

**Reactions of mountain pine beetles (*Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae) to diets presented with mutualist and non-mutualist fungi**

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## **Abstract**

The interaction between *Dendroctonus ponderosae* (mountain pine beetle) and an unknown black stain fungi is studied with an experiment that looked at whether beetles had a preference for entering diets that had been mixed with either black stain fungi, blue stain fungi (*Grossmania clavigera*), or simply malt extract agar. It was predicted that the unknown black stain fungi would be avoided by the beetles because it is not one of the fungal species that *Dendroctonus ponderosae* has a symbiotic relationship with. Analysis of the results with a chi square test for independence showed that the beetles showed no preference. Even though the beetles did not show a preference, it is possible that competition for subcortical resources and phloem between mutualist and non-mutualist fungal species results in a suppressed mountain pine beetle development. The potential for mountain pine beetle expansion will depend on whether the black stain fungus is more successful than the mycangial symbionts that the beetle invades with.

**Keywords:** *Grossmania clavigera*; *Dendroctonus ponderosae*; mountain pine beetle; symbiotic fungi; competition.

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## Introduction

### *History of the mountain pine beetle outbreak*

*Dendroctonus ponderosae* Hopkins (mountain pine beetle) is a bark beetle native to North America that operates at endemic levels and has the ability to achieve outbreak population levels (Safranyik et al. 2010). Any *Pinus* spp. (with the exception of *Pinus jeffreyi*) can function as the beetle's host; the mountain pine beetle is not limited by its host availability given the widespread distribution of pines across North America. The current epidemic in British Columbia began with outbreak populations that were triggered by homogenous stand compositions on a landscape level and warming temperatures associated with climate change. Epidemic mountain pine beetle populations were successful in new areas because of climate change increased the climatic suitability in formerly unsuitable areas. Later, the epidemic in British Columbia spread out of the province into Alberta by wind assisted long range dispersal over the Rocky Mountains, likely around 2002 (de la Giroday et al., 2012). It has expanded its range into Alberta, threatening the boreal jack pine forests. In 2010, the area of western Canada affected by the mountain pine beetle amounted to 14 million hectares (Safranyik et al., 2010).

## The epidemiology of the mountain pine beetle

### Endemic populations and their population dynamics

The mountain pine beetle carries out two main types of life strategies. They can exist as endemic or epidemic populations. As an endemic population, the mountain pine beetle does not have the densities required to sustain a mass attack on even a single large diameter tree (Safranyik and Carroll, 2007). At low population densities, mountain pine beetles are subject to the Allee effect. Populations of mountain pine beetles in this state must act as secondary bark beetles, needing to prey on diseased or suppressed trees if they hope to experience reproductive success. The

colonisation of non-healthy trees is riddled with trade-offs, even if it guarantees reproductive success when other options are not available. In most stands, diseased or suppressed trees are a rare resource that must be shared with competitors. The presence of these competitors and the poor quality of phloem in these suppressed trees ensures that the resulting mountain pine beetle brood in the next generation is low in number. Cohabitation of the same trees by different bark beetle species does not only reduce mountain pine beetle reproductive success through competition for the same resource, the presence of other species may also draw in addition predators (Boone et al., 2008). Predation pressure from the generalist Clerid beetles on endemic mountain pine beetles was found to have increased when *Ips pini* was present on the same trees as *Dendroctonus ponderosae*. The arrival of *Ips pini* after mountain pine beetles had finished their mating extended the time period when the pheromones that attract generalist predators persist. If *Ips pini* was not present, *Dendroctonus ponderosae* would be relatively safe from generalist predation. The combined effect of competition and predation led to a reduction in mountain pine beetle brood productivity. Endemic populations are also limited spatially by the rarity of suitable hosts (Safranyik and Carroll, 2007). They are unable to dominate a landscape because infestations are only able to persist in patches.

### **Release from the endemic state**

Mountain pine beetles are able to escape the negative effects of small populations on their population growth rate only when conditions increase the availability of potential hosts. This frees them from their reliance on the few suitable hosts in a stand. In British Columbia, our history of fire suppression allowed the growth of even aged, homogenous pine stands. As they aged, their resistance to beetle attack declined. Their widespread coverage of the landscape and homogeneity ensured that when age or environmental conditions caused a decline in resistance to mountain pine beetle, there would be more potential hosts available than in a heterogeneous stand

(Raffa et al., 2008). Along with a homogeneous landscape dominated by even aged stands, the occurrence of temporary climatic extremes like droughts or warm winters can aid mountain pine beetle populations. These stochastic events increase the reproductive success of beetles; warm winters reduce the overwintering mortality of larvae and droughts can increase the susceptibility of trees by reducing their defensive capabilities. These short term boosts to beetle productivity can allow beetle populations to cross size thresholds that allow positive feedbacks to act on the population. An incipient-epidemic population is in a precarious position; it still requires the continued presence of suppressed hosts to continue existing in this state. If the supply of suitable hosts runs out, then a population will return to an endemic state (Safranyik and Carroll, 2007). One of the sources of positive feedbacks is a large population of mountain pine beetles that can facilitate attacks on large diameter trees. If a population cannot grow past a threshold size, the depletion of suppressed or stressed trees from their resource pool can act as a negative feedback (Raffa et al., 2008).

### **The conditions required for epidemic populations**

Epidemic populations occur when incipient-endemic populations are able to grow large enough to defeat the defences of large diameter trees; they no longer need to opportunistically attack diseased or suppressed trees (Safranyik and Carroll, 2007). These populations also disperse over a landscape; infestations no longer need to occur in isolated patches. At epidemic levels, their life strategy is to preferentially attack large trees to avoid interspecific competition. One of the differences between endemic and epidemic populations is the cues that they rely on to determine the target of their mass attacks. Endemic populations look for signs of weak defences, sampling for low monoterpene concentrations in potential hosts (Boone et al., 2011). On the other hand, epidemic populations are drawn to high concentrations of monoterpenes, strong defensive capabilities is indicative of plentiful resources that can support large beetle broods. Mountain

pine beetles also use  $\alpha$ -pinene as a precursor of aggregation pheromones; higher concentrations of this monoterpene translate to more pheromones that draw in more attacking beetles that increase the likelihood of overcoming tree defences (Raffa et al., 2008, and Boone et al., 2011). Epidemic populations are free from the regulation of natural enemies of interspecific competition, only collapsing when there is a sufficiently lethal extreme event that drastically increases the mortality rate or when beetles run out of high quality hosts on the landscape (Safranyik and Carroll, 2007). Populations can return to an endemic state after epidemic populations collapse.

### **Tree reactions to attacks**

Plants are faced with a dilemma; they must choose between either devoting their resources to growth or to defence (Herms and Mattson, 1992). The respective metabolic processes that drive growth and defence development require many of the same precursors, meaning the choice of one option precludes acting out the other. Actively dividing cells also have suppressed secondary metabolisms that limit the production of defensive chemicals. The amount of energy devoted to defence will depend upon whether the resources in the environment are sufficient to allow regrowth. The high cost of failing to defend against beetle attacks creates the need for strong defences. Pine trees react to beetle attacks through a combination of constitutive and induced defences (Boone et al., 2011). The constitutive response is characterised by the release of resin from pitch tubes that serve as a physical obstacle to beetle intrusion. This resin can also slow the dispersal of aggregation pheromones released by invading beetles and delay the onset of mass attacks (Raffa and Berryman, 1983). At the same time as the constitutive defences slow beetle entry, the secondary metabolic processes kick in to produce the suite of monoterpenes and other toxic volatiles in the tissues surrounding the sites of beetle attacks. The parenchyma cells undergo autonecrosis, secreting their toxic contents into the location of the beetle attackers and

their fungal associates. This forms lesions that prevent the use of phloem by the beetles and fungi (Raffa and Berryman, 1982). The monoterpene concentrations in induced defences are much higher than in constitutive defences (Herms and Mattson, 1992, and Boone et al., 2011). In addition to monoterpenes, the secondary metabolism in lodgepole pines (*Pinus contorta*) also produces sesquiterpenoids and phenolics that act as toxins against microorganisms and insects; these can act as antifungal agents (Raffa and Berryman, 1983). The factor that leads to the failure of a tree to repel an attack is whether its ability to produce an induced defence is overwhelmed by the number of attacking beetles (Raffa and Berryman, 1982). Trees that successfully defend against endemic attacks tend to have higher concentrations of monoterpenes in their lesions. This in turn is dependent on the vigour of the tree. Older trees or suppressed trees are less likely to have the stores of photosynthates required for the synthesis of defensive secondary metabolites. More productive pines will be better defended than their weakened counterparts. Under epidemic conditions, beetles can ignore the monoterpene levels of even well defended trees by collectively exhausting the ability of the host to defend.

#### **Fungal species present in mountain pine beetle mycangia**

The mycangia of beetles carry with them the asexual propagative spores of mutualistic fungi that help the beetle attacks to succeed (Safranyik and Carroll, 2007). The two main species that are typically associated with mountain pine beetles are *Ophiostoma montium* and *Grossmania clavigera* (blue stain fungi). Both of these species can be found in the mycangia of mountain pine beetles (Lee et al., 2006). Both species function in a similar manner with respect to their relationship with their beetle vectors; they help kill the host tree. They attack the host by drying out the infected area after germination, disrupting the ability of the host to carry out water transport (Safranyik and Carroll, 2007). These efforts help contribute to the success of the beetle attack by limiting the host's ability to produce induced defences through the blocking of

transpiration (Solheim and Krokene, 1998). Between the two mycangial species, *Grossmania clavigera* is the more virulent of the two; it grows quickly in white pine (*Pinus monticola*), lodgepole pine (*Pinus contorta*) and ponderosa pine (*Pinus ponderosa*). *Grossmania clavigera* causes high tree mortality compared to the lack of mortality in trees inoculated with *Ophiostoma montium*. This is linked to the relative performance of *Grossmania clavigera* and *Ophiostoma montium* in fresh sapwood. The environment of fresh sapwood is characterised by high moisture and low oxygen. *Grossmania clavigera* is better suited for these types of environments than *Ophiostoma montium* (Adams and Six, 2007). The latter tends to grow better in relatively low moisture and high oxygen environments (Solheim and Krokene, 1998). *Grossmania clavigera* dominates in the sapwood, killing live tissues; this is later followed by the dominance in abundance of *Ophiostoma montium* as tree tissues die and dry out under the influence of *Grossmania clavigera* (Adams and Six, 2007). In addition to these sapwood environmental requirements, the two fungal species have different growth rates depending on the temperature. *Grossmania clavigera* dominates during cold to cool seasons while *Ophiostoma montium* dominates during warm to hot seasons. Therefore, the change in dominance in sapwood is tied to seasonal temperature fluctuations.

One of the other benefits mountain pine beetles can gain from their symbiotic relationship with their fungal associates is access to additional nutrients from the consumption of asexual spores (Safranyik and Carroll, 2007). Observations of larval feeding behaviour appear to show that mid-late instars return to old sections of their galleries where asexual spores have grown (Adams and Six, 2007). Both *Ophiostoma montium* and *Grossmania clavigera* have been found in the digestive systems of 3<sup>rd</sup> instars, indicating that the developing larvae feed on fungal material.

The experiment detailed in this paper will look at whether adult female mountain pine beetles will enter material that has been combined with cultured samples of black stain fungal species; beetles appear to avoid areas colonised by this fungal species in the field. This study will test the hypothesis that mountain pine beetles will avoid entering diets that have been mixed with black stain fungi cultures because the fungi is not one of the mutualist symbionts that the beetle relies on.

## **Methods**

### *Culturing of fungal samples*

The agar that was used in to prepare the fungal isolates in this experiment used a combination of 8.33 g of malt extract agar and 7.5 g of agar dissolved in 500 mL of distilled H<sub>2</sub>O. The 1 L bottles filled with the solution were autoclaved before being poured into petri dishes. Autoclave machines subject their contents to high temperatures and high pressures to ensure the agar in the petri dishes will be sterile. The filled petri dishes were left to cool and solidify.

The wood samples used to isolate the target wood fungi were contaminated with other microorganisms. In order to reduce the growth rate of the non-target organisms and ensure the black stain fungi can be isolated from the wood, an antibiotic, streptomycin, was added to the malt extract agar recipe. The streptomycin was prepared by adding 0.2 g of the solid form of streptomycin to 10 mL of distilled water. 2.5 mL of the dissolved streptomycin was then added to autoclaved agar before pouring the agar into the dishes.

Pieces of wood collected from wood colonised with black stain fungi were surface sterilised with by dipping the pieces of wood in ethanol for a few seconds and then moving the pieces into distilled water. These wood samples were then placed into the malt extract agar and

streptomycin plates. Initially, water agar plates were used in attempts to isolate the black stain fungus, but contamination by bacteria and time constraints required the use of plates filled with malt extract agar and antibiotics. After the plates had been successfully colonised by black stain fungus, pieces of the medium from these plates were placed onto multiple new malt extract agar plates. The blue stain (*Grossmania clavigera*) cultures were made with the MEA plates from agar plugs.

### **Mountain pine beetle experiment**

The diet used for each experimental unit was mixed with three different treatments: black stain fungus cultures, blue stain fungus cultures, and control. Experimental units that were in the blue stain treatment group had diet mixed with blue stain fungi cultures; black stain cultures were used for the black stain treatment group. The control group had agar mixed with diet. Small petri plates were divided in half, filling one side with the diet and leaving the other half open. The plates half filled with the diet were left to dry before adding monoterpenes to them. A mix of monoterpenes, including  $\alpha$ -pinene and limonene, was added to the diet to simulate the subcortical environment in lodgepole pines.

Mountain pine beetles were collected from lodgepole pine boles. Females were separated from the males and used for the experiment. In *Dendroctonus ponderosae*, females initiate attacks on trees, and only after the gallery is constructed does the male arrive to mate (Safranyik and Carroll, 2007). Males were identified by handling them and listening for stridulations (Michael and Rudinsky, 1972). The sex that initiates attacks for members of *Dendroctonus* spp. is also more likely to be larger (Foelker and Hofstetter, 2014).

The beetles were left alone for about 20 hours and their movements were recorded with time lapse video on a 15 minute interval. The plates with beetles that entered their diets and their

respective treatment type were recorded. Beetles were judged to have entered the diet if any of the images recorded by the camera showed a beetle entering the diet or showed an image without a beetle in the empty half of the dish. The images were taken with a monochrome setting. The trials used a red lamp because the mountain pine beetles are not sensitive to red light (Groberman and Borden 1980). They are, however, sensitive to the higher energy portion of the visible spectrum. If an incandescent light bulb was used instead, the beetles could have been possibly affected by the light source, given that it also emits in blue and green. In their study of adult *Dendroctonus pseudotsugae* and *Ips paraconfusus*, Groberman and Borden (1980) found that the beetles responded to light with a wavelength of 450 nm and between 510-530 nm. This suggests that they have dichromatic vision for the blue and green portion of the spectrum respectively. The results come from tests where authors exposed the compound eyes of beetles to wavelengths at 10-25 nm intervals between the range of 400 and 650 nm. The ERG responses of both *Dendroctonus pseudotsugae* and *Ips paraconfusus* to the light decreased in the red portion of the spectrum, with the lowest level of response at 650 nm. This indicates a lack of sensitivity to red light.

The use of a red lamp was to avoid a phototactic response; mountain pine beetles will fly into the direction of a light source (Shepherd 1966). Mountain pine beetles show a phototactic response when presented with a single light source. At moderate temperatures, their response to light is to fly in the direction of the source; at high temperatures, beetles show a negative phototactic response, moving away from the light source. At the temperatures present in the lab used for my experiment, positive phototaxis would have been a risk.

A chi-squared test for independence was used on Minitab 17 (Version 17.1.0) was used to determine whether the frequency of entrants was statistically associated with a treatment type (Bradley et al. 1979, and Sokal and James, 1981).

## **Results**

The collected data from all three trials are summarised in Table 1. The beetles exhibited no differences in preference for the different diets. The results of the chi square test for independence (Table 2) had a p-value of 0.322. A chi square test for independence that rejects the null hypothesis would have had a chi square table with observed cell frequencies that were deviated significantly from the expected values. The evidence suggests that we accept the null hypothesis, that the presence of the type of fungi has no effect on the entrance of beetles, and that we reject the alternate hypothesis, that the fungal species has an effect on the choice of beetles to enter the diet.

		Number of beetles that entered	Number of beetles that did not enter the diet
Trial 1 (Time period for experiment: 22 h 47 min)	Blue stain fungi	2	10
	Control	1	11
Trial 2 (Time period for experiment: 23 h 00 min)	Blue stain fungi	7	5
	Control	5	7
Trial 3 (Time period for experiment: 17 h 45 min)	Blue stain fungi	5	3
	Black stain fungi	4	4
	Control	3	5

Table 1. Summary of collected data from multiple trials. Tests with the black stain fungus were only done for the last trial. Blue stain fungus and the control treatments were done for all the trials. The total time for each trial is included in the table.

Entrance into Diet		Treatment Type			Total Observed
		Black Stain	Blue Stain	Control	
No	<i>Observed</i>	4	18	23	45
	<i>Expected</i>	5	20	20	
Yes	<i>Observed</i>	4	14	9	27
	<i>Expected</i>	3	12	12	
<b>Total Observed</b>		8	32	32	72

Table 2. Contingency table for the Pearson Chi-Square test for independence from Minitab 17

(Version 17.1.0). The test had a chi-square value of 2.267 and a p-value of 0.322. There was no difference between the different treatment types with regards to the frequency of beetles entering their diets. The table shows the observed number of entrants and the expected number of entrants if the number of beetles entering their diet was due to chance.

## Discussion

### Problems with the experiment

The methods used in this experiment have much room for improvement. Many of the problems were due to the time limitations of working with mountain pine beetles. The adult forms only survive for a short period of time. The mountain pine beetles that were collected from the boles were left in the refrigerator for several days. The sample sizes were limited because the experiments were run at the tail end of the period of beetle emergence from the sample boles. The study ran out of beetles to use for the experimental trials.

The use of cold storage to store beetles has been analyzed for the possible effects the process has on mountain pine beetles (Safranyik 1976). Safranyik (1976) found that with cold storage at relatively constant temperatures, beetle survival was dependent on sex and size. Males were more likely to die when placed in cold storage. Beetles that had a larger pronotal width – which is correlated with greater fat stores – were more likely to survive through cold storage. The beetles were stored at  $1 \pm 2$  °C, a few degrees colder than the temperatures used to temporarily store the beetles in this experiment. Given that the refrigerator used to store mountain pine beetles in my study was at a higher temperature than the storage used in Safranyik (1976), the beetles died much earlier than in Safranyik (1976) due to starvation and insufficiently suppressed metabolisms. The beetles in my study were stored for too long; Safranyik et al. (1976) recommended that studies of adult mountain pine beetle behaviour should only be done on freshly emerged beetles.

Female mountain pine beetles compose the majority of emergences at the beginning of the emergence period (Safranyik 1976). As time progresses, males begin making up a larger proportion of emergences. In addition, the sizes of beetles begin to decrease through the same

span of time. If my experiment was repeated, it would be better to use beetles from the beginning of the emergence period because of the greater supply of female beetles of larger sizes. This more closely resembles the beetles that initiate attacks in field conditions.

The wide variance between trials in the acceptance of the diet for each of the treatments points at the different terpene mix used in each trial as the culprit. I failed to do this experiment at an early enough time so that I could use a consistent method of adding the same recipe of monoterpenes. The two methods that were used to add monoterpenes were: 1) add the mixture to small circular filter papers and stick them to the cover of petri dishes; 2) add the monoterpenes directly to the diet. The shift from the first method to the second was because the entrances that resulted from the first method were low across both the control and blue stain treatment types. This opened that possibility that not enough monoterpenes were added to the pieces of filter paper. The acceptances of the diet that resulted from the shift to method 2 seem to suggest that this is indeed the case because of the attractiveness of monoterpenes to mountain pine beetles. Higher concentrations of monoterpenes make the acceptance of the diet more likely to occur because of they are precursors for bark beetle aggregation pheromones (Raffa et al. 1993). The use method 2, with its higher concentrations of volatiles, also resulted in the formation of condensation on the lids of the petri dishes after the volatiles had begun evaporating from the diet. The condensation that formed reduced ability to see into the dishes. The obfuscation made judging entrances vs. non-entrance more difficult to do. Method 1 did not have the same issues with condensation because of the lower concentrations of monoterpenes.

### **Low sample size effects on the validity of chi-square test for independence**

The low expected frequency in the black stain cell is a result of the low sample size for the black stain treatment. Low sample sizes are tied to reduced power. In addition the chi-square test uses

an approximation of exact multinomial probabilities (Bradley et al. 1979). The larger the sample size, the closer the approximation is to the true multinomial probabilities. The inclusion of cells with low expected frequencies could potentially make the decision to accept or reject a hypothesis invalid because of the higher value of Type I error due to a poor approximation of the distribution (Lewis and Burke 1949).

Therefore, I need to consider whether my use of the chi-square test for independence was invalidated by low expected frequencies that could have negatively affected the Type I error rate. The traditionally recommended minimum expected frequency has been 10, with 5 as the absolute minimum (Lewis and Burke 1949). This recommendation has been repeatedly challenged by studies that have concluded that even a minimum expected frequency of 5 is too conservative (Roscoe and Byars 1971, Camilli and Hopkins 1978, Bradley et al. 1979). These studies have found that at low expected frequencies, values even as low as 1 or 2 do not inflate the Type 1 error from  $\alpha = 0.05$  to 0.06. This means that the use of tests for independence will likely still be valid even when expected frequencies are low (Bradley et al. 1979). In Delucchi's (1983) review of Lewis and Burke's (1949) conclusions, he states that the use of expected frequencies that are less than 5 is fine, but that low associated sample sizes associated with low expected frequencies should still be avoided due to the negative effect it has on a study's power.

### **Possible reasons for the lack of response to the black stain fungi**

If the results can be trusted, they show that mountain pine beetles show no preference for either black stain, blue stain, or the control treatments. This would be explained by the fact that mountain pine beetles carry their blue stain fungal associates into prospective host trees. Control diets would represent uncolonised trees. Mountain pine beetles consider the complex of volatiles when they use their gustatory senses after landing on possible hosts to gauge the defensive ability

of the tree (Safranyik and Carroll, 2007). There would be no reason to expect entrances to respond to the presence of fungal associates within the diet.

The malt extract agar cultured fungi was in a vegetative asexual form. In the field, the fungal associates of mountain pine beetles grow after the beetle vectors inoculate the host tree upon attack. The fungus then spreads through the host at the same time as the beetle larvae grow (Safranyik and Carroll, 2007). The fungus also begins growing asexual spores; beetle larvae consume these spores for nutrients in addition to the resources they gain from feeding on the phloem (Adams and Six, 2007). Beneficial fungal associates begin growing after beetle entry and the proportional makeup of the fungal species complex changes as the time since colonisation increases. *Grossmania clavigera* functions as the primary coloniser, spreading quickly through the host tree and creating conditions that help the growth of the less virulent *Ophiostoma montium* (Solheim and Krokene, 1998, and Adams and Six, 2007). The asexual spores that are grazed by the growing larvae are not limited to one species because of the shifting dominance between the two main mycangial species. Beetle larvae appear to be able to feed on different species; unless the black stain fungi in this experiment has defences against beetle grazing, there should be no expectation of an avoidance of black stain spores.

If the beetles do not avoid entering the diet with the black stain fungi, what then can explain the observation that galleries are not found in black stain colonised areas? Different pathogens have differing levels of virulence, illustrated by *Grossmania clavigera*'s stronger ability to dry out the sapwood of the hosts compared to *Ophiostoma montium* (Solheim and Krokene, 1998). It could be possible that the avoidance of black stained areas is reflective of the fungus' possibly weaker ability to create amenable conditions for beetle development compared to the abilities of the beetle's mycangial associates.

## Competition between mutualistic and non-mutualist fungi and their effects on beetle success

Even if the black stain is not avoided by the mountain pine beetle, the presence of a non-mutualistic species can still have negative impacts on brood success. The effects of a non-mutualistic fungus species on the phloem environment have been studied for *Dendroctonus frontalis* broods (Ayres et al. 2000, and Lombardero et al. 2003). *Ophiostoma minus* is recognised to have an antagonistic relationship with *D. frontalis*. Lombardero et al. (2003) studied how *O. minus*' spread through the phloem environment occurs. They showed that *Tarsonemus* mites facilitate the dominance of *O. minus* through the phloem. When fungal inoculations were only due to the invasion of beetles, successful colonisation by *O. minus* regularly occurred only if there were high levels of inoculum. Without the high inoculum load, *O. minus* colonies only developed 10% of the time. Even when successful colonies did develop, the area that was colonised was limited to within 1-4 cm of the galleries. The presence of *Tarsonemus* mites allowed *O. minus* to spread 4 times the average area.

One of the possible mechanisms that have been considered by Lombardero et al. (2003) and Ayres et al. (2000) is that *D. frontalis*' mycangial associates, *Ceratocystiopsis ramaculosus* and *Entomocorticium* sp. A, perform their job of concentrating nitrogen from the phloem much better than *O. minus*. Mutualistic fungi appear to pull nitrogen from the sapwood and bring it to the spores in the phloem (Bleiker and Six 2007). This would explain how the phloem colonised by mutualistic fungi has a higher nitrogen concentration than phloem uncolonised by the fungi. The antagonism between *D. frontalis* and *O. minus* is a consequence of an inability of *O. minus* to support the nutritional requirements of *D. frontalis*. Because phloem is even more nutrient poor than foliage, beetles must have some strategy to obtain their limiting nutrients, nitrogen and phosphorus (Ayres et al. 2000). Ayres et al. (2000) found that *O. minus* had a significantly lower

nitrogen concentration than its fungal competitors (0.86% N for mycangial fungi vs. 0.70% N for *O. minus*). This has a direct effect on the success of beetle larvae through delays in developmental time as more feeding is required to reach the size necessary to complete larval development. In addition, the authors found meandering galleries in trees inoculated with *O. minus* that indicated failed broods. Less successful broods have larger galleries because the larvae need to feed on a larger area to compensate for poor availability of nutrients (Bleiker and Six 2007). Bleiker and Six (2007) found that mountain pine beetles emerging from fungal colonised trees were larger, as a result of better larval growth. Beetles that were reared on *Grossmania clavigera* were larger than ones that were reared with *Ophiostoma montium*, indicating the possibility that *G. clavigera* is better at concentrating nitrogen.

Phytophagous insects are unable to synthesize sterols (Six 2012). They are only able to acquire the sterols that they require for development and reproduction through feeding on their plant hosts. Sterols only occur at low concentrations in plants; this provides an incentive for bark beetles to find another source of sterols. Fortunately for the beetles with fungal associates, fungi are able to synthesize a supply of ergosterols. A beetle population whose symbionts must share phloem with a non-mutualistic competitor could possibly exhibit lower growth rate because of a limited access to needed sterols.

Access to mycangial fungi is also important for beetles even after they have reached the teneral adult stage. *D. ponderosae* appears to prefer feeding on its mycangial associates over just the phloem prior to their emergence as adults (Bleiker and Six 2007). Their reproductive success was tied to access to fungal associates (Six and Paine 1998). After raising *D. ponderosae* and *D. Jeffreyi* larvae with exposure to different mycangial associates, and a no fungus treatment, Six and Paine (1998) allowed pairs of male and female adult beetles to attempt to enter a bolt of

either *Pinus jeffreyi* or *Pinus contorta*. They found that for both species of beetles, the rejection rate was much higher when the beetles had not been exposed to mycangial associates as larvae. Beetle pairs that rejected bolts first entered and then emerged without constructing galleries. Beetles that were given access to fungi in their larval stage were more likely to enter and reemerge after several weeks. Six and Paine (1998) believe that the differences in behaviour were due to the effects of nutrients provided by mycophagy. Teneral *D. ponderosae* adults without access to mutualistic conidia must instead find the nutrients they need for reproduction by extensively mining the phloem (Bleiker and Six 2007). Even with multiple choices of mutualistic fungi, individual species of fungi will have differences in their ability to provide nutrients; this means that differences in fungal species dominance will have direct effects on beetle fitness (Six 2012). If an area of the phloem is covered by an unusable species of fungi, it is likely that mountain pine beetle adults will be less successful in creating large broods in the subsequent generation due to size and nutrient deficiencies.

### **Implications on the expansion of mountain pine beetle populations**

What do the results from this experiment and other studies of the effect of fungal communities on bark beetle reproductive success tell us? Mountain pine beetle adults appear to not exhibit a preference between different additions of fungi to their diet. The studies done by Lombardero et al. (2003) and Ayres (2000) show that there is a negative fitness cost to *D. frontalis* when the bark beetle lays its eggs in galleries that become colonised by an antagonistic fungi. Six and Paine (1998) showed that access to conidia before adult emergence is important for determining whether adults decide to stay within boles. The fungi used in this experiment would not appear to be a direct antagonist; the mountain pine beetles do not appear to have a negative reaction to it. But if it exists in the field as a competitor with mycangial fungi for subcortical resources, it could

possibly reduce the reproductive success of mountain pine beetles. Larval survival and their adult reproductive success is partly dependent on the availability of nutrients; the dominance of *O. minus* creates conditions for *D. frontalis* where populations may be suppressed. Ayres et al. (2000) predicted that the reduced nutritional value of *O. minus* will increase the larval developmental time and have observed high rates of mortality of *D. frontalis* when larvae have had to feed in phloem dominated by *O. minus*. Lombardero et al. (2003) note that if *D. frontalis* populations are both suppressed by *O. minus* and at replacement rates of population growth, relatively small decreases in the area colonised by the fungi could potentially allow positive population growth for the beetle. If these dynamics apply to *D. ponderosae*, the presence of a non-mutualistic competitor could mean that the mountain pine beetle becomes suppressed by competition for phloem resources when it encounters the black stain fungi, leading to lower rates of population growth. This will depend on whether the black stain fungi or *Grossmania clavigera* is more pathogenic.

## Acknowledgements

I would like to thank Allan Carroll for the help he gave for this experiment. He allowed me to use his lab and mountain pine beetles for this paper. I would also like to thank Richard Hamelin for the use of his lab for the culturing the fungi. Padmini Herath both taught me how to culture the fungi. Jordan Burke helped me with the mountain pine beetle with the experimental design and the carrying out of the experiment itself. He provided the diet and the terpene mix that was used in this experiment and I would not have been able to perform this experiment without his experience.

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