Ergosterol Content of Fungi Associated with *Dendroctonus ponderosae* and *D. rufipennis* (Coleoptera: Curculionidae, Scolytinae)

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**ABSTRACT**  Insects require sterols for normal growth, metamorphosis, and reproduction, yet they are unable to synthesize these organic compounds and are therefore dependent upon a dietary source. For phytophagous species, such as *Dendroctonus* bark beetles, whose food does not necessarily contain appropriate types or adequate quantities of sterols, fungal symbionts may provide an alternative source in the form of ergosterol. We determined and compared the relative amounts of ergosterol in the primary fungal associates of *Dendroctonus ponderosae* Hopkins and *Dendroctonus rufipennis* Kirby. Ergosterol content of host tree phloem naturally infested with larvae (and their fungal symbionts) of both species was also compared with ergosterol contents in uninfested phloem tissue. Mycelia of *Ophiostoma montium* (Bundock) von Arx and *Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington isolated from *D. ponderosae* mycangia, and *Leptographium abietinum* (Peck) Wingfield isolated from the exoskeleton of *D. rufipennis* contained relatively large quantities of ergosterol, although no significant differences in content were found among these fungal species. Phloem colonized by larvae of both species contained significantly more ergosterol than did uninfested host phloem tissue. Our results suggest that larval life stages of *D. ponderosae* and *D. rufipennis* may obtain vital nutrients not only from the host tree phloem but also from fungal symbionts, in the form of ergosterol, while emitting larval galleries.

**KEY WORDS** bark beetle, symbionts, mycangial fungi, mycophagy

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**Several bark beetle species within the genus *Dendroctonus* (Coleoptera: Curculionidae, Scolytinae) can exhibit dramatic population eruptions at periodic intervals, resulting in tree mortality across vast forested areas. Although the mechanisms are not always clear, both density-dependent and independent processes can contribute to these population fluctuations, including interactions with other organisms (Bridges 1983). Many *Dendroctonus* are symbiotically associated with at least one *Ophiostoma* fungal species that is mutually beneficial to the beetle host, whereas relationships with other fungal associates may be antagonistic or commensalistic (in 2003). The fungal benefits with association with the beetle host by consistent dissemination to an esophageal and relatively rare resource (a freshly killed tree) upon which they are dependent for growth and reproduction. In some associations, growth and reproductive success of beetle brood is increased in the presence of fungi, also because of a nutritional benefit (Whitney 1971, Bridges 1983, Golkhammar et al. 1990, Capcodge et al. 1995, Six and Paine 1998, Ayres et al. 2000). Although mycophagy may be obligatory for all bark beetle species, fungal feeding may provide vital nutrients not found in woody plant tissues or only present in inadequate concentrations (Harrington 2005). For example, most plant tissues have relatively low levels of nitrogen, and a fungal mutualist of *D. frontalis* Zimmermann was found to benefit larval growth by concentrating nitrogen in phloem tissue where feeding occurs (Ayres et al. 2000). Insects also require sterols for normal growth, metamorphosis, and reproduction, yet are unable to synthesize these organic compounds and are therefore dependent upon a dietary source (Clayton 1964, Richard and Thomas 1975, Svoboda et al. 1978). Sterols in woody plant tissues are typically present only in low concentrations (Kramer and Kaszowski 1960) or in forms not usable by insects (Clayton 1964). For insects that feed in woody tissues, fungal may provide an alternate source of sterols in the form of ergosterol (340-methylcholesterol, 5,7,22-trien-3β-ol) (Nörritz et al. 1969, Koik et al. 1978; Norris 1972, Mauser et al. 1990, Morales-Ramos et al. 2000). This major fungal sterol is produced by most fungi (Weeck 1973, 1989) and is highly usable by many insects for the production of hormones and cell membranes (Clayton 1964). Although ubiquitous in fungi, ergosterol varies in its concentration in mycelia by species, age of the culned.
ture, developmental stage, and growth conditions (Paisan et al. 1989).

In several insect-fungus symbioses, the insect associate has been found to be dependent upon stromata provided by the fungal associate (Norris et al. 1960, Kol 1972, Norris 1972, Maier et al. 1992, Morales-Barrus et al. 2000, Naar and Nolda 2003). The fruiting bodies are called sclerotia (Sclerotinia) that feed solely on fungi they harbor in galls induced by the tree (Norris 1972, Kol 1975, Mueller et al. 2005). Although these beetle species are uniquely adapted to their fungal partners, the specific functioning of the beetles in the symbiosis is not fully understood.
In several insect-fungus symbioses, the insect associate has been found to be dependent upon stolons provided by the fungal associate (Norris et al. 1969, Kolk 1978, Norris 1972, Moeller et al. 1992, Morales-Barron et al. 2000, Nair and Nolte 2003). These create ambushing galleries (Sobolovitzia) that feed solely on fungi they gather in galleries in the pupae of trees (Norris 1972, Kolk 1978, Moeller et al. 2005). Ambushing galleries are found upon emergent hyphae produced by their fungal symbionts for successful oviposition, oviposition, larval development, and pupation (Norris and Baker 1967, Norris et al. 1969, French and Roper 1972, Kolk 1979). This relationship holds for another ambusher, the coffee borer beetle, Heteroceras hampei (Ferrari), which feeds on, and feeds on, coffee borers but cannot nalt or reproduce without eggstrot and its symbiotic fungus Ficus morrisonensis (Morales-Barron et al. 2000).

Although almost all fungal produce eggstrot, the varying levels of eggstrot present in the mycelia of different species of fungi may account for the relative differences in the benefits that beetles exhibit for some fungal associates. For example, studies by Kolk et al. (1978) and Kolk and Norris (1979) found that the most beneficial species of fungi associated with Xylella absinths beetles was those that possessed the greatest concentrations of aggregates.

Dendroconus rufipes Kirby and Dendroconus sp. (Hymenoptera), two insect economically important bark beetle species in western North American forests, are closely associated with fungi. Leptographium abietis (Peck) Wingfield is commonly found in specialized pits on adult D. rufipes prepupa and eytra (Selbim 1985, Stroje and Bets 2006). Although the occurrence of aggregates is long known to wood borers, D. rufipes has been isolated from wood colonized by D. rufipes in some populations (Reynolds 1992, Selbim 1985). Six and Bets (2000) did not isolate this fungus from any D. rufipes adults collected in Alaska, Utah, Colorado, and Minnesota, suggesting that the association is incidental or accidental. Little research has been conducted on these fungi, and it is unknown whether they play a mutagenic or antagonistic role in D. rufipes population dynamics. D. rufipes is closely associated with two fungi, Ophiostoma montanum (Rumlin) von Arx and Ophiostoma clavigerum (Robinson, Jeffrey, & Davidson) Harrington. Both species can be found on the inoculated adult D. rufipes and in specialized structures of the mycelium termed mycangia that function in fungal transport (St 2004b, d). The mycangia are reutilized in subsequent infections, and their contents are also mycangia, suggesting that mycangia evolved to maintain close associations with specific fungi that provide source of nutrition for the beetle host (Harrington 2005). Effects of the two fungi on their host beetle are not well understood. Only the O. montanum mycangia is known to produce developmental nutritional effects of the fungi, and the results of these two studies seem to conflict (Six and Patte 1988, K. Blisker and D.L.S. unpublished data). In one study, D. rufipes were reared in logs colonized with patulin beetles associated with other O. clavigerum, O. montanum, or without fungi (Six and Patte 1998). Resulting beetles developed with O. clavigerum were more numerous and emerged earlier than those fed on bark that developed with O. montanum. Beetles introduced with mycangia obtained patulin from the bark. These results indicate that both fungal support brood production, but that O. clavigerum may be a superior associate. In experiments involving patulin production, O. montanum is most important in a recent study conducted in phylum sandblades (K. Blisker and D.L.S. unpublished data), beetles developing with O. montanum obtained significantly less pathogen than insects developing with O. clavigerum or no fungi, suggesting that O. montanum provides superior nutritional benefits. Beetles developing with O. clavigerum or no fungi had to consume more pathogen to meet their dietary requirements. As has been found with other mycophytic symbiotic beetles, we hypothesized that D. rufipes and D. rufipes obtain sterols, in particular ergosterol, from their close fungal associates. Because D. rufipes needs sterols for development and for eggstrot, and thus may not have as highly an evolved association with its fungi as or on a dependence on fungi for nutrients, we predicted that the fungal associates of D. rufipes may provide less of a beneficial nutrient than the beetles with O. montanum and compared the relative amounts of eggstrot contained in the primary fungal associates of D. rufipes and D. rufipes. Furthermore, the eggstrot content of phyla from host trees of both beetle species containing free-growing patulin was compared with that of uninfested phyla.

Materials and Methods

Fungal Identification and Growth in Extraction Resin. All fungal isolates were taken from the culture collection of D.L.S. and were initially identified by using morphological features in keys and descriptions contained in Upadhyay (1981), Cyrille and Sedjot (1990), and Jacobs and Wingfield (1994). Fungal isolates were then identified to species level using DNA sequencing and morphological comparisons with known strains (Lee et al. 2000). J. Kim and C. Brevi, personal communication.

Fungus Description. To test the effect of each mycelial and phaemal sample was scored to the nearest 0.1 mg. Freeze-dried fungal samples used in the extraction process weighed 0.05 to 0.1 g and phaemal samples 0.5 g to 1 g. Extracted content of the fungal mycelia and phaemal were analyzed using previously published methods (Neveil 1990, Koolman and Fraszy 1993). High-performance liquid chromatography (HPLC) grade methanol and water were used. For the sample extraction, the dried mycelium or phaemal were rehydrated with 95% EtOH for 3 h. Precise milliliters of 0.05% solution were added to the neutral BuOH:MeOH:EtOH:water:water extract containing the mycelium or phaemal was reconstituted with water to the desired concentration.

Table 1. Information of fungal isolates used in ergosterol analysis.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Beetles species</th>
<th>Location</th>
<th>Site name</th>
<th>OATD/CORL (Forest)</th>
<th>Collection year</th>
<th>Collection site</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Hall Boring</td>
<td>Squaw NF, ID</td>
<td>1080</td>
<td>2001</td>
<td>DL/R1317, DL/R1181</td>
</tr>
<tr>
<td>D. rufipes/zygotes</td>
<td>D. rufipes/zygotes</td>
<td>Tray Creek</td>
<td>Squaw NF, ID</td>
<td>1030</td>
<td>2001</td>
<td>DL/R1057</td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Wilkerson</td>
<td>Squaw NF, ID</td>
<td>1010</td>
<td>2001</td>
<td>DL/R1365</td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Lolo NF, MT</td>
<td>1000</td>
<td>2001</td>
<td>DL/R1365</td>
<td></td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Tray Creek</td>
<td>Squaw NF, ID</td>
<td>1090</td>
<td>2001</td>
<td>DL/R1365</td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Stetson</td>
<td>Squaw NF, ID</td>
<td>1050</td>
<td>2001</td>
<td>DL/R1365</td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Squaw NF, ID</td>
<td>1070</td>
<td>2001</td>
<td>DL/R1365</td>
<td></td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Squaw NF, ID</td>
<td>1090</td>
<td>2001</td>
<td>DL/R1365</td>
<td></td>
</tr>
<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Squaw NF, ID</td>
<td>1030</td>
<td>2001</td>
<td>DL/R1365</td>
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<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
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<td>2001</td>
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<tr>
<td>O. montanum</td>
<td>D. rufipes/zygotes</td>
<td>Squaw NF, ID</td>
<td>1000</td>
<td>2001</td>
<td>DL/R1365</td>
<td></td>
</tr>
</tbody>
</table>

Note: N 0.0%, F 0.0%, 40.0%, 30.0%, 20.0%, 10.0%, 5.0%, 3.0%, 2.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.03%,
takes from areas of host trees where larvae were most likely also included phosphorus uncollo-
ized by fungi.
Because of the presence of uncultured and known mycorrhizal in *D. ponderosae*, we hypothesized that its associated fungi would contain more ergosterol and thereby potentially confer a greater benefit to its host 

![Figure 3](image)

**Fig. 3.** Ergosterol content (milligrams per gram) quantified from unidentified and larval-infested wood samples of two tree species, *P. contorta* (*x* = 0.14) and *P. engelmannii* (*x* = 0.13), associated with *D. ponderosae* and *D. rubripes* respectively. Mycelia are the median (solid line), mean (dotted line), and 5th and 95th percentiles.

The fungus would be effective at the phylet in which the fungi grow. This may necessitate a greater amount of ergosterol per dry weight of mycelia for fungal colonization.

As expected, phylum-infested host trees contained no or only to zero ergosterol content. Conversely, phylum taken from trees colonized by *D. ponderosae* and *D. rubripes* and their fungal symbionts contained significantly higher ergosterol than the phylum taken from uncolonized trees. Samples were taken from infected trees where both *D. ponderosae* and *D. rubripes* were in larval life stages. Larvae of *D. ponderosae* are up to both fungi and phylum as they age (A.S. Adams and D.L.S., unpublished data). Consequently, they potentially gain nutrients from both the host tree and the fungal symbionts. Our results suggest that larval life stages of *D. ponderosae* and *D. rubripes* may obtain starches, in the form of ergosterol, while storing larval galleries.

Only one *saccaromyces* scottii, the coffee berry bovis, has been sequenced for fungal genomics associated with this fungal association (0.25-0.45) (Panizo et al., 1999) but it was a very small number of sequences from fungal associations of *Egyptris xenox.* (R.B. Baker et al., 2003). Therefore, the results of this study, while not definitive, indicate the potential for fungal colonization of the larvae feeding on E. xenox. In future studies, it is possible that the presence of ergosterol in fungal extracts was used in their analyses rather than extraction of RNA tissue directly apparent to bruce larvae, where colonization of the bruce larvae, and consequently ergosterol concentrations, are likely to affect the efficacy of the symbionts. In this study, we have collected wood samples, which contained less ergosterol than that of the uncolonized coffee berries (5 ± 0.005), were

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**BENTH AND SIE Fungi Associated with *Dendroctonus***


References Cited


Results

Mycoica of the three fungal species contained relatively large levels of ergosterol, as determined after 20 d of growth at 29°C. However, no significant differences were found among the fungal species in terms of ergosterol content (Fig. 1). The probability of containing ergosterol was significantly greater in P. oxalicum, P. oxalacetica, and D. racemosum (with the highest level) than in D. purpureum and D. radicisporum (with the lowest level) (mean 35.8%, 41.7%, and 32.5%, respectively). The results obtained were consistent with those of other studies (Abdul-Kareem and Abou El-Khair 1981; Jha et al. 1993; Pizzini and O'Connor 1994; Zayed et al. 1996). In addition, significant differences in ergosterol concentrations between the two host trees species or among sample dates were also found.

Discussion

We found no differences in the ergosterol content of mycoica of O. montana and O. crenata isolated from D. purpureum mycelia and L. abietinus isolated from the conks of D. radicisporum. Mean percentages of ergosterol (1.05%–1.50%) of dry weight of these fungal species were similar to levels found in filamentous fungi associated with decay aspen (1.02%–1.06%) (Abdul-Kareem and Abou El-Khair 1981; Jha et al. 1993; Pizzini and O'Connor 1994; Zayed et al. 1996). Yehoshua (1978) observed that some species of Phanoxytrichum (Phallaceae) contained 1.2% ergosterol as a dry weight. However, this level is lower than the ergosterol content of filamentous fungi isolated from deciduous and coniferous trees (1.02%–1.06%). Additionally, no significant differences in ergosterol concentrations between the two host trees species or among sample dates were also found.

One common scabby scolytus, the coffee berry borer, has been associated for dependence upon ergosterol provided by its symbiotic fungus (Morales-Bravo et al. 2000). In our laboratory experiments, it was observed that a diet containing 0.000% ergosterol resulted in lower growth and survival rates of the beetle. Although uninfected coffee beans contained no ergosterol, coffee beans colonized by the fungal symbiont contained levels of 0.000%. Although this level is slightly below that required by the beetle, the authors attributed this fact to the natural background levels of ergosterol in coffee beans. These results show that the fungus is required for the growth of the beetle, as a higher level of ergosterol is needed for the beetle to survive. However, no significant differences in ergosterol concentrations were found between the two host trees species or among sample dates.
Mitochondrial DNA from Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Suggests Cryptic Speciation and Pinpoints the Source of the Introduction to Eastern North America

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ABSTRACT
The hemlock woolly adelgid, Adelges tsugae Annand (Hemiptera: Adelgidae), is an introduced pest of unknown origin that is causing severe mortality to hemlocks (Tsuga spp.) in eastern North America. Adelges also occurs on other Tsuga species in western North America and East Asia, but these trees are not significantly damaged. The purpose of this study is to use molecular methods to clarify the relationship among hemlock adelgid worldwide and thereby determine the geographic origin of the introduction to eastern North America. Adelges were collected from multiple locations in eastern and western North America, mainland China, Taiwan, and Japan, and 125 bp of mitochondrial DNA was sequenced for each sample. Phylogenetic analyses suggest that the source of A. tsugae in eastern North America was likely a population of adelgids in southern Japan. A single haplotype was shared among all samples collected in eastern North America and samples collected in the natural range of T. sieboldii in southern Honshu, Japan. A separate adelgid mitochondrial lineage was found at higher elevations in the natural range of T. diversifolia. Adelges from mainland China represent a lineage that is closely diverged from insects in North America and Japan. In contrast to eastern North America, there is no conclusive evidence for a recent introduction of A. tsugae into western North America, where multiple haplotypes are found. Implications for hemlock woolly adelgid control, taxonomy, and plant-pest coevolution are discussed.

KEY WORDS: invasive pest, molecular systematics, Tsuga