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Saccharide-mediated antagonistic effects of bark beetle fungal associates on larvae

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Bark beetles are among the most destructive of pine forest pests and they form close symbiotic relationships with ophiostomatoid fungi. Although some fungi are considered to be mutualistic symbionts of bark beetles with respect to the supply of nutrients, detrimental effects of fungal symbionts on larval growth have also been frequently reported. The mechanisms of such antagonistic effects are hypothesized to be a decrease in nutritional resources caused by competition for saccharides by the fungi. Here, we provide experimental evidence that three beetle-associated fungi modify the nutritional content of an artificial phloem diet, leading to a detrimental effect on the growth of *Dendroctonus valens* larvae. When larvae were fed a diet of pine phloem in agar medium colonized with any of these fungi, feeding activity was not affected but weight significantly decreased. Additional analysis showed that fungi depleted the fructose and glucose concentrations in the phloem media. Furthermore, these detrimental effects were neutralized by supplementing the media with fructose or glucose, suggesting that fungi may affect larval growth by modifying diet saccharide contents. These data indicate that fungus-induced nutritional changes in bark beetle diet can affect larval growth, and that the mechanism involves fungus-induced saccharide depletion from the larval diet.

1. Introduction

Bark beetle–fungus symbioses are an integral part of pine forest ecosystems and play an important role in influencing the structure, composition and ecological succession of pine forest ecosystems [1]. Bark beetles, such as species in the genera *Dendroctonus* and *Ips*, are commonly associated with ophiostomatoid fungi in the genera *Ceratocystis*, *Ceratocystiopsis*, *Graphium*, *Leptographium*, *Grosmannia* and *Ophiostoma* [2], and some Basidiomycetes, such as *Entomocorticium* spp. and *Phlebiopsis* spp., also play an important role in bark beetle life cycles [2]. Fungal spores are carried in the adult beetle exoskeleton, and when beetles bore their galleries in the bark of pines, fungi are inoculated in the phloem layer. Once the larvae hatch, they start feeding on the fungus-colonized phloem. Fungi can have important effects on development, brood survival and even the population dynamics of bark beetles [3]. Bark beetles and their associated fungi provide a tractable system for the study of symbiosis in a context of wide ecological and economic significance [2].

Relationships between bark beetles and ophiostomatoid fungi are complex, because some fungi are beneficial while others are detrimental or have no evident effect on their bark beetle hosts [2]. Thus, bark beetle–fungi interactions have been classified as mutualistic, antagonistic and commensal [3]. Beetles associated with mutualistic fungi grow faster, with larger body size and lower progeny mortality [4]. Sugars and proteins are essential nutrients for most organisms, including insects, providing energy and precursors for metabolic processes and tissue building [5]. Not surprisingly, phloem colonized by mutualistic fungi has higher nutritional quality (amino acids and proteins) than phloem colonized by commensal or antagonistic fungi [6]. Antagonistic

fungi can cause reduced brood production and higher larval mortality [4,7]. In fact, larvae often fail to complete development when fed phloem colonized by these fungi [8]. Despite this evidence, it is still unknown whether or not this antagonism is a result of fungus-induced changes to the phloem–fungus substrate that larvae feed on.

Relationships between the red turpentine beetle, *Dendroctonus valens* LeConte (Curculionidae: Scolytinae) and its associated fungi have been studied recently [9], and some detrimental effects of symbiotic fungi were observed [10]. The red turpentine beetle is a phloem-feeding, invasive forest pest in China, where it has infested more than 500 000 ha of pine forest and killed over 10 million Chinese pines, *Pinus tabuliformis*, since 1999 [11]. The fungi *Leptographium sinoprocerum* (a newly described species associated with *D. valens* [12]) and *L. procerum* are the most frequently isolated species from *D. valens* and its galleries [9] over its range in China. *Ophiostoma minus*, a well-known detrimental fungal associate of *Dendroctonus frontalis* [6], can cause reduced brood production and higher mortality in *D. frontalis* larvae, and it also affects larval development in *D. valens* [10]. Detrimental fungi can reduce larval abundance in the gallery, which could ultimately influence bark beetle population dynamics [3].

In order to evaluate whether ophiostomatoid fungi play an important antagonistic role in their interactions with bark beetles through modification of nutritional resources, we first determined which ophiostomatoid fungi were most commonly associated with *D. valens* larvae in the field and then evaluated the effects of fungi on feeding activity and growth (i.e. body weight change) of *D. valens* larvae. We then tested whether specific fungi could modify the nutritional quality (sugars) of the bark beetle diet. Finally, we evaluated whether the nutritional modifications could influence the growth of larvae.

2. Material and methods

Adult *D. valens* were collected from infested *P. tabuliformis* in Tunlanchuan Forestry Farm (Shanxi, China). One female and one male beetle were introduced into newly cut bolts through a hole made using a 12 mm diameter cork borer and were covered with a metal screen to prevent escape [13]. One month later, beetle progeny, consisting of second and third instar larvae, were extracted from the bolts and used in the experiments.

Effects of fungus-colonized diet on larval weight and feeding were tested on phloem medium. Fungi used here were originally from China, but cultures of them were obtained from stock collections of the Forestry and Agricultural Biotechnology Institute, University of Pretoria (*O. minus* and *L. procerum*) and the Belgian Coordinated Collections of Microorganisms (*L. sinoprocerum*). Isolation and identification of fungi associated with larvae are given in the electronic supplementary material.

Phloem powder was made from phloem of *P. tabuliformis* (see the electronic supplementary material). Phloem medium (phloem powder 20 g, agar 10 g, water 300 ml) was prepared, autoclaved (30 min, 126°C, 0.14 Mpa), and poured into 15 ml Petri dishes. After cooling, the desired fungal isolate was inoculated and incubated at 25°C, RH 70 per cent in darkness for 35 days. Subsequently, 3 cm diameter media discs were made and transferred into six-well cell culture plates. Larvae were fed on the sterile medium for a week to avoid contamination [10], weighed (initial mass of larvae $\pm$ s.d. $= 8.17 \pm 0.21$ mg), and then randomly assigned to each treatment (medium with specific fungal isolate) and control (fungus-free media). Each treatment had 36 replicates. Larvae cannot complete their full development but can grow on these media (see the electronic supplementary material), therefore larvae were weighed after 6 days, and weight change was used to represent growth (dead larvae were excluded: four in control, six in *O. minus*, five in *L. sinoprocerum*, four in *L. procerum*). Boring activity was measured and compared across treatments (see the electronic supplementary material).

Effects of fungi on diet nutritional value and consequences of nutritional change on larval weight are given in the electronic supplementary material.

3. Results

Eleven fungal species, including *O. minus*, *L. sinoprocerum*, and *L. procerum*, were found in association with *D. valens* (see the electronic supplementary material, table S1). The rarefaction analysis shows that this number of fungal species is a good representation of the fungal flora (see the electronic supplementary material, figure S1).

Whereas body weight of larvae fed on control medium increased 2.27 per cent (figure 1*a*, one-way ANOVA, $F_{3,121} = 3.72$, $p < 0.05$). Boring activity was not significantly affected by fungi ($t = -0.79$, d.f. $= 139$, $p = 0.43$, electronic supplementary material, figure S2).

Fructose and glucose were the dominant sugars in the fungus-free medium, with contents of 64.82 per cent and 34.29 per cent, respectively, whereas other sugars (saccharose, maltose and lactose) accounted for only 0.89 per cent of the total sugar content. Fungi dramatically decreased the level of fructose (Welch method, $F_{2,6} = 1156.65$, $p < 0.001$) and glucose ($F_{3,8} = 96.46$, $p < 0.001$) in the media, from approximately 14 of fructose and 7 mg g^{-1} of glucose (in control) to nearly zero (table 1). Total sugar content was also significantly reduced in fungus-colonized media ($F_{3,8} = 223.43$, $p < 0.001$).

For each experimentally cultured fungus, the weight of larvae fed on media with added glucose or fructose increased significantly compared with the media without additional sugars (figure 1*b*: $F_{2,81} = 7.3$, $p < 0.05$; figure 1*c*: $F_{2,77} = 13.38$, $p < 0.001$; figure 1*d*: $F_{2,77} = 9.53$, $p < 0.001$).

4. Discussion

Our results show that sugars are important nutritional sources for both bark beetles and their associated fungi, and that fungi may be competing with beetles for this resource. Reduced body weight in larvae that fed on fungus-colonized media (figure 1*a*), and reduced fructose and glucose contents in these media (table 1) suggest that sugars are important nutritional elements in *D. valens* larval development and also that these fungi (*O. minus*, *L. sinoprocerum* and *L. procerum*) are detrimental to *D. valens* larvae. The importance of sugars in larval development was further confirmed by the increase in larval weight measured in larvae that were fed on sugar-supplemented phloem media (figure 1*b–d*). This might also be true with other bark beetles associated with fungi; a similar study also found that a detrimental fungal associate (*Ceratocystis minor*) of *D. frontalis* also reduced the sugar content (glucose and fructose) of phloem of live hosts (*P. taeda*) [14].

We provide evidence that may explain the mechanism underlying the antagonisms previously reported [6]. In

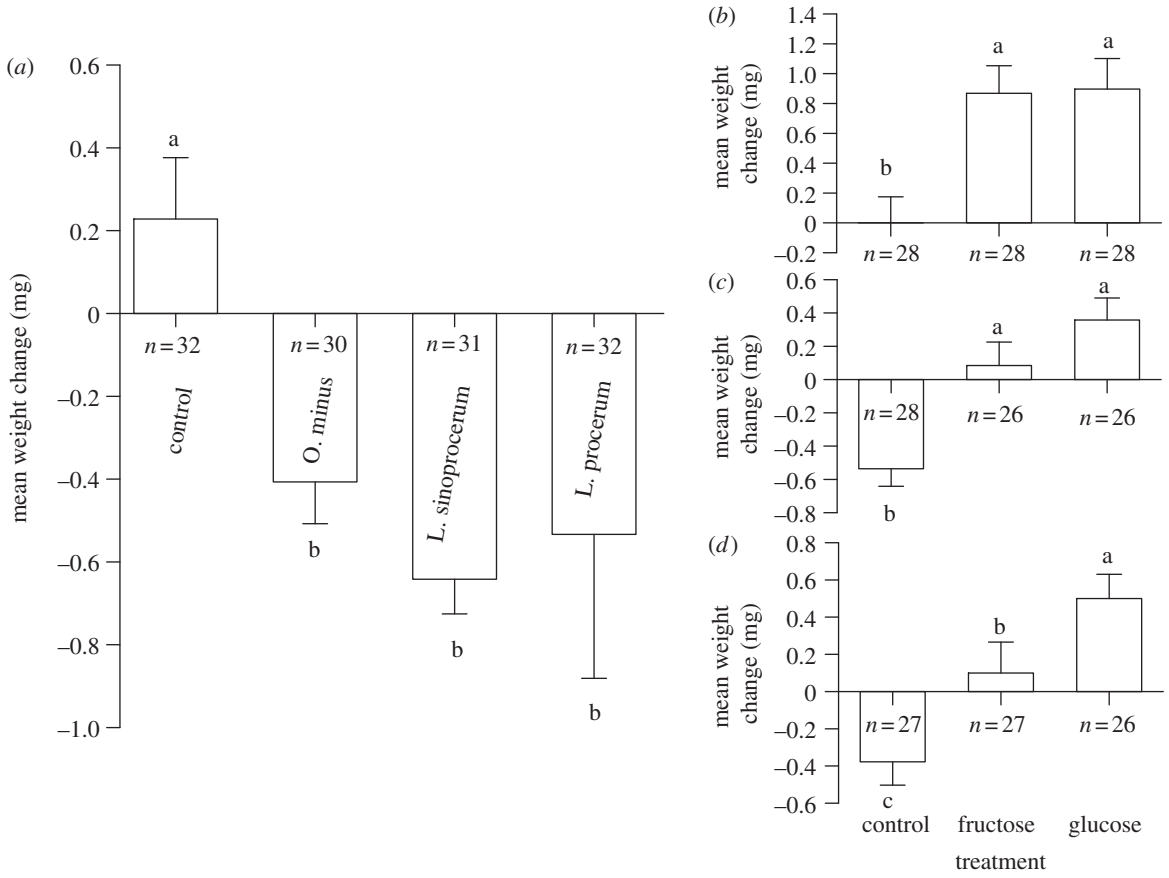

Figure 1. Average weight change of *Dendroctonus valens* larvae during a 6-day period after feeding on: (*a*) the control (sterile medium) and the medium with one of three fungal species (*Ophiostoma minus*, *Leptographium sinoprocerum* and *L. procerum*) (*b*) the control (*O. minus*-colonized medium) and *O. minus*-colonized medium with additional sugars (fructose or glucose) (*c*) the control (*L. sinoprocerum*-colonized medium) and *L. sinoprocerum*-colonized medium with additional sugars (fructose or glucose), (*d*) the control (*L. procerum*-colonized medium) and *L. procerum*-colonized medium with additional sugars (fructose or glucose). Data show mean ± s.e. Each letter indicates significant differences between treatments ($p < 0.05$ SNK multiple-comparison). n = sample size.

Table 1. Mean (±s.e.) contents (mg g^{-1}) of sugars in phloem medium. Control, fungus-free medium; *O. min, Ophiostoma minus*-colonized medium; *L. sin* , *Leptographium sinoprocerum*-colonized medium; *L. pro*, *L. procerum*-colonized medium. Values with the same superscript letter are not significantly different at $\alpha = 0.05$.

treatment	fructose	glucose	saccharose	maltose	lactose	total sugar
control	13.96 ± 0.37^{a}	7.38 ± 0.27^{a}	0.08 ± 0.01^{a}	0.07 ± 0.00^{c}	0.04 ± 0.00^{c}	21.53 ± 0.64^{a}
O. min	0.43 ± 0.01^{c}	0.86 ± 0.18^{c}	not detected	0.27 ± 0.04^{c}	0.02 ± 0.00^{d}	1.57 ± 0.20^{c}
L. sin	not detected	2.23 ± 0.49^{b}	0.06 ± 0.01^{b}	2.68 ± 0.15^{a}	0.09 ± 0.00^{a}	5.06 ± 0.45^{b}
L. pro	1.04 ± 0.01^{b}	1.01 ± 0.22^{c}	not detected	2.01 ± 0.34^{b}	0.06 ± 0.00^{b}	4.12 ± 0.14^{b}

particular, we suggest that fungi indirectly affect larval development by depleting sugar content in the phloem. Sugars are essential nutrients that provide energy and precursors for tissue building during larval development [5]. Slower larval development and lower larval weight affect adult beetle size, which in turn is strongly correlated with adult beetle survival, dispersal, pheromone production, mate choice and fecundity [13,15,16]. Antagonistic relationships with ophiostomatoid fungi via competition for nutritional resources could then indirectly affect bark beetle population dynamics. Effects of fungi on *D. valens* growth under field conditions need to be further tested, and environmentally sound control strategies should then seriously consider incorporating associated fungi in the development of new programmes of *D. valens* control.

Although our results support the existence of antagonistic relationships between *D. valens* larvae and their associated fungi in the galleries, there are also beneficial beetle–fungal relationships in other life stages of *D. valens* that should not be ignored. For example, fungal associates have been shown to induce the release of more attractive pine volatiles from infected host trees, and these volatiles facilitate aggregation of *D. valens* on host trees [8]. Thus, the relationships between *D. valens* and its fungal associates are highly context-dependent [17]. Furthermore, the artificial diet approach that we have employed here has its limitations, because

the beetle larvae can grow only for a short period but cannot complete full development on it. This type of variable symbiosis is likely to be found in similar symbiotic systems and may reflect the diverse living strategies of partners in symbioses under shifting circumstances. Such symbiotic variability could provide greater flexibility for bark beetles to adapt to a continually fluctuating environment and could involve fitness trade-offs between optimizing nutrition and optimizing aggregation.

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