2013

The effects of temperature on fungal symbionts in the mountain pine beetle-fungus multi-partite symbiosis

Melissa Lea Moore
The University of Montana

THE EFFECTS OF TEMPERATURE ON FUNGAL SYMBIONTS IN THE MOUNTAIN PINE BEETLE-FUNGUS MULTI-PARTITE SYMBIOSIS

By

Melissa Lea Moore

Bachelors of Science, University of California, Davis, CA, 2010

Thesis

presented in partial fulfillment of the requirements
for the degree of

Master of Science
in Forestry

The University of Montana
Missoula, MT

June 2013

Approved by:

Sandy Ross, Associate Dean of The Graduate School
Graduate School

Diana L. Six, Chair
Ecosystem and Conservation Sciences

Cory Cleveland, Co-Chair
Ecosystem and Conservation Sciences

John McCutcheon Co-Chair
Biological Sciences

Moore, Melissa, M.S., June 2013 Forestry

Chapter 1: Literature Review

Chairperson: Diana L. Six

The mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) (Coleoptera: Curculionidae, Scolytinae), is a tree killing bark beetle indigenous to North American conifer forests. This insect is capable of killing a wide range of hosts in *Pinus* (Paine et al. 1997), and has developed extensive outbreaks in recent years driven by a combination of climate change, fire suppression, and past logging practices (Safranyik and Carroll 2006, Raffa et al. 2008, Safranyik et al. 2010). MPB feed on tree phloem, a tissue which is relatively high in sugars yet poor in many other nutrients vital to the beetle's growth and reproduction (Six and Paine 1998, Ayers et al. 2000). To supplement what is provided by phloem tissue alone, MPB engage in symbiosis with two species of phloem- colonizing ophiostomoid fungi, *Grosmannia clavigera* and *Ophiostoma montium* (Whitney and Farris 1970, Lee et al. 2006, Six 2003). These fungi concentrate nitrogen from the sapwood and move it into the phloem where the beetle larvae feed (Bleiker and Six 2007, Cook et al. 2010, Goodsman et al. 2012). In addition, the fungi produce sterols that the beetles may use for hormone and egg production (Bentz and Six 2006).

To ensure the continuity of this relationship, newly eclosed MPB adults acquire fungal spores in specialized structures of the exoskeleton called mycangia prior to their dispersal to a new host tree (Whitney and Farris 1970, Paine et al. 1997, Six 2003, Bleiker et al. 2009). Once a host is located, MPB bore through the bark and into the phloem tissue, tunneling vertically upwards and introducing fungal spores onto the gallery walls. The fungi then grow longitudinally and horizontally through the phloem and transversely into the sapwood (Ballard et al. 1984, Solheim 1995, Paine et al. 1997). Beetle larvae tunnel horizontally through the phloem, perpendicular to parental galleries, feeding on fungi along with phloem throughout their development (Adams and Six 2007). After approximately one year of feeding, the larvae create chambers in the phloem where they pupate and eclose to teneral adults (Paine et al. 1997, Six and Paine 1998, Safranyik and Carroll 2006). In synchronicity with beetle pupation, the fungi

form a dense spore layer on the walls of the pupal chamber (Whitney 1971, Six and Paine 1996). When these spores are fed upon by teneral adults, their mycangia are charged with spores for transport to the next host tree (Whitney and Farris 1970, Whitney 1971, Six 2003, Bleiker et al. 2009). This spore feeding also appears to be critical for reproduction (Six and Paine 1998).

The population size of emerging adults each year is important to MPB population dynamics because larger beetle populations increase the potential for successful attacks on trees (Raffa and Berryman 1983). The amount of nitrogen provided by the fungi can affect beetle size (Six and Paine 1998, Bleiker and Six 2007) and therefore the number of eggs produced by a female (McGhehey 1971). While both fungi are capable of supporting MPB reproduction, they are not identical in their ability to concentrate nitrogen (Cook et al. 2010) and thus differ in their effects on beetle size and fecundity. In controlled experiments it has been shown that beetles reared on *G. clavigera* alone are larger than those reared on *O. montium* (Six and Paine 1998). MPB collected in the field carrying *G. clavigera* are also larger than those carrying *O. montium* (Bleiker and Six 2007). These differences could account for as much as a 38% difference in fecundity (Bleiker and Six 2007). Beetles do not appear to avoid or select phloem colonized by one or the other species of fungus (Bleiker and Six 2007), therefore, the potential for differential effects of the fungi on the MPB is more likely to be linked to conditions affecting the relative prevalence of the associates in a tree.

The factors affecting the growth of these fungi can be classified as biotic (e.g. tree chemistry and competition with other microbes) and abiotic (e.g. physical environment including weather). Tree chemistry is likely only important in the early stages of an MPB attack and for a few weeks afterwards (Solheim 1995). Competition with other microbes is likely important but poorly understood. One study found that yeasts and bacteria commonly found with MPB can either enhance or inhibit the growth of the two mutualistic fungi *in vitro* (Adams et al. 2008). Of the abiotic factors affecting the growth of the

two fungi, temperature appears to play a major role. For example, previous studies have shown that the optimal thermal range for growth of *G. clavigera* is approximately 5˚ C lower than that of *O. montium* (Six and Paine 1997, Solheim and Krokene 1998, Rice et al. 2008, Bleiker and Six 2009b). This indicates that as ambient temperatures fluctuate, the two species would be expected to grow at different rates at different times. This difference in growth rates under different conditions could account for the temporally variable pattern of growth of the two fungi observed in trees (Adams and Six 2007).

Temperature also likely influences sporulation. The fungi must sporulate in synchronicity with beetle eclosion to an adult stage in order to be acquired in mycangia and transported to the next tree (Whitney 1971, Six and Paine 1996). Sporulation of these fungi is likely temperature controlled as with other fungi (Carlile et al. 2001). However, there are currently no studies that have determined the effects of temperature on sporulation of these fungi. Interestingly, it has been observed that the relative prevalence of the two fungi carried by dispersing MPB is highly correlated with ambient temperature (Six and Bentz 2007). *G. clavigera* spores are more often carried in mycangia during relatively cool periods while *O. montium* is carried when it is relatively warm (Six and Bentz 2007). Therefore, temperature may influence not only which fungus is fed upon by larvae, but also which fungus is dispersed. This indicates that on occasion beetles will disperse a fungus different from that which it fed upon predominantly during development.

Interspecific competition between the two fungi could also have important impacts on fungal dynamics and thus on the host beetle. Fungi, in general, are known to be highly competitive with one another (Wicklow 1981, Rayner 1991, Shearer 1995, Carlile et al. 2001). However, the fungi associated with MPB appear to coexist without exhibiting strong direct competition (Solheim 1995, Bleiker and Six 2007, Bleiker and Six 2009a, Bleiker and Six 2009b). While the fungi do exhibit exploitation competition (scramble competition) where they capture space and then maintain it, they do not appear to

capture space from one another. Their hyphae often intermingle in tree phloem (Bleiker and Six 2007, Bleiker and Six 2009a) and on artificial media (Bleiker and Six 2009b), a phenomenon which is not expected among microbes subsisting on the same resource (Wicklow 1981, Rayner 1991, Shearer 1995, Carlile 2001).

Long-term coexistence of these two fungi with their host beetle indicates a mechanism that allows both species to remain in the system despite the presence of another symbiont. It has been suggested that differential temperature tolerances may allow them to capture substrate at different times (Six and Bentz 2007, Six 2012). In this case, as temperatures fluctuate over time, no one fungus can completely dominate and move to fixation with the host. Furthermore, by growing at different times they can avoid expending energy on direct competition (Six and Bentz 2007, Six 2012).

To test the hypothesis that different temperature tolerances allow coexistence of the two fungi by shifting dominance between them over time, I studied the effects of temperature on the ability of each species to grow and reproduce. In addition, I studied the effects of temperature on the ability of each species to capture resources when in the presence of the other species. To assess the effects of temperature on the growth of the fungi, I determined the growth rate of each species on artificial media over the range of temperatures at which they are able to grow (5, 10, 15, 21, 25, 30 and 35˚C), as well as the variability in growth rate responses to temperature within each species. To assess the effects of temperature on asexual reproduction of the fungi, I determined their ability to sporulate over these same temperatures. To assess the effects of temperature on resource capture, I determined the growth rate of and percent resource capture by each species when grown with the other fungus species compared to when grown with a subculture of itself at 10, 15, 21 and 25˚C. The results of my research allowed me to investigate whether the fungi associated with MPB exhibit differing thermal tolerances may allow each species to grow at different times, thus allowing both to capture space within the tree and facilitating the

maintenance of a multi-partite symbiosis with MPB (Walker 1995, Yachi and Loreau 1999, Wellnitz and Poff 2001, Stanton 2003, Six and Bentz 2007, Six 2012).

References Cited

Adams, A. S. and Six, D.L. 2007. Temporal variation in mycophagy and prevalence of fungi associated with developmental stages of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytinae, Curculionidae). *Environmental Entomology.* 26: 64-72.

Adams, A. S., Six, D. L., Adams, S. M., Holben, and W. E. 2008. *In vitro* interactions between yeasts and bacteria and the fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*). *Microbial Ecology.* 56(3): 460-466.

Ayers, M.P., Wilkens, R.T., Ruel, J.J, Lombardero, M.J. and Vallery, E. 2000. Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology.* 81(8): 2198-2210.

Ballard, R.G., Walsh, M.A., and Cole, W.E. 1984. The penetration and growth of blue stain fungi in the sapwood of lodgepole pine attacked by mountain pine beetle. *Canadian Journal of Botany.* 62: 1724-1929.

Bentz, B. J. and Six, D. L. 2006. Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus refipennis* (Coleoptera: Curculionidae, Scolytinae). *Annals of the Entomological Society of America.* 99(2): 189-194.

Bleiker, K. P. and Six, D. L. 2007. Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. *Environmental Entomology.* 36(6): 1384-1396.

Bleiker, K. P. and Six, D. L. 2009a. Competition and coexistence in a multipartner mutualism: interactions between two fungal symbionts of the mountain pine beetle in beetle attacked trees. *Microbial Ecology.* 57: 191-202.

Bleiker, K. P. and Six, D. L. 2009b. Effects of water potential and solute on the growth and interactions of two fungal symbionts of the mountain pine beetle. *Mycological Research.* 113: 3-15.

Bleiker, K. P., Potter, S. E., Lauzon, C. R., and Six, D. L. 2009. Transport of fungal symbionts by the mountain pine beetles. *Canadian Entomology.* 141: 503-514.

Carlile, M.J., Watkinson, S.C., and Gooday, G.W., 2001. The fungi 2nd Edition. *Academic Press (San Diego).*

Cook, S., S., Shirley, B.M. and Zambino, P. 2010. Nitrogen concentration in mountain pine beetle larvae reflects nitrogen status of tree host and two fungal associates. *Environmental Entomology.* 39: 821-821.

Goodsman, D.W., Erbilgin, N., and Lieffers, V.J. 2012. The impacts of phloem nutrients on overwintering mountain pine beetles and their fungal symbionts. *Environmental Entomology.* 41(3): 478-486.

Lee, S., Kim, J.J., and Breuil, C. 2006. Diversity of fungi associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. *Fungal Diversity.* 22: 91-105.

McGhehey, J. H. 1971. Female size and egg production of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins. *Northern Forest Research Centre, Edmonton, Alberta, Information Representative*. NOR-X-9.

Paine, T.D., Raffa, K.F., and Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology.* 42:179-206.

Raffa, K.F. and Berryman, A.A. 1983. The role of host plant resistance in the colonization behavior and ecology of bark beetles (Coleoptera: Scolytidae). *Ecological Monographs.* 53(1): 27-49.

Raffa, K.F., Aukema, B.H., Bentz, B.J., Carrol, A.L., Hicke, J.A., Turner, M.G., and Romme, W.H. 2008. Cross-scale drivers of natural disturbance prone to anthropogenic amplification: the dynamics of mountain pine beetle eruptions. *Bioscience.* 58: 501-517.

Rayner, A.D. 1991. The challenge of the individualistic mycelium. *Mycologia*, 83(1): 48-71.

Rice, A. V., Thormann, M. N., and Langor, D. W. 2008. Mountain pine beetle associated blue-stain fungi are differentially adapted to boreal temperatures. *Forest Pathology.* 38: 113-123.

Safranyik, L. and Carroll, A., 2006. The biology and epidemiology of the mountain pine beetle in lodgepole pine forests. Pp. 3-66, *In* Safranyik, L. and Wilson, B. (eds.) The mountain pine beetle: a synthesis of its biology and management and impacts on lodgepole pine. *Natural Resources Canada, Canadian Forest Service.*

Safranyik, L. Carrol, A.L., Regniere, J. Langor, D.W., Reil, W.G. Shore, T.L., Peter, B., Booke, B.J., Nealis, V.G., and Taylor, S.W. 2010. Potential for range expansion of mountain pine beetle into the boreal forests of North America. *Canadian Entomology.* 142: 415-441.

Shearer, C.A. 1995. Fungal competition. *Canadian Journal of Botany.* 73: S1259-S1264.

Six, D.L. and Paine, T.D. 1996. A technique for the introduction of fungi to bark beetle mycangia. *Journal of Entomological Science*. 31: 466-468.

Six, D. L. and Paine, T. D. 1997. *Ophiostoma clavigerum* is the mycangial fungus of the jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mycologia.* 89(6): 858-866.

Six, D. L. and Paine, T. D. 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus* ponderosae (Coleoptera: Scolytidae). *Environmental Entomology.* 27(6): 1393-1401.

Six, D.L. 2003. A comparison of mycangial and phoretic fungi of individual mountain pine beetles. *Canadian Journal of Forest Research.* 33: 1331-1334.

Six, D.L. and Bentz, B. J. 2007. Temperature determines symbiont abundance in a multi-partite bark beetle-fungus symbiosis. *Microbial Ecology.* 54: 112-118.

Six, D.L. 2012. Ecological determinants of bark beetle-fungus symbiosis. *Insects*. 3: 339-366.

Solheim, H. 1995. Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. *Canadian Journal of Botany.* 73: 70-74.

Solheim, H. and Krokene, P. 1998. Growth and virulence of mountain pine beetle associated blue stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. *Canadian Journal of Botany.* 76: 561-566.

Stanton, M.L. 2003. Interacting guilds: moving beyond the pairwise perspective on mutualisms. *The American Naturalist.* 162: S10-S23.

Walker, B. 1995. Conserving biological diversity through ecosystem resilience. *Conservation Biology.* 9(4): 747-752.

Wellnitz, T. and Poff, N.L. 2001. Functional redundancy in heterogenous environments: implications for conservation. *Ecology Letters.* 4: 177-179.

Wicklow, D.T. 1981. The fungal community: Its organization and role in the ecosystem 2nd Ed. *Marcel Dekker, Inc. (New York)*.

Whitney, H.S. and Farris, S.H. 1970. Maxillary mycangium in the mountain pine beetle. *Science* 167: 54-55.

Whitney, H. S. 1971. Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. *Canadian Entomology.* 103: 1495-1503.

Yachi, S. and Loreau, M. 1999. Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. *Proceedings of the National Academy of Science, USA*. 96: 1463-1468.

Moore, Melissa, M.S., June 2013 Forestry

Chapter 2: Effects of temperature on growth and sporulation and on resource capture during inter-specific and intra-specific competition of the fungal symbionts of the mountain pine beetle

Chairperson: Diana L. Six

The mountain pine beetle is an economically and ecologically important insect in western North American forests capable of killing millions of trees during outbreaks. This beetle depends on two fungi, *Grosmannia clavigera* and *Ophiostoma montium*, to provide the nutrients required for the beetle to develop and reproduce. Competition between these two fungal associates is expected because they use similar resources. Strong competition should lead to the eventual destabilization of the three-way symbiosis and fixation for the most competitive fungus. However, strong direct competition has not been observed, indicating that some mechanism likely allows the two fungi to coexist in a multi-partite symbiosis with the mountain pine beetle. These fungi exhibit different temperature tolerances, indicating that temperature may play a major role in determining the relative prevalence of the two associates over time as well as the outcome of competition between the two species. This, in turn, may support the long-term stability of the three-way symbiosis with the mountain pine beetle. To investigate the effects of temperature on the fungal symbionts, I collected 88 isolates from three locations in two states (50 *G. clavigera* and 38 *O. montium*) and measured their growth rates and sporulation at 5, 10, 15, 21, 25, 30, and 35˚C on artificial media. I also measured the growth rates of, and percent resource capture by, each fungus at 10, 15, 21, and 25˚C in the presence of the other species (inter-specific competition) or in the presence of the same species (intra-specific competition). My results indicate that *G. clavigera* excels at resource capture at 10˚C, while at 30˚C *O. montium* dominates. There was no significant effect of geographic origin on growth or sporulation of *G. clavigera*, supporting the findings of previous studies showing low genetic diversity in this species. In contrast, *O. montium* isolates from different locations exhibited significant differences in growth rate when grown alone and during competition, indicating population sub-structuring. *G. clavigera* sporulation was greatest at 30˚C while *O. montium* sporulated similarly across all temperatures. *G. clavigera* captured more resources than *O. montium* at most temperatures, and was able to capture a greater percentage of resources at a greater rate during inter-specific competition than during intra-specific competition at 10 and 15˚C. The reverse was true for *O. montium* which captured resources better during intra-specific competition, and captured a greater percentage of resources at the lower temperatures during intra-specific competition. These results show that temperature affects growth, sporulation and resource capture by these fungi and thus may influence the stability of the three-way symbioses between the fungi and the host beetle in a variable environment.

Introduction

The mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) (Coleoptera: Curculionidae, Scolytinae) is a bark beetle indigenous to pine forests in western North America. This insect is capable of killing a wide range of hosts in *Pinus* (Raffa 1988) and can kill millions of trees during outbreaks. Because of this, it is considered the most important insect pest in western North American forests. In recent years, damage caused by MPB has increased dramatically due to extensive outbreaks exacerbated by climate change, fire suppression and past logging practices (Safranyik and Carrol 2006, Raffa et al. 2008, Safranyik et al. 2010).

The beetle excels in the forest environment due, in part, to its participation in symbioses. While MPB carries a wide array of microbes (Lee et al. 2006), two fungi – *Grosmannia clavigera* and *Ophiostoma montium* – are consistently associated with the beetle across its entire geographic range (Whitney and Farris 1970, Six 2003, Lee et al. 2006). The two fungi are transported from tree to tree by adult beetles in exoskeletal structures called mycangia (Whitney and Farris 1970, Paine et al. 1997, Six 2003, Bleiker et al. 2009), ensuring continuity of the association from generation to generation.

MPB feed on tree phloem, which is a poor nutrient source relative to the needs of the insect (Six and Paine 1998, Ayers et al. 2000). *G. clavigera* and *O. montium* (Whitney and Farris 1970, Six 2003, Lee et al. 2006) provide nutritional supplementation by concentrating nitrogen (Six and Paine 1998, Bleiker and Six 2007, Cook et al. 2010, Goodsman et al. 2012) and producing sterols the beetles need for reproduction (Bentz and Six 2006). Beetle larvae feed on the fungi along with phloem throughout their development (Paine et al. 1997, Six and Paine 1998, Adams and Six 2007, Bleiker and Six 2009a). After completing development, the larvae create chambers in the phloem where they pupate and eclose to the adult form (Paine et al. 1997, Six and Paine 1998, Safranyik and Carroll 2006). New adults feed

on sticky fungal spore layers lining the pupal chamber (Six 2003). As they feed, their mycangia is passively filled with spores for transport to the next host tree (Whitney and Farris 1970, Whitney 1971, Six 2003, Bleiker et al. 2009).

Feeding on fungi has significant benefits for the beetles. Larvae that feed on phloem containing symbiotic fungi attain larger adult body sizes (Six and Paine 1998, Bleiker and Six 2007) and have higher survival rates (Safranyik 1976) than larvae that feed on uncolonized phloem. Furthermore, bark beetle adult body size is positively correlated with fecundity (McGhehey 1971). In addition, it appears that feeding on spores by new adults is essential for MPB reproduction (Six and Paine 1998). While both fungi are able to support beetle growth and development in trees, they differ in the degree of benefit they confer (Six and Paine 1998, Bleiker and Six 2007, Cook et al. 2010). Specifically, *G. clavigera* concentrates nitrogen better than *O. montium* (Cook et al. 2010). This difference likely accounts for observations that beetles developing with *G. clavigera* are bigger (Six and Paine 1998, Bleiker and Six 2007) and have higher productivity and survival rates (Six and Paine 1998) than those developing with *O. montium*. Differential supplementation provided by the two fungi translates directly to fitness effects on beetle productivity, suggesting that their relative prevalence in a population may influence MPB population dynamics

MPB must overwhelm tree defenses in order to kill and colonize a tree and to allow the fungi to establish. To accomplish this, the beetles initiate a mass attack using a complex attractant-antiattractant pheromone system that results in a regular spacing of attacks on the tree bole (Raffa and Berryman 1983). This also results in a regular pattern of inoculation of the fungal associates. As fungi grow out from their respective inoculation points they eventually encounter other fungal individuals, either hetero- or conspecifics, from nearby attack points. Most beetles carry only one fungal species in their mycangia, although under some circumstances both species may be carried (Six 2003, Six and Bentz

2007). The relative prevalence of each species of fungus inoculated into the tree depends upon the relative prevalence of the two symbionts being carried by attacking beetles which, in turn, is determined by temperature (Six and Bentz 2007). Once in the tree, both MPB development (Bentz et al. 1991, Jenkins et al. 2001) and fungal growth (Carlile et al. 2001) are processes controlled by temperature, which allows synchronization of their life stages.

Both fungi co-occur on the same substrate and both must gain access to a dispersing beetle for their spores to be transported to a new host tree, indicating a strong potential for competition. Two types of competition are possible. When microorganisms use the same substrate, they often exhibit interference competition (Wicklow 1981, Rayner 1991, Shearer 1995, Carlile et al. 2001) where one captures resource from the other and control of the resource once captured is often maintained by production of inhibitory compounds or by the creation of a barrage or necrotic zone (Wicklow 1981, Rayner 1991, Shearer 1995, Carlile et al 2001). The other type of competition exhibited by microbes is exploitation (scramble) competition (Wicklow 1981, Rayner 1991, Shearer 1995, Carlile et al. 2001). In this case, the outcome of competition is determined by how efficiently or rapidly each species captures resources. Each captures space at a particular rate until all resource is taken, but neither competitor captures resource from the other. In either case, strong competitive interactions are thought to be destabilizing to multi-partite mutualisms and are predicted to result in the loss of inferior competitors over time (Doebeli and Knowlton 1998). However, the three-way symbiosis between MPB and its two fungi has remained remarkably stable over long periods of evolutionary time (Six and Paine 1999).

Observational and experimental evidence indicates that the two fungi associated with MPB exhibit exploitation competition (Solheim 1995, Bleiker and Six 2007, Bleiker and Six 2009a, Bleiker and Six 2009b). The two fungi grow until individuals of the two species meet. At that time growth halts, although some intermingling of hyphae occurs at the margins of where the two fungi meet, both in

tree tissues, as well as *in vitro* (Solheim 1995, Bleiker and Six 2007, Bleiker and Six 2009a, Bleiker and Six 2009b). There is no development of barrage zones or necrosis, indicating that combative interactions between the two do not occur. The relative prevalence of the two fungi, therefore, appears to be determined mainly by how much resource each is able to capture within the tree. If one is better at capturing resourrce, it would be predicted to move to fixation over time; however, these two fungi have been in association with the beetle for a long period of evolutionary time, indicating the likelihood of an environmental mechanism that supports stability of both in the symbiosis over time (Six and Paine 1999, Six 2012).

Coexistence of competing organisms can arise when they respond differently to environmental conditions such that no one species is favored at all times (Chesson 2000). Few studies have directly addressed the effects of environmental conditions on the coexistence of these two fungi by examining potential outcomes of competition between them. In one study, conducted *in vitro* with varying water potential, both species exhibited reduced growth rates when grown with the other species. However, the growth rate of *G. clavigera* increased when it was grown in close proximity to *O. montium* under low water potentials (Bleiker and Six 2009b). This indicates that the fungi compete for resources, but that under certain conditions, *G. clavigera* may be able to utilize volatiles, by-products, or enzymes diffusing from *O. montium* to its advantage (Bleiker and Six 2009b). In another study, the relative ability of the two fungi to capture space in naturally-attacked trees was found to vary seasonally, and depended on the timing of their introduction to the host tree (Bleiker and Six 2009a). This study indicated that depending on the season, one or the other of the two species captures resources at a faster rate than the other. Together, these two studies suggest that these fungi compete and capture resources in a manner dependent on environmental conditions.

For non-motile organisms that use the same substrate, possessing differing environmental optima may allow a reduction in, or avoidance of, direct competition. Each species may therefore dominate at different times of the year as temperatures fluctuate seasonally, allowing temporal separation of resource capture (Hutchinson 1961, Six and Bentz 2007, Chesson 2012). For organisms exhibiting exploitation competition, differential thermal tolerances may allow multiple symbionts to capture resources at different rates ate different times, thus reducing the ability of any one to dominate over time (Walker 1995, Yachi and Loreau 1999, Wellnitz and Poff 2001, Stanton 2003). For the fungal symbionts of MPB, this may act to promote both the short- and long-term stability of their association with the beetle, enabling the existence a multi-partite symbiosis (Six and Bentz 2007, Six 2012).

Several studies have examined the growth rate responses to temperature exhibited by the two fungi. *G. clavigera* can grow at lower temperatures than *O. montium*, while the reverse is true for *O. montium* which can grow at warmer temperatures than *G. clavigera* (Six and Paine 1997, Solheim and Krokene 1998, Rice et al. 2008, Bleiker and Six 2009b). On artificial media, optimal growth of *G. clavigera* occurs approximately 5˚C lower than for *O. montium* (Solheim and Krokene 1998, Rice et al. 2008). Differential thermal tolerances may explain the shifting prevalence of the two fungi observed over time in naturally attacked trees (Adams and Six 2007, Bleiker and Six 2009a). For example, Adams and Six (2007) observed that *G. clavigera* was more often isolated from phloem adjacent to third and fourth instar larvae developing during spring and early summer when conditions were relatively cool, while *O. montium* was isolated more often from phloem adjacent to pupae, teneral adults, and eggs which develop in mid-to-late summer when temperatures are warmer. Temperature has also been correlated with the relative prevalence of the two fungi carried by dispersing beetles. Six and Bentz (2007) found that *O. montium* was isolated from the mycangia of dispersing MPB when temperatures were warm, while *G. clavigera* was isolated more often when temperatures were cool. These studies

collectively indicate that temperature determines the relative proportion of each fungus in a population at any point in time. They also indicate that this proportion shifts over time, with one fungus dominating during warm conditions and the other during cool conditions.

The dependence of the relative prevalence of the two symbionts on temperature has important implications for both fungal and beetle fitness. For example, at sites that are generally cool, or at times of the year when conditions are overall cooler, *G. clavigera* would be expected to be at an advantage and capture the most resources within the tree. In contrast, at sites where conditions are generally warm, or at during times of the year when conditions are warmer, *O. montium* would be expected to dominate. At sites where conditions fluctuate, the two fungi would be expected to capture resources at different rates at different times of the year, with highly variable outcomes in fungal resource capture and spore production. This variability in resource capture by the fungi may translate directly to corresponding variability in beetle fitness through differential nutrient availability during development. For example, an increase in the prevalence of *O. montium* during larval development could result in a decrease in adult female size, and therefore a reduction in fecundity relative to if the beetle developed with *G. clavigera*. For the fungi, this variability may support coexistence over time by never allowing any one fungus to move to fixation with the host (Six and Bentz 2007, Six 2012). The stability of this multi-partite symbiosis is thought to be important to MPB persistence under fluctuating environmental conditions. By having more than one symbiont, each with different temperature tolerances, MPB larvae may minimize the likelihood of being aposymbiotic as conditions shift through time (Six and Bentz 2007, Six 2012).

To further understand the effects of temperature on these fungi and their ability to maintain a stable symbiosis with the host, I hypothesized that different temperature tolerances will affect the rate of the rate of growth and resource capture and thus the outcome of competition among the fungi within a

tree. To test this hypothesis, I assessed the effects of temperature on the primary fitness parameters of the two fungi (growth rate, sporulation, and ability to compete with hetero- and con-specifics). For the MPB-associated fungi, these metrics determine their ability to capture resources over time and to disperse. While past studies have described temperature effects on growth rates of these fungi, they used relatively small numbers of isolates (Six and Paine 1997, Solheim and Krokene 1998, Rice et al. 2008). In this study, I included a large number of isolates from several locations in order to more accurately estimate growth rates and to capture the range of variability in responses to temperature within each species.

Methods

Fungal isolates. A total of 88 isolates (50 *G. clavigera,* 38 *O. montium*) were collected from two locations in MT and one location in UT. Sites in MT were Vipond Park (45°42.31 N, 112°55.54 W, elevation 2,438 m) located in the Beaverhead National Forest, and Lubrecht Experimental Forest (46˚53.30 N, 113˚26.03 W, elevation 1,263 m) located in Missoula County. The UT site, Stump Hollow (41˚57.34' N, 111˚31.47 W, elevation 2,136 m), is located in the Uinta-Wasatch-Cache National Forest. Multiple sites were used to the capture variability within each species, and also to detect if population sub-structuring occurred by geographic area different locations. Sites were chosen that were geographically distant and that varied by elevation. All fungal isolates were obtained either from dissections of MPB mycangia using methods described by Six and Paine (1997), or from phloem removed from MPB-colonized trees using a sterile cork borer. Pure fungal isolates were obtained using single spore isolations and then incubated at room temperature on 2% malt extract agar (MEA) enriched

with sterile pine twig cuttings to encourage sporulation. Isolates were identified using cultural characteristics and morphology.

Effects of temperature on growth rates of *G. clavigera* and *O. montium*. The 88 isolates were used to determine the growth rates of the two fungi associated with MPB at various temperatures as well as to determine the upper and lower temperature limits for the two species. The isolates were grown for five days on 2% MEA prior to use in each experiment to ensure that the cultures were in exponential growth phase. On the fifth day, a 13 mm ² circular plug of actively growing mycelium was extracted from the leading edge of the culture and placed mycelium side down onto the center of a 100 mm diameter Petri dish containing 2% MEA. The inoculated plates were incubated for 14 d at 5, 10, 15, 21, 25, 30 and 35˚C. Each treatment was replicated three times for each isolate. At the same time each day, beginning on the second day, the area colonized by each fungus was traced on the bottom of the Petri dish. On the tenth day, the bottoms of the Petri dishes were photographed with a digital camera and photos uploaded onto a computer where the daily growth area was measured using ImageJ 1.43u (ImageJ, Bethesda, MD). The cultures were held an additional 4 days to assess sporulation (below).

Data Analyses. Daily area measurements were regressed over time and then fit by regression analysis. The slopes of the regression lines for each replicate were averaged over isolate at each temperature, resulting in average absolute growth rates. The mean, range and standard error in growth rate for each species at each temperature were determined. A logarithmic transformation was used to obtain relative growth rate estimates (Osborne 2002). Mixed Model Analysis of Variance (ANOVA), maximum likelihood method with isolate as a random variable, was used to test for the fixed effects of temperature, species, site and their interactions on relative growth rate. Two similarly constructed Mixed Model ANOVAs for each species were used to examine the fixed effects of temperature and site on relative growth rate. Drop in deviance tests were used to compare the full model to a reduced model

lacking site as a fixed factor. Significant F-tests for all interactions were followed by Tukey-Kramer's honestly significant different (HSD) tests. For all tests, significance was accepted at the $P < 0.05$ level. Absolute growth rates for each isolate and the mean for each species at each temperature were plotted against temperature to produce growth curves comparing the two species at each temperature. Ninety-five percent confidence intervals were used to detect temperatures where growth rates for the two species overlapped. Lack of overlap by the 95% confidence intervals was determined to be a statistically significant difference between the two species at that temperature (Knezevic 2008).

The effects of temperature on sporulation of *G. clavigera* and *O. montium*. Cultures used in the growth rate study were also used to assess temperature effects on sporulation. After 14 d, two replicates of each isolate from each temperature treatment were removed from incubation. A known amount of sterile deionized water was added to the surface of each culture. A sterile bent glass rod was used to dislodge spores from the surface of the agar into the sterile DIH_2O. The resulting spore mixture was extracted from the surface of the plate, placed into a centrifuge tube, and vortexed for 30 s. A 10 µm aliquot of suspension was then injected into a hemocytometer and the number of spores in a subset of randomly chosen grids of the hemocytometer was counted.

Data analyses. The hemocytometer spore counts were converted to spores/ml using the dilution factor. Spores/ml was then scaled by the area of colonization at day 14 to produce an estimate of spores/mm². Replicates were averaged for each isolate at each temperature. The mean, range and standard error in spore production for each species at each temperature were determined. A logarithmic transformation was used to obtain relative spores/ml estimates (Osborne 2002). Mixed Model ANOVA, maximum likelihood method with isolate as a random variable, was used to test for the fixed effects of temperature, species, site and their interactions on relative growth rate. Observations from 35˚C were not included in the model because no growth was observed at this temperature. Significant F-tests for

all interactions were followed by Tukey-Kramer's HSD tests, with the exception of comparisons between the two species which were not conducted because the two fungi respond differently to growth on artificial media which could introduce bias (Bleiker and Six 2007, Bleiker and Six 2009b). For all tests, significance was accepted at the $P < 0.05$ level.

The effects of temperature on resource capture by *G. clavigera* and *O. montium* during intra- and interspecific competition. A total of 30 isolates (15 *G. clavigera,* 15 *O. montium*) were used in this experiment. To investigate the rate and percent of resource capture by the two species when grown together or with themselves, I created an arena that would allow estimations of linear growth of the fungi *in vitro.* To create the arena, I inserted a 55 mm diameter Petri dish inside a 100 mm diameter Petri dish. The space between the smaller dish and the larger dish was filled with 2% MEA forming a ring of growth medium (Figure 1). This design allowed me to measure how temperature affects resource capture in the context of a natural pattern of beetle attack and fungal inoculation, where the growth of an individual colony approaches either an intra- or an interspecific competitor from more than one direction.

The isolates used in this experiment were grown for five days on 2% MEA to ensure that they were in the exponential growth phase prior to use. On the fifth day, a 13 mm² circular plug of actively growing mycelia was extracted from the leading edge of the culture. Four plugs were placed mycelium side down, equidistant from each other on the surface of the ring of agar. For the intraspecific competition treatment, (*G. clavigera* growing towards *G. clavigera, O. montium* growing towards *O. montium)* four plugs of one isolate were used per plate. For the interspecific competition treatment, one isolate of each species from the same site were inoculated equidistant from each other and in alternation on the surface of the ring of agar. Three replicates of each treatment were incubated at 10, 15, 21, and 25˚ C. These temperatures were chosen because they represent the range of temperatures where both

species exhibit sufficient growth rates to test for competition (Solheim and Krokene 1998, Rice et al. 2008). Measurements were taken of daily fungal growth from each plug by marking the bottom of the dish parallel to hyphal extension and perpendicular to the edge of the Petri dish. The total distance captured by each isolate was recorded once the cultures met. A tape measure wrapped around the circumference of a 55 mm diameter Petri dish was used to measure the distance from the center of the plug to the leading edge of growth extending from each plug each day.

Data Analysis. To determine the effects of temperature on resource capture by the fungal symbionts of MPB, two metrics were analyzed: growth rate approaching competitor and the percent resource capture by each competitor once the arena was entirely colonized. Changes in rate of resource capture as an isolate approaches a competitor indicate that the colony senses the presence of the competitor, and therefore, and interference competition. In contrast, the percent resource captured by an individual isolate indicates the total amount of resource captured over the trial. The rates of resource capture by *G. clavigera* and *O. monitum* (during intra- and interspecific competition) were assessed by measuring daily growth rates, which were used in a regression analysis. The slopes of the regression lines formed using observations within individual plates were averaged to obtain an estimate of linear growth rate (mm/day). A logarithmic transformation was used to obtain relative mm/day estimates (Osborne 2002). Comparisons were made between species as well as within species using mixed model ANOVA (maximum likelihood method with isolate as a random effect). Within species, comparisons were made to detect differences in growth rates as affected by temperature, site, and competition treatment (intra- or interspecific competition), as well as the interactions between temperature and competition treatment, competition treatment and site, and temperature and site. Between species, comparisons were made to detect difference in growth rates as affected by temperature, site, species, as well as the interactions between temperature and species, and site and species.

The percent resource capture by each individual was measured by dividing the distance captured by the distance between opposing individuals and multiplying by one hundred. The percent distance captured was averaged within each plate for each competition treatment. Comparisons were made between species as well as within species using mixed model ANOVA (maximum likelihood method with isolate as a random effect). Within species, comparisons were made to detect differences in percent capture as affected by temperature, site, and competition treatment, as well as the interactions between temperature and competition treatment, and competition treatment and site, and temperature and site. Between species, ANOVA was used to detect differences in percent resource capture as affected by temperature, site, species, as well as the interactions between temperature and species, and site and species.

For the growth rate model, as well as the percent resource capture model comparing competition treatments within each species, significant F-tests for interactions (temperature*competition treatment, competition treatment*site, and temperature*competition treatment*site) were followed by Tukey-Kramer's HSD tests. In addition, the mean growth rates and percent resource capture were compared between species at each temperature. For all tests, significance was accepted at the $P < 0.05$ level.

Results

Growth rate experiment. Observations from 35˚C were not included in the model because no growth was observed at this temperature. The highest growth rates occurred at 21˚C for *G. clavigera* (283 mm²/day) and 30˚C for *O. montium* (295 mm²/day) (Table 1). The majority of isolates of both species grew between 5 and 30˚C. At 5˚C, four isolates of *G. clavigera,* and six isolates of *O. montium* showed no growth, but were able to reinitiate growth when held at room temperature. At 30˚C, four isolates of

G. clavigera failed to grow, while fifteen grew initially but stopped growing after day four; however all of the *G. clavigera* isolates were able to grow when moved to room temperature. None of the isolates of either species were able to grow when held at 35˚C; however, all were able to grow once again when moved to room temperature.

As expected, the effect of temperature on the growth of the two species of fungi was significant ($F = 214.716$; df 5,352; $P < 0.0001$). There was a significant interaction between temperature and species ($F = 14.458$; df 5,352; $P < 0.0001$). The 95% confidence intervals predicted by the model indicated that the growth rate of *G. clavigera* was significantly greater than *O. montium* at 10˚C, and that the growth rate of *O. montium* was significantly greater than *G. clavigera* at 30˚C (Figure 2). Results of HSD tests confirmed these predictions; mean growth of *G. clavigera* isolates was significantly greater than for *O. montium* isolates at 10˚C ($P = 0.0002$), and at 30˚C the mean growth of isolates of *O. montium* was significantly greater than for those of *G. clavigera* ($P < 0.0000$).

There was also a significant interaction between temperature and site ($F = 1.866$; df 10, 352; $P = 0.0488$). The HSD tests showed no significant differences between sites and within temperatures for either species, however differences were found between temperatures within site and within species (Table 1). In contrast, when the two species were modeled separately, drop in deviance t-tests confirmed a significant effect of site for *O. montium*, but not for *G. clavigera*. For the *O. montium* model, the mean growth rate for isolates collected from Vipond Park was significantly less than for isolates collected from Lubrecht Experimental Forest ($t = -2.55$; df 34; $P = 0.0156$) (Figure 3).

Sporulation experiment. The greatest mean number of spores/mm² was observed at 30˚C for both *G. clavigera* (4190.4 spores/mm², sem = 1,172.99) and *O. montium* (2401.0 spores/mm², sem = 1,599.61) (Table 2). The majority of isolates from both species produced spores at temperatures between 5 and 30˚C. Two isolates of *G. clavigera* and two isolates of *O. montium* failed to produce spores at 5˚C. The

same was true for one isolate of *O. montium* at 10˚C, one isolate of *O. montium* at 25˚C, and four isolates of *G. clavigera* at 30˚C. Peak mean sporulation for *G. clavigera* was observed at 30˚C; however, no mean peak in sporulation was observed for *O. montium,* which sporulated approximately equally across all temperatures (Figure 4).

ANOVA detected a significant effect of temperature on mean sporulation (F = 26.8939, df 5, 210, $P < 0.0001$) as well as a significant effect of species on mean sporulation (F = 30.1371, df 1, 210, $P < 0.0001$). The interaction between temperature and species had a significant effect on mean sporulation (F = 13.1630, df 5, 210, $P < 0.0001$). HSD tests showed significant differences in sporulation for *G. clavigera,* between 5 and 10˚C (P = 0.0210), and 15 and 21˚C ($P < 0.0001$); however, no significant differences in sporulation were found between any temperatures for *O. montium* (Table 2). The interaction between temperature and site had a significant effect on mean sporulation (F = 2.2860, df 10, 210, P = 0.0146). HSD tests found significant differences in mean sporulation between temperature at each site for *G. clavigera*; however, no significant differences were detected between sites within temperatures for either species (Table 2).

Resource capture during intra- and interspecific competition. In all replicates, slight intermingling of hyphae occurred when opposing isolates met. Interestingly, in the intraspecific competition treatment for *G. clavigera*, a zone lacking melanization (both *G. clavigera* and *O. montium* typically produce abundant melanin in wood and in culture) developed where the hyphae of the two isolates met. This did not occur in the *O. montium* intraspecific competition treatments, or in interspecific competition treatments. No barrage or necrosis zones were observed as a result of any interactions between any species combinations.

Temperature significantly affected mean growth rates of the fungi during competition for *O. montium* (F = 530.4125; df = 3, 279; $P < 0.0001$) and for *G. clavigera* (F = 196.1320, df 3, 294, $P <$

0.0001). In addition, for *O. montium,* mean growth rate overall was significantly greater during intraspecific competition than during interspecific competition (F = 10.9140; df 1, 279; P = 0.0011), however these results were not supported by comparisons of the means at each temperature (Figure 6). A significant effect was found for the interaction between competition treatment and site for *O. montium* (F = 5.8477, df 2, 279, P = 0.0033). HSD tests confirmed these results; isolates of *O. montium* collected from Vipond Park grew more slowly overall than isolates from Stump Hollow (P = 0.0001) and Lubrecht Experimental Forest (P = 0.0002), confirming the results of the drop in deviance test from the growth rate portion of the study (Figure 3).

The interaction between temperature and site had a significant effect on mean growth rates during competition for the combined competition treatments of *O. montium* (F = 3.5764, df 6, 285, P = 0.0020) as well as *G. clavigera* (F = 4.2802, df 6, 300, P = 0.0004). HSD tests showed that *O. montium* isolates collected from Vipond Park grew more slowly at 15˚C than isolates collect from Lubrecht Experimental Forest (P = 0.0025) and Stump Hollow (P = 0.0001). In addition, *G. clavigera* isolates collected from Stump Hollow grew more slowly at 10˚C than isolates from Lubrecht Experimental Forest (P < 0.0001). Of the two species, only *O. montium* was affected by the three-way interaction between temperature, competition treatment, and site (F = 2.7264, df 6, 279, P 0.0137). HSD tests confirmed that during intraspecific competition at 10˚C, *O. montium* isolates from Vipond Park grew less quickly than isolates from Lubrecht Experimental Forest (P = 0.0437) and Stump Hollow (P = 0.0200), and during intraspecific competition at 15˚C, *O. montium* isolates from Vipond Park grew less quickly than isolates from Stump Hollow (P = 0.0452).

Temperature had a significant effect on the percent substrate captured by *O. montium* (F = 7.9774, df 3, 278, P < 0.0001), but not for *G. clavigera* (F = 0.860, df 3, 293, P = 0.4621). *G. clavigera* captured significantly more resources during interspecific competition than during intraspecific

competition (F = 50.519, df 1, 293, P < 0.0001). The reverse was true for *O. montium* which captured significantly more resources during intraspecific competition than during interspecific competition (F = 36.0407, df 1, 278, P < 0.0001). The interaction between temperature and competition treatment had a significant effect on percent resource capture by *G. clavigera* (F = 2.781, df 3, 293, P = 0.0413) as well as by *O. montium* (F = 2.9972, df 3, 278, P = 0.0311) and HSD tests confirmed significant differences between competition treatments and within temperatures for both species. *G. clavigera* captured significantly more resources during interspecific competition than during intraspecific competition at 10˚C (P < 0.0001) and 15˚C (P = 0.0003) (Table 4, Figure 7). *O. montium,* in contrast, captured more resources during intraspecific competition than during interspecific competition at 10˚C (P = 0.0005) and at 15˚C (P = 0.0026) (Table 4, Figure 8), and less resource during interspecific competition at 10˚C than at 20 (P = 0.0005) and 25˚C (P = 0.0099) (Table 4). In addition, the interaction between temperature and site had a significant effect on percent resource capture by *O. montium* (F = 2.5871, df 6, 278, P = 0.0187). HSD tests found no differences between sites and within temperatures; however, isolates of *O. montium* from Lubrecht Experimental Forest captured significantly less resources at 10˚C than at 20 (P = 0.0084) and 25˚C (P = 0.0006) when competition treatment was not taken into account.

When the two species were compared during interspecific competition, temperature had a significant effect on the rate of resource capture (F = 344.85555, df 3, 258, P < 0.0001). *G. clavigera* captured a greater percentage of resources overall (F =27.2424, df 1, 24, P < 0.0001) as well as captured resources at a greater rate overall (F = 9.6675, df 1, 24, P < 0.0048) than *O. montium*. The interaction between temperature and site had a significant effect on the rate of resource capture during interspecific competition (F = 5.7020, df 6, 258, P < 0.0001). HSD tests found no differences in the rate of resource capture between sites compared within temperature; however, for both species, significant differences were found between temperatures within sites (Table 3). In addition, the interaction between

temperature and species had a significant effect on the percentage of resource captured (F = 7.8388, df 3, 258, $P < 0.0001$) as well as the rate of resource capture (F =4.9188, df 3, 258, P = 0.0024). HSD tests found that *G. clavigera* captured a greater percentage of resources than *O. montium* at 10˚C ($P < 0.0001$), 15˚C ($P < 0.0001$), and 25˚C ($P < 0.0231$) (Table 4), as well as captured resources at a greater rate than *O. montium* at 10 ($P < 0.0001$) and 15˚C($P = 0.0024$) (Table 3).

Discussion

Mutualisms are widespread and have profound effects on ecosystem functioning (Stachowitcz 2001, Palmer et al 2010). In the past, these interactions were often simplistically viewed as one-to-one interactions (Doebeli and Knowlton 1998); however, recent work indicates that most mutualisms involve multiple partners (Hoeksema and Bruna 2000, Ferriere 2002). This recognition has resulted in a shift in the focus of studies on mutualism from the single-partner/single-host paradigm towards a more realistic multi-partite model (Herre et al 1999, Hoeksema and Bruna 2000, Stanton 2003). Mutualisms between coniferous bark beetles and fungi typically involve at least two fungi and each partnership has remained remarkably stable over evolutionary time (Six and Paine 1999). This stability is most likely supported by environmental conditions, including temperature (Six 2012).

My results support the hypothesis that the effects of temperature on fungal fitness parameters may act to allow coexistence of *G. clavigera* and *O. montium* in a multi-partite symbiosis with MPB by shifting their relative dominance over time (Six and Bentz 2007, Six 2012). I found that the mean growth rates for *G. clavigera* significantly exceed those of *O. montium* at the lowest temperature tested (Figure 2). Low temperatures are most likely to occur during late autumn, early winter, and early spring, and would support greater resource capture for *G. clavigera* at those times of year (Adams and

Six 2007). In late spring and summer, when temperatures often reach 30˚C or more, *O. montium* should excel at resource capture (Adams and Six 2007) (Figure 2). Resource capture is also likely to be affected by the general thermal conditions at a site, with *G. clavigera* capturing more resource at sites that are overall cooler and *O. montium* capturing more resource at sites that are overall warmer. This possibility is supported by findings that *G. clavigera* dominates in association with beetles in cool sites while it is rare in hot sites (Six and Bentz 2007).

Although the growth rates observed for these fungi on artificial media differ from what would be observed in tree phloem, they are scalable to growth in trees (Addison et al. in review). The temperature ranges supporting growth of *G. clavigera* and *O. montium* observed in this study are in close agreement with those observed in previous studies (Six and Paine 1997, Solheim and Krokene 1998, Rice et al. 2008, Bleiker and Six 2009b); however, I found more variability within each species than has been found previously in studies using smaller samples (Table 1, Figure 2)(Six and Paine 1997, Solheim and Krokene 1998, Rice et al. 2008).

More variability between sites was observed for *O. montium* than for *G. clavigera* in terms of growth rate (Figure 3) as well as resource capture in response to temperature. My observations are in agreement with the findings of previous studies showing low nucleotide diversity (Roe et al. 2011) and high rates of gene flow for *G. clavigera* (Tsui et al. 2012). In contrast, differences between sites for *O. montium* suggests the existence of population sub-structuring in this species.. These differences between the two species may also be, in part, explained by their modes of reproduction. *G. clavigera* is predominantly asexual (Six and Paine 1999, Six et al. 2003, Lee et al. 2007) and exhibits a clonal population structure (Roe et al. 2011). In contrast, although *O. montium* also reproduces asexually, sexual reproduction is common. The ability to reproduce sexually could allow for greater genetic variability and a higher likelihood of local adaptation (Herre et al. 1999). It is not known how frequently

sexual spores of O. *montium* are disseminated by beetles; however, evidence of recombination has been found for *O. montium* (Roe et al. 2011) suggesting that dispersal of sexual spores does occur.

The spores produced by fungi in the Ophiostomatales are adapted for insect dispersal and are not wind-dispersed. Therefore, to be dispersed to another tree, the fungi must colonize a sufficiently large area of the tree to ensure they are present in locations where the beetles excavate pupal chambers ensuring that they are present for spore acquisition by new adults. Temperature determines the relative area captured by each fungus during the developmental period of the beetle; therefore, the greater the area captured, the greater the potential to maximize their fitness (i.e. the greater the number of hosts captured to disseminate spores). MPB either carries one fungus, the other fungus, both fungi, or none in their mycangia (Six and Bentz 2007, Bleiker and Six 2007). Which fungus is carried by a given beetle is likely due to whether the area in which it pupated is colonized by one fungus, the other fungus, or both fungi, and temperature effects on sporulation at the time of MPB eclosion. In this study, while *G. clavigera* sporulated at temperatures at and above 15˚C, the greatest mean sporulation occurred at 30˚C (Figure 4, Table 2). The beetle ecloses within this range of temperatures (Bentz et al 1991); therefore, if pupal chambers are constructed in areas of the trees captured by *G. clavigera*, spores should be available to the beetle for mycangial acquisition prior to dispersal. In contrast, sporulation of *O. montium* across temperatures was highly variable with no clear optimal temperature (Figure 4)(Table 2). *O. montium* can grow at higher temperatures than *G. clavigera*, which may account for its greater dispersal by adult beetle in summer and at hotter sites (Six and Bentz 2007). The co-occurrence of the two fungi in mycangia of individual beetles that has been observed when temperatures support the growth and sporulation of both fungi (Six and Bentz 2007) indicates that both fungi can co-occur in single pupal chambers, further supporting observations that the fungi do not exhibit strong antagonism toward one another.

Neither *O. montium* nor *G. clavigera* sporulate as readily on artificial media as they do on natural substrates (Bleiker and Six 2007, Bleiker and Six 2009b). Likewise, growth on artificial media is likely different than that occurring in trees due to differences in substrate structure, nutrients and secondary chemistry. However, this study was necessarily confined to artificial media to control variability in the system and due to the difficult nature of working with trees or logs. In future studies, comparison of the sporulation between these species should be done by conducting experiments using more natural substrates.

Results of the competition study confirmed the findings of previous studies indicating that interference competition does not occur between the two fungi (Bleiker and Six 2007, Bleiker and Six 2009a, Bleiker and Six 2009b). My results found that the growth rate of *O. montium* is not affected by the presence of *G. clavigera* more than the presence of conspecifics (Figure 6), and likewise, *G. clavigera* captured resources at a similar rate regardless of whether it was engaged in intra- or inter-specific competition (Figure 5). However, I did find that temperature, much like water potential (Bleiker and Six 2009a), affected competition outcomes and overall, *O. montium* captured more resources at the lower temperatures during intra- than inter-specific competition (Figure 8). In contrast, *G. clavigera* captured more resources at lower temperatures during inter- than intra-specific competition (Figure 7). These results indicate that the outcome of exploitation competition is affected by temperature.

Growth rates during competition (Table 3) differed from growth rates for the two fungi when grown without competitors (Table 1). These differences were likely due to the types of arenas used. In the competition experiment, fungal expansion was constrained to linear growth extending in two directions from the initial inoculation point (Figure 1). In the growth experiment, the fungi were able to grow out in all directions from inoculations made in the center of typical agar filled Petri dishes.

However, within experiments, outcomes were consistent for both studies; *G. clavigera* grew more quickly than *O. montium* at lower temperatures, and the growth rates of the two species were similar at intermediate temperatures (Figures 2 and 9).

My results indicate that *G. clavigera* and *O. montium* exhibit exploitation competition (where the outcome of competition is determined by the rate of resource capture), especially at lower temperatures. During inter-specific competition at the lower temperatures, *G. clavigera* captured resources at a greater rate than *O. montium* (Figure 9), and was also able to capture more resources at most temperatures than *O. montium* (Figure 10). Interestingly, *G. clavigera* was able to capture more resource than *O. montium* at 25˚C (Figure 10) even though the growth rate of *G. clavigera* was not significantly greater than that of *O. montium* at this temperature (Figure 9). *G. clavigera* is able to excel at resource capture at cooler temperatures because it grows faster than *O. montium* at those temperatures (Figures 2 and 9). However, at the higher temperatures, resource capture is much the same for each species whether growing with a conspecific or with the other species because the two species exhibit comparable growth rates at those temperatures (Figures 2 and 9). These results indicate that as long as temperatures remain relatively cool, *G. clavigera* will excel at resource capture and dominate within the system however, given environmental fluctuation, *O. montium* would not be expected to be outcompeted entirely.

As temperatures increase due to climate change, my results suggest that *O. montium* may become increasingly dominant and may over time move to fixation with the host beetle. These results are consistent with predictions from temperature-driven models, showing that *G. clavigera* will be lost from the symbiosis due to a warming climate (Addison et al, in review). At higher temperatures, as are expected to increasingly occur in the future, association with a symbiont that is tolerant of higher temperatures is advantageous at times when *G. clavigera* is incapable of capturing resources (Figure 2). However, given that MPB that develop with *O. montium* have overall lower fitness than those

developing with *G. clavigera* (Six and Paine 1998, Bleiker and Six 2007), this may translate to an overall negative impact on MPB population dynamics as the prevalence of *O. montium* increases due to a warming climate. Therefore, while warming is currently acting to exacerbate beetle impacts on forests (Safranyik et al. 2010), in the long term it may actually begin to reduce the ability of the beetle to develop widespread outbreaks.

In conclusion, the two fungal mutualists of MPB exhibit sufficient differences in temperature tolerances to support their ability to coexist under current environmental conditions. My data indicate that with a warming climate, *O. montium* is likely to become increasingly prevalent. Although temperature does not appear to affect the outcome of competition at the highest temperature tested in this study, warming could lead to the eventual exclusion of *G. clavigera* from the system due to greater resource capture over time by *O. montium*. However, the considerable variability in response to temperature exhibited by both species of fungi indicates a substantial ability to adapt to rapid warming at least over the short term.

Acknowledgements

Much gratitude is due to David Affleck and Brian Steele for their invaluable assistance with data analysis. Many individuals helped shape this project including Audrey Addison, Barbara Bentz, and Jim Powell, as well as my committee members, Cory Cleveland, John McCutceon, and Diana Six.
In addition, several volunteers assisted me in data collection. Thank you Caleb Craft, Joseph Caleb Dysthe, Pamela Erm, Brenna Hannapel, Dara McDevitt, Emily Newman, Austin Stewart, and Aspen Ward.

References Cited

Adams, A.S. and Six, D.L. 2007. Temporal variation in mycophagy and prevalence of fungi associated with developmental stages of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytinae, Curculionidae). *Environmental Entomology.* 26: 64-72.

Addison, A., Bentz, B.J., Friedman, M.L., Powell, J.A., Six, D.L. *In review*. The role of temperature variability in stabilizing the mountain pine beetle –fungus mutualism.

Atkins, M.D. 1967. The effect of rearing temperature on the size and fat content of the Douglas-fir beetle. *Canadian Entomology.* 99: 181-187.

Ayers, M.P., Wilkens, R.T., Ruel, J.J, Lombardero, M.J. and Vallery, E. 2000. Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology.* 81(8): 2198-2210.

Bentz, B.J., Logan, J.A., and Amman, G.D. 1991. Temperature-dependent development of the mountain pine beetle (Coleopteran: Scolytidae) and simulation of its phenology. *Canadian Entomology.* 123: 1083-1094.

Bentz, B.J. and Six, D.L. 2006. Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus refipennis* (Coleoptera: Curculionidae, Scolytinae). *Annals of the Entomological Society of America.* 99(2): 189-194.

Bleiker, K.P. and Six, D.L. 2007. Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. *Environmental Entomology.* 36(6): 1384-1396.

Bleiker, K.P. and Six, D.L. 2009a. Competition and coexistence in a multipartner mutualism: interactions between two fungal symbionts of the mountain pine beetle in beetle attacked trees. *Microbial Ecology.* 57: 191-202.

Bleiker, K.P. and Six, D.L. 2009b. Effects of water potential and solute on the growth and interactions of two fungal symbionts of the mountain pine beetle. *Mycological Research.* 113: 3-15.

Bleiker, K.P., Potter, S.E., Lauzon, C.R., and Six, D.L. 2009. Transport of fungal symbionts by the mountain pine beetles. *Canadian Entomology.* 141: 503-514.

Carlile, M.J., Watkinson, S.C., and Gooday, G.W., 2001. The fungi 2nd Edition. *Academic Press (San Diego).*

Chesson, P. 2000. Mechanisms of maintenance of species diversity. *Annual Review of Ecology and Systematics.* 31:343-366.

Cook, S.S., Shirley, B.M. and Zambino, P. 2010. Nitrogen concentration in mountain pine beetle larvae reflects nitrogen status of tree host and two fungal associates. *Environmental Entomology.* 39: 821-821.

Doebeli, M. and Knowlton, N. 1998. The evolution of interspecific mutualisms. *Proceedings of the National Acedemy of Science of the United States of America.* 95(15): 8676-8680.

Ferriere, R., Bronstein, J.L., Rinaldi, S., Law, R., Gauduchon, M. Cheating and the evolutionary stability of mutualisms. *Proceedings: Biological Sciences.* 269(1493): 773-780.

Goodsman, D.W., Erbilgin, N., and Lieffers, V.J. 2012. The impacts of phloem nutrients on overwintering mountain pine beetles and their fungal symbionts. *Environmental Entomology.* 41(3): 478-486.

Herre, E.A., Knowlton, N., Mueller, U.G., and Rehner, S.A. 1999. The evolution of mutualisms: exploring the path between conflict and cooperation. *TREE*. 14(2): 49-54.

Hoeksema, J.D. and Bruna, E.M. 2000. Pursuing the big questions about interspecific mutualism: A review of theoretical approaches. *Oecologia.* 125(3): 321-330.

Hutchinson, G.E. 1961. The paradox of plankton. *American Naturalist.* 95: 137-145.

Jenkins, J.L., Powell, J.A., Logan, J.A., and Bentz, B.J. 2001. Low seasonal temperatures promote lifecycle synchronization. *Bulletin of Mathematical Biology.* 63:573-595.

Knezevic, A. 2008. StatNews # 73: Overlapping confidence intervals and statistical significance. *Cornell University, Cornell Statistical Consulting Unit.*

Lee, S., Kim, J.J., and Breuil, C. 2006. Diversity of fungi associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. *Fungal Diversity.* 22: 91-105.

Lee, S., Hamelin, R.C, Six, D.L., and Breuil, C. 2007. Genetic diversity and the presence of two distinct groups in *Ophiostoma clavigera* associated with *Dendroctonus ponderosae* in British Columbia and northern Rocky Mountains. *Phytopathology.* 97: 1177-1185.

Mattson, W.J. 1980. Herbivory in relation to plant nitrogen content. *Annual Review of Ecological Syst*ems. 11: 119-161.

McGhehey, J.H. 1971. Female size and egg production of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins. *Northern Forest Research Centre, Edmonton, Alberta, Information Representative*. NOR-X-9.

Osborne, J. 2002. Notes on the use of data transformations. *Practical Assessment, Research and Evaluation,* 8(6). http://PAREonline.net/getvn.asp?v=8andn=6.

Paine, T.D., Raffa, K.F., and Harrington, T.C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology.* 42:179-206.

Palmer, T.M., Doak, D.F., Stanton, M.L., Bronstein, J.L., Keirs, E.T., Young, T.P., Goheen, J.R., and Pringle, R.M. 2010. Synergy of multiple partners, including freeloaders, increases host fitness in a multispecies mutualism. *PNAS.* 107(40):17234-17239.

Raffa, K.F. and Berryman, A.A. 1983. The role of host plant resistance in the colonization behavior and ecology of bark beetles (Coleoptera: Scolytidae). *Ecological Monographs.* 53(1): 27-49.

Raffa, K.F. 1988. The mountain pine beetle in western North America. *In* Dynamics of forest insect populations: patterns, causes, and implications. *Ed* A.A. Berryman, Plenum Press, New York. 505-553.

Raffa. K.F., Aukema, B.H., Bentz, B.J., Carrol, A.L., Hicke, J.A., Turner, M.G., and Romme, W.H. 2008. Cross-scale drivers of natural disturbance prone to anthropogenic amplification: the dynamics of mountain pine beetle eruptions. *Bioscience.* 58: 501-517.

Rayner, A.D. 1991. The challenge of the individualistic mycelium. *Mycologia*, 83(1): 48-71.

Rice, A.V., Thormann, M.N., and Langor, D.W. 2008. Mountain pine beetle associated blue-stain fungi are differentially adapted to boreal temperatures. *Forest Pathology.* 38: 113-123.

Roe, A.D., Rice, A.V., Coltman, D.W., and Cooke, J.E. 2011. Comparative phylography, genetic differentiation and contrasting reproductive modes in three fungal symbionts of a multi-partite bark beetle symbiosis. *Molecular Ecology.* 20: 584-600.

Safranyik, L. 1976. Size and sex-related emergence, and survival in cold storage, of mountain pine beetle adults. *Canadian Entomology.* 108: 209-212.

Safranyik, L. and Carroll, A., 2006. The biology and epidemiology of the mountain pine beetle in lodgepole pine forests. Pp. 3-66, *In* Safranyik, L. and Wilson, B. (eds.) The mountain pine beetle: a synthesis of its biology and management and impacts on lodgepole pine. *Natural Resources Canada, Canadian Forest Service.*

Safranyik, L. Carrol, A.L., Regniere, J. Langor, D.W., Reil, W.G. Shore, T.L., Peter, B., Booke, B.J., Nealis, V.G., and Taylor, S.W. 2010. Potential for range expansion of mountain pine beetle into the boreal forests of North America. *Canadian Entomology.* 142: 415-441.

Shearer, C.A. 1995. Fungal competition. *Canadian Journal of Botany.* 73: S1259-S1264.

Six, D. L. and Paine, T. D. 1997. *Ophiostoma clavigerum* is the mycangial fungus of the jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mycologia.* 89(6): 858-866.

Six, D. L. and Paine, T. D. 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus* ponderosae (Coleoptera: Scolytidae). *Environmental Entomology.* 27(6): 1393-1401.

Six, D.L. and Paine, T.D. 1999. Phylogenetic comparison of ascomycete fungi and *Dendroctonus* bark beetles (Coleoptera: Scolytidae). *Annual Entomological Society of America.* 92(2): 159-166.

Six, D.L. 2003. A comparison of mycangial and phoretic fungi of individual mountain pine beetles. *Canadian Journal of Forest Research.* 33: 1331-1334.

Six, D.L., Harrington, T.C., Steimel, J., McNew, D., and Paine, T.D. 2003. Genetic relationships among *Leptographium tenebrantis* and the mycangial fungi of three western *Dendroctonus* bark beetles. *Mycologia.* 95(5): 781-792.

Six, D.L. and Bentz, B. J. 2007. Temperature determines symbiont abundance in a multi-partite bark beetle-fungus symbiosis. *Microbial Ecology.* 54: 112-118.

Six, D.L. 2012. Ecological determinants of bark beetle-fungus symbiosis. *Insects*. 3: 339-366.

Solheim, H. 1995. Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. *Canadian Journal of Botany.* 73: 70-74.

Solheim, H. and Krokene, P. 1998. Growth and virulence of mountain pine beetle associated blue stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. *Canadian Journal of Botany.* 76: 561-566.

Stachowicz, J.J. 2001. Mutualism, facilitation, and the structure of ecological communities. *BioScience.* 51(3): 235-246.

Stanton, M.L. 2003. Interacting guilds: moving beyond the pairwise perspective on mutualisms. *The American Naturalist.* 162: S10-S23.

Tsui, C.K.M., Roe, A.D, El-Kassaby, Y.A., Rice, A.V., Alamouti, S.M., Sperling, F.A.H., Cooke, J.E., Bohlmann, J., and Hamelin, R.C. 2012. Population structure and migration pattern of a conifer pathogen, *Grosmannia clavigera,* as influence by its symbiont, the mountain pine beetle. *Molecular Ecology.* 21(1): 71-86.

Walker, B. 1995. Conserving biological diversity through ecosystem resilience. *Conservation Biology.* 9(4): 747-752.

Wellnitz, T. and Poff, N.L. 2001. Functional redundancy in heterogenous environments: implications for conservation. *Ecology Letters.* 4: 177-179.

Wicklow, D.T. 1981. The fungal community: Its organization and role in the ecosystem 2nd Ed. *Marcel Dekker, Inc. (New York)*.

Whitney, H.S. and Farris, S.H. 1970. Maxillary mycangium in the mountain pine beetle. *Science* 167: 54-55.

Whitney, H. S. 1971. Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. *Canadian Entomology.* 103: 1495-1503.

Yachi, S. and Loreau, M. 1999. Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. *Proceedings of the National Academy of Science, USA.* 96: 1463-1468.

Figure legends

Figure 1. Arena used to test for effects of temperature on resource capture by *G. clavigera* and *O. montium* during intra- and inter-specific competition.

Figure 2. Mean growth rate (mm^2/day) for *G. clavigera* (black) and *O. montium* (gray) isolates from two sites in Montana and one site in Utah (all sites combined) indicated by solid lines. Mean growth rate (mm^2/day) of individual isolates are indicated by points. Bars represent 95% confidence intervals.

Figure 3. Mean growth rate (mm^2/day) for *G. clavigera* and *O. montium* isolates from study sites in Montana (Lubrecht, Vipond Park) and Utah (Stump Hollow). Lines for Lubrecht Experimental Forest, Stump Hollow, and Vipond Park, respectively, are shown by increasing thickness.

Figure 4. Mean sporulation (spores/mm^2) for *G. clavigera* (thin line) and *O. montium* (thick line) (all sites combined) indicated by solid lines. Mean sporulation (spores/mm^2) of individual isolates are indicated by solid (•) points for *G. clavigera* and empty (°) points for *O. montium*.

Figure 5. Mean growth rates of *Grosmannia clavigera* during intra- and inter-specific competition (with *Ophiostoma montium*) at four temperatures. Error bars represent the standard deviation of the means.

Figure 6. Mean growth rates of *Ophiostoma montium* during intra- and inter-specific competition (with *Grosmannia clavigera*) at four temperatures. Error bars represent the standard deviation of the means.

Figure 7. Mean resource capture by *G. clavigera* in experiment testing for intra- and inter-specific (with *Ophiostoma montium*) competition at four temperatures. Different letters within temperatures indicate that means are significantly different. Error bars represent the standard deviation of the means.

Figure 8. Mean resource capture by *O. montium* in experiment testing for intra- to inter-specific competition at four temperatures. Different letters within temperatures indicate that means are significantly different. Error bars represent the standard deviation of the means.

Figure 9. Mean growth rates of *G.clavigera* and *O. montium* during inter-specific competition at four temperatures. Different letters within temperature indicate that means are significantly different. Error bars represent the standard deviation of the means.

Figure 10. Mean resource capture by *O. montium* and *G. clavigera* in experiment testing for inter-specific competition at four temperatures. Different letters within temperatures indicate that means are significantly different. Error bars represent the standard deviation of the means.