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Article

Fungal Frequency and Mite Load Trends Interact with a Declining Mountain Pine Beetle Population

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Abstract: The mite and fungal biota associated with the mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopk.) may not be stable throughout an irruptive event. In congeneric beetles, variations in the frequency of their associated organisms affect population trends and similar effects may occur in MPB. We studied fungi and mite trends in a declining irruptive MPB population as it attacked three different pine hosts in the Colorado Front Range. During the study, we found two new associates including one biologically relevant mite and one beneficial blue-stain fungus. Fungi hyperphoretic on mites were also documented. This included beneficial and potentially detrimental species to the MPB. The frequency of several organisms varied between some years or pine hosts but not within male or female beetles. A large increase of *Trichouropoda* sp. and *T. ips* mites trended inversely with the declining beetle population, while a decrease in the beneficial blue-stain fungi trended similarly to the declining beetle population. We discuss the interactions and potential effects of phoretic biota in relation to (1) the MPB associates' population trends, (2) the MPB incursions into cooler areas, and (3) the redundancy of blue-stain fungi carried by the MPB holobiont. These findings increase our knowledge of the mechanisms that influence MPB populations.

Keywords: antagonist; fungal biota; fungivore; mutualist; phoretic mite

1. Introduction

In western North America, episodic irruptions of mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopk.) can severely affect large forested areas [1]. On impacted ecosystems, tree mortality caused by the mountain pine beetle can promote important natural processes that invigorate them. Mortality events caused by the MPB foster changes in tree age structure by removing old and unhealthy trees while creating space for young and vigorous trees of the same species to grow [2]. These events can also alter ecological succession by facilitating the emergence of a different tree species composition on the landscape. The resulting tree composition may improve vegetation resiliency in these lands, especially in the face of changing climate. Tree mortality is not solely caused by the beetle's action and, as in other bark beetle species, several organisms use adult MPBs to gain transportation from one tree to the next. At any time, these organisms can affect the success of the beetle and the type of damage caused to trees.

Fungi are the most studied, and thus, are the best-known component of the MPB associated biota due to their contribution to a tree's rapid death [3–6]. These include unicellular yeasts and multicellular ascomycete fungi such as the blue-stain causing species. Yeasts are the most prevalent ascomycetes on many bark beetles [7–9], including the MPB [10–12]. Yeasts perform essential functions that impact MPB success in the newly attacked tree such as metabolizing alpha-pinene into verbenone, a semiochemical acting as an anti-aggregation pheromone during MPB mass-attacks and signaling attack

saturation [13]. Yeasts are reviewed elsewhere [14,15], and here we focus on filamentous blue-staining species. The blue-stain fungi prevalence varies among different MPB populations, but are generally reported to be highly frequent on beetle samples (e.g., 93–95% [16], 74–99% [17]). Blue-stain fungi growth causes a depletion of resinous defenses of infected pine trees [18], which improves the MPB attack success. However, more significant to the MPB is that the larvae deprived of all blue-stain fungi fail to complete metamorphosis [19,20]. Three blue-stain fungi are commonly associated with the MPB. *Leptographium longiclavatum* S.W. Lee, J.J. Kim and C. Breuil was first documented in Canada during the epidemic at the turn of the century. This species has been associated with cooler areas within the distribution of the beetle [21]. Another, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield can influence MPB success by obtaining its needed carbon from limonene [22], a component of the oleoresin that is toxic to the beetle [23–25]. By metabolizing limonene, *G. clavigera* converts the beetle's deterrent into a nutritional fungal mass beneficial to the insect [22]. The third associate, *Ophiostoma montium* (Rumbold) von Arx, may be a more recent associate [16] or it may be more closely associated with mites that are phoretic on the MPB. Yet other fungi may be cryptic and remain undescribed [26].

The blue-stain fungi prevalence varies among different MPB populations, but are generally reported to be highly frequent on beetle samples (e.g., 93–95% [16], 74–99% [17]). Within a tree, the variation in the relative abundances of blue-stain species may be influenced by the collection period after initial insect attacks. This variation may be due to site latitude, the beetle developmental state on the tree, and site temperature at the time of collection [17,21,27,28]. As an example, *Ophiostoma montium*, which is more tolerant of warmer temperatures, has been found predominantly in MPB populations attacking during warmer periods [17]. It has been suggested that under a warming climate scenario, *O. montium* could displace *G. clavigera* [29] as the most important blue-stain fungal beetle associate; however, both species are found in most MPB populations [10,12,16,17,20,30]. Analyses of the effects of temperature on the stability of the above two blue-stain fungi have shown that beetle movement between warm and cold habitats, as well as variations in their attack densities, can help explain the prevalence of both species in the symbiosis [31]. Thus, a fungal redundancy in the system may occur [10,21], but studies of fungal community trends during changing MPB populations have not been performed.

Adding complexity to the MPB's symbiosis, beetles also transport a community of several mite species, some of which are fungivorous like the beetle. Sixteen mite species have been reported phoretic in the MPB [32]. Fungi carried by phoretic mites are considered hyperphoretic, i.e., a phoretic within another phoretic [33–36]. In the congeneric southern pine beetle (*Dendroctonus frontalis* Zimm.), fungal species carried by hyperphoretic mites negatively affect this beetle by outperforming its beneficial fungal associates [37]. However, the effects of hyperphoretic fungi transported by mites in the MPB's symbiosis are unknown. We do not know whether their distribution within MPB populations is geographically and temporally stable or if it changes between tree host species or between population stages. In the southern pine beetle, mite frequencies respond to thermal changes within a single season [38]. It is possible that populations of fungus-feeding mite species are associated with the MPB change between tree species since a tree's chemical and ecological differences could affect the frequency of their phoretic fungi or affect mites directly. Exploring fungal dispersion by mites could contribute key information regarding the occurrence of multiple fungal associates and their fluctuations in this symbiosis.

In the USA, most of the research on MPB mite and fungal associates has been conducted in California and the Intermountain West, with little information available from the Southern Rockies. In Colorado, our knowledge comes from the original descriptions of organisms in both groups and recent documentations looking into the organisms' taxonomy and ecology [39,40]. Fluctuations of MPB populations are believed to be influenced by abiotic weather conditions. Here, we attempt to determine whether we can identify biological effects specific to an MPB population stage. We are only teasing out a small part of this complex component that involves predators, parasitoids, and other macro and

micro associates, including nematodes and bacteria, which may also be influenced by a community of associated insects that attack after the MPB. To gain a better understanding of how changes in the abundance of a particular fungal species may affect MPB populations, some understanding of the specificity of its association is important. We consider maxillary mycangial fungi as the most specific, followed by elytra fungi, and lastly, mite associated fungi as the least specific association present in the MPB. We refer to the entirety of the associations (i.e., fungi, mites, on MPBs) hereafter as the holobiont [8]. Therefore this work's goal was to examine the mite and fungal biota of the MPB present in different sub-populations of the insect within the Colorado Front Range (CFR). Specifically, (1) to determine potential variations of common mite species within years, on beetles attacking different pine hosts, and in beetles of different sex in the CFR, (2) to determine if MPB's blue-stain fungi species varied in frequency within beetle's maxillary mycangium or elytra, between years, or between hosts, and (3) to explore whether fungal dispersal by mites can help explain the redundancy of blue-stain fungi present in the MPB's symbiosis.

2. Materials and Methods

2.1. Study Area

Studies were conducted at the Arapaho-Roosevelt National Forest within the southern Rocky Mountains and north in the CFR, with a centroid at about 450,995.0, 4,503,158.0 (Zone 13N, UTM). Each year three rectangular plots of approximately 2 ha (200 × 100 m) were selected in pine forests dominated by three different pine hosts, i.e., *Pinus flexilis* E. James (limber pine), *P. contorta* and *P. ponderosa* (Figure 1). The plots were established within 300 m of trees with beetle activity, which was signaled by fading trees containing teneral adults in May. The attacked stands dominated or co-dominated by *P. ponderosa* and *P. contorta* species were examined during 2011–2013 and one *P. flexilis* dominated plot was examined in 2013.

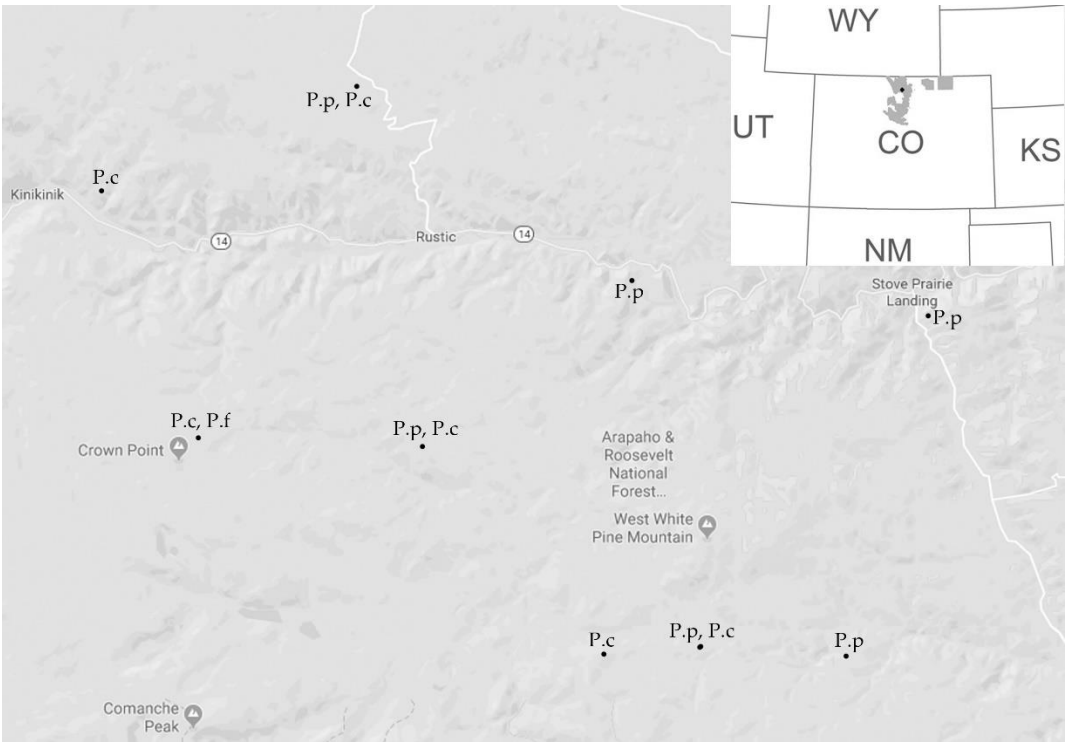

Figure 1. The map of study plots in *P. ponderosa* (P.p), *P. contorta* (P.c), and *P. flexilis* (P.f) dominated stands at the Roosevelt National Forest, Colorado, USA. The insert shows the location within the state of Colorado, U.S. The map is a terrain relief layer modified from Google Earth.

At each plot, healthy trees were monitored one to three times per week beginning in the third week of May, which is the earliest recorded emergence date in the region [41]. Twelve-funnel Lindgren traps baited with a synthetic set of attractants including alpha-pinene, myrcene, and trans-verbenol (Synergy Semiochemicals, Burnaby, BC, Canada) were used to monitor beetle activity in each plot and to determine when to intensify beetle survey and collection efforts. Beetles were collected as they landed on trees and before they began excavating egg galleries. A defense mechanism used by curculionids–thanatosis or death-feigning—in which these drop from a substrate in the presence of potential danger, was used to capture individual beetles in a sterile microcentrifuge tube, preventing the transfer of mites and fungi between individuals. Every year collections became increasingly more difficult, which corresponded to the reduced access to study areas near the High Park fire in 2012 and a drop in beetle activity in Larimer County, culminating in endemic population levels in 2013. Specimens were transported on ice-coolers and kept refrigerated until processed.

2.2. The Sampling and Identification of the Studied Organism

All beetles were examined under a dissection microscope (Leica MZ16, Heerburg, Germany) and identified using diagnostic keys [42,43]. Additionally, all mites from all beetles were quantified and identified based on the original descriptions, keys for the documented mites from the MPB, and various related subcortical insects [44–54]. The studied morphological characters from cultures grown in MEA included the colony color and the shape of its margin, as well as the microscopic characters of the conidiophore production region and conidia type from these and the phloem chip cultures. These characters were compared to published descriptions [55–59]. The fungi were sampled from all MPBs and a subsample of beetles was selected to sample the hyperphoretic fungi on their phoretic mites. To obtain the fungi from mites, a single specimen plate was prepared by placing a whole mite on the plate that was previously frozen to prevent their escape. For the study of fungi transported by the MPB, we concentrated on their presence in the elytra, where shallow pits trap fungal spores, and the maxillary mycangia which is considered a specialized dissemination structure where fungal material including spores are carried [12,60]. To obtain fungi from the maxillary mycangia, the entire maxilla was dissected from the beetle, the maxillary cardine (possessing the maxillary mycangia) was then removed, rinsed twice in distilled water, and then placed in a 2% malt extract agar plate (MEA). To grow the fungi from the elytra, the MPB were streaked dorsally against the MEA plate and then removed. Studies looking at the competitive interaction between *O. montium* and *G. clavigera* have determined that the growth of the two species is similar and does not restrict fungi on either phloem or MEA [61,62]. The plates were kept at room temperature (21–22 °C). This temperature could have given a slight growth advantage to *G. clavigera,* and *L. longiclavatum,* over *O. montium*, given their advantage at these cooler temperatures. Copies of these fungi were observed every two days for a month and all different colonies were again plated on new MEA from which hyphal tips were selected to obtain pure colonies. In occasions when multiple organisms were difficult to separate, these were transferred to a new plate, as well as to a phloem section, to help their separation.

2.3. Molecular Identification of Fungi

Molecular data were used to confirm the morphological identification of blue-stain species; methods of DNA extraction and sequencing are detailed in Reference [40]. The regions analyzed included the nuclear ribosomal ITS2-LSU (partial 5.8 + ITS 2 + partial 28S) and the protein-coding gene β-tubulin (partial gene). The DNA extraction and amplification were performed at the Center For Forest Mycology Research (CFMR) while the sequencing was performed at the University of Wisconsin Biotechnology Center (UWBC) in Madison, WI. Newly generated sequences were compared with other ophiostomatoid sequences through a BLAST Search, and some were also used in a previous phylogenetic study [40], which confirmed the identity of the ophiostomatoid species. Sequences of *G. clavigera* and *L. longiclavatum* were deposited in GenBank (KY940824-KY940843).

2.4. Statistical Analysis

A negative binomial generalized linear model (GLM) was used to determine whether beetle mite loads (i.e., the mite counts per beetle) of the combined species *Tarsonemus ips* Lindquist, *Tarsonemus endophloeus* Lindquist, *Trichouropoda* sp. Berlese, and *Proctolaelaps* sp. Berlese differed by beetle sex or on beetles attacking *P. contorta*, *P. ponderosa*, and *P. flexilis* in 2013. Additionally, we described the effects of *Tarsonemus ips*, *Trichouropoda* sp. and *Proctolaelaps* sp. species-specific mite loads temporally by fitting a negative binomial generalized linear mixed model (GLMM) to data from all three years in *P. contorta* and *P. ponderosa*. Our model included the fixed categorical variables year, tree species, and mite species, as well as a random effect of individual beetle, to account for repeated observations from the same beetle. Lastly, we evaluated the prevalence of different fungal species on beetles across time and tree host species by fitting a binomial GLMM with a logit link function. The fixed effects included fungal species (i.e., *O. montium*, *L. longiclavatum*, *G. clavigera*), tree host species (i.e., *P. contorta*, *P. ponderosa*), year (i.e., 2012, 2013), year-by-fungi interaction, and a random effect for individual beetles. In all models, post-hoc Tukey pairwise comparisons were used to control for the familywise error rate incurred by conducting multiple comparisons and determine trends within fixed effects, where appropriate. All analyses were carried out using the statistical software R version 3.4.2 using the following packages: *MASS* [63] for fitting the GLM, *lme4* [64] for fitting the GLMMs, and *multcomp* [65] and *emmeans* [66] for post-hoc comparisons.

3. Results

3.1. Mite Trends

3.1.1. Phoretic Mite Diversity and Yearly Trends

Within the collection period, we documented five mite species phoretically carried by MPBs attacking *P. contorta* and *P. ponderosa* dominated stands and found the same species from a beetle attacked *P. flexilis* stand in 2013 (Table 1). *Tarsonemus ips*, and species of *Trichouropoda* and *Proctolaelaps* were prevalent during the three years. For unknown reasons, *Proctolaelaps* sp., a nematophagous mite, was not found on any beetle population in 2012. A fourth species, *Tarsonemus endophloeus*, was only detected in 2013 when a canal between the flight muscles and the clavicle and coracoid processes was first examined. This fungivore was the least abundant of the four common mite species detected that year. A fifth mite, *Histiogaster arborsignis* Woodring, occurred only rarely in our samples.

Table 1. Total number of phoretic mites on mountain pine beetles (MPB) attacking *P. ponderosa*, *P. contorta*, from 2011 to 2013 and *P. flexilis* in 2013 (*n* = MPBs sampled). Samples from beetles arriving at *P. flexilis* were only obtained in 2013. NA—*T. endophloeus* was only sampled in 2013. Percent of MPBs carrying mites shown in parentheses.

MPB Mites	Feeding Guild	2011 *n* = 95	2012 *n* = 69	2013 *n* = 248
Histiogaster arborsignis Woodring	fungivore	2 (2.1%)	1 (1.4%)	2 (1.2%)
Proctolaelaps sp. Berlese	nematophagous	18 (7.4%)	0 (0%)	127 (18.1%)
Tarsonemus endophloeus Lindquist	fungivore	NA	NA	95 (16.1%)
Tarsonemus ips Lindquist	fungivore	51 (21.1%)	91 (42%)	330 (14.9%)
Trichouropoda sp. Berlese	omnivore	30 (7.4%)	38 (26.1%)	747 (40.3%)

Overall, phoretic mites were present on 56.8 percent of all beetles (n = 412). The average mite load found during the study, excluding *Tarsonemus endophloeus*, was 3.4 (SD = 6.8; range = 0–60). Mite loads of *Proctolaelaps* sp. were lower than those of either *Tarsonemus ips* ($p < 0.001$) or *Trichouropoda* ($p < 0.001$), between which there was no difference ($p = 0.893$). In 2013, beetles carried more mites than in 2011 ($p < 0.001$) or in 2012 ($p < 0.001$). The increase that year was, in part, due to a large percentage point increases in the prevalence of *Proctolaelaps* sp., and *Trichouropoda* mites (Table 1), when these were also more often found aggregated than singly (69% vs. 31%). During this period, an inverse trend line was observed between mite abundance and the area killed by the MPB (Figure 2).

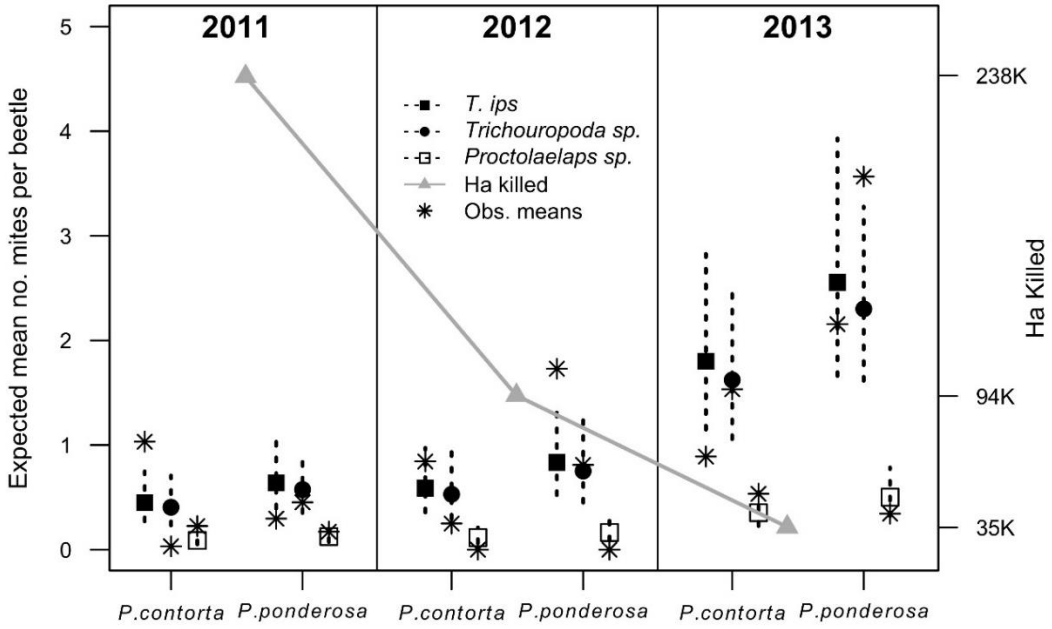

Figure 2. The expected mean mite load on the mountain pine beetles (MPB) attacking *P. ponderosa* and *P. contorta* from 2011 to 2013. There was an increasing trend in MPB mite load that was inverse to a decreasing MPB population trend (gray line) in Larimer County, CO (USDA FHT, online). See Table 1 for the sums of the observed mite counts. The asterisks indicate the observed frequencies.

3.1.2. Influence of Attacked Pine Host on Mite Trends

We removed samples from MPBs attacking the host *P. flexilis* and the counts of *T. endophloeus* mites to examine the overall trend of *Proctolaelaps* sp., *T. ips*, and *Trichouropoda* sp. during the three years in MPBs arriving at *P. ponderosa* and *P. contorta* (Figure 2). There was a modest effect of pine host species when relating the species-specific mite load between samples from *P. contorta* and *P. ponderosa* ($p = 0.080$), where beetles within *P. contorta* had smaller mite loads. Similarly, in 2013 there was a lower total mite load in beetles arriving at *P. contorta* in contrast to those arriving at either *P. flexilis* ($p = 0.016$) or *P. ponderosa* ($p = 0.008$). No differences were found between mite loads in beetles arriving at *P. flexilis* and *P. ponderosa* ($p = 0.984$) (Figure 3).

Figure 3. The boxplots of MPB phoretic mite trends within beetles attacking three pine hosts (left panel) and within beetle sexes (right panel) in 2013. The total number of mites per beetle was lower in MPBs attacking *P. contorta*, but was not substantially different between male and female MPBs. Differences among groups are identified by the letter under each boxplot within a panel using a significant level of 0.05.

3.1.3. Influence of Beetle Sex on Mite Trends

We determined the sex of 75% of all beetles and found that the percent of female MPBs carrying *Proctolaelaps sp.*, *Trichouropoda* sp., and *T. ips* mites was barely higher than in males (58% (n = 231) vs. 52% (n = 78)). The average number of mites per beetle was also similar among the two sexes ($\bar{x}_{female}$ = 3.91 vs. $\bar{x}_{male}$ = 4.28). In 2013, we included the mite *T. endophloeus* in our analysis and found no difference in the total number of mites carried between male and female beetles (p = 0.439) (Figure 3).

3.2. Trends of Transported Fungi

3.2.1. Fungal Transport Trends in Different Beetle Structures

All MPBs (n = 273) carried fungi, but only 40.7% carried the mutualistic blue-stain fungal species; although the samples were smaller in 2012 (n = 38 beetles vs. n = 235). Most beetles carried a single species of blue-stain (25.5%), but sometimes two (13.9%) or even three (2.2%) could be found on a few insects. The prevalence of different fungal species varied in different beetle structures where *O. montium* and *L. longiclavatum* were the most common maxillary mycangial associates (Table 2). Differences in species transport were apparent, where *L. longiclavatum* was most commonly found in the maxillary mycangia and *O. montium* occurred more often on the elytra. *Grosmannia clavigera* was found to be surprisingly scarcer than either of the other two species and was not distinctly abundant in any beetle structure. (Table 2). A high proportion of *O. montium* species in 2012 found in the maxillary mycangia were also found in the elytra. In fact, only one beetle with *O. montium* presence in the maxillary mycangia did not also have *O. montium* presence in the elytra. On the other hand, in 2013, only half of the beetles with *O. montium* presence in the maxillary mycangia also had *O. montium* presence in the elytra.

Table 2. Number and percent of beetles with presence of *O. montium*, *L. longiclavatum*, or *G. clavigera* in the maxillary mycangia, elytra, both structures, or either structure (columns) across and within years (rows). * indicates significance difference at the 0.05 level.

Year	Blue-Stain	Maxilla	Elytra	Max. & Ely.	Any
2012 n = 38	*O. montium*	15 (39%)	25 (66%)	14 (37%)	* 26 (68%)
	L. longiclavatum	15 (39%)	4 (11%)	4 (11%)	15 (39%)
	G. clavigera	5 (13%)	4 (11%)	0 (0%)	9 (24%)
2013 n = 235	*O. montium*	26 (11%)	28 (12%)	13 (6%)	* 41 (17%)
	L. longiclavatum	29 (12%)	15 (6%)	4 (2%)	40 (17%)
	G. clavigera	9 (4%)	13 (6%)	1 (0.004%)	21 (9%)
2012–2013 n = 273	*O. montium*	41 (15%)	53 (19%)	27 (10%)	67 (25%)
	L. longiclavatum	44 (16%)	19 (7%)	8 (3%)	55 (20%)
	G. clavigera	14 (5%)	17 (6%)	1 (0.004%)	30 (11%)

3.2.2. The Year and Host Effects on Fungal Trends

Overall, the probability of blue-stain fungal presence in "any" mountain pine beetle structure was lower in 2013 than in 2012. This difference was substantial for *O. montium* ($p < 0.001$), but less so for *L. longiclavatum* ($p = 0.061$) and *G. clavigera* ($p = 0.065$). In 2012, *O. montium* was more prevalent than either *G. clavigera* ($p < 0.001$) or *L. longiclavatum* ($p = 0.041$). In 2013, *O. montium* was again more prevalent than *G. clavigera* ($p = 0.041$), but it was not different from *L. longiclavatum* ($p = 0.776$). A modest difference in prevalence was found between *L. longiclavatum* and *G. clavigera* in 2013 ($p = 0.053$), and no difference was found in 2012 ($p = 0.249$; Table 2). We did not observe a pine host effect on the fungal presence ($p = 0.658$) in the frequency of any blue-stain fungi between *P. ponderosa* or *P. contorta* within the same year (Figure 4).

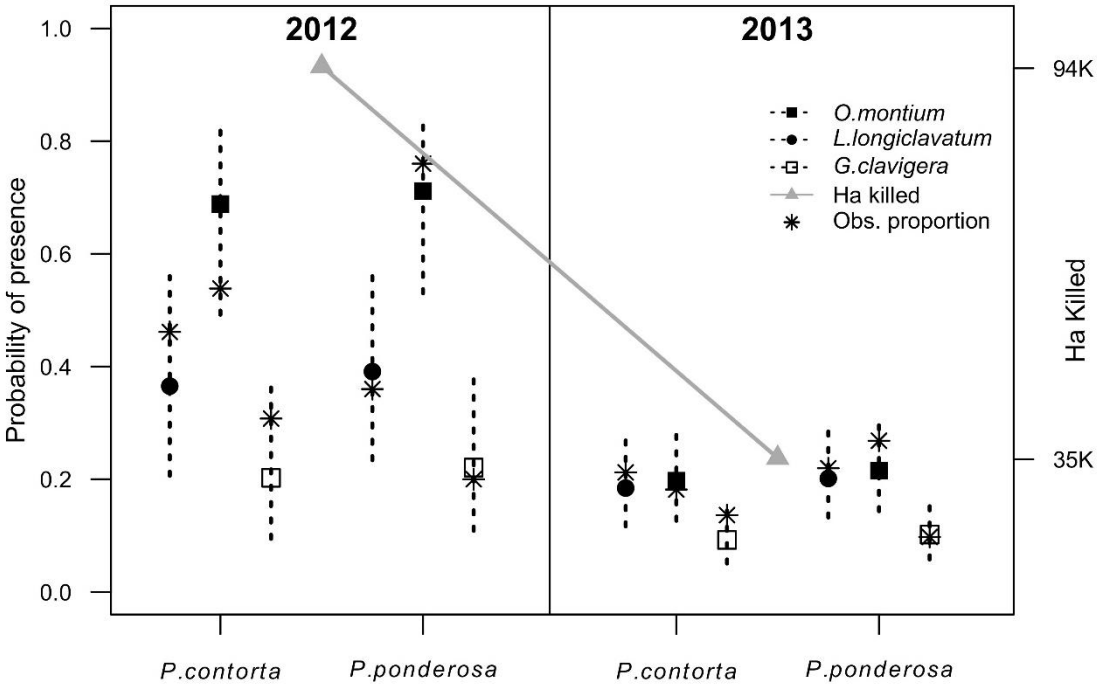

Figure 4. The probability of blue-stain fungal species being transported by mountain pine beetles during the two years. A decrease in the probability of presence occurred in 2013 and was parallel to a reduction in the measured beetle activity (Hectares killed) at Larimer County, CO. The asterisks indicate observed frequencies.

3.2.3. Fungal Trends Observed in Phoretic Mites

Mites contributed to slight increases of some blue-stain species transported by the whole symbiotic association or the holobiont. Among the 47 beetles carrying *Tarsonemus ips* that were evaluated for fungal presence, just one holobiont increased its carried *G. clavigera* frequency in 2012 due to this species (Figure 5). Similarly, no beetle holobiont increased their proportion of transported *L. longiclavatum* or *O. montium* by the effect of *Tarsonemus ips* alone in 2012 or 2013 (Figure 5). Likewise, among the 93 beetles with *Trichouropoda* sp. evaluated for fungal presence, two holobionts increased their proportion of *O. montium*, three increased their proportion of *G. clavigera*, and one increased their proportion of *L. longiclavatum* due to mites in 2012. In 2013, just three beetles increased their proportion of *O. montium* by the *Trichouropoda* sp. Additionally, none increased their proportion of *L. longiclavatum* or *G. clavigera.* Therefore, *Trichouropoda* sp. played a larger role in increasing the presence of blue-stain on the holobiont than *Tarsonemus ips,* although neither appears to add any large contribution to the overall fungal abundance (Figure 5). In addition to their overall reduction in blue-stain carried by mites, other general trends observed on mites between the two years were an increase in their carried yeast and *Penicillium* sp., as well as an increase in the number of mites without any recoverable fungi in MEA. Not much is known about yeast carried by mites, but these may be different species than those associated with MPB's maxillary mycangia.

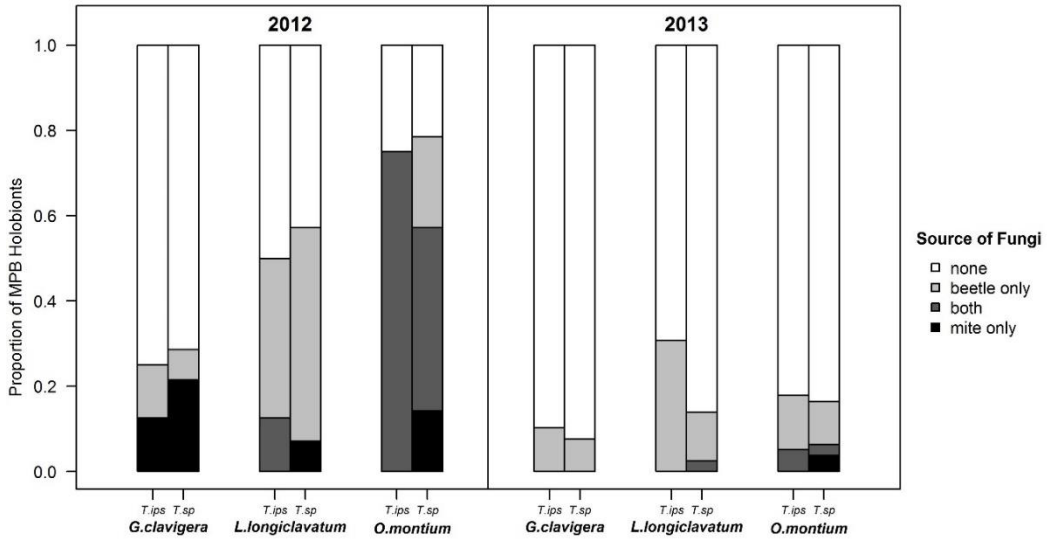

Figure 5. The frequency of three blue-stain fungi in mountain pine beetles (MPBs) that carried mites. The fractions represent the absence of blue-stain fungi (white), the fungus detected only on MPB (light gray), on *Tarsonemus ips* and *Trichouropoda* sp. (*T.* sp.) mites alone (black), or on both MPB and mites (dark gray). In 2012, *Tarsonemus ips* contributed largely more *G. clavigera* into the MPB holobiont, but this effect was reduced in 2013 when a larger sample was examined. The overall fungal presence decreased largely in 2013 when mites only added *O. montium* to the holobiont. The proportions are shown in different columns for each mite.

4. Discussion

The study of the interactions between bark beetles and the numerous associated organisms living in subcortical habitats is challenging. Therefore, few studies have attempted to describe how these interactions can influence beetle populations [38,67,68]. Understanding which organisms are involved in these interactions and their potential contributions is fundamental to pursue complex questions about their effects on irruptive *Dendroctonus* population trends. The current theory suggests MPB populations trends are directly affected by temperature and the abundance of suitable hosts, which are traditional top-down and bottom-up population constraints, respectively. Although temperature

can explain a significant part of population trends for several species, it does not always explain complex situations involving the movement of species into novel areas. In fact, some results have been mixed, e.g., species from cool areas moving into warmer areas following warm climate spells or their incursions into unusually cold areas. Investigating complex interactions between beetles and their symbiotic associates may help explain these unusual occurrences.

In this study, we looked at the population trends of mite and fungal associates of male and female MPBs attacking three pine hosts. Like other bark beetles, MPB often carries several phoretic mites both externally and under their elytra. We still do not understand what attracts phoretic mites to this insect, but our findings suggest that the cues used by the different mite species might not be of a sexual nature. Although in stands dominated by a single tree species, we may expect MPB attacking trees come from the same tree species, we cannot infer the phoretic mite susceptibility to a different host chemistry. All five mite species arrived with MPBs to all pine species but their resistance to each pine oleoresins components needs to be studied. One of the most common mite species we found was an oribatid in the genus *Trichouropoda*, this mite has only recently been reported in association with the MPB [69,70]. *Trichouropoda* sp. mites were previously found to be rare in an MPB population that was reaching its epidemic climax in South Dakota [71]. Here at later stages of the MPB population, we found this mite's abundance trended increasingly, from being uncommon when the MPB population was at its climax to becoming the most abundant mite when this collapsed to endemic levels (Table 1). Moreover, the species was not found in an incipient population in Canada [69], suggesting its population may have a type of development that lags behind a rapid expanding host population [72]. The aggregated occurrence of this mite on some beetles also gives us a clue to its relationship with the MPB. A theory describing natural laws applicable to all parasites suggests that macroparasites, such as mites, have an aggregated behavior that adversely affects host as their density increases within its population [73]. The effect of this macroparasite on the MPB needs to be investigated to understand whether it is direct (e.g., affecting flight, dispersion, etc.) or indirect (e.g., resulting in an increase of antagonistic fungi in the holobiont) to better evaluate whether is an antagonist to the MPB (Figure 6).

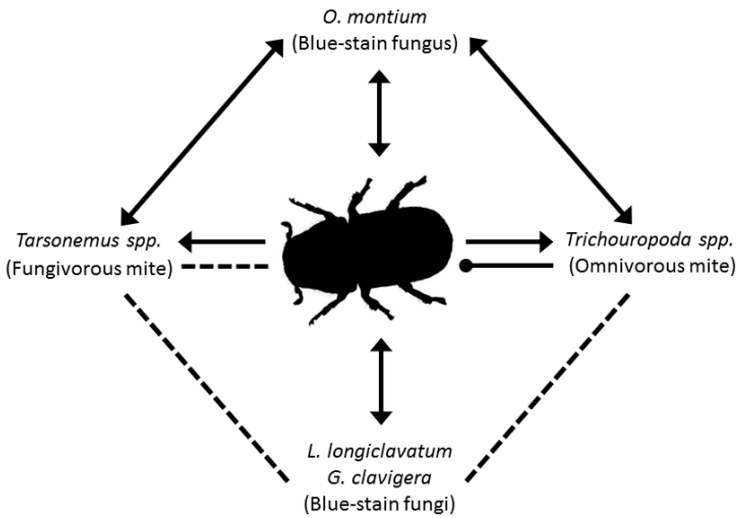

Figure 6. The interactions between the mountain pine beetle (MPB) phoretic and hyperphoretic fungi transported by mites. This illustration presents a hypothetical range of interactions among a phoretic fungivorous and an omnivorous mite genus in the horizontal axes and among the three blue-stain fungi in the vertical axes and the MPB. The transverse lines indicate the interactions among the blue-stain fungi and the two common mite genera found during the decline of an MPB population. The arrows point to the affected organism in the relationship and the sign indicates the type of effect. The solid lines indicate a direct effect and the dotted lines indicate an indirect effect of the interaction.

In relation to the fungi carried by phoretic mites, we do not know whether these establish any strong mutualistic association with any of the blue-stain fungi associated with the MPB holobiont. In the SPB, *Tarsonemus* mites have established a strong relationship with *Ophiostoma minus*. In fact, *Tarsomenus ips* is a mycetophagous found in both SPB and MPB and may obtain nutrition from ophiostomatoids present in the MPB symbiosis, but this will have to be demonstrated experimentally. We did not identify any substantial blue-stain fungi increase in the MPB holobiont as a result of mites. However, we underestimated their contribution by limiting fungal sampling to only one mite of each species per beetle, even when some beetles transported over 60 mites of a single species. This reduced not only the probability of finding any given fungal species in the MPB holobiont, but also the effective measurement of any fungal population shift effect caused by mites. Nevertheless, mites more often transported *O. montium* than the other two blue-stain fungi (Figure 5). A logic follow-up study would quantify the effects of fungi transported by all mites on a beetle to better comprehend the potential mite contribution to the MPB holobiont's fungal biota. Fungal dissemination by mites may be important to *O. montium*, as it is in other symbioses in which their sexual spores are the type transported by mites. Sexual spores benefit fungi by increasing the genetic diversity and foster further sexual reproduction by increasing the distinct mating types to occur in the new host. It is probable that mite dissemination of the *O. montium* sexual spores may contribute to the greater genetic recombination found in that species [21,74].

The *Ophiostoma montium* effects on the MPB may be cryptic or not as direct as the effects in *O. minus* with the southern pine beetle symbiosis. For instance, although *Ophiostoma montium* is a proven nutritional symbiont of the MPB, it was shown that during its growth it entangled and killed portions of the MPB's larvae [19]. Some potential outcomes of having an *O. montium* dominated fungal association in a population are (1) an increase on mites feeding on it, (2) a reduction on the growing area in which the other two better nutritional symbionts of the beetle grow. Under this scenario, we could expect that the reduced development speed on beetle larvae feeding on this fungus could have negative effects on its success (Figure 6). On the other hand, by promoting a shorter development time, the other two blue-stain species can allow MPBs facing an early fall onset of cold weather to reach it at a more resilient developmental stage. A faster MPB development may allow its incursion into cooler latitudes and elevations, as well as population increments in these areas as a greater portion of the population survives the shorter season. Specifically, the blue-stain *L. longiclavatum* promoted a faster MPB larvae development in Canada [19], where the fungus was shown to increase in frequency in direct relation to the increase in latitude [21]. Thus, in northerly latitudes, the MPB may be benefitting from its more frequent association with *L. longiclavatum* [21]. Although the 2000's MPB epidemic expansion to new latitudes and elevations in both the Colorado Rockies and Alberta is considered to have been caused by warmer winter temperatures, there is no good quantification of non-prevailing MPB incursions into novel areas. It is possible that lethal cool winter temperatures may not be the only limiting factor keeping the beetle outside of these areas. *Leptographium longiclavatum* has only recently been described from MPB populations in Colorado [39,45], where the beetle's incursions to unusually high elevations were observed during this and another study [75].

The theory interpreting the common occurrence of multiple fungal associates in the MPB suggests that fungal associate redundancy may foster climate adaptability to an insect that moves relatively fast across an elevational gradient in which warmer "loving" fungi flourish at lower, warmer elevation areas and vice versa [17]. Nevertheless, occasionally an unexpected blue-stain fungus is the dominant species during thermally inversed conditions to those considered to be their most favorable [62]. This could indicate that during beetle emergence, the frequencies of the different fungi that these carry may not always be determined by a site's temperature but that a phenotypical flexibility is innate to these species. This phenotypic variability might be affected by the transmission of the sexual fungal spores that are packed in a specialized structure (i.e., sporotheca) in some mites [76]. Finding *L. longiclavatum* in the Southern Rockies supports the phenotypic plasticity of this blue-stain associate and makes us think about the other functions of this instrumental symbiont. Since both the beetles and their phoretic

mites can carry all three blue-stain fungal symbionts of the insect, we contemplate the possibility that the multipartite relationship between beetles, mites, and fungi could alter the population frequencies of each of these fungi in addition to environmental effects. Additionally, certain frequencies may contribute to the expansions of this irruptive species into atypical latitudes and elevations of cooler temperatures. Furthermore, the frequencies of its most nutritional symbiont, *L. longiclavatum*, may affect the overall population stage fluctuations of the insect.

5. Conclusions

We document that a reduction on the total blue-stain disseminated by the MPB holobiont (i.e., MPB and its phoretic mites) trended similarly with a population decline of the insect's epidemic, which may elucidate a new mechanism to explain MPB epidemic population declines. This reduction departs from the typical high-frequency reported from this insect and was obtained using similar sampling methods. However, our findings may be confounded by an increase in the population of two mite species. These phoretic mites carried blue-stain fungal species known to be mutualistic to the beetle, but we did not find out whether these made any substantial contribution to the overall fungal abundance in the holobiont. The blue-stain mite fungi transmission was already known from other areas [70], however; here we describe that the fungal frequency followed a similar decreasing pattern in both organisms, suggesting that, given their mentioned increase, phoretic mites may be only facultative beneficiaries from blue-stain fungi. All common phoretic mite species carried by the MPB arrive with the beetle to *P. ponderosa*, *P. flexilis*, and *P. contorta* in the Colorado Front Range in mixed as well as in single-host dominated stands. Thus, it is probable that, as well as MPB, these mites are adapted to the different tree host chemistries. We argue that phoretic mites may alter blue-stain abundances in the MPB holobiont, although we were not able to show this since our sampling methods likely underestimated their overall contribution. The factors affecting shifts in irruptive bark beetle populations are yet to be fully understood. Here, we present two potential factors during an exceptionally difficult to predict stage of an insect population: its epidemic collapse. Although the mite effect needs to be further investigated, the nutritional requirement of blue-stain fungi by the MPB is known and may represent valid evidence for predicting MPB population declines.

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