Microbial Ecology

Temperature Determines Symbiont Abundance in a Multipartite Bark Beetle-fungus Ectosymbiosis

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Abstract

In this study, we report evidence that temperature plays a key role in determining the relative abundance of two mutualistic fungi associated with an economically and ecologically important bark beetle, *Dendroctonus ponderosae*. The symbiotic fungi possess different optimal temperature ranges. These differences determine which fungus is vectored by dispersing host beetles as temperatures fluctuate over a season. *Grosmannia clavigera* is the predominant fungus carried by dispersing beetles during cool periods but decreases in prevalence as daily maximum temperatures approach 25°C, and becomes extremely rare when temperatures reach or exceed 32°C. In contrast, *Ophiostoma montium* increases in prevalence as temperatures approach 25°C, and becomes the predominant symbiont dispersed when temperatures reach or exceed 32°C. The possession of different optimal growth temperatures may facilitate the stable coexistence of the two fungi by supporting growth of each fungus at different times, minimizing direct competition. Furthermore, the beetle may reduce its risk of being left aposymbiotic by exploiting not one, but two symbionts, whose combined growth optima span a wide range of environmental conditions. The possession of multiple symbionts with different temperature tolerances may allow the beetle to occupy highly variable habitats over a wide geographic range. Such temperature-driven symbiont shifts are likely to have major consequences for both the host and its symbionts under current temperature regimes and those predicted to occur because of climate change.

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Introduction

Mutualisms are predicted to be inherently unstable and prone to erosion because of cheating by established symbionts or invasion by exploiters [7]. Obligate mutualisms are also viewed as risky given that if one associate is lost, the other cannot survive [13]. However, despite these predictions, many mutualisms, including many obligate associations, appear to have existed in a relatively stable state over long evolutionary periods. Most studies attempting to detect mechanisms of stability in mutualisms have focused on pairwise interactions (one host–one symbiont) ([8] and references therein). However, many mutualisms involve multiple symbionts, several of which may fulfill similar roles with the host (symbiont redundancy). Whereas redundant symbionts impart the same *type* of benefit to a host, it is highly unlikely that any two will provide exactly the same *degree* of benefit. That "redundant" symbionts may differ in the degree of benefit they confer raises a number of questions unique to multipartite symbioses. For example, do such symbionts differ in their relative prevalence among host populations, and if so, what factors influence this variability? Do differences in the prevalence of symbionts within and among populations affect host fitness and host population dynamics? If the answer to the last question is yes, then why and how apparently inferior symbionts are maintained in stable symbiosis becomes an especially intriguing question.

To address these questions, we are investigating a multipartite mutualistic ectosymbiosis occurring among a tree-killing bark beetle, *Dendroctonus ponderosae*, and two fungi, *Grosmannia clavigera* (formerly *Ophiostoma clavigerum* [31]) and *Ophiostoma montium*. Phylogenetic studies indicate that *G. clavigera* has a long, shared evolutionary history with the host beetle, whereas *O. montium* appears to be a relative newcomer [27; D.L. Six et al., unpublished observation]. Although *O. montium* is

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a more recent associate, it is widespread in its occurrence and is likely to have also been associated with the host over a relatively long period of evolutionary history.

The symbiosis between the beetle and the two fungi is considered mutualistic. The fungi receive benefit from association with the beetle through reliable transport to food resources and habitat [24]. Before emergence from the host tree, new adult beetles feed on spore layers produced by the fungi on the walls of beetle pupal chambers [24]. While feeding, the beetles pack their mycangia (structures of the integument specialized for dissemination of fungi) with spores, and then disperse to colonize new trees where they inoculate the fungi into tree tissues and lay eggs [24]. In return, the beetles gain nutritional benefit from feeding on the fungi, both as larvae and adults. The larvae of the beetles develop and feed in the phloem layer of trees [1]. Phloem is a nutrient-poor food, and symbiotic fungi are believed to supplement the tree-based diet of *Dendroctonus* beetles by concentrating nitrogen [2] and/or providing sterols required for molting and reproduction [4]. The two fungi associated with *D. ponderosae* appear to differ in their relative contributions to host nutrition. *D. ponderosae* reared in logs with *G. clavigera* develop faster and produce more brood than when associated with *O. montium* [26]. In addition, feeding on fungi by new adult beetles before dispersal appears to be critical for beetle reproduction and both fungi appear to be adequate for this purpose. New adults allowed to feed on spores of either fungus, will enter logs, construct galleries and lay eggs. In contrast, beetles that do not feed on spores, will not enter logs, and do not produce galleries or eggs [26].

In addition to sharing the same host, the two fungi exploit the same habitat and the same nutritional resources within the tree. Such a broad overlap of niches should lead to strong direct competition between the two fungi for space, nutrients, and hosts, destabilizing the multipartite symbiosis. In addition, differential benefits conferred to the host by the two symbionts should also act to destabilize the symbiosis by increasing selection for the symbiont that confers superior benefits and selecting against the symbiont that confers inferior benefits. Despite these predictions, this multipartite symbiosis appears to be relatively stable, indicating the existence of a mechanism that allows both fungi to coexist with the host.

In this research, we investigated whether the relative proportions of the two fungal symbionts associated with *D. ponderosae* vary seasonally among and within populations, and from year to year within a population, by monitoring the fungi carried in mycangia of dispersing new adults at several sites in Montana and Idaho (Table 1). We then investigated whether the environmental variable, ambient temperature, influenced the patterns of symbiont prevalence that we detected. By investigating patterns of symbiont prevalence and the factors that affect variability in prevalence, we can gain insight in to the proximate mechanisms by which multipartite associations maintain stability.

Methods

Adult beetles were collected in Lindgren traps [16] baited with a commercially available aggregation pheromone bait specific to *D. ponderosae* and containing exo-brevicomin, *cis*- and *trans*-verbenol, and myrcene (Phero-Tech, Delta, British Columbia) set at six sites in Montana and three sites in Idaho. Sites in Montana were located in the west-central portion of the state and ranged from 25 to 170 km apart. The three Idaho sites were located in the Stanley Basin of the Sawtooth National Recreation Area,

Table 1. Year, total numbers of beetles captured in pheromone-baited traps, and total numbers (percentages) of beetles from which isolations were made of *G. clavigera* and/or *O. montium* from mycangia

Site	*Year*	*Total beetles trapped*	*Number of isolations*	*Number carrying G. clavigera (%)*	*Number carrying O. montium (%)*	*Number carrying both fungi (%)*
Montana sites						
Twelvemile Creek	2001	1208	413	80 (19.4)	333 (80.6)	0
	2002	3772	810	154 (19.01)	656 (80.99)	0
	2003	3323	459	27 (5.9)	432 (94.1)	0
Point Six	2001	353	269	3 (1.1)	266 (98.9)	0
Lubrecht Forest	2002	2147	373	119 (31.9)	254 (68.1)	0
Cold Creek	2002	490	111	29 (26.1)	82 (73.9)	0
Hidden Valley	2003	4714	321	36 (11.2)	285 (88.8)	2 (0.01)
Thompson Falls	2003	6789	364	41 (11.3)	323 (88.7)	8 (0.03)
Idaho sites						
Hell Roaring	2002	7665	217	104 (47.7)	80 (36.7)	33 (15.1)
Vienna	2002	6036	237	145 (61.2)	68 (28.7)	24 (10.1)
Nip & Tuck	2002	7118	289	131 (45.3)	120 (41.5)	38 (13.1)

each located approximately 15 km apart, and at least 275 km south of the southernmost (closest) collection site in Montana. Sites ranged in elevation from 988 m (Twelvemile Creek) to 2414 m (Point Six). All collection sites were located in stands dominated by lodgepole pine (*Pinus contorta*) with the exception of Lubrecht Forest, which consisted primarily of ponderosa pine (*Pinus ponderosae*) and Point Six, which was dominated by whitebark pine (*Pinus albicaulis*).

One (Idaho) to three (Montana) traps were set per site for the entire dispersal period of emerging brood adults when possible (snow prevented early/late entry to some sites). Trap catches were collected every 2 to 7 days, with collections beginning on different dates among the sites. Up to 100 live beetles from each trap per collection date were used for isolating fungi from beetle mycangia. For one site in Montana, Twelvemile Creek, we trapped beetles for three consecutive years. At all other sites, beetles were trapped for a single season.

Fungi were isolated from mycangia by first decapitating beetles and surface-sterilizing the heads, either by using three sequential 1-min rinses in sterile water, or by immersion in modified White's solution [3] for 30 s followed by two 1-min rinses in sterile water. Both methods yielded similar results in their ability to remove surface contaminants. The maxillae (containing the mycangia) were then dissected from the heads and placed onto the surface of 2% malt extract agar (MEA) in petri dishes to allow fungi to grow out of the mycangia and onto the isolation media [25]. Isolation cultures were held at 21–22°C for at least 2 weeks under natural light to allow growth and sporulation of fungi. Fungi were then identified using morphological characteristics [10, 29]. Sequencing of DNA of isolates grown from single spores of select cultures was used to verify identifications based on morphology. To extract DNA, a small amount of mycelium and conidia was scraped from the surface of colonies growing on 2% MEA, macerated in 200 µl PrepMan Ultra (Applied Biosystems, USA), incubated at 95°C for 10 min, and then centrifuged. The supernatant containing the extracted DNA was then removed and used in polymerase chain reaction (PCR).

PCR amplification was carried out using the primers Bt2b [9] and T10 [21] to amplify a portion of the β-tubulin gene. Each PCR reaction mixture (50 µl total volume) consisted of 25 µl Master Mix (Promega, USA), 0.5 µl of each primer (10 pmol concentration), 23.5 µl water, and 1 µl of the DNA extract. PCR conditions were one cycle of denaturation at 95°C for 3 min, followed by 36 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, and one final cycle of extension at 72°C for 4 min.

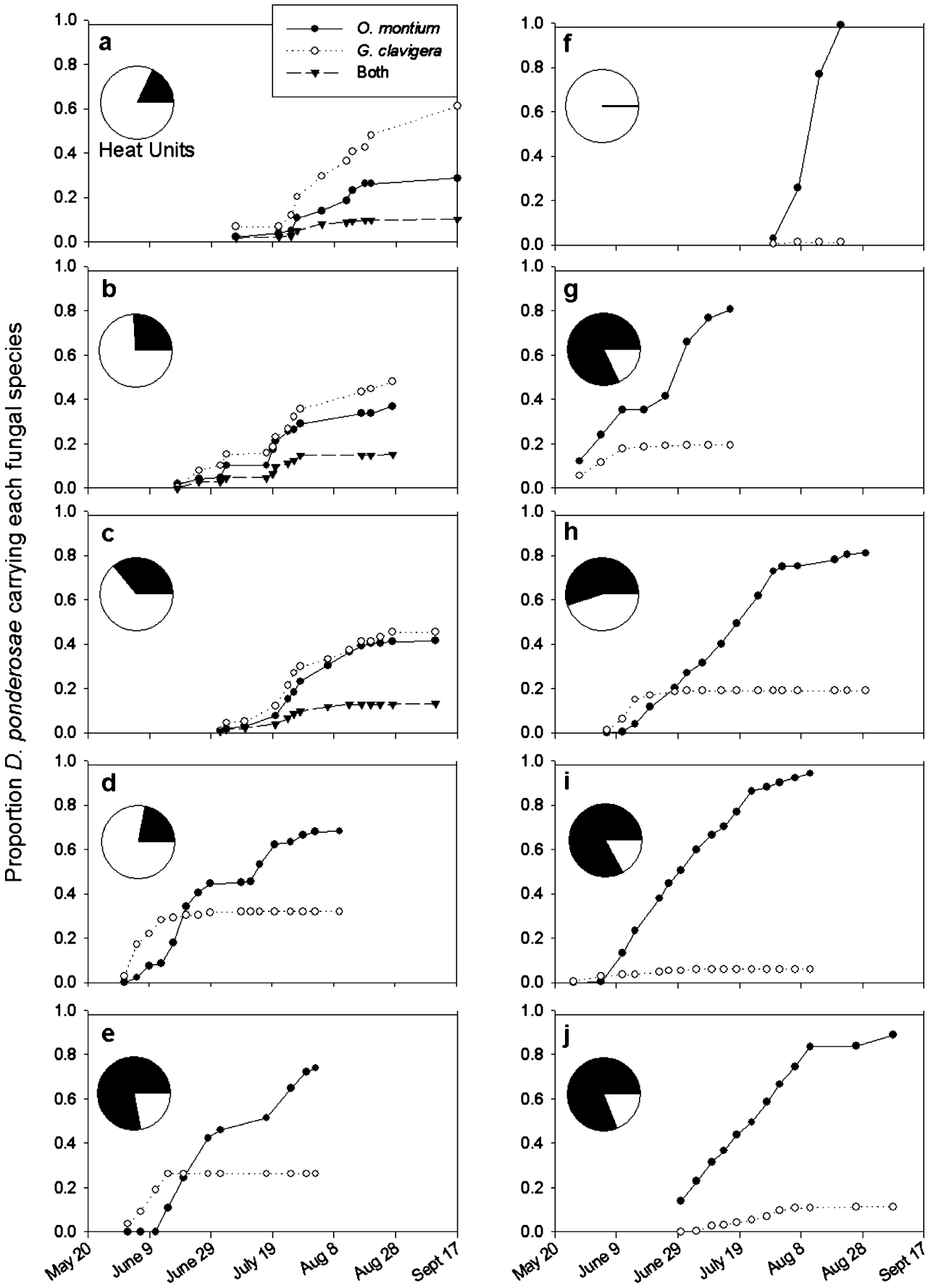
PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Germany) and sequencing was performed on an ABI 3130X2 automated sequencer (PerkinElmer Inc., USA). Sequences of isolates from this study were aligned with sequences in GenBank obtained using BLAST searches http://ncbi.nlm.nih.gov/BLAST).

From previous work we knew that the two fungi possess different temperature tolerances [25, 28]. *G. clavigera* grows more rapidly than *O. montium* at temperatures between 3 and 22°C, but at temperatures at or over 25°C, its growth declines rapidly until ceasing altogether at 31°C. *O. montium*, on the other hand, grows nearly and *G. clavigera* at 22–25°C. However, it continues to grow well at temperatures ranging from 25 to 34°C but ceases growth at 37°C [25, 28]. Therefore, we analyzed the effect of daily maximum and minimum air temperature at each site on the seasonal distribution of beetles carrying each fungal species. Daily temperature data acquired from NOAA (http://www.ncdc.noaa.gov), SNOTEL (http://www.wcc.nrcs.usda.gov/snotel), and temperature dataloggers (Campbell Scientific, Logan, UT, USA) (Idaho sites only) were used to predict daily maximum and minimum air temperatures at each trap location during the beetle dispersal period (~1 May through 30 September) using the BioSIM system [22]. Daily maximum and minimum temperature and collection date were then tested for significance in explaining the proportion of beetles carrying each fungal species using a random coefficient mixed model with a binomial error distribution specified [17]. Using mixed models, we also tested the significance of the number of days with a maximum temperature >25°C (the temperature at which growth of *G. clavigera* slows) and >32°C (the absolute upper threshold of growth for *G. clavigera*) in explaining the mean proportion of beetles at each site carrying either species of fungus. Differences among sites and years were tested using Tukey's honestly significant difference multiple comparison procedure.

Results

We found that the relative prevalence of the two fungi with dispersing adult beetles varied substantially from population to population, and within a population, over the dispersal period (Table 1 and Fig. 1). Proportions of the two fungi also differed significantly among years for beetles collected at Twelvemile Creek (the only site sampled for multiple consecutive years) (df=2,35, F=63.3, P<0.0001) and among sites (df=9,109, F=77.46, P<0.0001).

In Montana, *O. montium* was the most prevalent fungus in all populations, and was isolated from 74 to 99% of beetles (Fig. 1). At all Montana sites, *G. clavigera* was carried only by early-flying beetles and *O. montium* only by late-flying beetles in all populations in all years (Fig. 1). At two sites, Thompson Falls and Hidden Valley, a very small percentage of individual beetles captured carried both fungi (0.01% Hidden Valley, 0.03% Thomp-


Proportion D. ponderosae carrying each fungal species
O. montium
G. clavigera
Both
Heat Units
a
b
c
d
e
f
g
h
i
j
1.0
0.8
0.6
0.4
0.2
0.0
May 20
June 9
June 29
July 19
Aug 8
Aug 28
Sept 17

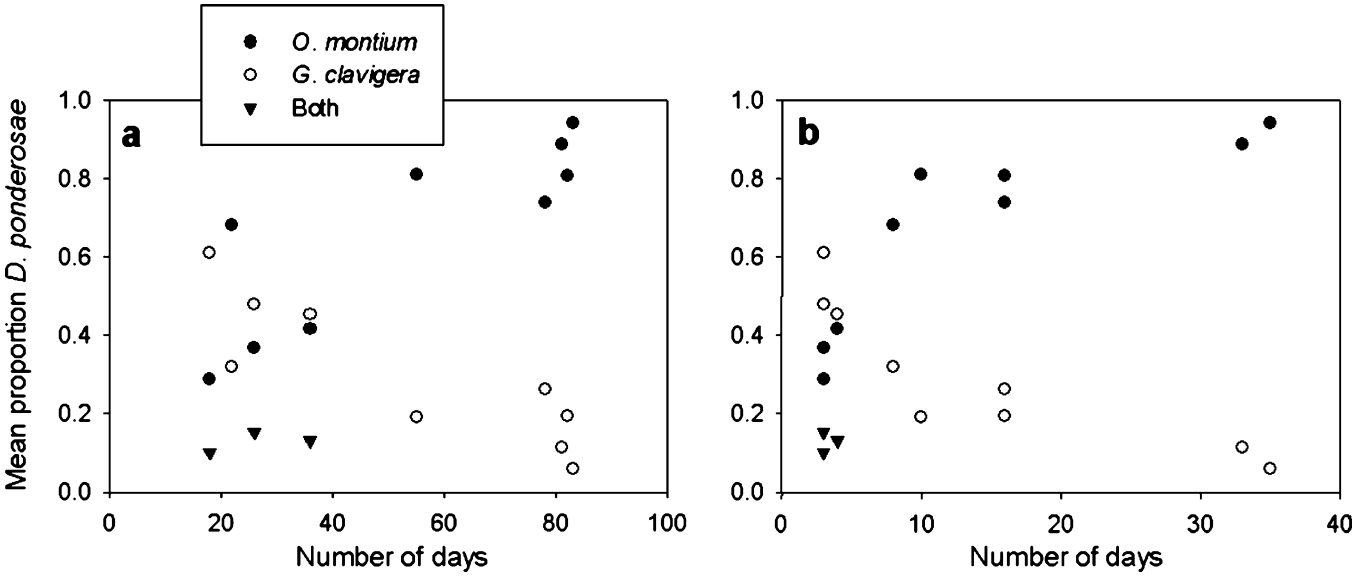


Figure 2. Mean proportion *D. ponderosae* carrying *O. montium*, *G. clavigera* or both fungi as a function of the number of days at each collection site between 1 May and 30 September with a maximum temperature of (a) >25°C and (b) >32°C.

son Falls) (not included in Fig. 1 because of low numbers).

In contrast to the Montana populations, *G. clavigera* was the most prevalent fungus isolated from beetles captured at all three Idaho sites (Fig. 1). At the two warmest Idaho sites, Hell Roaring and Nip & Tuck, both fungi were isolated from beetles over the entire dispersal period. However, at the coldest Idaho site, Vienna, fewer beetles overall carried *O. montium*, and by mid-August nearly all beetles captured were carrying *G. clavigera* (Fig. 1). Compared with beetles in Montana, a relatively high percentage of individual beetles at the Idaho sites carried both fungi in their mycangia (15% Hell Roaring, 10% Vienna, and 13% Nip & Tuck) (Fig. 1).

Maximum daily temperature (df=1,117, F=66.77, P<0.0001) and collection date (df=1,31.43, F=130.16, P<0.0001) were highly significant in explaining the proportion of beetles carrying *O. montium*, the fungus known to grow at warmer temperatures. At all but one site, Point Six, significantly more beetles carrying *O. montium* were sampled from sites that experienced a greater number of days when the maximum temperature was >25°C (df=1,7, F=17.54, P<0.0041), the temperature at which *G. clavigera* growth is suppressed, but at which growth of *O. montium* is still highly supported (Fig. 2a). This trend was even more striking when proportion of beetles carrying the two fungi was plotted against number of days with a maximum temperature >32°C (df=1,7, F=32.25, P<0.0008), the upper threshold for *G.* clavigera growth (Fig. 2b).

Figure 1. Cumulative proportion *D. ponderosae* carrying *O. montium*, *G. clavigera*, or both fungi over the adult dispersal period at nine collection sites: (a) Vienna, ID 2002; (b) Hell Roaring, ID 2002; (c) Nip & Tuck, ID 2002; (d) Lubrecht Forest, MT 2002; (e) Cold Creek, MT 2002; (f) Point Six, MT 2001; (g) Twelvemile Creek, MT 2001; (h) Twelvemile Creek, MT 2002; (i) Twelvemile Creek, MT 2003, and (j) Thompson Falls, MT 2003. Also shown for each site are heat units expressed as number of days between 1 May and 30 September when the maximum daily temperature was >25°C.

Discussion

Our results strongly suggest that differences in the relative prevalence of the two fungi among sites and over the dispersal period of the host beetle are driven by ambient temperature. At only one site did beetles carry a proportion of the two fungi different than would be predicted by the temperature tolerances of the two fungi. At Point Six, our coolest and highest elevation site, beetles carried greater than 99% *O. montium*, the warm-tolerant fungus. We believe these results can be explained by patterns of beetle activity and topographically mediated wind patterns in the general area surrounding this site. Beetle activity at Point Six was very low, whereas outbreak levels of the beetle existed in nearby, relatively hot, lower elevation ponderosa pine stands. Summer orographic weather patterns often serve to transport beetles from low elevation and spatially separated populations to high elevation sites. We believe, therefore, that the beetles captured in traps at Point Six may actually have originated from the outbreak in low elevation ponderosa pine.

Shifts in prevalence of the two fungi with dispersing beetles are likely to be due to effects of temperature on

sporulation by the fungi in pupal chambers at the time that brood adults eclose and begin to feed on and pack their mycangia with spores. The two fungi are not highly antagonistic with one another when grown in culture [K. Bleiker and D. L. S., unpublished observation] and have often been observed or isolated together from phloem or from the same pupal chamber [23, 30; Adams and Six, in press]. The ability of the fungi to intermingle in tree substrates and the rarity of fungus-free dispersing beetles indicates that both fungi are probably present in many pupal chambers, but depending upon temperature, typically only one will sporulate and be acquired in mycangia at a particular point in time.

Temperature-driven symbiont shifting may provide a mechanism that has allowed both fungi, long-established symbiont and more recent exploiter alike to persist in a long-term symbiosis with their host. By growing at different temperatures, and thus at different times, the fungi minimize competition with one another except at a narrow range of temperatures where the growth of both fungi is equally supported. In turn, the beetle may benefit by reducing its risk of being "left alone" by exploiting not one, but two symbionts, whose combined growth optima span a wide range of environmental conditions. For bark beetles, such as *D. ponderosae*, which inhabit a broad geographic range and inhabit highly variable habitats, possessing multiple symbionts may be especially important. Few fungi have broad environmental tolerances. Therefore, possession of multiple fungal symbionts with differing but overlapping environmental tolerances may allow a host to more successfully exploit its environment and to be less likely to be negatively affected by environmental fluctuations over time.

Temperature-driven symbiont shifts are likely to have major consequences for beetle and fungal fitness and population dynamics under conditions predicted to occur because of climate change. The results of our study suggests that temperature determines the degree of vectoring of each fungus within a population, which would greatly affect fungal fitness. If the two fungi differ in their relative benefits to the host, as suggested by Six and Paine [26] and recent research [K. Bleiker and D.L. Six, unpublished data], then which fungus predominates in a population will affect host fitness as well. Surface temperatures in western North America are projected to warm in coming decades [14]. Increasing temperatures will not only affect *D. ponderosae* directly [18], with predicted range expansion in some areas [6] and a decrease in the forested areas suitable for population success in others [11], but also indirectly through effects on both fungal species. In the absence of genetic adaptation to rapidly changing temperature, concomitant shifts in the beetle's range will result in dramatic shifts in the relative prevalence of both fungal species. *O. montium* should increase in the warmest portions of the *D. ponderosae* range, whereas the relative prevalence of *G. clavigera* would be limited to the coolest portions of the range, with the fungus possibly going extinct in some areas where it is currently found. Thus, it is likely that anthropogenic effects on climate may act to substantially destabilize and alter the outcomes of this symbiosis.

The potential effects of such a change in the symbiont community of *D. ponderosae* are unknown. In the long-term, a reduction in the symbiont community (loss of *G. clavigera*) may lower the beetle's ability to buffer environmental extremes. In the short-term, changes in the symbiont community may translate to effects on beetle population dynamics. In at least one other multipartite *Dendroctonus*–fungus symbiosis, the differential effects of symbionts on their hosts have been shown to translate to strong effects on host outbreak dynamics [5, 12, 15, 19, 20]. In the *D. ponderosae*–fungus symbiosis, if one symbiont supports higher beetle fitness, then an increase in that symbiont might support a more aggressive host population. In contrast, if an inferior symbiont becomes more prevalent, host populations may be negatively affected.

D. ponderosae is considered the most important forest insect pest in western North America and kills millions of trees annually. It is both ecologically and economically significant in the long-term sustainability, function, and productivity of western North American pine ecosystems. Therefore, climate change induced alterations of the symbiont community of this beetle could have considerable ramifications for North American coniferous forests.

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