DISSERTATION

# MITE AND FUNGAL ASSOCIATES ON MOUNTAIN PINE BEETLES ATTACKING THREE PINE SPECIES IN NORTHERN COLORADO

Submitted by

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ABSTRACT

MITE AND FUNGAL ASSOCIATES ON MOUNTAIN PINE BEETLES ATTACKING THREE PINE SPECIES IN NORTHERN COLORADO

During its life cycle, mountain pine beetle (*Dendroctonus ponderosae*) interacts with phoretic organisms such as mites, nematodes, fungi, and bacteria. The types of associations these organisms establish with the mountain pine beetle vary from mutualistic to antagonistic. The most studied of these interactions are those between beetle and fungi. The least studied are interactions with bacteria, but these have received increased attention recently. Nematodes remain little studied. During 2011 to 2013, I studied phoretic mites arriving to limber (*P. flexilis*), lodgepole (*P. contorta*), and ponderosa (*P. ponderosa*) pines. Species of blue-stain fungi can be hyperphoretic by being carried on mites transported by beetles. Therefore, I studied phoretic fungi transported by mountain pine beetle and the mites arriving to limber, lodgepole, and ponderosa pines. On average, 57% of mountain pine beetles carried phoretic mites, a percentage that increased from 32 to 65% over three years in which mountain pine beetle population declined in our plots. Overall, I found that four of five mite species were common (>10%) on beetles arriving to the three pine hosts, but only *T.ips* and *T. hirsuta* were present during all years. The uncommon fifth species, *Histiogaster arborsignis*, was more frequently found on mountain pine beetle predators in the family Cleridae. Mountain pine beetle phoretic mites were not found on co-arriving insect predators including three species of clerids, *Medetera aldrichii*, or on parasitic hymenoptera *Coeloides sympitys*. Co-arriving *Dendroctonus* beetle species to ponderosa (*D. valens*) and lodgepole (*D. murrayanae*) pines also carried a different

mite fauna than mountain pine beetles. I report a new species of omnivorous mite, *Trichouropoda* cf. *hirsuta* for the Colorado Front Range. The percent of beetles carrying mites increased significantly from 2011 (32%) to 2012 (62%) but did not increased singnificantly from 2012 to 2013 (65%). The average number of mites per beetle did not increased significantly between 2011 (1.23) to 2012 (1.32), but it was significantly greater between 2012 and 2013 (5.19). Within the three years mite assemblages of three species common to mountain pine beetle arriving to lodgepole and ponderosa pines changed significantly on ponderosa pines but did not change significantly on mountain pine beetle arriving to lodgepole pine. Mite assemblages arriving to all hosts within years were the same on male and female mountain pine beetles. During 2012 and 2013 I examined mountain pine beetle associated blue-stain fungi and fungi hyperphoretic on its phoretic mites. Mountain pine beetle carried *Grosmannia clavigera* and *Ophiostoma montium*, the two blue-stain species reported from western USA, but also *Leptographium longiclavatum* reported previously only from mountain pine beetle in Canada. Beetles transported the three blue-stain species to all three pine hosts during the three years. While four common mountain pine beetle phoretic mite species carried some blue-stain fungal species, along with other fungi such as *Alternaria*, *Ceratocystiopsis*, *Entomocorticium,* and *Penicillium*, among others; only the two most common species, *Tarsonemus ips* and *Trichouropoda hirsuta* transported all three blue-stain species present on mountain pine beetle. Overall, the contribution mites made to the total blue-stain transport was approximately 2.0 % of the total transported by the beetle-mite complex in the symbiosis. A general and significant reduction in occurrence of blue-stain transported by both, beetles (77 to 34 %) and mites (77 to 4 %) was found between 2012 and 2013. These two years were, respectively, a warm dry and average temperature and humid years. Mites carrying *O. montium* significantly increased the

probability of finding that fungus on beetles. Although, the overall transport of *O. montium* by *T. ips* and *T. hirsuta* during the two years was small, the type of spores these carry were sexual (ascospores) which could benefit the fungus by increasing the proportion of recombinant sexual types on that species.

# ACKNOWLEDGEMENTS

In a multidisciplinary effort such as the study of multipartite symbiosis, we rely on the expertise of many specializing on the fields relating our study. My mentors Drs. Jose F. Negrón William R. Jacobi, and Ned Tisserat provided guidance and expert support during my attempts to learn something about fungal pathogens. Multidisciplinary entomologists such as Richard Hofstetter help elevate the mind to consider new lines of thought while still making it fun. Danielle Reboletti, who shared data, images and contributed to joint publications on related topics. Tree physiologists introducing me to their exciting and complex field Drs. Bill Bauerle and Rob Hubbard provided needed insight to understand tree complexities. Lab partners from CSU always shared their ideas and were example of dedication while keeping an always friendly and positive environment including Dr. Daniel West, Christy Cleaver, and Megan Dudley. Colleagues Lance Asherin, John Frank, Laurie Huckabee, Sparkle McCoy, Ben Gannon, John Popp, and many others in the RMRS staff lend a hand at one time or where there to encourage me when situations seemed difficult. And many more that I can name due t space. This work was funded by the USDA, Forest Service and Rocky Mountain Research Station and the SRI Program. I'm very grateful to my mentors and examples of life Drs. José F. Negrón, and William R. Jacobi who provided not only great ideas all over the study but hand on hand help at work. Rebecca Powell, and Laurie Huckabee who dedicated many hours of their valuable time during exiting field and often less exiting laboratory duties specially during times of heavy work load. I remain grateful and at your service always.

# DEDICATION

To my wife Yesenia for always being there when I needed her, her love and support kept me going. To my children that became teenagers during this period, for their unconditional love and support and understanding. To my parents that always encourage me to pursue broader horizons. And to Drs. Don Bright and John Moser whom taxonomic expertise and important life long contributions have inspired many, for being an inspiration source to me as well during my journey.

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# Chapter 1. Phoretic Symbionts of the Mountain Pine Beetle (*Dendroctonus ponderosae* Hopkins).

## Introduction

The mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins) is a natural disturbance agent in western North American coniferous forests, which uses various species of *Pinus* as hosts. Eruptive populations can cause extensive levels of tree mortality. When beetles arrive at a tree they carry a large array of ecto- and endosymbiotic organisms, which exhibit highly complex interactions that can contribute to the success or failure of population establishment in the new host. These include several species of mites (Reboletti 2008, Mori et al. 2011), external and internal nematodes (Reid 1958, Massey 1974), fungi and yeasts (Whitney 1982, Paine et al. 1997, Six 2003), and bacteria (Cardoza et al. 2009, Winder et al. 2010, Hulcr et al. 2011). This collection of organisms comprises an entire functioning community including fungivores, herbivores, detritivores, scavengers, parasites, and predators. The roles of some microorganisms, particularly certain fungi, are relatively well understood in the MPB community (Six and Paine 1998, Six 2003, Bentz and Six 2006). Some fungi and bacteria may facilitate digestion of host tissues, aid pheromone synthesis, or serve as a food source for beetles (Six 2003, Harrington 2005, Bentz and Six 2006). Bacterial and yeast symbionts may benefit beetle hosts by modifying the microbial community, particularly by inhibiting antagonistic fungi (Cardoza et al. 2006a). Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.-Criv.) Vuill., can easily infect MPBs, especially during epidemics, playing a role in population dynamics (Hunt et al. 1984, Safranyik et al. 2001). Mites are commonly associated with bark beetles and

have a suite of interactions (Cardoza et al. 2008, Hofstetter 2011). Many of these associates probably exert both positive and negative effects on beetles (Eckhardt et al. 2004, Klepzig and Six 2004, Kopper et al. 2004). For example, in the southern United States, *Tarsonemus ips* Lindquist carries *Ophiostoma minus* (Hedgc.) H. and P. Syd., which is antagonistic to southern pine beetle (SPB) (*Dendroctonus frontalis* Zimm.); however, in Chiapas, Mexico, the same mite is associated with *Ceratocystiopsis ranaculosa* T.J. Perry & J.R. Bridges (Moser and Macías Sámano 2000) a known mutualist of SPB. Hence, the effects of phoretic mites may be context-dependent, or they can be considered conditional mutualists.

Species that attach to other organisms, called phoronts, are highly adapted for transport in or on other organisms and often have highly modified structures in their phoretic stage. In this form of symbiosis, the organisms often go through behavioral changes (such as cessation of feeding and host searching) or morphological changes that are quite different from those of nonphoretic individuals of the same species. Under most conditions, phoretic organisms can be classified as commensal, in that they do not cause any direct harm or benefit to the carrier but benefit by being transported to a new habitat (Houck 1994). When these phoronts are abundant, these may interfere with carrier movement, reduce travel distances, and be energetically costly (Kinn 1971, Kinn and Witcosky 1978). Phoronts may provide direct or indirect benefits or harm to their carrier or influence ecological interactions within host trees. Thus, the relationship of the phoront and its carrier may be mutualistic, neutral (e.g., commensal, benefiting the phoront), or antagonistic (predatory, parasitic, or toxic), resulting in a loss of fitness to the carrier.

## The Phoretic Mite Fauna

Trees colonized by bark beetles often become home to a large variety of other invertebrates. Mites (Acari: Acariformes) are common phoronts on bark beetles (Kinn 1971, Moser and Roton 1971), and their numbers on individual beetles can vary greatly from none to hundreds of mites on a single beetle (Hofstetter 2011). Mites of bark beetles are now known to have strong interactions with associated organisms, are major components of biological diversity, and can have an impact on bark beetle population dynamics and fungal interactions (e.g., Hofstetter et al. 2006a, 2006b).

An extensive body of literature exists on phoretic mites associated with several bark beetle species (reviewed by Hofstetter et al. 2013) such as SPB (Moser 1976, Kinn and Witcosky 1978, Hofstetter et al. 2007), spruce beetle (SP) (*Dendroctonus rufipennis* Kirby) (Cardoza et al. 2008), European spruce bark beetle (*Ips typographus* L.) (Takov et al. 2009), species of *Pityokteines* Fuchs (Pernek et al. 2008), and *Scolytus* Geoffroy (Moser et al. 2010). Most mites associated with bark beetles are in the order Sarcoptiformes (Kinn 1971, Moser and Roton 1971, Hofstetter et al. 2013). Tarsonemid mites in the order Trombidiformes include parasites of beetle eggs (Lindquist 1986) and fungivores that often have intricate relationships with fungi associated with beetles (Moser 1985, Bridges and Moser 1986, Moser et al. 1989a, 1989b, Cardoza et al. 2008). Mesostigmata mites, including many genera found in decaying fungi, are especially prominent as predators of nematodes and other mites and as phoronts on adult bark beetles (Kinn 1971, Moser and Roton 1971, Lindquist 1975, Lindquist and Wu 1991). Oribatid mites that often associate with bees and wasps are also common on bark beetles and may act as commensal

organisms, mutualists, or predators (Kinn 1971). Phoretic mites may be specific on bark beetle species or found on multiple insect species, including predatory insects (Hofstetter et al. 2013).

There are published records of mites associated with MPB (Lindquist and Hunter 1965, Lindquist 1969, 1971, Moser and Roton 1971, Mori et al. 2011), including 13 phoretic mite species (Table 1.1). Studies in Alberta, Canada (Mori et al. 2011), and South Dakota (Reboletti 2008) showed that the percentage of adult beetles carrying phoretic mites varied by collection method but typically averaged 30 –50% of beetles. The mean numbers of mites varied by site and time of year (Mori et al. 2011), ranging from 0.93 to 2.75 mites per beetle (Reboletti 2008). Reboletti (2008) recorded the location of several phoretic mite species on the beetle exoskeleton. *Proctolaelaps subcorticalis* Lindquist were found under the elytra, whereas *Tarsonemus endophloeus* Lindquist were found on the metathoracic wing origin or the sternum. Other mite species were found on various places on the beetle's exoskeleton (Reboletti 2008, Mori et al. 2011) (Figure 1.1). Despite the best efforts of past investigators, our understanding of MPB mite fauna remains incomplete, partly because of understudied areas of their geographic distribution.

Table 1.1. Feeding guild and abundance of phoretic mites found on adult mountain pine beetle in North America. Omnivorous = feeds on a variety of organisms: fungi, bacteria, dead invertebrates, etc. Mycetophagous = feeds on fungi, often transports and disperses reproductive structures of fungi. Predacious = feeds on living organisms such as nematodes, invertebrate eggs, or larvae. We categorize phoretic mites abundance on beetles as rare (< 1% have the particular mite species), Infrequent (1–5%), common (5–20%), and frequent (>20% have the species).

| **Mite symbiont** | **Feeding guild** | **MPB abundance** |
|---|---|---|
| *Histiogaster arborsignis* Woodring | Mycetophagous | Infrequent |
| *Macrocheles schaeferi* Walter | Predacious | Rare |
| *Nanacarus* sp. | Omnivorous | Rare |
| *Parawinterschmidtia* (Khaustov) sp. | Unknown | Rare |
| *Proctolaelaps hystricoides* Lindquist & Hunter | Predacious | Common |
| *P. subcorticalis* Lindquist | Predacious | Frequent |
| *Schweibea* sp. | Unknown | Rare |
| *Tarsonemus endophloeus* Lindquist | Mycetophagous | Rare |
| *T. ips* Lindquist | Mycetophagous | Frequent |
| *Trichouropoda* sp. | Omnivorous | Rare |
| *Winterschmidtia* sp. | Unknown | Rare |
| *Tydeidae* (undetermined genus) | Unknown | Rare |

Mite Feeding Guilds and Diversity

The phoretic mite diversity associated with MPB seems moderate to low compared with that of the collection of phoretic mite species existing within beetle populations (hereafter assemblages) found on other species of *Dendroctonus* Er. (Moser and Roton 1971, Cardoza et al. 2008, Hofstetter et al. 2013). For instance, only five species of phoretic mites were associated with MPB in Alberta, Canada. Mori et al. (2011) suggested that this may be due to recently established relationships on the leading edge of the MPB outbreak spreading to novel range expansions in Alberta. However, sampling intensity and methodology may influence levels of species diversity reported in various studies. For example, in South Dakota, Reboletti (2008) catalogued 10 mite species on MPB when sampling was conducted over multiple years. As found for the SPB, additional differences in mite diversity could be associated with MPB population levels or environmental conditions that may affect species differentially, among other factors (Hofstetter et al. 2006b).

In terms of trophic effects and community interactions, several mite species are known to be predators of other invertebrates or early MPB developmental stages. *Macrocheles* Latr. and *Proctolaelaps* Berlese mites are known to feed on nematodes, other mites, and bark beetle eggs and early larvae (Lindquist and Hunter 1965, Moser and Roton 1971, Kinn 1983, Hofstetter et al. 2013). *Tarsonemus* Canestrini and Fanzago and *Histiogaster* (Griffiths) are known to be fungal feeders (Moser and Roton 1971, Moser 1985, O'Connor 1990, Cardoza et al. 2008, Moser et al. 2010, Hofstetter et al. 2013). The feeding habits of other mite genera found on MPB, such as

*Parawinterschmidtia* (Khaustov), *Schweibea* Oudemans, *O. montium* Berlese, and *Winterschmidtia* Oudemans, are unknown, but they may be omnivores (Hofstetter et al. 2013).

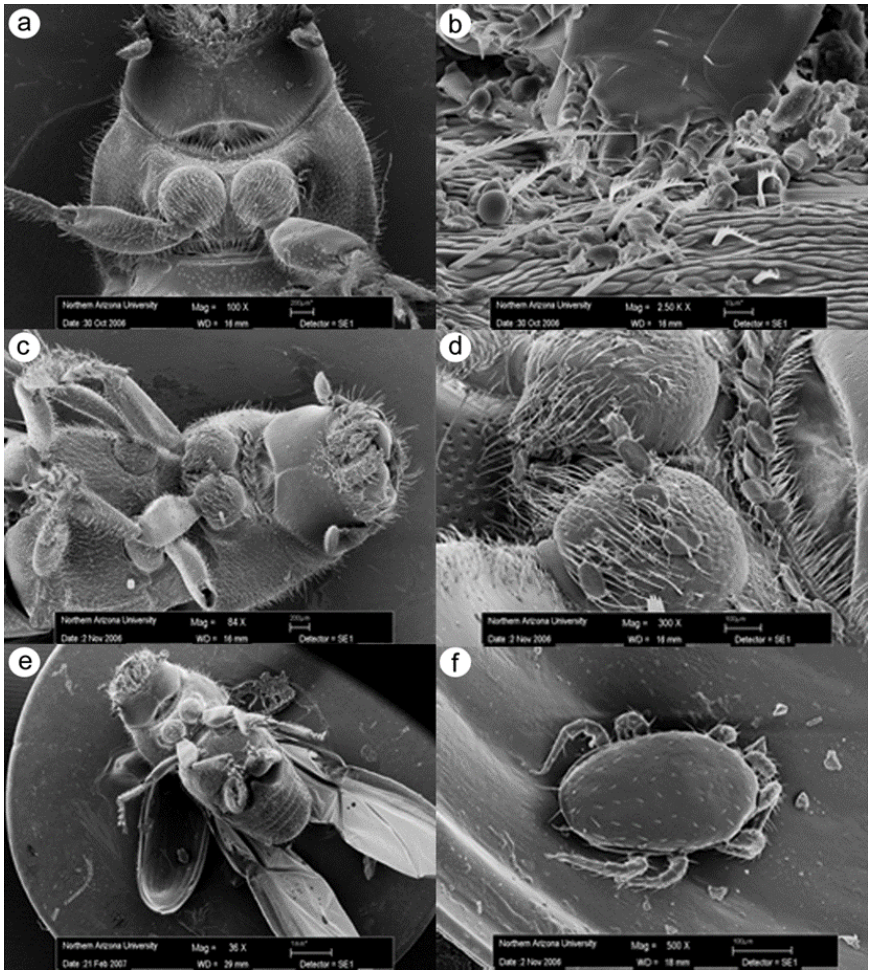


Figure 1.1. Scanning electron microscope images. (a) Phoretic *Tarsonemus* mites located near the first coxa of an adult MBP. (b) Closer look at the first two sets of legs of *Tarsonemus*; note that there is a spore located in the left-hand portion of the image. (c) Species of *Tarsonemus* located on the thorax of an adult MPB. (d) A closer image of (c). The phoretic mites appear in a necklace-like fashion on the beetle. (e) *Proctolaelaps* located in the elytra. (f) A closer image of the body surface of *Proctolaelaps*. (Images by R.W. Hofstetter and D.M. Reboletti.)

Seasonal changes and environmental conditions, among other factors, may influence the bark beetle-associated mite fauna (Hofstetter et al. 2006b). Like MPB, mites are subject to the effects of weather, predation, and disease. Mite abundance, from other bark beetle species studied, is known to fluctuate seasonally (e.g., Hofstetter et al. 2007), develop at rates mediated by temperature (e.g., Lombardero et al. 2000), and experience mortality from extreme temperatures (e.g., Evans et al. 2011) or diseases (Schabel 1982).

## Interactions between Mites and MPB

Phoretic mites can have a direct impact on MPB by effects on beetle free movement or by predation on immature stages. Mites can directly reduce the flight velocity of individual SPBs (Moser 1976). The presence of clusters of mites at the tips of the elytra of Douglas-fir beetle (DFB) (*Dendroctonus pseudotsugae* Hopk.) reduced its wing beat frequency (Atkins 1960). Although no specific studies have been conducted on MPBs, the findings of Moser (1976) and Atkins (1960) suggest that effects may potentially occur by decreasing dispersal and colonization by the beetle.

Mites can also be predators of eggs and larvae of species of *Dendroctonus* (Lindquist and Bedard 1961, Moser and Roton 1971, Kinn and Witcosky 1978, Lindquist 1986). Moser (1975) studied mite species found associated with brood of SPB and concluded that eight species could be useful as natural control agents in reducing field infestations. These included *Histiogaster arborsignis* Woodring, *Proctolaelaps dendroctoni* Lindquist & Hunter, *Macrocheles boudreauxi* Krantz, *Dendrolaelaps neodisetus* Hurlbutt, *Eugamasus lyriformis* McGraw & Farrier,

*Dendrolaelaps neocornutus* Hurlbutt, *Dendrolaelaps isodentatus* Hurlbutt, and *Proctolaelaps fiseri* Samsinak. Species of mites belonging to these genera are associated with MPB (Reboletti 2008, Mori et al. 2011), yet it is not known what affect they may have on its population dynamics.

Mites can indirectly affect MPB by altering the presence and abundance of antagonistic or mutualistic fungi, yeast, bacteria, nematodes, or other invertebrates. For instance, *Tarsonemus* spp. are known to influence the abundance of fungi in SPB-infested trees (Lombardero et al. 2003) and can potentially affect the population dynamics of beetle populations (Hofstetter et al. 2006a). Such interactions may exist in the MPB subcortical environment since mites have been observed in areas of blue-stain fungi such as *Ophiostoma montium* (Rumbold) Arx and *Grosmannia clavigera* (Rob.- Jeffr. and R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wing. in MPB-infested trees.

Figure 1.2. Four *Trichouropoda* spp. (tortoise mites) transported around the front coxae of a MPB that rests on a pine needle. (Photograph by Javier E. Mercado.)

Some of the most common phoretic mite species on MPB are known to carry fungal spores, suggesting that they could vector fungi between trees and within trees. Mori et al. (2011) observed fungal spores attached directly to the cuticle of *P. subcorticalis*, and Reboletti (2008) observed *P. subcorticalis* and *T. ips* carrying spores. In South Dakota, where both mites disseminated spores, Reboletti (2008) found that approximately 70% of the spores transmitted by the two mites were *O. montium*, whereas approximately 30% were *G. clavigera*. Mites in the genus *Histiogaster* Berlese are also known to carry fungal spores when associated with SB (Cardoza et al. 2008) and thus may vector and feed on the beetle's associated fungi as well. Similarly, a species in the genus *O. montium*, a genus found on MPB in at least South Dakota (Reboletti 2008), Alberta (Knee et al. 2012), and Colorado (J.E. Mercado, USDA Forest Service, unpubl. observ., July 2013) (Figure 1.2), is one of the principal vectors of Ophiostomatoids in *Protea* L. flowers in South Africa (Roets et al. 2011). It is possible that MPB-transported *O. montium* mites introduce fungi that could alter the fungal composition in subcortical environments. *T. ips* could help augment the frequency of *O. montium*, the mycangial fungus that prefers warmer temperatures (Six and Bentz 2007), throughout the season in the subcortical niche. Mites thus have the potential to influence fungal communities and abundance within MPB-infested trees and potentially the frequency of mycangial fungi dispersed by MPBs.

Indirect negative effects can also occur between MPB and its phoretic mites. Mites have been shown to carry *B. bassiana* on their surfaces (Renker et al. 2005) and transmit it to the pales weevil (*Hylobius pales* [Herbst]) (Peirson 1921). Another fungal species, the green muscardine fungus (*Metarhizium anisopliae* [Metschn.] Sorokin), was effectively transmitted by a species of *Macrocheles* mite to the pales weevil, causing widespread mortality to the beetles (Schabel

1982). Thus, it is pertinent to examine the potential of mite symbionts in vectoring entomopathogens of MPB.

Changing densities of MPBs within trees can affect the abundance of mycetophagous mites by influencing the fungal species composition within the host trees. The overall phoretic mite community assemblage and abundance may increase with augmentation of MPB densities. As MPB density increases within the tree, total phoretic mite abundance on emerging beetles could increase. Mites that remain within habitats after MPBs have left probably have the greatest mortality or must find other phoretic hosts, such as clerid beetles, secondary bark beetles, or woodborers, to locate new habitats. Thus, mutualism may better explain the relationship between mites that both transport and feed on MPB beneficial fungi.

## The Symbiotic Nematode Fauna

Nematodes are one of the most diverse groups of invertebrates and include many functional groups (Bongers and Bongers 1997) that are common internal and external symbionts in many subcortical beetles (Massey 1974). However, the interactions between bark beetles, nematodes, and other associated organisms have received little attention. The nematode fauna of *Dendroctonus* species in North American has been described for the roundheaded pine beetle (*Dendroctonus adjunctus* Blandf.) (Massey 1966), DFB (Furniss 1967), SB (Cardoza et al. 2008), SPB (Massey 1956), and MPB (Steiner 1932, Thorne 1935, Reid 1958, Massey 1974). Steiner (1932) made the first contribution to the taxonomy of nematode fauna associated with MPB by describing three species collected from MPBs colonizing western white pine (*Pinus*

*monticola* ex D. Don) from northeastern Washington. Subsequently, nine species were described by Thorne (1935) from beetles collected on lodgepole pine (*Pinus monticola* ex Loudon) from northeastern Utah and British Columbia, Canada (Reid 1958). Massey (1974) summarized the biology and taxonomy of species associated with North American bark beetles, adding one species from central New Mexico to that of the MPB. Currently, the associated nematode fauna of MPB includes 13 species that are known to establish ecto- and endosymbiotic relationships with the beetle (Table 1.2).

Table 1.2. Feeding guild and transport site of 13 species of ecto- and endosymbiotic nematodes described for *Dendroctonus ponderosae* in North America.

| Nematode symbionts | Feeding guild | MPB transport site |
|---|---|---|
| *Aphelenchoides tenuidens* Thorne, 1935 | Endoparasite | Digestive tract |
| *Bursaphelenchus conurus* (Steiner, 1932) Goodey, 1960 | Mycetophagous | Under elytra |
| *Bursaphelenchus talonus* (Thorne, 1935) Goodey, 1960 | Mycetophagous | Under elytra |
| *Contortylenchus reversus* (Thorne, 1935) Rühm, 1956 | Endoparasite | Hemocoel |
| *Cryptaphelenchus latus* (Thorne, 1935) Rühm, 1956 | Mycetophagous | Under elytra |
| *Ektaphelenchus josephi* Massey, 1974 | Mycetophagous | Under elytra |
| *Ektaphelenchus obtusus* Massey, 1956 | Mycetophagous | Elytra, hemocoel |
| *Mikoletzkya inedia* Massey, 1966 | Egg predator | Under elytra |
| *Mikoletzkya pinicola* (Thorne, 1935) Baker, 1962 | Egg predator | Under elytra |
| *Neoditylenchus pinophilus* (Thorne, 1935) Goodey, 1963 | Mycetophagous | Undet. |
| *Panagrolaimus dentatus* (Thorne, 1935) Rühm, 1956 | Unknown | Under elytra |
| *Parasitaphelenchus acroposthion* (Steiner, 1932) Rühm, 1956 | Endoparasite | Hemocoel |
| *Sphaerulariopsis hastatus* (Khan, 1957) Nickle 1963 | Endoparasite | Hemocoel |

Nonparasitic nematodes are typically transported externally on the beetle, whereas parasitic species are usually transported internally. Phoretic and parasitic nematodes transported by bark beetles undergo an alternate third larval state known as a dauerlarvae, which is a resting or diapausing state (Poinar 1969). In MPB, dry clusters of dauerlarvae travel at the inner base of

each elytron (Cardoza et al. 2006b) (Figure 1.3). Those in the genus *Ektaphelenchus* Fuchs (Figure 1.4) build a leathery cocoon in which up to 75 immature females have been found (Massey 1974) (Figure 1.5). *Ektaphelenchus obtusus* Massey has been found in pocket-like structures, termed "nematangia" in the hind wing of the SB, but in MPBs collected from Utah, nematangia were not found (Cardoza et al. 2006b).

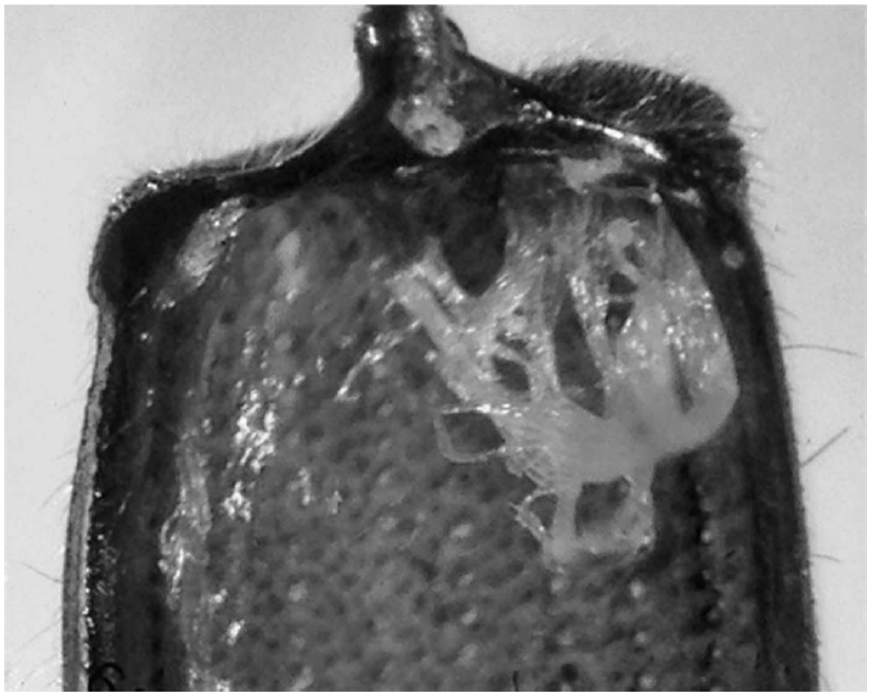

Figure 1.3. A mass of unidentified nematode dauerlarvae travels inside a MBP's elytral base. (Photograph by Javier E. Mercado.)

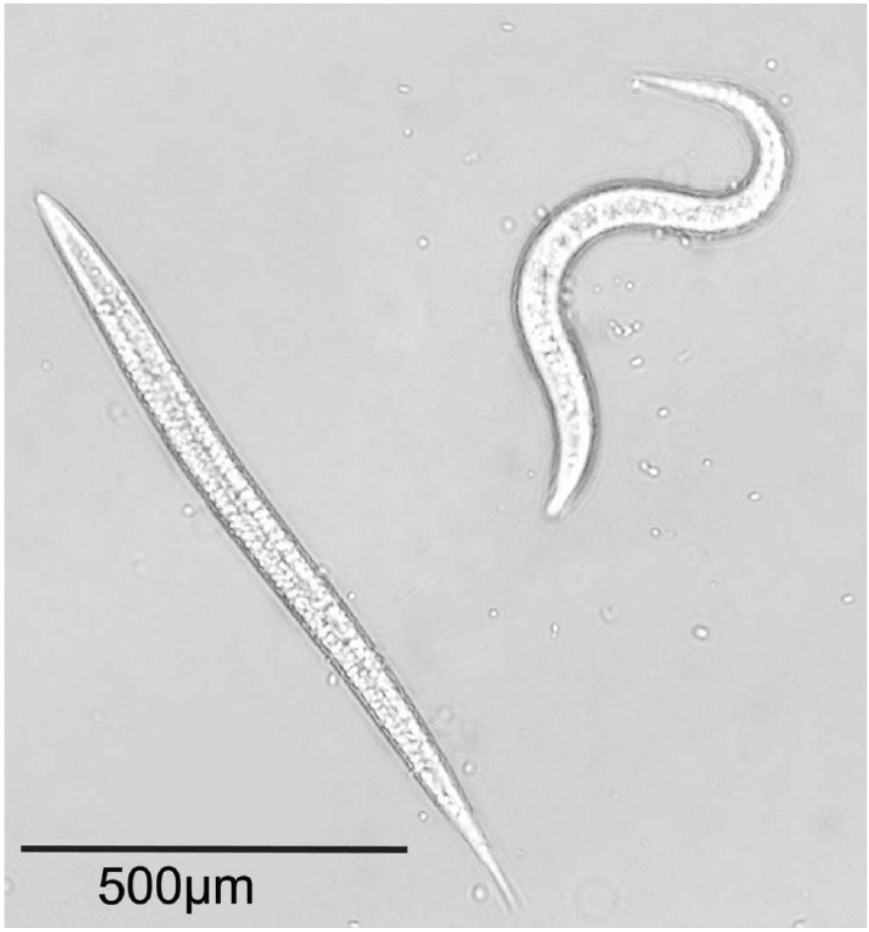


Figure 1.4. Unidentified nematode from inside the elytra of a MPB. Note the spores floating on the medium around the nematode. (Photograph by Javier E. Mercado.)

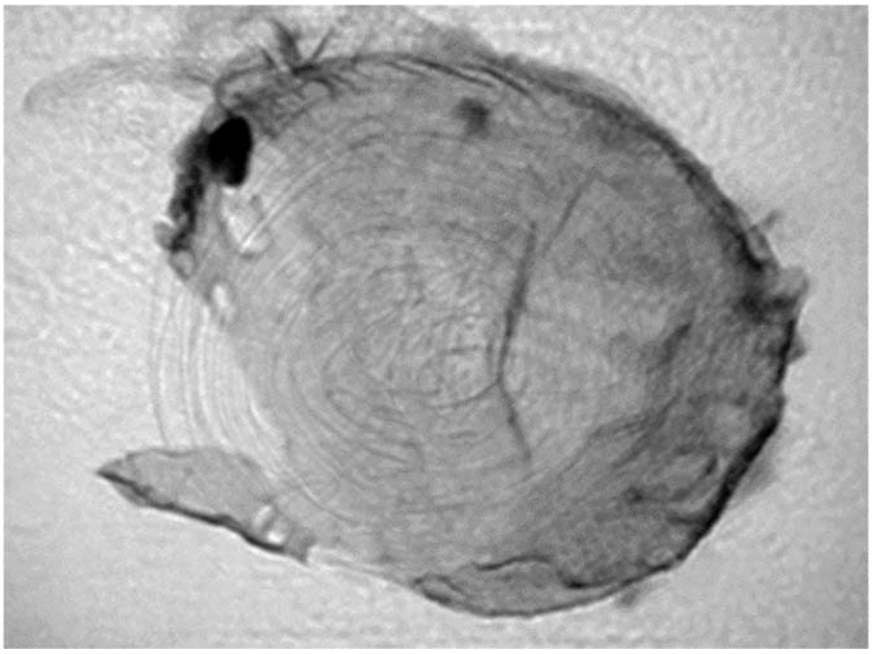

Fig. 1.5. Cocoon-like structure (<1 mm in diameter) created by a phoretic nematode. The structure was removed from under the elytra of a SPB caught in a flight trap. Note that the nematode is still within the structure. (Photograph by Richard W. Hofstetter.

Effects of Nematodes on MPB Populations

Nematodes can develop an array of symbiotic strategies with their transporting hosts. These strategies can be phoretic, parasitic, necromenic (completing development after natural death of host), or predatory (Massey 1974). Studies of parasitic nematode symbionts occurring in *Dendroctonus* species have shown that both null and negative effects can occur. In laboratory experiments in British Columbia, Canada, Atkins (1961) found in general a null effect of phoretic nematodes on DFB during its dispersal to new host trees. The results suggested that the overall flying range capacity of 90 DFBs was not different between beetles lacking nematodes or those with any combination of ecto- and endoparasitic nematodes; however, the first of several

induced flights during a period of 8 hours was significantly shorter for beetles carrying nematodes. Physiologically, nematodes can negatively affect the reproductive success of their beetle hosts. Adult female DFB showed a reduction of 20% of total protein and size of their oocytes when harboring the endoparasitic nematode *Contortylenchus reversus* (Thorne) Rühm (Thong and Webster 1975). Southern pine beetles serving as hosts to *Contortylenchus brevicomi* (Massey) showed a 74% reduction in brood in contrast with that of uninfested individuals (MacGuidwin et al. 1980) and Massey (1956) indicated a reduction in egg production in SB in northern Colorado infested by nematodes in the genus *Sphaerulariopsis* Wachek. In British Columbia, females with the nematode *Sphaerulariopsis hastatus* (Khan) Nickle produced approximately 33% less brood than females lacking these. The infested individuals also exhibited lethargic behavior unlike that of those uninfested by the nematode (Reid 1958). Amman and Cole (1983) found that *Mikoletzkya pinicola* (Thorne) Baker was the principal cause of egg mortality through predation. In addition to reducing egg numbers, nematodes can directly affect the development of various insect stages. MacVean and Brewer (1981) indicated that *Steinernema carpocapsae* (Weiser) can infect all developmental stages of MPB, but only at very high concentrations per individual beetle. They found that early developmental stages were more susceptible, but inoculations of 3,000 nematodes were needed to kill 44 and 66% of larvae and pupae, respectively. The nematode *C. reversus* has a potentially high rate of transmission into MPB brood from parent MPBs. For instance, in Utah, females of *C. reversus* were reported to produce hundreds of eggs in both the larvae and adult hemocoel (Thorne 1935).

Nematodes may indirectly affect MPB through interactions of the associated microbe biota of both organisms. In SB and SPB, mycetophagous nematodes in the genera

*Ektaphelenchus* and *Parasitorhabditis*, genera also found in MPB, associate with fungi as well as bacteria different from those normally associated with their beetle carriers (Cardoza et al. 2006b, Carta et al. 2010). Fungal spores have been observed on nematodes from Colorado (J.E. Mercado, USDA Forest Service, unpubl. observ., July 2013.) (Figure 1.4). However, the interactions between MPB microbes and those of their phoretic nematodes have not been studied.

Little has been published on the direct effects of nematodes on bark beetles, and with the exception of Cardoza et al. (2008), no recent studies have been conducted with nematodes in bark beetles. The available literature indicates that nematodes can reduce the fitness of bark beetles, including MPB. Consequently, nematodes may contribute to maintenance of bark beetle populations at endemic levels. Moreover, the decline of an outbreak of the SB in Colorado during the late 1950s was attributed in part to nematode infections of a species in the genus *Contortylenchus* Rühm due to reduced female fecundity as quantified by McCambridge and Knight (1972). To better understand the population dynamics of MPB, it is important to examine the interaction of nematodes with other associated organisms. Nematode-vectored microorganisms could influence the microbial composition found in carrier beetles and, therefore, that of their subcortical niche.

## Symbiotic Fungi of the MPB

Fungal symbionts are common in the Scolytinae in which they can contribute to beetle nutrition in MPB (Six and Paine 1998, Bleiker and Six 2007) and SPB (Ayres et al. 2000) as well as to important physiological processes, such as metamorphosis and sexual maturation of beetles

(Bentz and Six 2006). Fungal groups in the MPB system include filamentous Ascomycetes (blue-stain fungi), unicellular Ascomycetes (yeasts), and filamentous Basidiomycetes. Many Scolytinae have evolved mycangial harboring structures to transport fungi, suggesting that benefits are derived from these microorganisms (Batra 1967, Whitney and Farris 1970, Farrell et al. 2001). Symbiotic fungi can sometimes negatively affect the health of the beetle's host tree (Brasier 1991, Kolařík et al. 2011).

The most studied group of symbiotic fungal associates of bark beetles are the Ascomycota in the class Sordariomycetes (Linnakoski et al. 2012). This group of fungi is responsible for some of the most severe impacts to plant communities in the United States. The widespread mortality in chestnut (*Castanea dentata* [Marshall] Borkh.) caused by the fungus *Cryphonectria parasitica* (Murrill) M.E. Barr and the mortality caused by the fungi *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier in elms (*Ulmus* L. species) are two classic examples of fungal diseases vectored by bark beetles. Within the Sordariomycetes, all species found in MPB belong to the family Ophiostomataceae. Three sexual genera in this family are associated with MPB: *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Grosmannia* Goid. (nonsexual form *Leptographium* Lagerberg & Melin), and *Ophiostoma* H. & P. Syd. (De Beer et al. 2013). Although the sexual form of *Leptographium longiclavatum* Lee, Kim, and Breuil has not been described, it is considered part of the *G. clavigera* species complex (De Beer and Wingfield 2013). It appears that this species does not reproduce sexually (Roe et al. 2011).

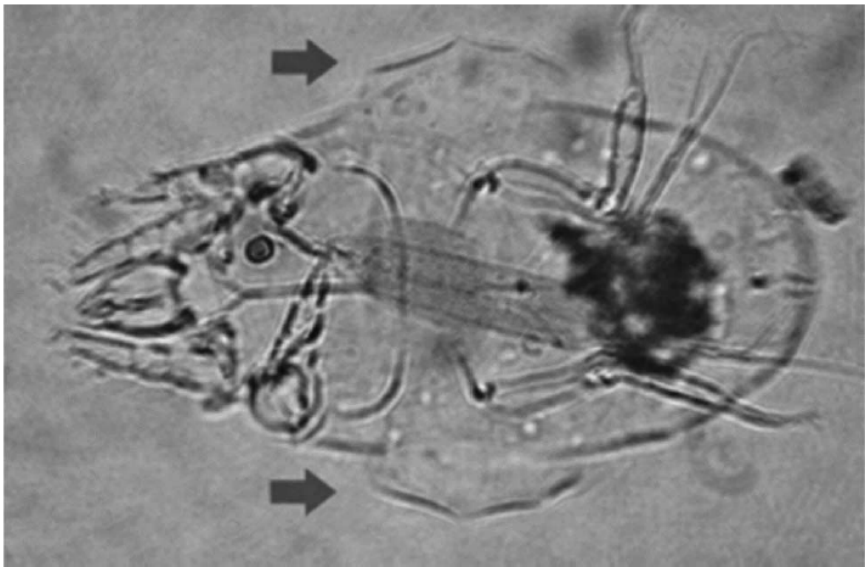

Figure 1.6. *Tarsonemus ips* with view of sporothecae (arrows). Fungal spores are carried and stored in each sporotheca. The mite was collected from a live MPB in the Black Hills National Park, South Dakota. (Image by D. Reboletti.)

Almost 40 years after the first observation of blue-stain fungi symptoms on infected pines (Von Schrenk 1903), the first of the three mycangial fungi associated with MPB, *O. montium*, was described (Rumbold 1941). This species is morphologically similar to *Ophiostoma ips* (Rumbold) Nannf., a species with a much broader distribution that has been collected on rare occasions externally on MPBs (Six 2003). The second MPB mycangial fungus to be described was *G. clavigera*. Both *O. montium* and *G. clavigera* are found throughout the MPB's distribution, from Northern Baja California in Mexico (Mock et al. 2007) to areas where MPB has recently expanded into northwestern Alberta, Canada (Cullingham et al. 2012). As suggested by studies looking at numerous loci, *G. clavigera* may actually be a complex of two cryptic sibling species; each better equipped to inhabit either lodgepole or ponderosa pines (Alamouti et al. 2011). The most recently described mycangial blue-stain fungus was *L. longiclavatum*. Since

its discovery in lodgepole pine from Canada (Lee et al. 2005), it has been found in the northern range of MPB across Canada where it also occurs in lodgepole X jack pine (*Pinus banksiana* Lambert) hybrids in areas of Alberta (Rice and Langor 2009). In the United States, this species has been found in AZ, CO, ID, MT, NV, OR, SD, UT, and WA (Dario Ojeda, University of British Columbia, pers. comm., Feb. 24, 2014). It is probable that because its optimal growing temperature is similar to that of *G. clavigera* (Lee et al. 2005) and because of its ability to grow in widely distributed lodgepole and ponderosa pines, *L. longiclavatum* may be as widely distributed as *G. clavigera*.

These fungi have evolved to be transported by bark beetles and their phoronts in several ways of varying complexity. Sexual and nonsexual spores of the Ophiostomatales are dispersed in sticky secretions that adhere to the exoskeleton of insects living in their subcortical niches (Malloch and Blackwell 1993). The asexual spores or conidia of *O. montium* and *G. clavigera*, two mutualistic Ophiostomatoids, are commonly transported in the beetle's mycangia (Bleiker and Six 2009). Externally, phoretic fungi are transported by beetles in several ways. Their spores can attach to setae or to exoskeletal pits. The pits in the elytra are thought to work as simple mycangia and have been shown to carry *G. clavigera* and *O. montium* (Six 2003, Bleiker and Six 2009). In addition, fungi can be transported by phoretic mites (Moser 1985, Moser et al. 1989b, 2010, J.E. Mercado, USDA Forest Service, unpubl. observ., July 13, 2013) (Figure 1.6) and nematodes (Cardoza et al. 2008, Suh et al. 2013) (Figure 1.4). The proportion of fungal spores that adhere to beetles can differ between un-emerged and emerged beetles (Six 2003). Six (2003) reported that MPBs contained higher spore loads of *O. montium* when still in their galleries compared with after emergence.

Table 1.3. Common fungal symbionts of *Dendroctonus ponderosae* in North America.

| Fungal symbiont | Symbiotic | Beetle transport site |
|---|---|---|
| OPHIOSTOMATALES (BLUE-STAIN FUNGI) | | |
| *Ophiostoma montium* (Rumbold) Arx | Mutualist | Mycangia, exoskeleton |
| *Grosmannia clavigera* (R.C. Rob. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. | Obligate mutualist | Mycangia, exoskeleton |
| *Leptographium longiclavatum* S.W. Lee, J.J. Kim & C. Breuil | Mutualist | Mycangia, exoskeleton |
| SACCHAROMYCETALES (YEASTS) | | |
| *Ogataea pini* (Holst) Y. Yamada, M. Matsuda, K. Maeda & Mikata | Mutualist | Gut, Exoskeleton |
| *Kuraishia capsulata* (Wick.) Y. Yamada, K. Maeda & Mikata | Mutualist | Gut, Exoskeleton |
| *Nakazawaea holstii* (Wick.) Y. Yamada, K. Maeda & Mikata | Mutualist | Gut, Exoskeleton |
| *Yamadazyma scolyti* (Phaff & Yoney.) Billon-Grand | Mutualist | Gut, Exoskeleton |
| RUSSULALES (FILAMENTOUS YEAST) | | |
| *Entomocorticium dendroctoni* Whitney | Mutualist | Mycangia |

Ophiostomatales follow the nomenclator in De Beer et al. (2013).

Other non-staining Ascomycetes are also vectored by MPB. A species similar to *Ceratocystiopsis minuta* (Siemaszko) H.P. Upadhyay & W.B. Kendr. but genetically close to *Cop. ranaculosa* (Plattner et al. 2009) has been documented on MPBs in Colorado (Upadhyay 1981) and British Columbia, Canada (Robinson 1962, Kim et al. 2005, Lee et al. 2006a). Khadempour et al. (2012) found that in British Columbia, probably that same species (*Ceratocystiopsis* sp. 1 until formal description) was the only fungal species having a development cycle that positively correlates significantly with developing MPBs. *Cop. ranaculosa* is a nutritionally important mycangial mutualist of SPB (Klepzig et al. 2001), and similar relationships are possible in other *Dendroctonus* species.

Other fungi, including Basidiomycetes and yeasts, have been found in MPB mycangia. Whereas yeasts are frequently found in the mycangia, the associations with Basidiomycetes in the genus *Entomocorticium* H.S. Whitney, Bandoni & Oberw. (perhaps not a true symbiont but treated as such in this article) seem looser, because these have not been found in that specialized transporting structure (Lim et al. 2005, Lee et al. 2006a, Khadempour et al. 2012). The agaricomycete *Entomocorticium dendroctoni* Whitney and another undescribed species in that genus were found less frequently than *G. clavigera* but more abundantly than *L. longiclavatum* in parts of Canada (Lee et al. 2006a, Khadempour et al. 2012). Other species of *Entomocorticium* as well as species in the genus *Phlebiopsis* Jülich have been documented on MPBs from California, Colorado, and Canada (Hsiau and Harrington 2003), but it is not known whether these represent frequent phoretic associates.

Several yeasts that were originally placed in the genus *Pichia* Hansen (Table 1.3) and that are now placed in the genera *Ogataea* Yamada, Maeda, et Mikata; Kuraishia Yamada, Maeda, et Mikata; Nakazawaea Yamada, Maeda, et Mikata; and Yamadazyma Billon-Grand (Billon-Grand 1989, Yamada et al. 1994, 1995) have also been collected externally from MPB, although these are typically found in the insect's gut. A variety of Ascomycota and Basidiomycota including saprobes have been collected externally from MPB (Six 2003, Kim et al. 2005, Lim et al. 2005) but are not usually associated with the beetle and could represent opportunistic "hitchhikers".

## Virulence of MPB Fungal Symbionts

Phytopathogenicity is the ability of an organism to cause disease to plants (Shaner et al. 1992). In some Ophiostomatales associated with bark beetles, phytopathogenicity is indicated by the staining of the wood. Wood stain diseases are not always a cause of death in mature trees, as is evidenced by *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. which is used as biological control of related virulent fungal species (Dunn et al. 2002). However, on rare occasions symbiotic Ophiostomatales are virulent, contributing to or killing the trees in which they are vectored by beetles (Parker et al. 1941, Solheim and Safranyik 1997, Kolařík et al. 2011). For instance, the most destructive tree-killing bark beetle species in the northern hemisphere, the MPB and the European spruce bark beetle (*Ips typographus* L.), vector at least one tree-killing fungal associate (*G. clavigera* and *Ophiostoma polonicum* [Siemaszko] C. Moreau, respectively). This has been shown under artificial inoculations while trying to remove the beetles' tree killing effect (Christiansen and Solheim 1990, Solheim and Krokene 1998).

The virulence of the mycangial Ophiostomatoids associated with MPB has been suspected for a long time. Von Schrenk (1903) first described the fungus development in sapwood. His depiction of the blue-stain fungus growing into the parenchyma ray cells and into the tracheids (Figure 1.7) may represent *G. clavigera* or *O. montium* and not *O. piliferum*, the species he considered as the pathogen affecting ponderosa pine in the Black Hills. MPB vectors three pathogenic (blue-stain) Ophiostomatoids: *G. clavigera*, *O. montium*, and *L. longiclavatum* to their conifer hosts, each exhibiting different degrees of virulence. After being introduced into a tree, these fungi grow into the phloem, penetrating the xylem through the parenchyma rays and destroying them (Ballard et al. 1982, 1984). After this, hyphae extend radially, reaching the heartwood margin, where radial growth stops. The hypha grows through the half-bordered pits into adjacent tracheids (Von Schrenk 1903, Rumbold 1941) where it displaces the tori (Ballard et al. 1982, 1984). Both *O. montium* and *G. clavigera* have been shown to reach the heartwood of lodgepole pines in Canada in a period of about 5 weeks; however, *G. clavigera* is usually the first to reach the heartwood (Solheim 1995) because of a greater tolerance of that species to the high moisture and less oxygenated properties of healthy sapwood (Solheim and Krokene 1998). Blue-stain fungi growing into pine sapwood is believed to occlude it with fungal material or resin (Tyree and Sperry 1989, Wullschleger et al. 2004). A symptom of sapwood occlusion may be the decline in transpiration usually observed within 10 days after infection (Yamaoka et al. 1990, Hubbard et al. 2013). In experiments in Colorado, Hubbard et al. (2013) observed that transpiration in infected trees declined to 40% of that observed in control trees 2 months after the infection.

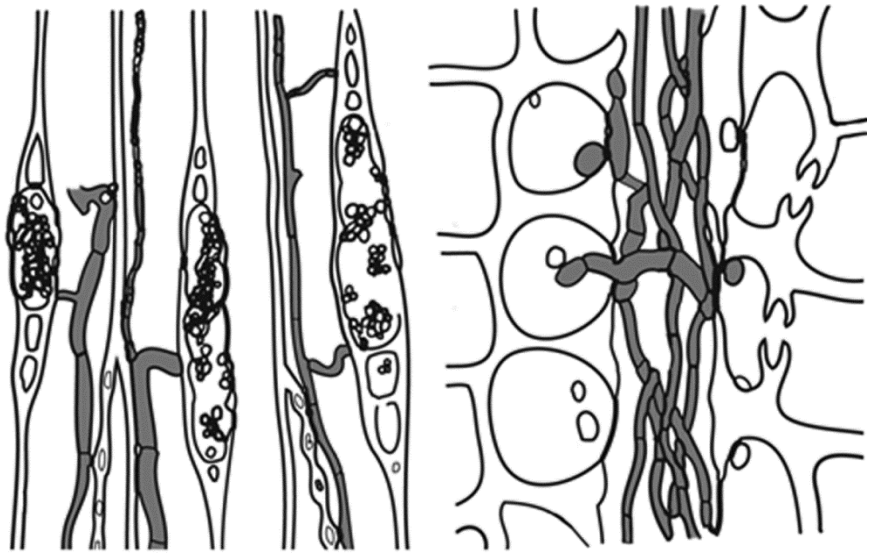

Figure 1.7. A tangential (left) and a radial (right) view of the blue-stain fungal hyphae (gray) growing on the tracheids and on the xylem's parenchyma rays as described from ponderosa pine in the Black Hills in the early 1800s. (Modified from Von Schrenk 1903.)

A fascinating aspect of the MPB-fungi interactions is teasing out the contribution that these two organisms may have in causing tree death. The plurality of the research addressing this question has been driven by what Six and Wingfield (2011) elegantly called the "classic paradigm." As they describe it, the paradigm itself is composed of two hypotheses. The first hypothesis suggests that tree mortality is the result of fungal invasion into the xylem, disrupting water flow; alternatively, the second suggests that fungal invasion of the phloem results in the depletion of tree defenses, which allows a successful bark beetle colonization (Six and Wingfield 2011), resulting in girdling and tree death.

Several studies have examined the separate impact of fungal inoculation and girdling to trees: the physiological effect of the fungus (i.e., xylem blockage) and the "mechanical effect" of the beetle (i.e., girdling). The impacts of fungal inoculations are difficult to isolate because these

usually require some amount of damage to the phloem. The virulence to pines caused by blue-stain Ophiostomatoids found on MPB was tested during artificial inoculations (Mathre 1964, Strobel and Sugawara 1986, Yamaoka et al. 1995, Lee et al. 2006b, Solheim and Krokene 1998) with different amounts of phloem removal. Virulence of *O. montium* was tested in 10- to 15 year-old ponderosa pines by inoculating trees on top of a girdled 40 cm band (Mathre 1964). Tree mortality caused by *O. montium* infection occurred within 15–22 days. Although this method did not remove the effects of girdling, it caused a much faster tree death than otherwise would have been observed by girdling alone (see Mathre 1964). In other studies, the killing capacity of *O. montium* in lodgepole pine was shown to be significant by Strobel and Sugawara (1986), only when a considerably large portion of the phloem was removed, for which they recorded 88% mortality; but death occurred two seasons after treatment. However, in the same study, a method leaving most of the phloem intact only killed one of six infected trees. Yamaoka et al. (1990) measured sap flow under a healthy section of lodgepole nested between two girdled bands that were reattached after they were inoculated with *G. clavigera* and *O. montium*, among other fungi. Their results showed that *G. clavigera* reduced sap flow more significantly than the other fungi. Yamaoka et al. (1995) artificially inoculated *G. clavigera* and *O. montium* under phloem flaps, carefully leaving alternate portions of phloem intact. In these experiments, the capacity of *G. clavigera* to kill mature lodgepole pines was found to be greater than that with *O. montium*. The virulence of the mycangial fungus *L. longiclavatum* was inferred by the length of lesions caused during artificial inoculations to lodgepole pine (Lee et al. 2006b), jack pine, and to jack X lodgepole pine hybrids in Canada (Rice et al. 2007). Jack pine appeared to be more susceptible than lodgepole pine to the three mycangial MPB fungal associates (Rice et al. 2007). In addition to carrying the cold-tolerant *G. clavigera*, the novel host association of MPB with *L.*

*longiclavatum* may have contributed to the beetle's expansion into new areas in Alberta, Canada and may increase its chance of dispersal through the boreal forest and into the eastern United States (Safranyik et al. 2010).

If we hypothetically remove the fungi from the beetle, the sole effect of MPB to tree death is the girdling damage of the phloem. However, even the complete girdling of a pine tree does not cause death in less than 1 year or "rapid death" (Craighead 1928, Hubbard et al. 2013). Girdled trees are capable of maintaining healthy physiological activity related to transpiration. For example, girdled lodgepole pines maintained transpiration rates well into the growing season after a beetle attack (Hubbard et al. 2013), and girdled ponderosa pine presented changes in xylem embolism and conductivity similar to those of control trees during the growing season (Domec and Pruyn 2008). The time it takes for a girdled conifer to die is highly variable, but often exceeds 1 year (Noel 1970, Wilson and Gartner 2002). However, pines completely and successfully colonized by the MPB and its associated blue-stain fungi typically die within 1 year after the attack. One of the first symptoms presented in trees colonized by MPB is a rapid reduction in transpiration (Yamaoka et al. 1990, Hubbard et al. 2013).

In summary, studies on the separate effects of fungal inoculation and girdling suggest that no single component causes rapid tree mortality but that perhaps there is a synergistic effect achieved by the combined impacts of both organisms. We may need to pursue new thinking and creative avenues of research to explain these interactions. For example, the loss of sapwood conductivity recorded by Yamaoka et al. (1990) and Hubbard et al. (2013) is a symptom of embolism in the sapwood (Tyree and Sperry 1989). A mechanism of embolism repair proposed

by Salleo et al. (1996, 2004) involves the translocation of sugars in addition to water to embolized sapwood by healthy phloem (Salleo et al. 1996, 2004). The disruption of this mechanism, caused by damage to the phloem, could explain the rapid tree mortality caused by the combined effect of MPB and its blue-stain fungi.

## Beneficial and Antagonistic Relationships between MPB and Its Fungal Symbionts

Many insects have evolved intimate relationships with fungi and derive benefits from species they culture and protect. The culture of fungi or fungiculture has evolved in three different groups of insects: ants, termites, and bark beetles (Mueller et al. 2005). Recently, behaviors such as mycocleptism, the stealing of fungi (Hulcr and Cognato 2010), and the use of certain bacteria as selective fungicides (Cardoza et al. 2006a, Scott et al. 2008) that protect the bark beetle's beneficial fungi have been documented.

The presence of protective mycangia, specialized for the transport of fungi in MPB, suggests a long-established mutualism. Fungi may benefit MPB by providing hospitable conditions in areas occupied by the beetle's developing brood. For instance, the ophiostomatoid *G. clavigera* may aid in the establishment of the beetle by exhausting tree defenses (Lieutier et al. 2009, but see Six and Wingfield 2011), and the fungi benefit from the beetles rapid vectoring through a recently attacked tree where little competition with other fungi occurs. Both blue-stain fungi and MPB have life strategies that mutually benefit their establishment. These have probably co- evolved to develop in a tree before tree death. Killing a tree too rapidly (i.e., before MPB saturates the available phloem space) is probably detrimental to both fungi and MPB as the

two colonizers benefit from low competition during early attack stages until their successful establishment (Kim et al. 2005, Khadempour et al. 2012).

*Grosmannia clavigera* can also benefit MPB by detoxifying the terpenoids present in the defensive oleoresin of attacked trees (DiGuistini et al. 2011), creating a safer environment for its developing brood. The two primary mycangial fungi can also benefit the beetles by redistributing nutritional components, such as nitrogen, where concentrations are not adequately available on the tree's phloem (Bleiker and Six 2007). *G. clavigera* is better at concentrating nitrogen in the beetle's developing area than *O. montium* (Cook et al. 2010). In addition, these fungi may provide sterols that are required for the synthesis of pheromones by adult beetles (Bentz and Six 2006) and potentially serve as a maturation resource for the insect (Six 2003).

*Dendroctonus* individuals with a greater body mass (10.8 mg versus 10.0 mg) were found to have better reproductive fitness (Elkin and Reid 2005), a greater flying capacity (Williams and Robertson 2008), and a greater tolerance against host tree defenses (Reid and Purcell 2011) than smaller conspecifics. The fitness of MPB can be affected by the species of fungal associates consumed during its development. In laboratory experiments MPB brood that fed on stem sections inoculated with *G. clavigera* developed more rapidly and in greater numbers than those in stem sections inoculated with *O. montium*, and broods were not produced in the absence of any of these fungi (Six and Paine 1998). The symbiotic relationship between *O. montium* and MPB could be less specific than the one with *G. clavigera*, because it has been found to be less restricted to the mycangia (Six 2003). Mutualistic organisms benefit from the synchronization of their development (Boucher et al. 1982). Although the development of *O. montium* overlaps with

all developmental stages of MPB, that of *G. clavigera* was found to be more common during the teneral stage (Khadempour et al. 2012). The synchronization of *G. clavigera* with the dispersal stage of MPB suggests a stronger affinity of that fungus with the beetle. Because *O. montium* has been documented from *ips* pini (Say) (Lim et al. 2005), *Ips* perturbatus (Eich.) (Alamouti et al. 2007, Rudski 2011), and mites carried by MPB (Reboletti 2008, J.E. Mercado, USDA Forest Service, unpubl. observ., July 2013), this species does not rely exclusively on MPB for its establishment on new trees, although the beetle is perhaps its most important vector. Several findings suggest that *O. montium* is less important to MPB than *G. clavigera*. MPB can survive and reproduce successfully with *G. clavigera* alone (Six and Paine 1998), making the mutualistic relationship between MPB and *O. montium* not obligate but facultative. *O. montium* is considered to have established an association with MPB more recently than *G. clavigera* (Bleiker et al. 2009). In addition, *O. montium* may provide fewer nutritional benefits to MPB, making it less favorable to the beetle, as suggested by the smaller beetle brood size produced in association with this fungus versus that with *G. clavigera* (Six and Paine 1998, Bleiker and Six 2007). However, both species appear to benefit MPB, given their ability to grow during different environmental conditions (Six and Bentz 2007), providing a nutrition source in a changing environment (Six 2012), a scenario that may indicate adaptive characteristics of MPB-fungal associations.

Yeasts also make important contributions to the establishment and development of MPB on newly attacked trees. The species *Ogataea pini* (Holst) Y. Yamada, M. Matsuda, K. Maeda & Mikata can indirectly benefit MPB by promoting the growth of at least one of the mycangial fungi, *O. montium* (Rumbold 1941). The yeasts *Kuraishia capsulata* (Wick.) Y. Yamada, K.

Maeda & Mikata and *O. pini* were found to benefit MPB both indirectly and directly by oxidizing tree terpenes that are harmful to both fungus and beetle (Hunt and Borden 1990). They may also contribute to the attack behavior of MPB by regulating the conversion of cis- and trans-verbenone into verbone (Hunt and Borden 1990), an anti-aggregation pheromone that fosters secession of attack, which may provide benefits by reduced competition. In addition to the benefits provided by the Ascomycota, Basidiomycetes are considered important nutritional mutualists of MPB (Whitney et al. 1987). For example, *Entomocorticium* species contribute to the nutrition and brood success of *Dendroctonus* species such as the SPB (Klepzig et al. 2001, Hofstetter et al. 2006a) and the western pine beetle (*D. brevicomis* LeConte) (Paine et al. 1997, Davis et al. 2011). Species in this genus enrich the beetles' developing substrate more efficiently than other associated fungi (Ayres et al. 2000). *Entomocorticium dendroctoni* was found to increase the egg production of MPBs by 19% (Whitney et al. 1987). Species of *Entomocorticium* are known to positively interact with yeasts in other *Dendroctonus*-fungal systems. The yeast *O. pini* was shown to increase the growth of the beneficial *Entomocorticium* sp. B in the western pine beetle system (Davis et al. 2011), and similar interactions may occur in the MPB system.

Antagonistic relationships have also been identified between beetles and their microbial biota. Entomopathogenic fungi have been mentioned in the literature as being widely distributed along with MPB (Safranyik et al. 2001); however, this information has not been quantified. One pathogen from this group, *B. bassiana*, has been collected from oral secretions of beetles from Colorado and Utah (Cardoza et al. 2009). The pathogenicity of a sympatric wild strain of this entomopathogen was tested against MPB under laboratory conditions in British Columbia, Canada, and although the pathogen had a low germination rate on the beetle's body surface, it

was effective in killing (Hunt et al. 1984). It is probable that entomopathogenic fungi reach MPB surfaces indirectly through phoretic mites (see Mites section); however, this phenomenon remains undetermined.

Indirect Multitrophic Effects of Fungal Symbionts of MPB Phoronts

Some phoretic mites and nematodes transported by MPBs are mycetophagous, carrying the fungus on which they feed to freshly attacked trees. Like their insect vectors, several phoretic mites have coevolved life histories with mutualistic fungi and transport them in specialized "flap-like" structures, called sporothecae (Figure 1.6) (Moser 1985). The fungi vectored by MPB's phoretic mites may affect MPB fitness. For example, in the SPB, *T. ips* transports *O. minus*, an antagonistic species that diminishes the reproductive success of SPB by outperforming the growth of its beneficial mycangial fungi and *Entomocorticium* sp. A (Klepzig et al. 2001, Lombardero et al. 2003). It will be important to examine whether *T. ips* vectors antagonistic fungi in the MPB system as well.

As in mites, the effects to trees and to the beetle of fungi associated with phoretic nematodes are unknown. Nematodes transported by other phytophagous beetles can vector fungi; the most notable example might be the pine wood nematode (*Bursaphelenchus xylophilus* [Steiner and Buhrer] Nickle), transported by species of *Monochamus* Dejean. This nematode was found associated with *O. ips* and *O. minus* in parts of the United States (Wingfield 1987). The ectosymbiotic nematode genus *Ektaphelenchus* also has been found in association with *Ophiostoma*toid fungi. Cardoza et al. (2006b) cultured species of *Ophiostoma* different from

those typically associated with the SB from the nematangia (a nematode harboring structure) of *E. obtusus*. The potential indirect effects of fungi that may be carried by species of *Bursaphelenchus* Fuchs and *Ektaphelenchus* on MPB are not known.

## MPB Bacterial Associates

Other important microbial symbionts of MPB are bacteria, which are usually transported internally but can also be excreted in the beetle's frass or secreted orally (Cardoza et al. 2006a, 2009). Their roles are not well understood, but some species have been found to have fungicidal, nutritional, and antagonistic effects on the fungal fauna present in bark beetle systems (Cardoza et al. 2006a, Adams et al. 2008).

All insects are associated with bacteria that can either be transported phoretically outside the body or as primarily gut symbionts where they perform different functions. The study of bacteria in bark beetles has expanded recently, including that with MPB. The associations between bacteria and MPB's blue-stain fungi were first noticed by Rumbold (1941). A single actinobacterium in the genus *Microbacterium* Lehmann and Neumann was isolated from the gut of MPB specimens from Colorado and Utah, whereas more recently two species of *Streptomyces* Waksman & Henrici were recovered from the body surface of 14% of sampled MPBs (Hulcr et al. 2011). In addition, 12 species of bacteria were cultured from living MPB larvae in British Columbia (Winder et al. 2010). Recently, six bacteria in the genera *Serratia* Bizio; *Rahnella* Izard, Gavini, Trinel, and Leclerc; *Pseudomonas* Migula; and *Brevundimonas* Segers were documented from MPB in Alberta and British Columbia, Canada (Boone et al. 2013). Because

bacteria are some of the most diverse groups of microorganisms on earth, the current knowledge probably represents only a small fraction of the total diversity and interactions associated with MPB.

## Beneficial and Antagonistic Effects of Bacterial Symbionts

Complex interactions occur in the insect groups practicing fungiculture. For example, species of *Acromyrmex* Mayr leaf-cutting ants have evolved the capacity to transport bacterial species of *Streptomyces*. These ants exploit the antifungal properties of *Streptomyces* by using them as fungicides to control the growth of antagonistic fungi in their fungal gardens (Currie et al. 1999). As for the leaf-cutting ants, SPBs have been shown to use bacteria in their oral secretions to kill unwanted fungi (Cardoza et al. 2006a). The common soil bacterium, *Micrococcus luteus* (Schr.) Cohn inhibited the growth of a species of *Aspergillus* Micheli found invading the galleries of SBs in Alaska (Cardoza et al. 2006a).

Some species of bacteria have been found to inhibit the growth of mycangial symbionts of MPB. *Bacillus subtilis* (Ehrenberg) Cohn was found to inhibit the growth of *G. clavigera* and *O. montium*. This bacterium was collected from portions of the phloem that were neither attacked by MPB nor colonized by mycangial symbionts in Montana (Adams et al. 2008). Moreover, *B. subtilis* was found in oral secretions of MPB, suggesting it may be an associated bacterium (Cardoza et al. 2009). Whether the MPB avoids areas infected by this bacterium or whether there is a type symbiosis between MPB and this bacterium warrants further investigations. Several species of bacteria have been shown to cause different effects on the two most common MPB

mycangial Ophiostomatoids. In western Montana, a species of *Micrococcus* Cohn antagonized the growth of MPB's most beneficial blue-stain fungi, *G. clavigera*, whereas it benefited the growth of *O. montium* in bioassays in which the three organisms were grown together (Adams et al. 2008). Conversely, *Pseudomonas fluorescens* Migula and *Pectobacterium cypripedii* (Hori), recovered from the beetle's mouthparts and larvae in another Montana study, significantly increased the growth and spore formation of *G. clavigera* in the presence of α-pinene, whereas *P. cypripedii* significantly decreased the growth of *O. montium* (Adams et al. 2009). A situation in which the most affected blue-stain fungus was *O. montium* was observed in British Columbia in areas of significant larval mortality, where *Serratia liquefaciens* (Grimes and Hennerty) and *Serratia plymuthica* (Lehmann and Neumann), isolated from 16% of live cultured MPB larvae, decreased the growth of *O. montium* by about 70% and that of *G. clavigera* by 40% (Winder et al. 2010). Similar to fungi, bacteria may also help detoxify terpenoids in the MPB host tree, which may contribute to the successful establishment of the insects. Adams et al. (2013) and Boone et al. (2013) found that bacteria in the genera *Serratia*, *Rahnella*, and *Brevundimonas* can metabolize tree defensive terpenoids such as diterpene abietic acid, a terpenoid shown to inhibit the growth of *G. clavigera* and *O. montium* (Boone et al. 2013). These important findings indicate that the study of bacteria in MPB and their potential use by the beetle is of importance and deserves more attention.

## Concluding Remarks

An array of symbiotic organisms are carried by and shares life histories with MPB. Our general knowledge is based on a limited number of places across the vast distribution of the

beetle. Studies to date suggest that mites affect colonization and dispersal and can drive populations of related bark beetle species. The most common mites found in the MPB may be mutualistic because they vector blue-stain fungi species beneficial to the beetle, but the prevalence of these associations vary under different population and climate scenarios. Nematodes can affect flight capacity and reproductive potential and thus have an impact on MPB, but to become effective, these may require high numbers. Fungi and bacteria often synergize, modifying the otherwise inhospitable subcortical niche where beetles develop. After more than 100 years of learning about MPB, many questions on the association among these organisms and the MPB still remain. For example, although we have considerable knowledge about the association between *G. clavigera* and *O. montium*, it still needs further clarification. In addition, the growing study field of bacterial associates suggests specialized interactions between these and beetles; for instance, their use in fungiculture, in which some aid mutualistic fungi while simultaneously limiting the growth of antagonistic species.

Multitrophic interactions in the subcortical niche are rich and complex and many remain unexplored. Obtaining a broader understanding of the multitrophic interactions in different contexts may uncover a wealth of information concerning ecological factors that may drive population fluctuations of this important landscaping agent. The cryptic habitat that MPB and its microscopic symbionts use presents challenges to investigation. Slow-growing species that do not invade the sapwood such as *Ceratocystiopsis* sp. 1 may provide new information on the nutritional requirements of the MPB, yet the identity of this species remains unresolved. The mechanisms explaining the synergistic effects between the girdling caused by MPB and the sapwood invasion by blue-stain fungi, demand research that satisfies the different theories about

their impact on rapid tree mortality. Among the most common blue-stain fungi, *G. clavigera* has been found to be more beneficial to the MPB than *O. montium*, but how its benefit compares with those of *L. longiclavatum* and *Ceratocystiopsis* sp. 1 remains to be explored. Khadempour et al. (2012) initiated this endeavor by connecting the developmental synchronicity of fungi with the beetle and the dietary significance for the insect.

The type, strength, and reliability of the symbiotic relationships can greatly influence MPB dynamics. Palmer et al. (2003) outlined three general factors that influence mutualism strength or specificity: the variability in the "quality" (in terms of benefits) of alternative partner species; the reliability/dependence of mutualist species; and the effectiveness of partner selections. Thus, partnership consistency is a key element of long-term mutualist associations, and mycangial fungi and MPB are a good example of it (Klepzig and Six 2004). It is thought that the relative strength and importance of most mutualisms vary temporally and spatially with respect to the extent that they confer reciprocal benefits (Bronstein 2001). This hypothesis implies that some level of context dependence is inherent in many mutualisms (Bronstein 1994), and, in fact, many symbiotic interactions in the MPB community range from mutualistic to commensal to antagonistic, given various sets of environmental conditions, resource quality, and the presence of particular species (Klepzig and Six 2004). We are beginning to see a picture in which MPB symbionts can be consistently found in association with the insect and filling the gaps of information will clarify the symbiotic characters of these organisms.

A missing piece of the puzzle is how interactions may be affected under a climate change scenario. The main blue-stain fungal species composition seems to be sensitive to temperature

changes (Six and Bentz 2007), but whether this relates solely to the fungi growing requirements or to population variations of other phoretic symbionts that also vector the fungi still needs to be explored. Extreme climatic events and migration of host trees will undoubtedly have an effect on the distribution of climate-sensitive associates and dynamics of the interactions. Expanding our knowledge base about these effects will further increase our capacity to explore potential ways of using the actions of MPB-associated organisms as methods of biological control. We hope that the information presented will help students, managers, and scientists in formulating and addressing key ecological processes among these fascinating organisms.

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Chapter 2. Phoretic mite assemblages on mountain pine beetles (*Dendroctonus ponderosae* Hopk.) attacking limber, lodgepole, and ponderosa pines in northern Colorado.

## Introduction

Since 1996, Colorado's pine forests have been impacted by a large mountain pine beetle (*Dendroctonus ponderosae* Hopkins) epidemic. Lodgepole pine (*Pinus contorta* Douglas) forests have sustained the largest impact as most of the State's 1.5 million acres of this cover type have experienced mortality above the normal endemic levels (CO St. FS 2013). In addition to lodgepole pine in northern Colorado, other principal hosts of mountain pine beetle include limber pine (*Pinus flexilis* E. James) and ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson) (Wood 1982). Mountain pine beetle tree attacks involve the introduction of nematode, fungal, mite, and bacterial associates of the beetles (Thorne 1935, Lee et al. 2005, Mori et al. 2011, Cardoza et al. 2009). Of these associated organisms, fungi have been most studied given their capacity of growing into the sapwood clogging tracheids contributing to the tree's death (Ballard et al. 1982, 1984, Solheim 1995, Hubbard et al. 2013). Studying trophic and non-trophic interactions between multiple organisms living in a subcortical habitat is challenging due to their cryptic habitat; therefore, few studies have attempted to describe how interactions between beetles and their associates can influence beetle populations (Lombardero et al. 2000, 2003, Hofstetter et al. 2006). Understanding which organisms are involved in these interactions and their potential contributions represents the foundation to pursue complex questions in relation to their effects on beetle populations. Until recently, our knowledge about fungal dispersal on this symbiosis was that mountain pine beetle was the sole carrier and disperser of blue-stain fungi;

but now recent evidence suggests fungal dispersion can be also carried by mites (Mori et al. 2011, Reboletti 2008).

Phoresis is the transport of an organism by a larger animal in which the small one does not feed on the carrier (Torre-Bueno 1962). Phoretic organisms benefit from their carriers by obtaining transport to otherwise limited spatiotemporal resources (Klepzig et al. 2001; Cardoza et al. 2008; Mercado et al. 2014) and can benefit their carrying hosts. For example, bacteria in beetle secretions have antibiotic properties that regulate the growth of fungal species antagonistic to the beetle-fungus symbioses (Adams et al. 2008, Cardoza et al. 2006). Certain fungi and yeasts enhance beetle's subcortical habitat by altering its moisture, concentrating N, and detoxifying terpenoids (Reid 1961, Bentz and Six 2006, Wang et al. 2014). Moreover, mountain pine beetle reproduction did not occur in the absence of its associated blue-stain fungi (Six and Paine 1998). Nematodes are also common phoretics of bark beetles, often travelling under the elytra or in nematangia, a specialized structure for phoresy (Cardoza et al. 2006).

Mites (Arachnida: Acari) is another group of organisms frequently found associated with bark beetles. Estimated at over one million species (Walter & Proctor 1999), mites are diverse and many species have evolved symbiotic relationships with xylophagous insects, facilitating their incursion into its shared niche with the insects. Within a single bark beetle gallery one can find, predatory, omnivore, and mycetophagous mite species (Hofstetter 2011), affecting beetles and its associates in different ways. For instance, predatory mites typically feed on nematodes transported by bark beetles (Cardoza et al. 2008) controlling nematode populations that can affect the reproductive success of the beetle (Thong and Webster 1975, MacGuidwin et al. 1980,

see review Mercado et al. 2014). Omnivorous mites can feed on invertebrates, including immature stages of bark beetles, as well as fungi. In the southern pine beetle (*Dendroctonus rufipennis* Kirby) the relationships with fungivorous mites in the genus *Tarsonemus* Canestrini and Fanzago can change through time from mutualistic to antagonistic (Hofstetter et al. 2006) affecting carrier beetle populations. Different phoretic mite species may have important effects on their transporters. For example, *Tarsonemus* mites are important carriers of ophiostomatoid fungi (Bridges and Moser 1983, Roets et al. 2007), and can affect the proportion of mutualistic and antagonistic fungi on the subcortical niche of the southern pine beetle (Hofsttter et al. 2006). It is important to understand if different frequencies of mites like *Tarsonemus* may be in part responsible of different frequencies of species of blue-stain fungi reported on ecosystems with different thermal characteristics (Six and Bentz 2007) like those present within the mountain pine beetle's landscape distribution along different tree forest types.

Mites associated with mountain pine beetle were first documented in Colorado during the late 1960's (Lindquist and Hunter 1965; Lindquist 1969, 1971). Some of these mites were obtained from beetles and their galleries in ponderosa pine and not directly from beetles flying to their host. Therefore, it is possible that either non-phoretic mites or species transported to that substrate by other insects were reported instead. Also, reports of mites from Colorado do not describe beetle population levels during their collection; this information is needed to interpret potential effects on mites on beetle populations. Currently 13 phoretic mite species are known to use mountain pine beetle as the carrying host, (Mercado et al. 2014), five of which might occur phoretically in Colorado beetles.

The goal of this study was to examine phoretic mite assemblages on mountain pine beetle in the Colorado Front Range among three different host species in order to further increase our knowledge about these potentially influential associates. I expected to find different species of mites on beetles arriving to different hosts; particularly, I expected to find *Tarsonemus* mite species to be more prevalent on ponderosa pine than in lodgepole pine. The principal objectives were (1) to determine how the number of species of phoretic mites or their assemblages differed on beetles arriving to different tree hosts or travelling on beetles of different sex, (2) to determine if different phoretic mite feeding guilds dominated on beetles attacking different pine hosts, and (3) to determine transport strategies of the different mite species by analyzing mite species on mountain pine beetle and simultaneously arriving insect associates.

## Study area and Methods

Studies were conducted in the Arapaho-Roosevelt National Forest in the Colorado Front Range (CFR). Plots were established along three transects in pine forests occurring at elevations of 1,800 m to 3,050 m [Fig. 2.1]. Since pure stands of a given species were not always found meeting our selection attributes, I was able to examine beetles and their phoretics arriving to lodgepole and ponderosa pines during the three years but to limber pine only during 2013. The centroid of the study area was located at about (Zone 13N, UTM) 450995.0, 4503158.0. Mountain pine beetle was active within 300 m of all plot centers as suggested by fading trees containing teneral adults.

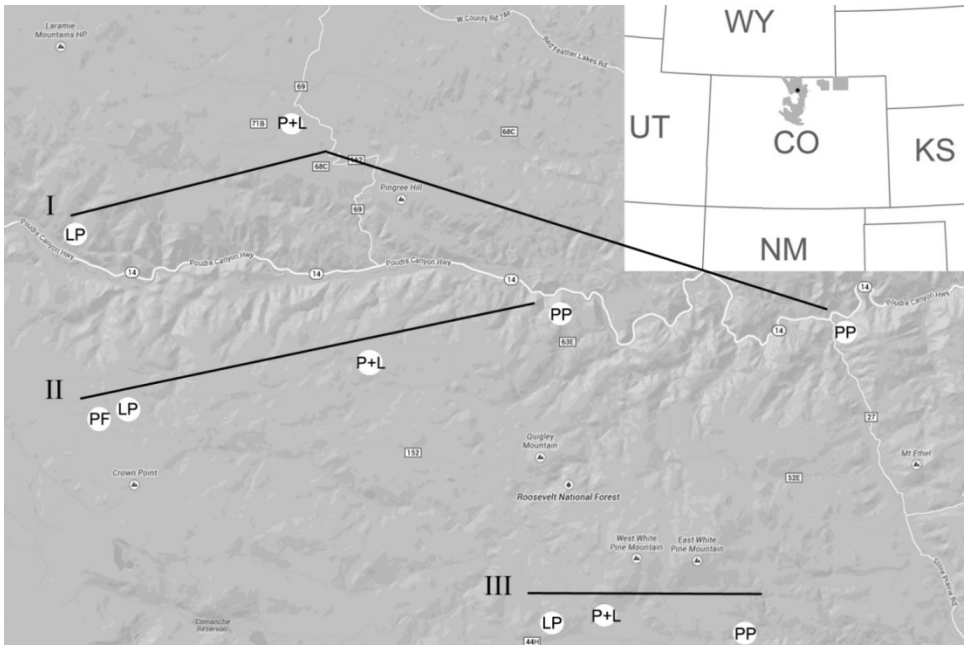


Figure 2.1. Map of study plots along three transects containing the three tree species. Ponderosa pine (PP), ponderosa and lodgepole pine (P+L), lodgepole pine (LP), and limber pine (PF). Circles denote study plots within the Roosevelt National Forest, Colorado, USA. Map relief layer modified from Google Earth (map view).

Trees were monitored from one to three times per week beginning the 3rd week of May, the earliest that beetles begin emerging from infested trees (McCambridge 1964). Twelve-funnel Lindgren traps baited with a commercial set of attractants including alpha-pinene, myrcene, and trans-verbenol (Synergy Semiochemicals, Burnaby, BC) were used to monitor beetle activity in each stand and help determine when to intensify beetle collection efforts. During the duration of the study, the mountain pine beetle population, as suggested by the amount of newly affected area (acres), showed a decreasing trend both at state and county levels [Fig. 2.2]. We collected montain pine beetles and simulataneously arriving insects at trees during their arrival. We exploited a defense mechanism of bark beetles and clerids called thanatosis (death-feigning) in

which these insects drop from a substrate in the presence of potential danger by placing a sterile micro centrifuge below the beetle to collect the startled beetle. Other insects were collected by simply being carefully approaching these and launching fast and accurate strikes with the vials. One beetle was collected in each tube to prevent mite transfer among beetles. Once beetles were collected these were placed on an ice-cooler for transport to the laboratory. Beetles were then examined and the number and place of attachment of the externally carried mite species and of those concealed under the elytra were recorded; also the sex of the beetle was recorded.

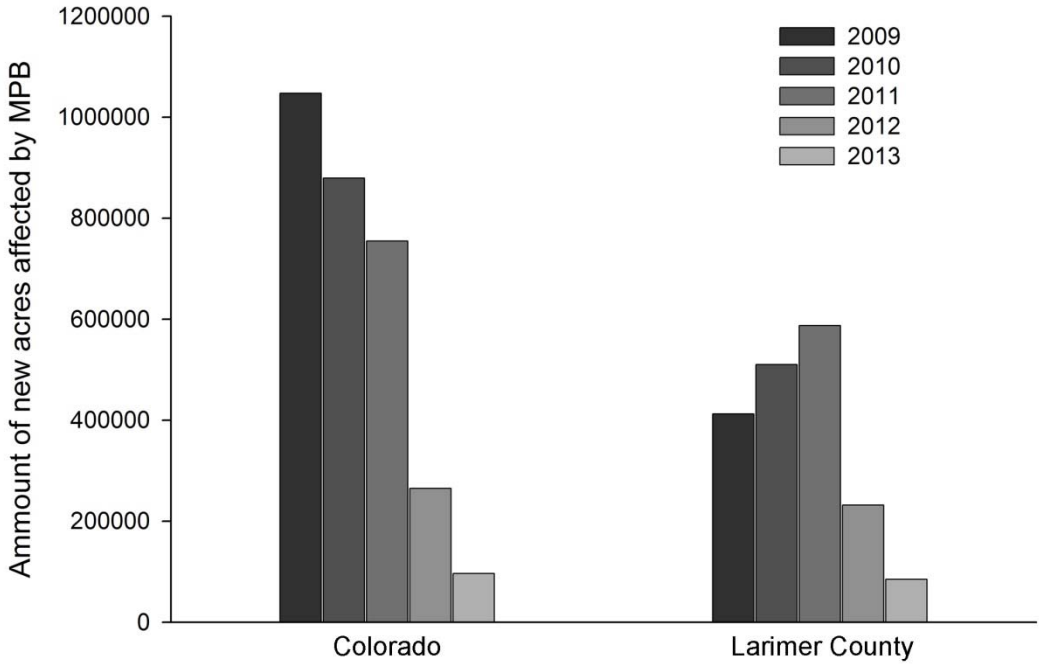


Figure 2.2. The area of new affected pine forest (in acres) by mountain pine beetle in Colorado (left) and Larimer County (right) decreased during the duration (2011-2013) of this study (Data from FHTET).

## Mite identification

Mites were mounted on microscope slides and identified using published species descriptions (Lindquist and Hunter 1965; Lindquist 1969, 1971). Determination keys for the documented species from mountain pine beetle and related subcortical beetles such as the southern pine beetle and long horned beetles (Kinn and Swanston 1976; Kinn and Linit 1989) were also used in the identification of the *Trichouropoda* species.

## Statistical analysis

Summary statistics (R version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) were used to explain frequencies of three species present during the three years in two hosts and among four mite species occurring on three pine hosts in 2013. Overall the significance of similarities (p-value) are explained based on α of 0.05, significances between 0.05 and 0.1 are stated as borderline. Analysis of the average number of mites per beetle (load) during different years was done using ANOVA with a Tukey-Kramer Pairwise Comparisons (R, mltcomp package). Non-parametric multiple-response permutation procedure (MRPP, R; Vegan package) using Euclidean distances and 10k permutations was used to help determine if mite species assemblages varied on beetles of different sex and on beetles attacking two different pine species. MRPP post-hoc analyses reported p-values are adjusted ($p_{adj}$) by the Holm method (R, Vegan). Among limber, lodgepole, and ponderosa in 2013 these analyses were used to describe similarities between three pine hosts and four species of mites. Chi square (prop.test, R) was used to compare proportions between paired observations.

Transport strategies of mountain pine beetle phoretic mites

We evaluated carrier preference of mite species found on mountain pine beetle by examining their presence on other potential carriers. Assuming mites benefit from reaching a new host ahead of competitors as does their carrying host, we sampled mountain pine beetle associated predators including clerid beetles (*Enoclerus sphegeus* Fabricius, *E. lecontei* (Wolcott), and *Thanasimus undatulus* Say), Diptera (*Medetera aldrichii* Wheeler), parasitoid Hymenoptera (*Coeloides sympitys* Mason), and other *Dendroctonus* bark beetles (i.e. *Dendroctonus murrayanae* Hopkins and *D. valens* LeConte), arriving within three days of initial mountain pine beetle attack to determine if its phoretic mites used other insects to reach trees. The phoretic mite attachment site (Binns 1982) can reflect travel specialization of mites selecting these versus an arbitrary niche. Mountain pine beetles can fly long distances in search of a host tree. After choosing a tree the insects need to overcome the trees' oleoresin defenses. Transport site specialization can give us a sense of how adapted these organisms are for transporting on this beetle.

Results

Phoretic mites and community composition on beetles attacking three pine species

Over the three years, 412 mountain pine beetles were individually collected arriving to all plots and tree species. Phoretic mites were present on 234 (56.8 %) collected beetles. The proportion of beetles carrying mites showed an increasing trend along the years (31.6, 62.3, and

64.9 %), but was only significantly different between 2011 and the next two years ($\chi^2$= 32.1, df = 2, p < 0.001). Beetles carried a total of 1,368 mites ranging from none to 60 mites per beetle ($\bar{x}$= 3.3). In all, the five mite species found included the fungivores *Tarsonemus endophloeus* Lindquist, *Tarsonemus ips* Lindquist, and *Histiogaster arborsignis* Woodring; a nematode predator, *Proctolaelaps subcorticalis* Lindquist; and an omnivore, *Trichouropoda hirsuta* Hirschmann [Fig. 2.3]. During 2011 and 2012, when mountain pine beetles arriving at lodgepole and ponderosa pine hosts were sampled, three species *T. hirsuta*, *T. ips*, and *P. subcorticalis* were found. During 2013 a fourth mite species, *T. endophloeus* was found on beetles arriving to all hosts, together with the previous three species these represented 99.8 % of the phoretic mite fauna on all beetles that year. *Histiogaster arborsignis*, occurred only rarely with only two specimens collected in 2011, one in 2012, and two during 2013. Of two species of *Tarsonemus* mites found, *T. endophloeus* and *T. ips*, none was particularly abundant on any particular pine species stands. *Tarsonemus ips* was found on lodgepole and ponderosa pines the three years during which it was only significantly more frequent on beetles arriving to ponderosa pine than in lodgepole only in 2013 ($\chi^2$=5.38, p= 0.020).

Figure 2.3. The deutonymph (phoretic stage) of phoretic mites found during this study. From left to right *Histiogaster arborsignis*, *Tarsonemus endophloeus*, *T. ips*, *Proctolaelaps subcorticalis*, and *Trichouropoda* sp. (cf. *hirsuta*), a new mountain pine beetle phoretic mite record from northern Colorado. Images are not to scale.

The average number of mites per beetle (load) and the species assemblages on arriving beetles did not vary by the sex of carrier beetles; however, it varied on beetles arriving to different pine hosts. Looking at all hosts, the mite load per beetle also varied during some years 2011 (1.2), 2012 (1.3), and 2013 (5.2), which were significantly different between 2011 and 2013 ($t= 21.9$, $p< 0.001$) as well as between 2012 and 2013 ($t= 12.9$, $p< 0.001$). *Trichouropoda hirsuta* was the main driver of the increasing trend. Overall, assemblages of the three mite species were significantly different in beetles arriving to ponderosa and lodgepole pines during 2011-2013 ($A= 0.032$, $p= 0.001$).

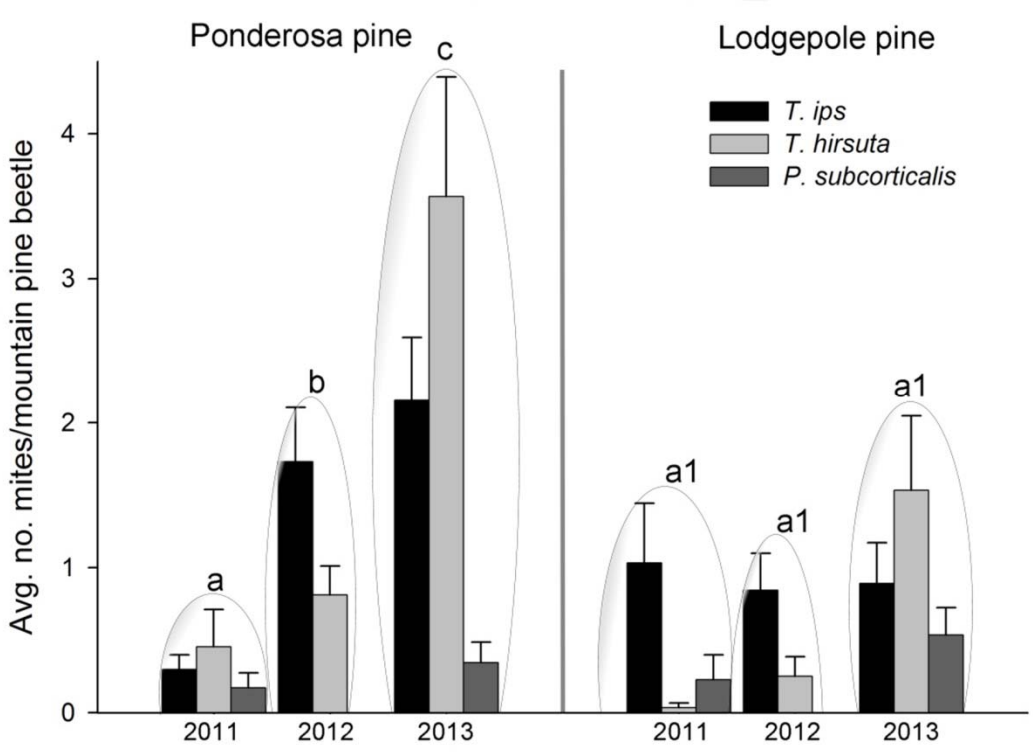


Figure 2.4. The average number of three common mite species found on mountain pine beetles arriving at ponderosa (left) and lodgepole (right) pines from 2011 to 2013. Only comparisons within the same pine species were performed. Bars represent the standard error (SE). Similarities (a, b, c, a1; $\alpha = 0.05$) of the three species assemblage were analyzed with MRPP (R version 3.1.2 R Foundation for Statistical Computing, Vienna, Austria).

When contrasting assemblages of three mites, *T. ips*, *P. subcorticalis*, and *T. hirsuta* occurring commonly during all years between pair of hosts, we found their communities were significantly different between ponderosa and lodgepole pines (A= 0.006, $p_{adj}$= 0.031), lodgepole and limber (A= 0.023, $p_{adj}$< 0.001), and between ponderosa and limber pines (A= 0.005, $p_{adj}$= 0.033). The newly recorded species *T. hirsuta* showed a generally increasing trend both in frequency on the population sample and in mite load per beetle in beetles arriving to ponderosa

and lodgepole pines [Fig. 2.4]. In 2012 beetle samples, *P. subcorticalis* was absent and *T. ips* was the most common in both lodgepole and ponderosa pines [Fig. 2.4]. The most common phoretic mite species in 2013 was the newly recorded species *T. hirsuta* [Fig. 2.4].

Mite assemblages of 4 species arriving on beetles attacking the three host pines in 2013 were not affected by the sex of the mountain pine beetle carrier. Assemblages of four common mites on beetles arriving to three pines hosts in 2013 were significantly different [Fig. 2.5]. Looking at similarities between all pairs of hosts in 2013 lodgepole and ponderosa pines were determined to be significantly different using MRPP statistical analysis (A= 0.015, $p_{adj}$= 0.015), as well as between lodgepole and limber pines (A= 0.012, $p_{adj}$= 0.030), but not significant between limber and ponderosa pines (A= 0.002, $p_{adj=}$ 0.176) [Fig. 2.5]. However with the ADONIS analysis only comparisons between lodgepole and ponderosa were found borderline significant [$R^2$= 0.024, $p_{adj}$= 0.062].

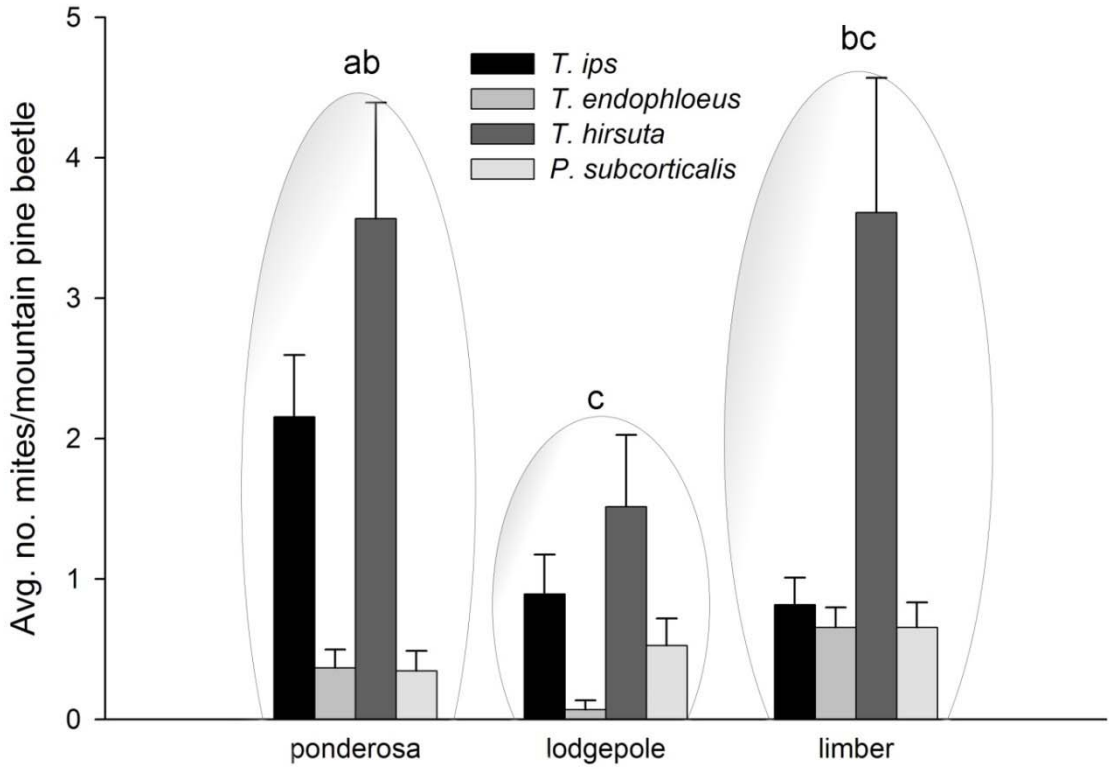


Figure 2.5. The average number of four common mite species found on mountain pine beetles arriving at ponderosa, lodgepole, and limber pines during 2013. Bars represent the standard error (SE). Similarities (ab, c, bc) of the four species assemblage were analyzed with MRPP (R version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria).

Contrasting phoretic mite feeding guilds frequencies across different pine species

Mite feeding guilds were differently represented between beetles arriving to the three pine hosts (A= 0.029, $p_{adj}$< 0.001) but not on beetles of different sex (A= -0.002, $p_{adj}$= 0.9). Feeding guilds present on the collected mites included fungivores, predators, and omnivores, of which the last is represented by the new documented species. In 2011 the predatory guild represented by the single species *P. subcorticalis* was the dominant feeding guild found in ponderosa pines followed by the fungivorous (*T. ips*); however sample size during that year was

low. In contrast on lodgepole pine the dominant feeding guild that year was the fungivore (*T. ips*). In 2012, the predator feeding guild was absent from both hosts. In 2012, the dominant feeding guild was the fungivore (i.e. *T. ips*), while the omnivore guild represented by *T. hirsuta* increased in both hosts during 2012 becoming the most common feeding guild present in 2013 on all three hosts.

Use of alternate carrier by mountain pine beetle phoretic mites

Both the beetle predators and other *Dendroctonus* species carried a different mite fauna than mountain pine beetles ($\chi^2$= 5.018, df= 1, p< 0.001). At all collection sites the three species of predatory clerid beetles carried only *H. arborsignis*. The average numbers of this phoretic mite were 6.8 (0-42, n= 98) on *E. lecontei*, 7.0 (0-31, n= 30 beetles) on *E. sphegeus* (ponderosa pine), and 0.5 (0-6, n= 16) on *T. undatulus*, these mites attached primarily to their coxae. All 30 parasitic *M. aldrichii* lacked any mites. In all pine species, parasitic hymenoptera lacked the mite genera phoretic on the mountain pine beetle, 13 % carried only a tiny unidentified species which was absent in other insects. On co-arriving red turpentine beetles in ponderosa pines (n= 40) as well as at high elevation on lodgepole pine beetles (n= 30) none of the mite species present on mountain pine beetles were found. In ponderosa pines, the associated red turpentine beetle mite fauna was completely different from that of the mountain beetle. A larger predatory mite in the genus *Proctolaelaps* was the dominant species on red turpentine beetle followed by a nematophagous species of *Dendrolaelaps* Halbert found in the analogous attachment niche of *T. ips* on mountain pine beetle. Also, twenty percent of lodgepole pine beetles carried mites

including species in the genera *Macrocheles* Latreille and *Dendrolaelaps* all different taxa of that present on mountain pine beetles.

Carrier attachment niche specialization

During the study the four common phoretic mites on mountain pine beetle did not change their attachment site or transport strategies. The two fungivores *T. endophloeus* and *T. ips* were similar in size but preferred distinct phoretic niches in mountain pine beetle bodies. *T. ips* was typically seen anteriorly or posteriorly of the procoxae, but when highly numerous they were found in other coxae as well. During 2011 and 2012 *T. endophloeus* was not recorded since the specific attachment site was cryptic, therefore it wasoverlooked those years. These small mites were often hidden under sclerites of the wing base (93 %). Near all *P. subcorticalis* were found under the elytra (95 %) whereas *T. ips* (82 %) attached to the procoxae where they grasped the inter-segmental rows of setae (Figure in Mercado et al. 2014). When this niche was saturated they attached to both the anterior and posterior basis of procoxae were setae was present. When few *T. hirsuta* (six or less) attached to a beetle these were more frequently found on the space between the head and the procoxae (72 %). Greater numbers saturated that niche, which made these attach using their pedicels to the declivity or other external sclerites including the legs. [Fig. 2.6].

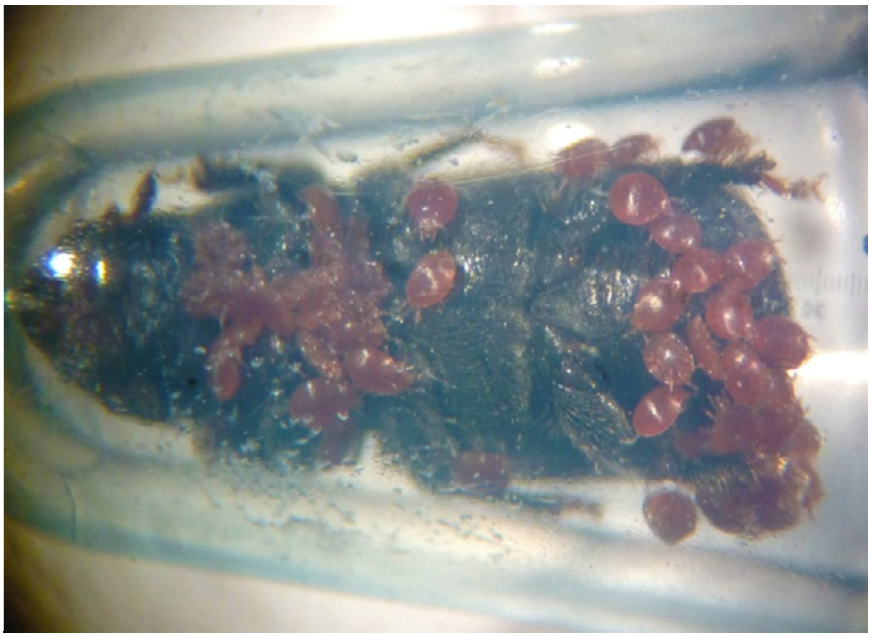

Figure 2.6. Phoretic mites overcrowding its mountain pine beetle carrier. Heavily infested beetles were collected in 2013 transporting over 50 *Trichouropoda hirsuta* phoretic mites attaching to many parts of the beetles' exoskeleton. The direct collection of individually arriving specimens on microcentrifuge vials allowed documenting this type of occurrence and to accurately determine number of mites per beetle.

## Discussion

Previous knowledge about phoretic mites on mountain pine beetle derived from studies that examined two different beetle population levels attacking two different pine hosts (i.e. epidemic in ponderosa pine, Reboletti 2008, i.e. incipient in lodgepole pine, Mori et al. 2011). The present study increases mountain pine beetle phoretic mite knowledge by describing mite fauna present during a declining population level (Colorado State Forest Report 2013, Forest

Heath Protection Technology Enterprise Team, FHTET) affecting three pine hosts simultaneously.

As in other bark beetles mites are common phoretic associates of the mountain pine beetle. The percent of mountain pine beetles carrying mites found in this study (32-62%) is within the range documented from beetle populations arriving to ponderosa pine in South Dakota (50%, Reboletti 2008) and to lodgepole pine in AB, Canada (30%, Mori et al. 2011). It is also similar to other species of bark beetles such as *Ips pini* (78%, Pfammatter et al. 2013) suggesting mites have evolved relationships with bark beetles to reach specific subcortical environments. Mori (et al. 2011) considered that a low mite number of species on arriving mountain pine beetles in Alberta represented recent relationships between mites and beetles in the new impacted range of the beetle. Backing his hypothesis, low mite diversity was also reported from studies examining mites on southern pine beetle in areas were a population of that beetle was expanding in Arkansas (Stephen and Kinn 1980). However, the number of mite species carried by mountain pine beetle found during this and previous studies is similar to species with a semi- to univoltine life cycle such as the spruce beetle (8 spp. Cardoza et al. 2008), *Dendroctonus rhizophagous* Thomas and Bright (7 spp., Chaires-Grijalva et al. 2013), red turpentine beetle (4 spp., this study), and the lodgepole pine beetle (3 spp., this study). It can; however, be considered low if compared to the species diversity found on bark beetles with multivoltine life cycles such as southern pine beetle (>100 species, Hofstetter 2011) and *Ips pini* (20 spp. Pfammatter et al. 2013). Therefore, it is probable that the number of phoretic mite species on the mountain pine beetle is typical of the species and others with similar life histories.

For the transporting insect population, mite load (mites/mpb) is an important factor when considering fungivores or predatory mite species which may affect the ecology of the subcortical environment by changing ratios of beneficial and antagonistic fungi or feeding on nematodes or beetle immature stadia. The increasing mite load we observed from 2011 to 2013 (1.23 to 5.19) corresponded with a reduction in the beetle activity in the area but we did not gather information to test if this was an important factor to the beetle' epidemic decline. The increase was only significant from 2012 to 2012 when it was driven largely by increasing populations of an omnivorous mite *T. hirsuta*. Mites in the genus *Trichouropoda* have been found to be reproductive successful when feeding on species of *Ophiostoma* (Roets et al. 2007) but have been observed feeding on *I. pini* eggs (Hofstetter et al. 2009). Given the significant decrease of ophiostomatoid fungi in 2013 but the increase of these mites that year is possible that they switched diet affecting the beetles, something that should be further investigated. In AB, Canada sampled beetles in front of the epidemic had a mite load of less than 1 mite/beetle (Mori et al. 2011), however their numbers might have been affected by their wet trap collecting method (Lindgren+ethanol). Mite loads from South Dakota are difficult to interpret, these ranged from near three mites per beetle to about one during the peak of an epidemic. In summary, as found on the three studies the principal mites of the mountain pine beetle *T. ips* and *P. subcorticalis*, are a fungivore and a nematode predator, respectively.

The new species for the area, *T. hirsuta* may represent a mite associate occurring during late or declining levels of mountain pine beetle epidemics, as species in this genus have not been found on incipient populations (Mori et al. 2011) and only have been found rarely on areas in which beetle populations were still at the height of the epidemic (Reboletti 1998); however,

further studies including examinations during endemic beetle levels are needed to further support this hypothesis. There is however, the possibility that this species was collected before in northern Colorado. DeLeon (1939 unpublished) documented an oribatid mite from mountain pine beetle during a previous epidemic near our study area; we were unable to locate this specimen to confirm its identity. One unreported species from recent studies in Canada *Tarsonemus endophloeus* Lindquist (Mori et al. 2011) is often concealed under sclerites at the base of the wing (Mercado et al. 2014). During our first two years, sampling methods did not properly search for that mite species and we might have missed recording it. Another species *Tarsonemus subcorticalis*, previously documented from *Dendroctonus* sp. in ponderosa phloem near the study area (Lindquist 1969), was not phoretic on beetles and its presence in phloem was not determined in this study; thus its presence on mountain pine beetle remains unconfirmed. The last species found during our studies, *H. arborsignis*, is either rare in declining beetle populations or simply uncommon as described from South Dakota (Reboletti 1998, Mercado et al. 2014) and in field samples from AB, Canada (Mori et al. 2011). Our findings suggest that this species preferred checkered beetles for transport; therefore, is possible that laboratory studies reporting it as a common phoronts of *Dendroctonus* bark beetles may be overestimating the natural frequency of these mites. For example Mori et al. (2011) found that species to be the most abundant (71% of beetles with mites) of three common mites in laboratory experiments, but to be uncommon (< 4%) on field caught beetles. Also, Cardoza et al. (2008) described this was the most common mite obtained from beetle galleries in phloem sandwiches.

Mite assemblages on the mountain pine beetle may change throughout different population levels and their presence could be used to characterize these levels and even help

predict the faith of the population. For instance, only two common phoretic mites (*P. subcorticalis*, *T. ips*) were reported from Alberta (natural conditions), Canada in areas where the population was still growing (Mori et al. 2011) from which the nematophagous species *P. subcorticalis* was the most common. In South Dakota, were studies were done during ongoing beetle epidemics, these two species were also the most common but their frequencies shifted and *Trichouropoda* mites were rare (0.3%) in the population (Reboletti 2008). These two common species may represent well-adapted associates that may include mutualistic, neutral or situational antagonistic symbionts of the mountain pine beetle; however, their exact relationship remains uncertain. In this study, were mountain pine beetle were sampled during a declining period, the frequency of *P. subcorticalis* was slightly lower than during epidemic peak period sampled in South Dakota and the frequency of *T. ips* fell to second place after the omnivore *Trichouropoda*.

Distinct feeding guilds of phoretic mites found on eruptive bark beetles may impact the ecology of their carriers (Lombardero et al. 2000) or their host tree (Moser et al. 2010). In 2011 the predatory guild was more frequent in beetles attacking ponderosa pines at low elevation. Wildfires during 2012 burned ponderosa pine plots reducing mountain beetle activity in that low elevation forest limiting our sampling. Lastly, no beetle activity was found in low elevation ponderosa pines in 2013. Although we cannot attribute a collapse of beetle populations due to the dominance of that feeding guild, this should be considered in future studies. Whereas, the direct (trophic) impact by predatory mites (i.e. beetle predation) can be determined with relative ease, the indirect (non-trophic) effect of mycetophagous species is more difficult to determine. Mycetophagous mites carry fungal spores within protected structures similar in function to beetles' mycangia, termed sporotheca (Moser 1985). Fungi vectored by mites can affect the

development of their insect carriers differently. Bridges and Moser (1983) described how *Tarsonemus* mites, phoretic on the southern pine beetle significantly increased the probability of recovering *Ophiostoma minus* (Hedgc.) H. et P. Syd. from beetles placed on agar plates. *Ophiostoma minus* was later found to negatively affect southern pine beetle brood development and was antagonistic to *Entomocorticium* sp. a fungus beneficial to the beetle (Barras 1970, Barras and Perry 1972, Lombardero et al. 2000, 2003, Klepzig et al. 2001). For this reason, increases in the population of that mite species resulted in the decline of southern pine beetle populations (Hofstetter et al. 2006). This phenomenon should be further explored in other bark beetles including the mountain pine beetle. During 2013 it was difficult to find beetle activity since the epidemic was declining. The most common mite feeding guild during 2013 was the omnivores represented by *T. hirsuta*. Thus future studies should look at what is the direct effect of this species on the mountain pine beetle. This species was absent from the leading edge of an epidemic (Mori et al. 2011) but present on an ongoing one in South Dakota (Reboletti 2008). As our findings suggest, this might indicate that this species population develops during a late period of the epidemic.

Similarly to studies in AB, Canada (Mori et al. 2011) we found that mites may cue on signals common to both sexes of the beetle. Although, the nature of this attraction remains unresolved, it is possible that mites are attracted first to physical stimuli, such as beetle vibrations, and later to pheromones or semiochemicals of plant or fungal origin present on the beetles. The four common phoretic mites used different attachment sites and transport strategies something that may relate to their physiology in addition to their ecology. The two species of *Tarsonemus* found in our samples were small enough to travel under sclerites of the base of the

wing, however these didn't. Only *T. endophloeus* used this mode of transportation suggesting perhaps a more specialized association with the beetle. *Proctolaelaps* species have evolved flattened bodies that allow it to travel under the elytra but also to remain flattened against its surface during flight. They also find some of their nematode food source under the elytra. *Tarsonemus ips* attachment niche anterior to the procoxae allowed it to grasp a row of setae that borders the space between the thorax and the head (Fig. 1.1d). This newly documented species in Colorado is also the largest and can only attach externally to the beetle [Fig. 2.6]. Species in this genus secrete a sticky substance through their anus, which hardens forming an attachment structure called the pedicel. The least common phoretic mite species, *H. arborsignis* is considered a generalist (O'Connor 1990, Pfammatter et al. 2013). This species uses not only bark beetles but buprestids, cerambycids, and in our study all three predatory clerids known from the area. The absence of mountain pine beetle phoretics on co-arriving insects may suggest mites cue on species-specific signals, such as a pheromone. Factors such as: utilization degree of simultaneously arriving insects, the use of a safe or specialized attaching site, and the degree of carrier host saturation by mites could be used to build affinity indices of mites on mountain pine beetle or other insects.

Future studies looking at mountain pine beetle and their phoretic mite faunas across the beetles' geographic distribution may provide clues that may shed light to help better understand beetle population fluctuations. For example, different mite species could be associated with different fungi with specific capacities to interact with distinct resin compounds in different tree hosts occurring throughout the beetle's distribution, something that may benefit the beetle given the variety of oleoresin compounds found on different pines (Smith 2000). Although its effects

were not quantified or measured in our studies, heavy mite loads may affect carrier's dispersal (Drummond 1988) as well as their landing site on a tree (Kinn and Witcosky 1978). We recorded an increase of an omnivore and a fungivore mite within successive years during a period of declining mountain pine beetle activity in the area as estimated from aerial surveys (CSFS, 2013 Forest Health Annual Aerial Survey Report). The number and mite taxa found on beetles arriving to the three hosts were not different. Phoretic mites can have different effects on populations of transporting hosts. Predatory phoretic mites feeding on beetle nematode parasites are mutualistic as long as they don't affect beetle populations by feeding on the beetle's immature stages. Also, fungivorous species can potentially drive populations of the fungal species this may disseminate on particular tree species. Studies in this area could be fundamental in understanding population fluctuation on this and related forest insects. Our results improves the previous knowledge by providing information about the phoretic behavior of mites and by contrasting it across the three common host pines of mountain pine beetle in Colorado.

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Chapter 3. Fungal associates of mountain pine beetle (*Dendroctonus ponderosae* Hop.) and its phoretic mites arriving to limber, lodgepole, and ponderosa pines in the northern Colorado Front Range.

## Introduction

For nearly 20 years a widespread mountain pine beetle (*Dendroctonus ponderosae*, Hopk) epidemic has affected North America's western pine forests including those in Colorado. The primary hosts lodgepole (*Pinus contorta* Douglas ex Loudon), ponderosa (*P. ponderosa* Douglas ex C. Lawson), and limber pines (*P. flexilis* E. James) have been impacted during the recent epidemic (CO St. FS 2013). Insect epidemics provide research opportunities to study often understudied interactions between the insect and a diversity of associated organisms. Among the most fascinating ecological processes needing attention are the interactions between mountain pine beetle (and related bark beetles) and the phoretic biota these carry. Beetles and these organisms live in apparent balance in the host tree, helping create conditions in the phloem that allow their establishment and reproduction before competing organisms reach that habitat. Mountain pine beetle symbionts include bacteria, fungi, nematodes, and mites. Phoretics benefit by gaining transport to ephemeral niches in beetle-killed trees (Six and Klepzig 2004; conversely, phoretic organisms can aid bark beetles transform the subcortical niche into one more hospitable. For example, bacteria on mountain pine beetle's oral secretions detoxify harmful terpenoids in the tree's resin (Adams et al. 2013). In the spruce beetle (*Dendroctonus rufipennis* Kirby), bacteria have also been found to suppress antagonistic fungi arriving opportunistically at the tree, which could potentially out-grow beneficial fungal species (Cardoza

et al. 2008). Unicellular ascomycetes like the yeasts, *Kuraishia capsulata* (Wick.) Y. Yamada, K. Maeda & Mikata and *Ogataea pini* (Holst) Y. Yamada, M. Matsuda, K. Maeda & Mikata (Whitney and Farris 1970, Lee et al. 2006, Bleiker et al. 2009) carried by mountain pine beetle metabolize a tree terpenoid, α-pinene, into verbenone. This pheromone, emitted by beetles during mass attack informs late arriving beetles of the saturation state of a tree under colonization (Hunt and Borden 1990). Multicellular ascomycetes such as the blue-stain fungi, *G. clavigera*, (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield, *O. montium* (Rumbold) von Arx, and *Leptographium longiclavatum* S.W. Lee, J.J. Kim & C. Breuil grow into the phloem and xylem causing a resinous reaction, analogous to a mammalian allergy (see reviews by Paine et al. 1997, Raffa et al. 2005, Six and Wingfield 2011) inducing a fast depletion of the tree's mechanical defenses (Reid 1967). And *Grosmannia clavigera* has recently been found to detoxify limonene (Wang et al. 2014), the bark beetle toxic and deterrent component of oleoresin (Smith 1975, Coyne and Lott 1976, Raffa and Berryman 1982).

Different relative abundances of particular blue-stain species are often documented on the mountain pine beetle. These variations may be related to sampling time after beetle attack or its developmental state on the tree, collecting site temperature, or latitude (Kim et al. 2005, Six and Bentz 2007, Roe et al. 2011, Khadempour et al. 2012). For reasons yet unknown, of two common blue-stain fungal associates found in western US, *G. clavigera* provides better reproductive fitness to the mountain pine beetle than *O. montium* (Six and Paine 1998, Bentz and Six 2006, Bleiker and Six 2007). Therefore, we could expect *G. clavigera* to be the most common fungal associate present during epidemics. However, *O. montium*, a species tolerant of warmer temperatures, has been found more often during warmer periods (Six and Bentz 2007,

Moore 2013). It has been suggested that under a warming climate scenario it may displace *G. clavigera* (Moore 2013). Recent models analyzing the effects of temperature variation on the stability of these two blue-stain fungi have shown that the beetle movements between warm and cold habitats, as well as variations on the attack density of the beetles help explain the prevalence of both fungi in the beetle-symbiosis (Addison et al. 2013). However, effects of fungal dispersal by non-trophic associates could be an important factor explaining the existence of multiple fungal associates in this symbiosis and their varying frequency found in nature. It is therefore of relevance to determine the contribution of other fungal dispersers to the beetle-fungi symbiosis under different conditions, including those existing among the beetle's different host pines.

One frequent group of associate fungal dispersers that has received little attention in the mountain pine beetle is the phoretic mites. Thirteen species of phoretic mites have been documented in association with this beetle (Lindquist and Hunter 1965; Lindquist 1969, 1971; Mori et al. 2011, Reboletti 2008, and see review Mercado et al. 2014). In several bark beetle species, fungi are hyperphoretic (a phoretic within another phoretic) on mites (Bridges and Moser 1983, Levieux et al. 1989, Moser et al. 1989, Pernek et al. 2008, Moser et al. 2010). More importantly, the type of spore carried by species of *Tarsonemus* mites has been determined to be sexual or ascospore (Moser 1985, Moser and Bridges 1986, Lombardero et al. 2003). As recently documented in mountain pine beetle populations from South Dakota (Rebolleti 2008), fungi are also carried by its phoretic mites. Yet we lack a thorough understanding of the significance and extent of fungal transport by phoretic mites, or whether mite populations are distributional and temporally stable or vary under different conditions such beetle population level or tree host species being attacked by the insect. In the southern pine beetle (*Dendroctonus frontalis* Zimm.),

mite frequencies respond to seasonal changes (Hofstetter et al. 2006). It is possible that mountain pine beetle' phoretic mite population fluctuates across different tree species often growing at different elevations affecting the overall frequency of their hyperphoretic fungi.

In the USA, the plurality of the research on mountain pine beetle's mite and fungal associates has been conducted in California and the Intermountain West with little information available from the Southern Rockies. Our knowledge on blue-stain fungal associates in Colorado comes from small collections that were non-level specific to mountain pine beetle attacking populations (Rumbold 1941, Robinson-Jeffrey and Davidson 1968). Also, these collections of fungi were made from beetle galleries or beetles already in the galleries creating uncertainties about their mode of arrival to the trees. Similarly, few collections exist of its associated mites and these were not specific to phoretic species (Lindquist and Hunter 1965; Lindquist 1969, 1971), and the fungi these mites carry are largely unknown. The goal of this work was to examine fungal species found on beetles and mites in the Colorado Front Range (CFR) and the contribution mites make as fungal dispersers to the different host pines in the area. The main objectives of this study were: to examine if blue-stain fungi species associated with mountain pine beetle in the CFR are consistent with those documented in the rest of its geographical distribution; if phoretic mite species on mountain pine beetle in the CFR transport blue-stain fungi; and to contrast blue-stain species being transported to the different native hosts by phoretic mites and mountain pine beetle in the CFR. This information can begin establishing the foundation to further examine the role of mountain pine beetle associates in its population dynamics.

Study site

The study area was located in the Arapaho-Roosevelt National Forest in the northern CFR [Fig. 3.1]. East facing forest stands dominated by ponderosa from 1,700 m to 2,500 m in elevation, and lodgepole pine is from 2,400 m to 3,200 m in elevation with mixed stands in their ecotone. Limber pine grows across all elevations mainly in scattered exposed rocky sites (Peet 1981).

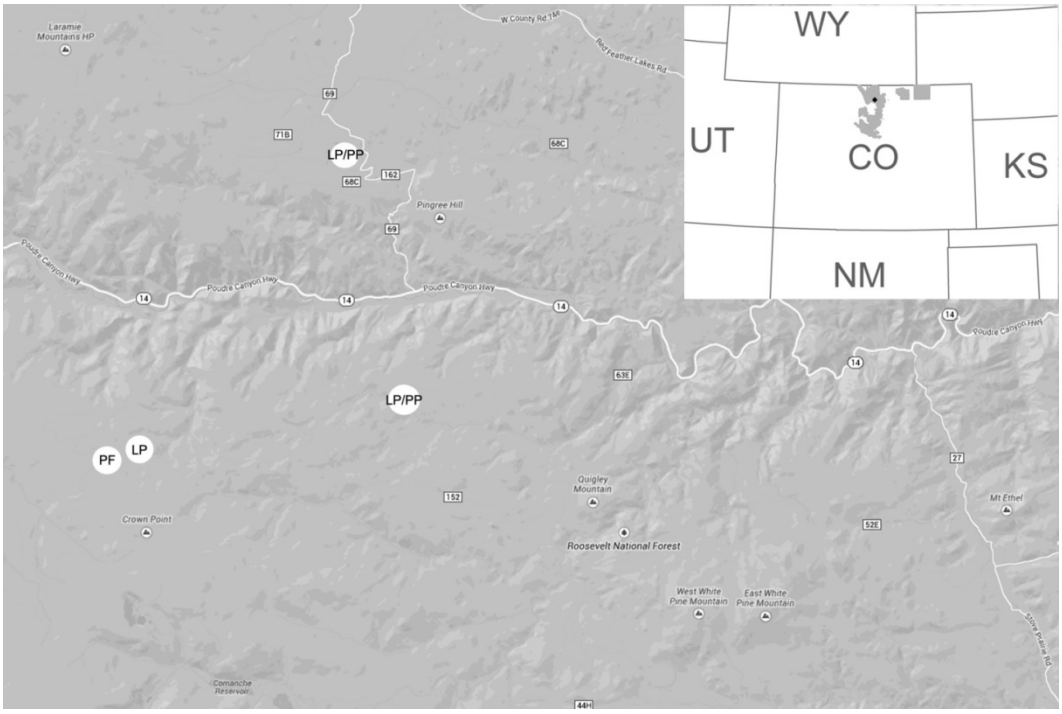


Figure 3.1. Study sites at the Arapaho-Roosevelt National Forest in northern Front Range, Colorado. Circles indicate the location of study plots; ponderosa (PP), lodgepole pine (LP), and limber pine (PF). Map relief layer modified from Google Maps [map view] (https://maps.google.com/; ©2014 Google).

Methods

Three weeks before the historically documented flight period of mountain pine beetle initiated (McCambridge 1964) we located symptomatic trees containing live beetle brood. On those stands containing similarly sized conspecific trees, we established circular plots of 100 m radius around symptomatic trees. Plots were monitored weekly to collect attacking beetles beginning the third week of May. In 2012, 4 plots were established at 2,300 m elevation in mixed ponderosa-lodgepole forests [Fig. 3.1]. In 2013, one plot in each forest dominated by ponderosa (2,300 m), lodgepole (3,050 m), and limber (3,100 m) were established. Attacked trees within these plots were tagged and their position recorded with GPS (Garmin 62 S). After an attacked tree had been located, trees within the plot were carefully inspected for landing beetles. Arriving beetles were collected individually in clean 1.5 mL microcentrifuge vials to prevent cross-contamination by fungi and mite movement between beetles. Death-feigning (thanatosis), is a defense mechanism by many insects (Miyatake et al. 2004), including *Dendroctonus* bark beetles, in which beetles drop in presence of a stimuli. This reaction was used to collect individual beetles by using a clean micro centrifuge tube to collect the dropping beetles. Specimens collected were kept in a cooler in the field. At the lab externally carried mites were counted and their attachment site on the beetle was recorded, then these were refrigerated at 4° C until ready for culturing. To obtain the fungal associates carried by mountain pine beetles, their maxillary mycangia, specialized structure to carry fungi (Whitney & Farris 1970) were removed and serially washed in two drops of water before placed on a petri dish containing1.5 % MEA (Malt Extract Agar (Difco) without antibiotics. Dorsal surfaces of the elytra were streaked against the agar surface in a second plate containing same media. Lastly, the beetles were sexed

by removing abdomen and examining their genitalia. To determine fungal associates carried by mites, single specimens from each species were carefully removed from the beetle with a fine point and placed on a petri dish with the same culturing media used with beetles. Live mites were killed to keep them from running out of agar surface. During 2012 only fungi from the mites *T. ips* and *T. hirsuta*, were sampled and in 2013 the mites, *P. subcorticalis* and *T. endophloeus*, a mite only collected in 2013, were also sampled for fungi. Fungal colonies were incubated at 21° C with 12 hrs. of fluorescent light and 12 hrs. of dark. After approximately two weeks, small plugs of each different colony were transferred to water agar (2 % agar/L). After 2-4 days single hyphal tips were harvested and placed on a fresh 1.5 % MEA plate.

Identification of mites

Morphological determinations of mite species were performed using characters from original descriptions (Lindquist and Hunter 1965; Lindquist 1969, 1971) as well as from published keys for documented species on mountain pine beetle, congeneric southern pine beetle, and long horned beetles (Kinn and Swanston 1976, Kinn and Linit 1989).

Identification of fungi

To identify fungi we used morphological characteristics of color, colony margin, sexual and non-sexual spores and their aggregation patterns based on published descriptions (Rumbold 1941; Robinson-Jeffrey and Davidson 1968; Grylls and Seifert 1993; Lee et al. 2005; Upadhyay 1981). In culture, *Leptographium longiclavatum* differed from similar clavate conidia possessing

*G. clavigera*, by the colony's irregular margins, dark-olive green color, and the growth of conidiophores closer to the center of the colony; whereas, *G. clavigera* had an entire margin, brown-olive coloration, and growth of sparser conidiophores across the colony. Pure colonies from sexual spores of *Ophiostoma montium* obtained from our mite cultures were used as a reference for determining this species. *Ophiostoma montium* was distinguished from the other two blue-stain species by brown hyphal growth in agar and its production of dense centers of aggregated mycelium (apparent as scattered dark spots) across the agar surface.

Statistical analysis

Summary statistics (R version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) were used to explain frequencies of three species present during the three years in two hosts and among four mite species occurring on three pine hosts in 2013. Overall the significance of similarities (p-value) are explained based on α of 0.05, significances between 0.05 and 0.1 are stated as borderline. Non-parametric multiple-response permutation procedure (MRPP, R; Vegan package) using Euclidean distances and 10k permutations was used to help determine if mite species assemblages varied on beetles of different sex, and on beetles attacking two different pine species. In addition a non-metric multi-dimensional scaling analysis [ADONIS, R] was used to help understand the effect of the same variables individually. In [ADONIS] and (MRPP) post-hoc analyses reported p-values are adjusted ($p_{adj}$) by the Holm method (R, Vegan). Among limber, lodgepole, and ponderosa in 2013 these analyses were used to describe similarities between three pine hosts and four species of mites. Chi square was used to compare proportions between paired observations.

## Results

### Fungi isolated from beetles

A total 273 mountain pine beetles were collected attacking the three principal host pines during 2012 (38) and 2013 (235). Overall, 92 % of all sampled beetles carried at least one fungal species including the blue-stain *G. clavigera*, *L. longiclavatum*, and *O. montium*, other ascomycetes such as *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. sp. yeasts, as well as *Alternaria* Nees, *Entomocorticium* H.S. Whitney, Bandoni & Oberw., and *Penicillium* Link species. Mountain pine beetles collected in 2012 were more frequently found carrying blue-stain fungi (76.9 %) than beetles collected in 2013 (33.6 %) for a two year average of 39.9%. During both years the blue-stain species, *G. clavigera*, *O. montium*, and *L. longiclavatum* were detected from maxillary mycangia and elytral surfaces of beetles arriving at all pine hosts. Overall, 25.2 % of beetles carried one species of blue-stain, 13.5 % carried two, and only 1.1 % carried all three species. The most common blue-stain fungal associate found on arriving beetles (mycangia + elytra) in both years and on to all pine hosts was *O. montium* (24.5 %).

During both years, the most frequent blue-stain fungi in the maxillary mycangium were *L. longiclavatum* (16.1 %), and *O. montium* (15.0 %). *Grosmannia clavigera* was identified from 5.1 % of beetles' mycangia. Fungi recovered from elytral streaking cultures showed a different species prevalence with *O. montium* being the most frequent (19.3 %), followed by *L. longiclavatum* (6.9 %), and *G. clavigera* (6.2 %).

In beetles attacking ponderosa pines during 2012, *O. montium* was the most common blue-stain present on both mycangia and elytra, being more frequently recovered from the beetle's elytra than from the mycangium (Table 3.1). *Leptographium longiclavatum* was the second most common blue-stain associate found in mycangia, found three times more frequently (Table 3.1) than *G. clavigera* in that structure. In 2013, *L. longiclavatum* and *O. montium* remained the dominant blue-stain fungi recovered from beetle elytra and maxilla. The two fungi did not show a marked segregation among structures of beetles arriving to any of the three hosts when sampled in 2013 (Table 3.1).

*Leptographium longiclavatum* was most frequently found on the maxillary mycangium of beetles arriving to lodgepole pine; whereas, *O. montium* was more frequently found on beetles arriving to ponderosa pine (Table 3.1). Also, each species was more prevalent on a distinct beetle structure with *L. longiclavatum* being more frequently found on beetle mycangium and *O. montium* on the beetles' elytra. On mycangial cultures from beetles arriving to lodgepole pine *L. longiclavatum* was found more commonly associated with *G. clavigera*. Non blue-stain fungi were also documented from these samples with yeasts being some of the more frequently isolated associates. In 2013, the most abundant fungal associates were yeasts recovered from about 33, 50, and 60 % of beetles arriving to ponderosa, lodgepole, and limber pine. These organisms were not identified to species but yeasts documented from mountain pine beetle include *Ogataea pini* (Holst) Y. Yamada, M. Matsuda, K. Maeda & Mikata, *Kuraishia capsulata* (Wick.) Y. Yamada, K. Maeda & Mikata, and *Nakazawaea holstii* (Wick.) Y. Yamada, K. Maeda & Mikata (Rivera et al. 2009).

Table 3.1. Percent of fungal (blue-stain in **bold**) associates found on beetle sampled body parts in 2012 and 2013 (SEMs in parenthesis). Comparisons were between same blue-stain species found on same structure over years. Primed and non-primed letters indicate performed comparisons, same letters were not significantly different by $\chi^2$ ($\alpha = 0.05$).

| Ponderosa pine<br>Fungal associate | 2012 Maxillae<br>(N=25) | 2013 Maxillae<br>(N=82) | 2012 Elytra<br>(N=25) | 2013 Elytra<br>(N=82) |
|---|---|---|---|---|
| ***Ophiostoma montium*** | **56.0 (10.1) [a]** | **13.4 (3.8) [b]** | **72.0 (9.2) [a1]** | **19.5 (4.4) [b1]** |
| ***Grosmannia clavigera*** | **12.0 (6.6) [a]** | **2.4 (1.7) [b]** | **8.0 (5.5) [a1]** | **7.3 (2.9) [b1]** |
| ***Leptographium longiclavatum*** | **36.0 (9.8) [a]** | **19.5 (4.4) [a]** | **8.0 (5.5) [a1]** | **6.1 (2.7) [a1]** |
| *Ceratocystiopsis* sp1 | 4.0 (4.0) [a] | 6.1 (2.7) [a] | 4.0 (4.0) [a1] | 7.3 (2.9) [a1] |
| *Entomocorticium* sp. | 4.0 (4.0) [a] | 1.2 (1.2) [a] | 12.0 (6.6) [a1] | 1.2 (1.2) [b1] |
| Yeast | 12.0 (6.6) [a] | 32.9 (5.2) [b] | 4.0 ( 4.0) [a1] | 35.4 (5.3) [b1] |
| *Alternaria* sp. | 0.0 [a] | 13.4 (3.8) [a] | 0.0 [a1] | 1.2 (1.2) [a1] |
| *Penicillium* sp. | 4.0 (4.0) [a] | 19.5 (4.4) [a] | 0.0 [a1] | 28.0 (5.0) [b1] |
| Lodgepole pine | (N=13) | (N=66) | (N=13) | (N=66) |
| ***Ophiostoma montium*** | **7.7 (7.7) [a]** | **13.6 (4.3) [a]** | **58.3 (14.4) [a1]** | **12.1 (4.0) [b1]** |
| ***Grosmannia clavigera*** | **15.4 (10.4) [a]** | **7.6 (3.3) [a]** | **15.4 (10.4) [a1]** | **7.6(3.3) [a1]** |
| ***Leptographium longiclavatum*** | **46.2 (14.4) [a]** | **12.1 (4.0) [b]** | **15.4 (10.4) [a1]** | **10.6(3.8) [a1]** |
| *Ceratocystiopsis* sp1 | 23.1 (12.2) [a] | 6.1 (3.0) [b] | 15.4 (10.4) [a1] | 4.5 (2.6) [a1] |
| *Entomocorticium* sp. | 15.4 (10.4) [a] | 4.5 (2.6) [a] | 15.4 (10.4) [a1] | 3.0 (2.1) [a1] |
| Yeast | 7.7 (7.7) [a] | 27.3 (5.5) [a] | 15.4 (10.4) [a1] | 28.8 (5.6) [a1] |
| *Alternaria* sp. | 7.7 (7.7) [a] | 3.0 (2.1) [a] | 0.0 [a1] | 0.0 [a1] |
| *Penicillium* sp. | 0.0 [a] | 7.6 (3.3) [a] | 0.0 [a1] | 6.1 (3.0) [a1] |
| Limber pine | **Not Surveyed** | (N=87) | **Not Surveyed** | (N=87) |
| ***Ophiostoma montium*** | | **6.9 (2.7)** | | **4.6 (2.3)** |
| ***Grosmannia clavigera*** | | **2.3 (1.6)** | | **2.3 (1.6)** |
| ***Leptographium longiclavatum*** | | **5.7 (2.5)** | | **3.4 (2.0)** |
| *Ceratocystiopsis* sp1 | | 4.6 (2.3) | | 1.1 (1.1) |
| *Entomocorticium* sp. | | 13.8 (3.7) | | 6.9 2.7) |
| Yeast | | 43.7 (5.3) | | 58.6 (5.3) |
| *Alternaria* sp. | | 10.3 (3.3) | | 4.6 (2.3) |
| *Penicillium* sp. | | 12.6 (3.6) | | 14.9 (3.8) |

Fungi isolated from mites

Twenty-nine (42 %) of the beetles (n= 69) carried mites in 2012, and 163 (65 %, n= 251) in 2013. The mite load per beetle in 2013 was almost three times greater than in 2012 (5.2 vs. 1.9). Five mite species were recovered from sampled mountain pine beetles during the two years

[Table 3.2]. Beetles carried fungivorous, predatory, and omnivorous mites visible externally and under the elytra and wing bases. Overall, 23 (11.0 %) of sampled mites (n= 209) carried at least one blue-stain species. As with beetles, more mites were sampled in 2013 (186 vs. 23) blue-stain fungus detection was significantly lower that year (4.1 %) than in 2012 (75.0 %) ($\chi^2$= 77.6, df= 1, p< 0.001). During both years *T. ips* and *T. hirsuta* were found carrying the three principal blue-stain associates known from the mountain pine beetle. The most common blue-stain associate found on mites was *O. montium* [Table 3.2].

Few mites were sampled from beetles arriving to ponderosa and lodgepole pines in 2012; however, *Ophiostoma montium* was recovered from four out of five *T. ips* in ponderosa and one of two in lodgepole pines [Table 3.2]. Also, *O. montium* was recovered from six of eight *T. hirsuta* and two of five mites arriving beetles to ponderosa and lodgepole pines respectively [Table 3.2]. In 2013, none of 18 *T. ips* from ponderosa, one of four from lodgepole and none from 15 on beetles arriving to limber carried *O. montium*. Three of 30 *T. hirsuta* in ponderosa, two of nine from lodgepole pine and none from 39 from limber arriving beetles transported *O. montium*, the most common associate the previous year. On the four mite species sampled in 2013, yeasts were the most common fungus type present. Other frequently found fungi found on these mites included yeasts and *Penicillium* this second had a greater incidence on mites during 2013.

Half of the mites also carried non-staining fungal associates such as two potentially alimentary associates of the beetle, *Ceratocystiopsis* sp.1, and *Entomocorticium* sp. (Table 3.1). In general, at least one fungal associate was recovered from 30 % of the mites. Fungal associates

recovered from mites included undetermined species of yeasts, *Penicillium* sp., *Alternaria* sp. and ophiostomatoid fungi, among other less common associates. In general, mites were not a significant source of recovered blue-stain fungi.

Table 3.2. Percent of common fungal associates (blue-stain in **bold**) recovered from four phoretic mites during 2012 and 2013. Comparisons were between the same fungus and mite species between years. Primed and non-primed letters indicate performed comparisons, same letters were not significantly different by $\chi^2$ ($\alpha$= 0.05).

| Ponderosa pine<br>Mite fungal associate | 2012 *T. ips*<br>(N=7) | 2013 *T. ips*<br>(N=19) | 2012 *T. hirsuta*<br>(N=9) | 2013 *T. hirsuta*<br>(N=29) | 2013 *P. subcorticalis*<br>(N=7) | 2013 *T. endophloeus*<br>(N=9) |
|---|---|---|---|---|---|---|
| *O. montium* | **85.7 (14.3) $^{a}$** | **5.2 (5.3) $^{b}$** | **66.7 (16.7) $^{a1}$** | **10.3 (5.8) $^{a1}$** | **0.0** | **0.0** |
| *G. clavigera* | **14.3 (14.3) $^{a}$** | **0.0 $^{a}$** | **33.3 (16.7) $^{a1}$** | **0.0 $^{a1}$** | **0.0** | **0.0** |
| *L. longiclavatum* | **14.3 (14.3) $^{a}$** | **0.0 $^{a}$** | **11.1 (11.1) $^{a1}$** | **6.9 (4.8) $^{a1}$** | **0.0** | **0.0** |
| *Ceratocystiopsis* sp.1 | 14.3 (14.3) $^{a}$ | 10.5 (7.2) $^{a}$ | 0.0 $^{a1}$ | 3.4 (3.4) $^{a1}$ | 0.0 | 0.0 |
| *Entomocorticium* sp. | 14.3 (14.3) $^{a}$ | 0.0$^{a}$ | 0.0 $^{a1}$ | 6.9 (4.8) $^{a1}$ | 14.3 (14.3) | 11.1 (11.1) |
| Yeast | 0.0 $^{a}$ | 42.1 (11.6) $^{b}$ | 0.0 $^{a1}$ | 62.1 (9.4) $^{b1}$ | 71.4 (8.4) | 33.3 (16.7) |
| *Alternaria* sp. | 0.0 $^{a}$ | 0.0 $^{a}$ | 0.0 $^{a1}$ | 0.0 $^{a1}$ | 0.0 | 0.0 |
| *Penicillium* sp. | 0.0 $^{a}$ | 10.5 (7.2) $^{a}$ | 11.1 (11.1) $^{a1}$ | 44.8 (9.0) $^{a1}$ | 42.8 (20.2) | 11.1 (11.1) |
| Lodgepole pine | 2012 *T. ips*<br>(N=2) | 2013 *T. ips*<br>(N=4) | 2012 *T. hirsuta*<br>(N=5) | 2013 *T. hirsuta*<br>(N=11) | 2013 *P. subcorticalis*<br>**(N=12)** | 2013 *T. endophloeus*<br>(N=0) |
| *O. montium* | **50.0 (50.0) $^{a}$** | **25.0 (25) $^{a}$** | **40.0 (24.5) $^{a1}$** | **18.2 (12.2) $^{a1}$** | **8.3 (8.3)** | **0.0** |
| *G. clavigera* | **0.0 $^{a}$** | **0.0 $^{a}$** | **0.0 $^{a1}$** | **0.0 $^{a1}$** | **0.0** | **0.0** |
| *L. longiclavatum* | **0.0 $^{a}$** | **0.0 $^{a}$** | **0.0 $^{a1}$** | **0.0 $^{a1}$** | **0.0** | **0.0** |
| *Ceratocystiopsis* sp.1 | 50.0 (50.0) $^{a}$ | 0.0$^{a}$ | 0.0 $^{a1}$ | 18.2 (12.2) $^{a1}$ | 8.3 (8.3) | 0.0 |
| *Entomocorticium* sp. | 50.0 (50.0) $^{a}$ | 0.0$^{a}$ | 0.0 $^{a1}$ | 0.0 $^{a1}$ | 0.0 | 0.0 |
| Yeast | 50.0 (50.0) $^{a}$ | 50.0 (28.9) $^{a}$ | 20.0 (20.0) $^{a1}$ | 36.4 (14.1) $^{a1}$ | 33.3 (13.1) | 0.0 |
| *Alternaria* sp. | 0.0 $^{a}$ | 0.0 $^{a}$ | 0.0 $^{a1}$ | 0.0 $^{a1}$ | 0.0 | 0.0 |
| *Penicillium* sp. | 0.0 $^{a}$ | 25.0 (25) $^{a}$ | 60.0 (24.5) $^{a1}$ | 0.0 $^{b1}$ | 8.3 (8.3) | 0.0 |
| Limber pine | **Not Sampled** | 2013 *T. ips*<br>(N=16) | **Not Sampled** | 2013 *T. hirsuta*<br>(N=39) | 2013 *P. subcorticalis*<br>(N=24) | 2013 *T. endophloeus*<br>(N=23) |
| *O. montium* | | **0.0** | | **0.0** | **0.0** | **0.0** |
| *G. clavigera* | | **0.0** | | **0.0** | **0.0** | **0.0** |
| *L. longiclavatum* | | **0.0** | | **0.0** | **0.0** | **0.0** |
| *Ceratocystiopsis* sp.1 | | 0.0 | | 2.6 (2.6) | 8.3 (5.8) | 4.3 (4.3) |
| *Entomocorticium* sp. | | 0.0 | | 2.6 (2.6) | 8.3 (5.8) | 0.0 |
| Yeast | | 31.3 (12.0) | | 58.9 (8.0) | 45.8 (10.4) | 34.8 (10.2) |
| *Alternaria* sp. | | 0.0 | | 0.0 | 0.0 | 4.3 (4.3) |
| *Penicillium* sp. | | 0.0 | | 15.4 (6.2) | 12.5 (6.9) | 4.3 (6.0) |

Beetle and mite association effect on blue-stain on the system

Overall, the added contribution of mite and beetle carriers increased total *O. montium* abundance by 2 % [Fig. 3.2]. While the contribution to other blue-stain was very small it is known that these are sexual spores. During 2013 three *Tarsonemus* with spores were found to carry over 30 externally [Fig. 3.3]. Although, we can't confirm these spores were sexual, this is are the type known to be carried by these mites (seen introduction) and only cultures from mites resulted in the production of ascocarps of *O. montium*. The presence of *O. montium* on the elytra increased significantly with the presence of mites carrying *O. montium* on the beetles ($\chi^2$= 42.62, df = 1, $p < 0.001$).

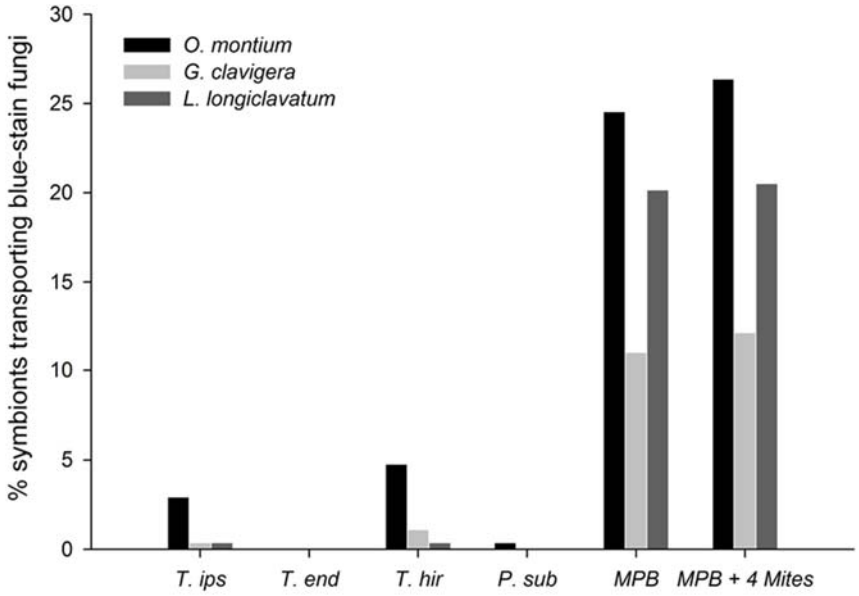


Figure 3.2. Percent of blue-stain fungi carried by four mite species (*Tarsonemus ips*, *T. endophloeus*, *Trichouropoda hirsuta*, and *Proctolaelaps subcorticalis* MPB, and their complex into all species from 2012-13. The overall contribution of *O. montium* by mites into the symbiosis was approximately 2.0 %.

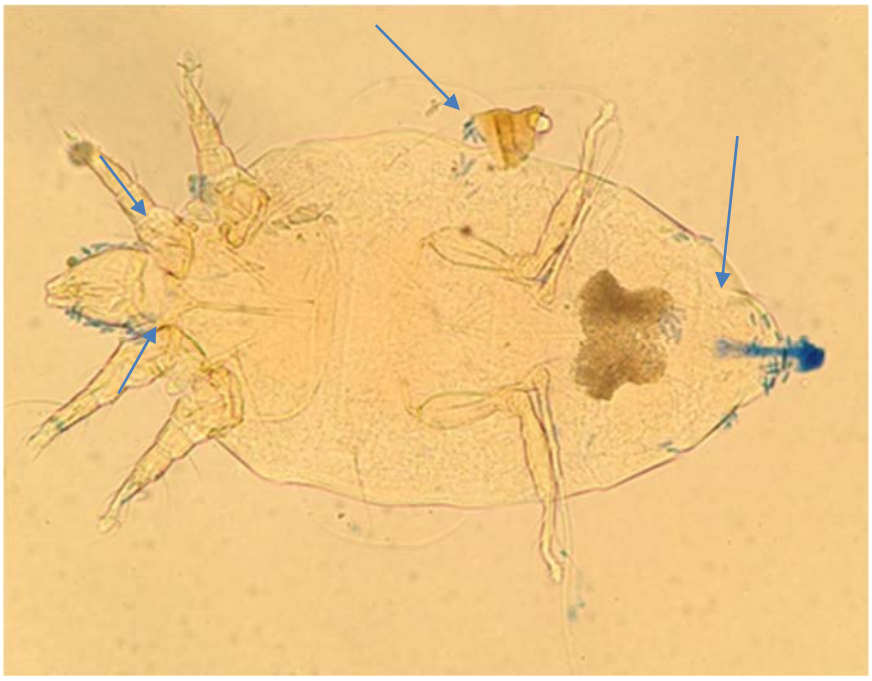

Figure 3.3. *Tarsonemus ips* carrying cylindrical spores (stained with lactophenol blue) not only in its sporotheca, but over its body surface and hind gut (120X magnification).

Discussion

The current knowledge of blue-stain fungi suggests that *G. Clavigera* and *O. montium* dominate the scenario in western United States (Six 2003). A third blue-stain associate, *Leptographium longiclavatum* has thought to be a northern fungal associate possessing a colder optimal growth temperature allowing it to survive colder winters occurring at the beetles' northernmost distribution (Rice et al. 2008, Safranyik et al. 2010). The inverse climatic restriction has been suggested for *G. clavigera* and *O. montium* in Canada (Rice et al. 2008); however, these species occur across the beetle's distribution in the US. Finding *L. longiclavatum* at the warmest sites of our study during a record warm year in Colorado reveals the great

phenotypic plasticity that *L. longiclavatum* shares with *G. clavigera* and *O. montium*. As the other main blue-stain species *L. longiclavatum* was found across the three mountain pine beetle hosts sampled. With its ability to grow and cause damage to Jack pine (*Pinus banksiana* Lamb.) hybrids in Canada is probable that it may occur in other mountain pine beetles' hosts across its distribution. Given the similarity of non-sexual clavate spores among *G. Clavigera* and *L. longiclavatum* it is also probable that this species has been overlooked in the past, possibly being lumped with *G. clavigera*.

Mite fungal dissemination influences the fungal species arriving to a host tree and consequently to the southern pine beetle (Hosftetter et al. 2006). In this study the overall contribution mites made in transporting three important blue-stain associates carried also by mountain pine beetle may seem too small to suggest these were the primary disseminators of any of these fungi. Nevertheless, mite fungal dissemination may be particularly important to the fungi, since sexual spores are the type carried by mites phoretic in other beetles such as: the southern pine beetle (Bridges and Moser 1983, Moser and Bridges 1986, Moser 1985, Moser et al. 1995), species of *Scolytus* (Moser et al. 2010), and other bark beetles (see review by Hofstetter and Moser 2014). Despite the mites' low contribution to the total *O. montium* on the beetle-mite complex found in this study, mite contribution may still be important for the sexual spore dissemination of that fungus. In fact, mite dissemination of *O. montium* sexual spores may contribute to greater genetic recombination found on that species in other areas (Roe et al. 2011, Tsui et al. 2013). Sexual spores benefit fungi by fostering greater genetic diversity and providing a greater frequency of distinct mating types to occur.

The new mite associate recorded for the CFR during this study, *Trichouropoda hirsuta*, is on a genus only documented from mountain pine beetles during the outbreak that started 16 years ago. Species of *Trichouropoda* are the primary fungal disseminators of ophiostomatoid fungi on phoretic mites carried by chaffer beetles to *Protea* sp. inflorescences in South Africa (Roets et al. 2011). Teneral, molting, and adult *T. hirsuta* were observed aggregated under the bark near the reproductive structures of *O. montium* and *Cop*. sp.1 [Fig. 3.4] three times in ponderosa pines. Sexual forms of those fungi were only obtained from mite fungal cultures, confirming the importance of mites to these fungi. A potential relationship between *Cop* sp. 1 and the mountain pine beetle was suspected based on developmental synchrony (Khadempour et al. 2012). *T. hirsuta* mites were seen on stained wood beneath the bark between beetle galleries. On this wood the reproductive structures or perithecia were present in large numbers. [See Fig. 3.4 with *O. montium* and *Cop*. sp. 1 perithecia in gallery]. It is possible that *Cop*. sp. 1 is beneficial to phoretic mites developing in synchrony with beetle carriers and not necessarily to the beetles. The true importance of this fungus remains uncertain.

The low fungal transport on mites found in this study could also reflect an overall reduction of blue-stain in the mite-beetle symbiosis that may relate to beetle population levels. The only other study looking at mite-fungal association on this beetle in South Dakota (Reboletti 1998) found a greater amount of mites transporting blue-stain fungal spores. However, that study sampled an incipient mountain pine beetle epidemic unlike our apparently declining populations in the CFR. Finding that the overall fungal proportions on mites varied between 2012 (warmer and dry year) and 2013 (cooler and wetter year) and a similar trend on the proportions from beetle populations, suggest that blue-stain was decreasing for other reasons, such as

competitiveness of blue-stain with other fungi growing on a wetter period. It will be appropriate to sample similar information from contrasting beetle population such as endemic, incipient, epidemic, and declining to obtain a better understanding on how mite blue-stain contribution into the symbiosis can affect the overall percent and composition of blue-stain fungi introduced to trees and how this may affect beetle populations.

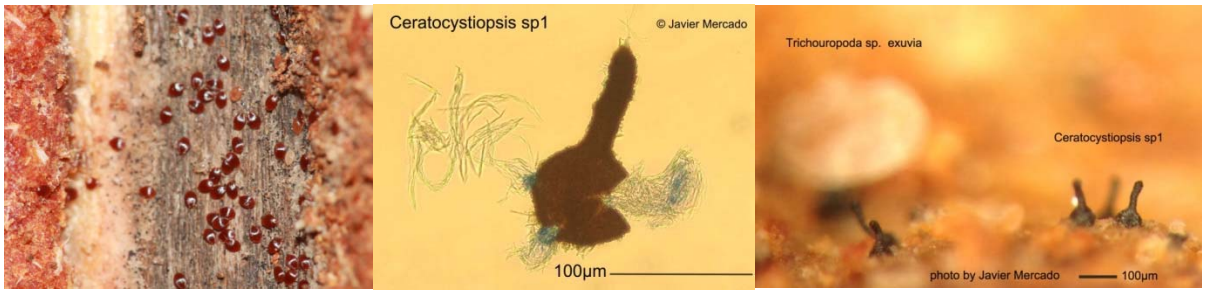


Figure 3.4. Mites including the new documented associate, *Trichouropoda hirsuta* Hirschmann, occurred under the bark between mountain pine beetle galleries where reproductive structures of *Ophiostoma montium* and *Ceratocystiopsis*. sp. 1 developed and produced spores.

Contrasting fungi found on beetles and mites, *O. montium* was more frequently found than *G. clavigera* and *L. longiclavatum* on mites, which in turn were proportionally more frequent on beetles. Recent findings support a closer mutualism of *O. montium* with mites and *G. clavigera* with the beetles. Synchronization of fungal sporulation during periods of beetle flight was studied by Moore (2013). She found that sporulation of *G. clavigera* initiated at 15° Celsius increasing to a peak at 30° Celsius at temperatures that are close to the earliest flight reports of mountain pine beetle and peak flight periods of this species in Colorado, respectively (McCambridge 1964; McCambridge 1971; Gray et al. 1972). Although, *O. montium* had no

apparent sporulation peak, it began sporulating earlier than *G. clavigera*; this may be associated to mite activity during beetle-host search, which occurs prior to beetle emergence.

Although, *G. clavigera* is capable of reproducing sexually (Tsui et al. 2012), the sexual structures or perithecia from these species are rarely seen (Lee et al. 2005). Sexual spores have been documented rarely on mountain pine beetle surfaces (Bleiker et al. 2009) or mycangia, in contrast to the documentation of non-sexual spores or conidia (Bleiker et al. 2009). This could be related to its primarily non-sexual reproductive strategy. It is possible that in *G. clavigera* the ubiquity of long conidiophores bearing elongate asexual spores or conidia and the rarity of short perithecia containing smaller, rounded sexual spores has resulted from fungal adaptations to exploit the beetle as its primary vector. Also, that other fungal associates of mountain pine beetle-mite symbioses use different strategies to obtain dispersal benefits from different taxa.

Fungal composition seemed similar on mites and in beetles on both years with few exceptions. Generally the frequencies found on mites resembled more closely that found on beetle's elytra. At least for the blue-stain fungi these similarities might be explain at least in part by the main reproductive methods of the different species. *Ophiostoma montium* sexual structures are more commonly found under bark than those from the other two blue-stain species. This species spores are secreted on a sticky tendril at the tip of a long-necked pear-shaped structure called a perithecia. These secretions might somehow result attractive to these, whereas dissemination by the beetle may results as an accident as the beetle exterior comes in contact of a structure growing into the gallery, but also indirectly by the action of phoretic mites that carry them over the beetle. Bleiker et al. (2009) found that spores of *O. montium* occurred in clusters

on the beetles elytra but exclusively non-sexual spores on the mycangia. Fungi adapted for mycangial dissemination might need to be of a determined size, shape, or growing substrate to become intercepted by the beetle. Both *G. clavigera* and *L. longiclavatum* produce asexual spore on conidiophores of about 1.5 mm that grow surrounding galleries, these structures become easily intercepted by feeding beetles and are the ones seen on mycangia (Bleiker et al. 2009). Some species frequencies did not differed among beetle structures or mites. *Penicillium* sp. was the second most common non blue-stain associate found on mites on the cooler second year, but it was similarly common on both sampled beetle structures that year this might be due the air disseminated spores.

Apart from blue-stain other fungi found might be important to the beetles diet (Khadempour et al. 2012). We found no relation with the presence of *Penicillium* sp. on mites and the frequency of blue-stain fungi on the beetle-mite complex; however, this is an important topic that should be investigated under different mountain pine beetle population levels.

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