

# Ergosterol Content of Fungi Associated with *Dendroctonus ponderosae* and *Dendroctonus 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 phloepagous 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 also was compared with ergosterol contents in uninfested phloem tissue. Mycelia of *Ophiostoma montium* (Rumfold) 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 mining larval galleries.

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

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 filamentous fungal species that is mutually beneficial to the beetle host, whereas relationships with other fungal associates may be antagonistic or commensalistic (Six 2003b). The fungi benefit from association with the beetle host by consistent dissemination to an ephemeral 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, often because of a nutritional benefit (Whitney 1971, Bridges 1983, Goldhammer et al. 1990, Coppedge et al.

1995, Six and Paine 1998, Ayres et al. 2000). Although mycophagy may not 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, Richmond and Thomas 1975, Svoboda et al. 1978). Sterols in woody plant tissues are typically present only in low concentrations (Kramer and Kozlowski 1960) or in forms not usable by insects (Clayton 1964). For insects that feed in woody tissues, fungi may provide an alternate source of sterols in the form of ergosterol (24 $\beta$ -methylcholesta-5,7,trans 22-trien-3 $\beta$ -ol) (Norris et al. 1969, Kok et al. 1970, Norris 1972, Maurer et al. 1992, Morales-Ramos et al. 2000). This major fungal sterol is produced by most fungi (Weete 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 cul-

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ture, developmental stage, and growth conditions (Pasanen et al. 1999).

In several insect-fungus symbioses, the insect associate has been found to be dependent upon sterols provided by the fungal associate (Norris et al. 1969, Kok et al. 1970, Norris 1972, Maurer et al. 1992, Morales-Ramos et al. 2000, Nasir and Noda 2003). These include ambrosia beetles (Scolytinae) that feed solely on fungi they garden in galleries in the sapwood of trees (Norris 1972, Kok 1979, Mueller et al. 2005). Ambrosia beetles are dependent upon ergosterol produced by their fungal symbionts for successful oocyte development, oviposition, larval development, and pupation (Norris and Baker 1967, Norris et al. 1969, French and Roeper 1975, Kok 1979). This relationship holds for another scolytine, the coffee berry borer, *Hypothenemus hampei* (Ferrari). This beetle bores in, and feeds on, coffee berries but cannot molt or reproduce without ergosterol from its symbiotic fungus *Fusarium solani* Martius (Morales-Ramos et al. 2000).

Although almost all fungi produce ergosterol, the varying levels of ergosterol present in the mycelia of different species of fungi may account for the relative differences in benefits to host beetles exhibited by some fungal associates. For example, studies by Kok et al. (1970) and Kok and Norris (1973) found that the most beneficial species of fungi associated with *Xyleborus* ambrosia beetles were those that possessed the greatest concentrations of ergosterol.

*Dendroctonus rufipennis* Kirby and *Dendroctonus ponderosae* Hopkins, two economically important bark beetle species in western North American forests, are closely associated with fungi. *Leptographium abietinum* (Peck) Wingfield is commonly found in specialized pits on adult *D. rufipennis* pronota and elytra (Solheim 1995, Six and Bentz 2003). Although *Ceratocystis rufipenni* (Wingfield) Harrington & Solheim has been isolated from wood colonized by *D. rufipennis* in some populations (Reynolds 1992, Solheim 1995), Six and Bentz (2003) did not isolate this fungus from any *D. rufipennis* adults collected in Alaska, Utah, Colorado, and Minnesota, and they consider it to be a minor or incidental associate. Little research has been conducted on these fungi, and it is unclear whether they play a mutualistic or antagonistic role in *D. rufipennis* population dynamics.

*D. ponderosae* is closely associated with two fungi, *Ophiostoma montium* (Rumfold) von Arx and *Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington. Both species can be found on the exoskeleton of adult *D. ponderosae* and in specialized structures of the maxillae termed sac mycangia that function in fungal transport (Six 2003a,b). Sac mycangia are relatively rare in bark beetles, and those that do possess them are also mycophagous, suggesting that mycangia evolved to maintain tight associations with specific fungi that provide sources of nutrition for the beetle host (Harrington 2005). Effects of the two fungi on their host beetle are not well understood. Only two studies have been conducted investigating potential nutritional effects of the fungi, and the results of these two studies seem to conflict (Six and Paine 1998;

K. Bleiker and D.L.S., unpublished data). In one study, *D. ponderosae* were reared in logs colonized with parental beetles associated with either *O. clavigerum*, *O. montium*, or without fungi (Six and Paine 1998). Resulting brood that developed with *O. clavigerum* were more numerous and emerged significantly earlier than brood that developed with *O. montium*. Beetles introduced without fungi did not produce brood. These results indicate that both fungi support brood production but that *O. clavigerum* may be a superior associate in conferring nutritional benefit. However, in a recent study conducted in phloem sandwiches (K. Bleiker and D.L.S., unpublished data), beetles developing with *O. montium* consumed significantly less phloem than insects developing with *O. clavigerum* or no fungi, suggesting that *O. montium* provides superior nutritional benefits. Beetles developing with *O. clavigerum* or no fungus had to consume more phloem to meet their dietary requirements.

As has been found with other mycophagous scolytine beetles, we hypothesized that *D. ponderosae* and *D. rufipennis* obtain sterols, in particular ergosterol, from their close fungal associates. Because *D. rufipennis* does not have sac mycangia, as has been found for *D. ponderosae*, and thus may not have as highly an evolved association with its fungus or as strong a dependence on fungi for nutrients, we predicted that the fungal associate of *D. rufipennis* may provide less of a beneficial nutrient resource in the form of ergosterol compared with one or both of the fungi associated with *D. ponderosae*. Our objective was to determine and compare the relative amounts of ergosterol contained in the primary fungal associates of *D. ponderosae* and *D. rufipennis*. Furthermore, the ergosterol content of phloem from host trees of both beetle species containing feeding larvae (and symbiotic fungi) also was compared with that of uninfested phloem.

## Materials and Methods

**Fungal Identification and Growth for Extraction.** Fungal isolates used in this study were taken from the culture collection of D.L.S. and were initially identified by using morphological characteristics as well as keys and descriptions contained in Upadhyay (1981), Grylls and Seifert (1993), and Jacobs and Wingfield (2003). The identity of select isolates was further confirmed using DNA sequencing and morphological comparisons with known strains (Lee et al. 2003) (J. Kim and C. Breuil, personal communication) (Table 1).

Three replicates of each isolate of each of the three fungal species were analyzed for ergosterol content. Each replicate was grown on 2% malt extract agar for 10 d. Then, a small plug of mycelium of each isolate was placed into a 123-ml Erlenmeyer flask containing 2% malt yeast extract broth and allowed to grow for an additional 10 d. Fungal mats were removed from the liquid broth, placed on a filter, and rinsed with distilled water while a vacuum was applied to remove culture broth residue. Mycelial mats were then freeze-dried in individual vials. After lyophilization, vials were topped

Table 1. Collection information for fungal isolates used in ergosterol analysis

Fungal species	Beetle species/location of isolation	Site name USDA National Forest (NF)	Collection yr	Isolate no.	Latitude/longitude
<i>O. montium</i>	<i>D. ponderosae</i> /mycangium	Hell Roaring Sawtooth NF, ID	2002	DLS1117, DLS1121	N 44° 2' W 114° 51'
		Truckee Sierra Nevada NF, CA	1996	DLS1057	N 39° 20' W 120° 11'
	<i>D. ponderosae</i> /mycangium	Williams Sawtooth NF, ID	2001	DLS1109	N 44° 6' W 114° 50'
<i>O. clavigerum</i>	<i>D. ponderosae</i> /mycangium	12 mile Lolo NF, MT	2002	DLS1095, DLS1126	N 47° 23' W 115° 14'
		Truckee Sierra Nevada NF, CA	1996	DLS1061	N 39° 20' W 120° 11'
	<i>D. ponderosae</i> /mycangium	Smiley Sawtooth NF, ID	2001	DLS1125	N 43° 53' W 114° 48'
<i>L. abietinum</i>	<i>D. rufipennis</i> /exoskeleton	Floyd Mountain Routt NF, CO	2001	DLS1122, DLS1106	N 40° 67' W 106° 60'
		North Lake Routt NF, CO	2002	DLS1123, DLS1124, DLS1107	N 40° 75' W 106° 60'

with nitrogen and held in the dark at 2°C until ergosterol extraction.

**Phloem Sampling.** To compare concentrations of ergosterol estimated from known fungal isolates in culture with actual ergosterol concentrations consumed by larvae feeding in trees colonized by fungal associates, we collected and extracted ergosterol from phloem from trees infested by the two host beetles. Samples of phloem were collected on 16 April 2003 from four *D. rufipennis*-infested *Picea engelmannii* Parry and four uninfested *P. engelmannii* at three sites on the Fishlake National Forest, Utah. Phloem samples from one *D. ponderosae*-infested *Pinus contorta* Douglas and four uninfested *P. contorta* were collected 28 January 2003 on the Wasatch Cache National Forest, Utah. Additional phloem samples were collected 23 April 2003 from four *D. ponderosae*-infested *P. contorta* and two uninfested *P. contorta* on the Sawtooth National Forest, Idaho. Phloem was collected by using a sterile knife to remove a 100-cm<sup>2</sup> section of phloem from live and beetle-infested tree boles 1.37 m above the ground. Samples removed from trees infested with *D. rufipennis* and *D. ponderosae* were taken from areas where larvae were mining. Phloem samples were transported to the laboratory on ice, oven-dried for 24 h, and stored at -30°C until lyophilization.

**Ergosterol Determination.** Before transfer to extraction vials, the dry weight of each mycelial and phloem sample was recorded to the nearest 0.1 mg. Freeze-dried fungal samples used in the extraction process weighed ≈0.03 g and phloem samples ≈1.0 g. Ergosterol content of the fungal mats and phloem was analyzed using previously published methods (Newell et al. 1988, Padgett and Posey 1993). High-performance liquid chromatography (HPLC)-grade methanol (20 ml) was added to a round-bottomed flask containing the dried mycelium or phloem and refluxed for 2 h at 80°C. Five milliliters of a 4% solution of KOH in 95% ethanol was added to the neutral extracts obtained from the methanolic pathway and refluxed for 30 m at 80°C to hydrolyze the esters. Sterols were removed from the alcoholic base by partitioning into pentane after cooling to 25°C and adding

10 ml of distilled water. A series of three pentane additions (10, 5, and 5 ml) were mixed by repeatedly inverting tightly sealed flasks. After each addition and mixing, the solution was placed in a separatory funnel. The pentane layer was withdrawn, and pentane fractions from each sample were pooled. The pentane samples were evaporated in open glass flasks at 30°C under a stream of nitrogen. Each sample was redissolved in 2 ml of methanol, and the vials were tightly sealed and sonicated for 5 min to enhance the rate of dissolution. Redissolved samples were filtered through 0.22-μm polytetrafluoroethylene membranes and stored in the dark at 2°C for 1–7 d until HPLC analysis.

Samples were analyzed by isocratic liquid chromatography on an Agilent 1100 (Agilent Technologies, Palo Alto, CA) by using a 250-by 4.6 mm 5-μm Supelco LiChrospher RP18 column (Supelco, Bellefonte, PA) held at 45°C. Samples (5 μl) were injected by a thermostatted autosampler cooled to 5°C by using methanol (2 ml/min) as a mobile phase. Ergosterol was detected by an Agilent diode array detector at 282 nm and quantified using an ergosterol four point external standard calibration curve (Sigma, St. Louis, MO). Three replicates of each extracted fungal and phloem isolate were analyzed. Final ergosterol concentration of each sample was expressed as milligrams of ergosterol per gram of fungal or phloem dry mass.

**Statistical Analysis.** Differences in ergosterol concentration among the fungal species were analyzed using mixed model analysis within SAS (Littell et al. 1996). Fungal species was included as a fixed effect, and replicate and isolate within species were included as random effects. Because the phloem data violated normality assumptions, and our main objective was to test for presence or absence of ergosterol in uninfested and infested phloem, we transformed the data to a binomial format. Samples with an ergosterol quantity >0 were coded as 1 and as 0 otherwise. GLIMMIX, a SAS procedure for fitting generalized linear mixed models (Littell et al. 1996), was used to accommodate the random effects of replicate and isolate within species, with host tree species included as a fixed effect and a binomial error distribution.

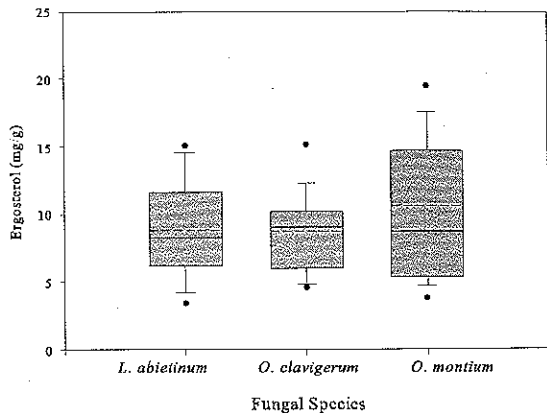


Fig. 1. Ergosterol content (milligrams per gram) quantified from mycelium of three bark beetle-associated fungal species grown for 10 d on 2% malt yeast extract followed by 10 d on 2% malt yeast extract broth (*L. abietinum*:  $\bar{x}$  = 8.80,  $n$  = 16; *O. clavigerum*:  $\bar{x}$  = 8.15,  $n$  = 12; *O. montium*:  $\bar{x}$  = 10.57,  $n$  = 13). Shown are the median (solid line), mean (dotted line), and 5th and 95th percentiles.

## Results

Mycelia of the three fungal species contained relatively large levels of ergosterol, as determined after 20 d growth at 20°C. However, no significant differences were found among the fungal species in total ergosterol content (Fig. 1). The probability of containing ergosterol was significantly greater in *P. engelmanni* and *P. contorta* phloem infested with *Dendroctonus* larvae (and their fungal symbionts) than uninfested phloem samples which contained either no detectable ergosterol or only trace amounts ( $F$  = 8.92;  $df$  = 1, 21.56;  $P$  = 0.0069) (Fig. 2). We found no significant differences in ergosterol concentrations between the two host tree species or among sample dates.

## Discussion

We found no differences in the ergosterol content of mycelia of *O. montium* and *O. clavigerum* isolated from *D. ponderosae* mycangia and *L. abietinum* isolated from the exoskeleton of *D. rufipennis*. Mean percentage of ergosterol (0.88–1.06% dry weight) of these fungi was similar to levels found in filamentous fungi associated with decaying building materials (0.26–1.40%) (Pasanen et al. 1999), but it was much greater than levels quantified from fungal associates of *Xyleborus* ambrosia beetles (0.12–0.24%) (Kok and Norris 1973). *Xyleborus* feed almost entirely on concentrated spore layers of their associated fungi, and the ergosterol obtained from these layers is vital to successful development and reproduction (Baker and Norris 1968). Because *Dendroctonus* larvae feed on phloem colonized by fungal hyphae (A.S. Adams and D.L.S., unpublished data), they consume fungal biomass in much lower concentrations than do ambrosia beetles. Consequently, the ergosterol in the mycelium

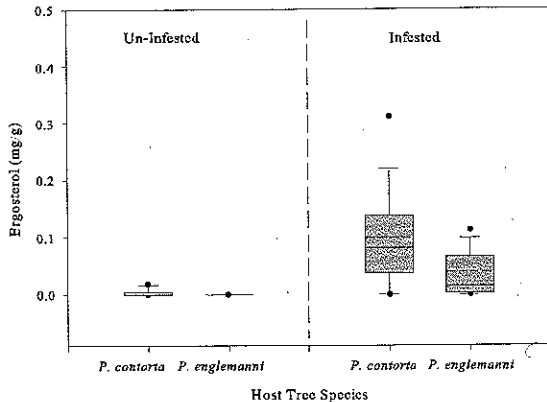


Fig. 2. Ergosterol content (milligrams per gram) quantified from uninfested and larval-infested phloem samples of two tree species, *P. contorta* ( $n$  = 14) and *P. engelmanni* ( $n$  = 12), associated with *D. ponderosae* and *D. rufipennis* respectively. Shown are the median (solid line), mean (dotted line), and 5th and 95th percentiles.

of the fungi would in effect be diluted by the phloem within which the fungi grow. This may necessitate a greater amount of ergosterol per dry weight of mycelium to fulfill the basic sterol requirements of the host beetles.

As expected, phloem of uninfested host trees contained zero to only trace levels of ergosterol. Conversely, phloem taken from trees colonized by *D. ponderosae* and *D. rufipennis* and their fungal symbionts contained significantly more ergosterol than did phloem taken from uninfested trees. Samples were taken from infested trees when both *D. ponderosae* and *D. rufipennis* were in larval life stages. Larvae of *Dendroctonus* feed upon both fungi and phloem as they mine (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. rufipennis* may obtain sterols, in the form of ergosterol, while mining larval galleries.

Only one nonambrosia scolytine, the coffee berry borer, has been assessed for dependence upon ergosterol provided by its symbiotic fungus (Morales-Ramos et al. 2000). In laboratory experiments, it was determined that a diet containing an ergosterol concentration between 0.015 and 0.020% (dry weight) was required to achieve normal reproduction by the beetle. Although uninfested coffee berries contained no ergosterol, coffee berries colonized by the fungal symbiont contained levels of  $\approx$ 0.011%. Although this level is slightly below that required by the beetle, the authors attributed this to the fact that whole berry extractions were used in their analyses rather than extractions of berry tissues directly adjacent to larvae where colonization of the berry by the fungus, and consequently ergosterol concentrations, are likely to be the greatest. Similarly, our infested phloem samples, which contained less ergosterol than did the fungal-colonized coffee berries ( $\bar{x}$  = 0.006%), were

taken from areas of host trees where larvae were mining, but most likely also included phloem uncolonized by fungi.

Because of the presence of sac mycangia and known mycophagy in *D. ponderosae*, we hypothesized that its associated fungi would contain more ergosterol and thereby potentially confer a greater benefit to its host than would *L. abietinum* associated with *D. rufipennis*, which possesses only rudimentary pit mycangia. Sac mycangia are specialized structures thought to have evolved specifically to carry fungi, thereby suggesting a more obligate relationship between beetle host and fungal associate (Six 2003a). We also hypothesized that *O. clavigerum* would provide greater quantities of ergosterol to *D. ponderosae* than would *O. montium*. *O. clavigerum* has a long shared evolutionary history with *D. ponderosae* (Six and Paine 1998), whereas *O. montium* is considered a more recent associate (Six and Paine 1999). However, although we found no difference in the amount of ergosterol among any of the primary fungi, our results suggest that these associates of *D. ponderosae* and *D. rufipennis* do make relatively large quantities of ergosterol available to their beetle hosts. Future studies should investigate the degree of mycophagy and the use and degree of dependence of *Dendroctonus* beetles on ergosterol provided by their fungal symbionts.

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