in western Canada and the United States. These studies have focused on (1) how environmental and climatic factors in the jack pine boreal forest will impact the growth and survival of the mountain pine beetle and its fungal associates; (2) the impact of phenotypic and genotypic resistance of jack pine trees on reproduction, development, and survival of the mountain pine beetle and its associated pathogenic fungi; and (3) how interspecific interactions between the mountain pine beetle, tree diseases, and other insect herbivores will mediate the outcome of mountain pine beetle-jack pine interactions. Although these studies have provided useful predictions of the potential impacts of the mountain pine beetle in jack pine boreal forests, information about how the mountain pine beetle's microbial associates will perform in these emerging regions and hosts was lacking. In the current thesis, I focused on this component because (1) bacterium-fungus interactions can affect all aspects of mountain pine beetle ecology and therefore should be integrated into more robust models incorporating natural enemies, weather, species composition, and tree defenses; (2) this host expansion represents a particularly pertinent biological invasion, in that it comprises a bridge into a new biome, and signifies an unprecedented climate change-induced epidemic; and (3) regardless of the extent to which projected range expansions are realized, an understanding of bacterium-fungus interactions will improve our ability to predict and manage

populations of bark beetles, which are the most important insect group affecting North American coniferous forests.

Our results supported earlier studies that the bacteria and fungi associated with the mountain pine beetle and its host trees do not constrain the invasion of jack pine by the beetle. Further, we demonstrated a possible switch in the roles of some bacteria and fungi in jack pine as compared to lodgepole pine, so that beetles were able to obtain a reproductive capacity in jack pine equivalent to that in lodgepole pine. This result has important implications for managers, in that management strategies should incorporate means of dealing with potential mountain pine beetle invasion of jack pine boreal forests, regardless of whether the beetle has been present historically, since neither climate nor microbial associates have proven to be sufficient barriers for mountain pine beetle host and range expansion.

Although the current project focused on the mountain pine beetle system, considering the behavioural similarity of the mountain pine beetle to other tree-killing species, the results may apply to other conifer-bark beetle systems as well. The results of this project have improved our knowledge of the bacterial community associated with tree-killing bark beetles, provided a quantification of the relative sources of variation in bacteria at the beetle, tree, and stand scales, indicated how bacteria affect symbiotic and opportunistic fungi, established how the interactions between bacteria and fungi are impacted by host tree compounds, and demonstrated the relative importance of beetle-associated versus endophytic bacteria. In general, these results demonstrate that understanding micro-scale interactions, such as those that exist between bark beetles and their microbial associates or between the microbial associates of bark beetles and their host trees, can help us to understand beetle invasion dynamic processes at various spatial scales, ranging from small-scale processes, such as successful tree colonization, to large-scale processes, such as beetle outbreaks. For example, an ecological mismatch between plants and the microbial associates of bark beetles can reduce

beetle fitness under the bark. A reduction in beetle fitness diminishes successful host location and mate finding, and thus reduces host colonization success, which can eventually reduce the size of the local beetle population. This process is particularly important for beetles at the endemic level, when they are highly vulnerable to biological and abiological pressures, and therefore are also vulnerable to local extinction due to Allee effects. These effects stem initially from such a small-scale process as an upset of the community of microorganisms present in the system, and can negatively impact large-scale processes including beetle invasion dynamics and beetle establishment in a new range. Therefore, understanding key processes at the micro-scale will help to predict the invasion success of an organism in a new range.

Opportunities for Future Research

Although the results of the comparisons of teneral adult pronotum widths were statistically significant, the small number of emerged teneral adults makes it difficult to draw any definite conclusions. Low beetle emergence from assay units was mainly due to the experimental design. Although the phloem sandwich assay allow for a high number of replications per treatment and is suitable for observing development from egg to larva, it is not the most optimal host substrate for observing the complete beetle life cycle. Perhaps future studies should use cut bolts to allow beetles to complete their life cycles and to investigate the effects of bacteria and fungi on mountain pine beetle emergence, although this form of assay may not allow for a high number of replications per treatment.

The jack pine trees used in this study were collected from northeastern Alberta. Since tree genetics and phytochemistry vary within tree species, including in jack pine (Lusebrink et al. 2011), our results may not necessarily be indicative of jack pines in other geographic locations across Canada and the United States. It has been speculated that jack pines on the western edge of their Canadian range may contain genes that have been infiltrated from lodgepole pine (Lusebrink et al.

2011), and this may alter the results obtained in the current study. It is important, therefore, to conduct similar experiments using jack pines from across a geographic gradient.

This study was conducted in the laboratory under a constant temperature regime, but various species of fungus, and likely bacterium as well, reach optimum growth at different temperatures. For instance, *G. clavigera* was shown to grow better at lower temperatures than did *O. montium*, but at higher temperatures, *O. montium* was superior to *G. clavigera* (Six and Paine 1997). Under natural conditions, temperatures fluctuate, and these fluctuations may impact the growth of bacteria and fungi under the bark, therefore altering the relative impacts of these microorganisms on mountain pine beetle reproduction. To gain a better understanding of the effects of various combinations of bacteria and fungi in different host tree species on mountain pine beetle reproduction, it will be important to study this system under temperature fluctuations similar to those that occur throughout a beetle's life cycle.

Conclusions

Our study has filled an important knowledge gap in the invasion dynamics of the mountain pine beetle. We have shown that the bacteria and fungi interact to impact the reproductive potential of the beetle, and that the effect of these interactions changes depending on tree species, so that mountain pine beetle's invasion of jack pine forests will not be constrained by an inability of its associated microorganisms to assist the beetle within this novel host. On a broader scale, our results have increased our knowledge of the complex interactions between bark beetles, their hosts, and the associated bacteria and fungi, and have improved our understanding of the roles of these microorganisms in different hosts and during different beetle life stages.

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Chapter 4: Appendix

A table summarizing the means and standard errors for each variable and each bacterium-fungus combination in lodgepole-jack pine hybrids is shown. Bacterium-fungus combinations were ranked for each treatment combination based on the length of the maternal galleries constructed by beetles under each treatment, time required for beetles to construct maternal galleries, rate at which maternal beetles constructed galleries, number of larvae present in each assay, and length of larval galleries in each treatment.

A table showing the teneral adult dry weight data is also presented. Due to the small number of replicates available for teneral adult dry weight, these data were not used to address the objectives of this experiment.

Table 4-1. *Dendroctonus ponderosae* (mountain pine beetle) maternal gallery length, time required to reach maximum maternal gallery length, number of emerged larvae, and larval gallery length within *Pinus contorta* (lodgepole pine) x *P. banksiana* (jack pine) hybrids under various bacterium-fungus combinations. Means (± standard errors) were ranked from lowest to highest for maternal gallery length.

Bacteria	**Fungus**	**Maternal Gallery Length (mm; mean ± se)**	**Time to Maximum Gallery Length (days; mean± se)**	**Maternal Gallery Length Per Day (mm/day; mean± se)**	**Number of Larvae (larvae; mean ± se)**	**Larval Gallery Length (mm; mean ± se)**
AbA1	*G.clavigera*	No data[a]	No data	No data	No data	No data
D4-22	*O.montium*	No data	No data	No data	No data	No data
D4-22	*Aspergillus*	No data	No data	No data	No data	No data
D4-22	Yellow	No data	No data	No data	No data	No data
Hy4T4/1	Yellow	No data	No data	No data	No data	No data
AbA1	*O.montium*	70.4±65.4	42.0±14.0	1.3±1.1	1.0±0.0	0
AbA1	Yellow	86.0±12.5	28.0±7.0	3.6±1.1	6.0±1.5	45.9±8.7
Hy3TC5	*Aspergillus*	104.1±42.0	14.0±0.0	7.4±3.0	4.0±1.0	33.1±8.4
D4-22	*G.clavigera*	131.4±46.5	35.0±8.1	3.8±1.2	7.3±4.9	30.8
AbA1	*Aspergillus*	153.3±42.1	33.7±7.8	5.1±1.8	6.8±3.3	24.4±4.3
Hy4T4/1	*O.montium*	177.0±117.9	24.5±3.5	6.7±3.9	9.0±1.0	38.1±5.9
Hy3TC5	*O.montium*	183.9±19.0	38.5±17.5	5.7±2.1	15.5±9.5	31.3±8.2
Hy4T4/1	*G.clavigera*	183.9±45.0	35.0±10.7	7.0±3.0	6.3±3.5	36.0±6.1
Hy3TC5	*G.clavigera*	190.5±61.2	38.5±3.5	5.1±2.1	6.0±3.0	29.5±5.3
Wild-type	**Wild-type**	**194.0±40.2**	**40.6±4.9**	**4.3±0.8**	**7.9±0.6**	**36.5±3.1**
Hy4T4/1	*Aspergillus*	227.3±56.6	33.3±6.0	6.7±1.0	8.3±2.5	35.4±3.6
Hy3TC5	Yellow	334.5±92.7	43.4±8.1	7.7±1.5	10.4±3.8	41.1±3.6

[a] No data indicates that there were no assays from the specified treatment group that were successful

Table 4-2. Dry weights of *Dendroctonus ponderosae* (mountain pine beetle) teneral adults within *Pinus contorta* (lodgepole pine), *P. banksiana* (jack pine), and *P. contorta* (lodgepole pine) x *P. banksiana* (jack pine) hybrids under various bacterium-fungus combinations. Means are displayed.

Tree Species	Bacteria	Fungus	Dry Weight (mg; mean)
Lodgepole Pine	**Wild-type**	**Wild-type**	**0.80**
Lodgepole pine	Hy3TC5	*O.montium*	1.46
Lodgepole pine	Hy4T4/1	*G.clavigera*	2.82
Lodgepole pine	Hy4T4/1	*O.montium*	1.62
Lodgepole pine	Hy4T4/1	*Aspergillus*	1.67
Lodgepole pine	Hy4T4/1	Yellow	2.85
Lodgepole pine	D4-22	*G.clavigera*	2.72
Lodgepole pine	D4-22	*O.montium*	2.49
Lodgepole pine	Hy3TC5	Yellow	0.97
Jack pine	AbA1	*G.clavigera*	0.78
Jack pine	AbA1	*O.montium*	2.62
Jack pine	AbA1	Yellow	2.59
Jack pine	AbA1	*Aspergillus*	2.36
Jack pine	Hy3TC5	*O.montium*	1.69
Jack pine	Hy3TC5	Yellow	2.72
Jack pine	Hy4T4/1	Yellow	1.81
Jack pine	Hy4T4/1	*Aspergillus*	1.52
Jack pine	D4-22	*G.clavigera*	2.06
Jack Pine	D4-22	*Aspergillus*	0.67
Hybrid	**Wild-type**	**Wild-type**	**0.78**
Hybrid	AbA1	Yellow	1.11
Hybrid	Hy4T4/1	*Aspergillus*	2.40
Hybrid	Hy3TC5	Yellow	1.49

[a] No data indicates that there were no assays from the specified treatment group that were successful