Mutualism with aggressive wood-degrading *Flavodon ambrosius* (Polyporales) facilitates niche expansion and communal social structure in *Ambrosiophilus ambrosia* beetles

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Abstract

Most wood-boring insects compete with wood decaying basidiomycetes for woody biomass.

One clade of ambrosia beetles gained access to rotten wood – an abundant resource unsuitable to most wood-boring insects – by evolving a farming-like mutualism with a white-rot polypore.

Here we show the mutualism between *Ambrosiodmus/Ambrosiophilus* and the polypore

Flavodon ambrosius is superior in lignocellulolytic capacity compared to Ascomycota ambrosia

fungi and other white rot Basidiomycota. This mutualism facilitated the evolution of large, long-

lived, communal colonies with overlapping generations and egg-laying by pre-dispersal progeny

females. *Flavodon ambrosius* resembles other white-rot Polyporales in that it causes significant

weight loss in wood decay assays and strong polyphenol oxidase reactions, indicative of lignin-

modifying enzymes. The symbiosis is asymmetrical: there are many species of *Ambrosiodmus*

and *Ambrosiophilus* but all use a single known species of *Flavodon*, which determines the

ecological strategy of the entire insect clade.

Index descriptors: *Ambrosiophilus*, *Ambrosiodmus*, *Flavodon ambrosius*, Polyporales,

communality, wood decay, lignin degradation, fungus farming, symbiosis

Introduction

Wood is one of the most abundant natural organic compounds on Earth and a huge potential source of energy for heterotrophic organisms. However, very few animals have evolved the capacity to digest the lignocellulosic matrix, and thus lack access to a diverse number of polymeric carbohydrates. Many fungi do have the necessary enzymatic machinery, but lack the ability to access wood in living trees and often rely on wound sites to gain ingress into living trees (Pearce 1996). Due to the complementary capabilities of animals and fungi, one of the most commonly evolved solutions for access to wood and utilization of plant substrates is mutualistic association (Mueller et al. 2005). In wood boring insects, association with fungal and bacterial mutualists is widespread. This includes associations with wood decay fungi, as with the fungus-growing termites *Macrotermes* and their mutualistic fungi, *Termitomyces* sp. (Hyodo et al. 2000). Although wood borers are capable of producing their own lignocellulolytic enzymes allowing for microbial-independent digestion of cellulose (Martin et al. 1991, Watanabe and Tokuda 2010), association with fungi can provide superior or at least complementary catabolic capacity (Geib et al. 2008, Norris 1980) and access to a larger volume of wood by taking advantage of fungal enzymes that selectively decompose the principal components of wood: cellulose, hemicellulose, and lignin. Access to the energy and nutrients sequestered in wood has, in part, facilitated the ecological and evolutionary success of wood borers, including adaptive species radiations and the development of an evolutionarily novel trait (i.e., mycangium). Access to recalcitrant compounds in wood has also led to the evolution of complex societies with hierarchical structure, and has helped establish wood borers as one of the foundational guilds and ecosystem engineers (Ellison et al. 2005) that exert strong positive

effects on secondary colonizers (Calderón-Cortés et al. 2011). Insect-fungus symbioses specializing in using plant material, such as termites and leaf-cutter ants, support some of the largest colonies of animals on earth (Mueller and Gerardo 2002).

One of the most well-known symbioses between wood boring insects and fungi are the fungus farming ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae). However, most ambrosia beetles are associated with fungi that are not capable of digesting lignocellulose and instead utilize less recalcitrant carbon compounds (Licht and Biedermann 2012). These easily digestible carbon sources are typically available in a dead tree for only a short time after tree death. Consequently, the majority of ambrosia fungi and beetles are only able to colonize declining and freshly dead trees, and are not competitive in trees colonized by general wood-decay fungi. Furthermore, once a tree senesces and dies, the supplantation of mutualistic fungi by wood decaying basidiomycetes (Frankland 1998) significantly limits the time the insects can remain in the wood. That in turn constrains the evolution of family and social dynamics among the insects: nearly all ambrosia beetle species are only able to develop a single generation on a given tree, and all new individuals must disperse. Very few ambrosia beetles display any more advanced levels of sociality (Kent and Simpson 1992, Biedermann and Taborsky 2011).

One exception among the subfamily Scolytinae is the recently discovered association between saproxylic *Ambrosiodmus* ambrosia beetles (Scolytinae: Xyleborini) and their mutualistic fungal symbiont *Flavodon ambrosius* (Basidiomycota: Polyporales) (Li et al. 2015, Simmons et al. 2016). This symbiosis between a wood borer and a wood-decay polypore led to several evolutionary and ecological advances: (1) *Ambrosiodmus* galleries and *Flavodon* persist

and thrive even in wood so decayed that it is no longer usable by other ambrosia beetles and fungi; (2) a single species *Flavodon ambrosius* appears to be conserved across different species of *Ambrosiodmus* on multiple continents; and (3) and the association has led to successful colonization of new regions by the symbiotic partners, as evidenced by the multiple Asian *Ambrosiodmus* now widespread across the U.S.A. (Atkinson 2016). Field observations of the genus *Ambrosiophilus*, which is phylogenetically a sister genus to *Ambrosiodmus* (Hulcr and Cognato 2009, Cognato et al. 2011), indicated that these beetles are widespread and may also carry a symbiotic fungus that is capable of decomposing lignocellulose. Similar to *Ambrosiodmus*, *Ambrosiophilus atratus* has spread rapidly throughout the Eastern U.S.A. since it was first detected in Maryland in 1983; it is now reportedly present in 29 states (Atkinson 2016). In 2013, a second and previously undescribed *Ambrosiophilus* species, *A. peregrinus*, a species presumed to be native to Asia, was reported from northern Georgia (Smith and Cognato 2015). Similar to *Ambrosiodmus* (Faccoli et al. 2009, Li et al. 2015), the fungal mutualist of *Ambrosiophilus* appears to sustain large beetle colonies even in the presence of advanced decay and co-colonization by other wood decay fungi, long after tree death.

The novelty of *Ambrosiophilus* documented in this work is that the long-term availability of resources seems to have facilitated the largest colonies recorded from ambrosia beetles, overlapping generations, and a communal level of sociality (Kukuk and Schwarz 1987), which is unique compared to behavior of all other known ambrosia beetles. Here we specifically address the identity of the *Ambrosiophilus* symbiont, its capacity to decompose lignocellulose compared to other ambrosial and non-ambrosial fungi. We also tested whether the *Ambrosiophilus* system satisfies the definition of a communal social system, by testing for the presence of

overlap of generations, communal reproduction, and delayed dispersal by the new generation of females.

Methods

Sampling and isolation of fungi from host beetles and galleries

To isolate and identify the dominant fungal symbionts in *Ambrosiophilus atratus*, individuals representing all life stages were carefully removed from an expansive multigenerational gallery found inside highly decayed wood of an *Ailanthus altissima* on the campus of West Virginia University in Morgantown, West Virginia, USA (39°38' 01"N, 79° 57' 02"W) (Fig. 1). A total of 67 mature adult females, 23 progeny females, 6 males, 15 larvae, 4 pupae, and 36 eggs were recovered from the active gallery. Many (possibly hundreds) additional individuals, representing all life stages were observed inside the wood but were not sampled. Sampling depth for the various life stages of *A. atratus* used in culture-dependent studies were as follows: four pupae, 15 larvae, three adult males, and 29 adult females. Sampling was carried out on WVU property and did not require permits.

Live extracted beetles and colonized gallery sections were transported to the laboratory in clean containers. Twenty nine females (a mix of parental and progeny females), three males, 15 larvae, and 4 pupae were surface-disinfested and used for serial dilution plating as previously described (Kasson et al. 2013). Adult males and females were separated into three body regions (head, pronotum, and abdomen) to resolve patterns of association between specific fungal morphotypes and body regions (Fraedrich et al. 2008, Kasson et al. 2013). The pupae and larvae were crushed whole. Colony forming units (CFUs) were tallied for each fungal morphotype and representative cultures were retained for long-term storage and DNA-based

studies. Concurrent to serial dilution platings, six separate gallery scrapings of green cream-like fungal growth visible throughout the gallery system (Fig. 1A area of detail) were removed aseptically using a sterile teasing needle and added to 500 µl of sterile distilled water, vortexed, serially diluted, and plated on Glucose Yeast Extract Agar (GYE). Dominant morphotypes were quantified and purified by the methods described above. Following serial dilution plating, CFUs were tallied for each morphotype and representatives sub-cultured and retained for DNA studies. Additional subcultures were retained on Potato Dextrose Agar (PDA) slants for long-term storage in both the Kasson and Hulcr labs.

DNA extraction, amplification, and sequencing

Fungal DNA was extracted from dominant fungal morphotypes recovered from both beetles and the gallery substrate, as well as representative fungi used in wood decay assays (Table 1). Fungal isolates were transferred to Difco potato dextrose broth (PDB; BD and Co., Franklin Lakes, NJ, USA) and incubated for 7-10 d as previously described (Short et al. 2015). Following incubation, genomic DNA was extracted using a Wizard kit (Promega, Madison, WI, USA). DNA was suspended in 75 µl Tris-EDTA (TE) buffer preheated to 65°C.

Sequences of the nuclear internal transcribed spacer region ITS1-5.8S-ITS2 (ITS) and nuclear 28S ribosomal DNA (rDNA) regions were amplified with the primer combinations ITS5/ITS4 (White et al. 1990) and LR0R/LR5 (Vilgalys and Hester 1990) using BioLine PCR kits (BioLine USA Inc., Taunton, MA, USA) or ExTaq polymerase kits (Clontech Laboratories, Inc., Mountain View, CA, USA), respectively. Amplified products were visualized with either SYBR gold (Invitrogen, Grand Island, NY, USA) or SYBR1 Green I Stain (Lonza Group Ltd., Basel,

Switzerland), which then were loaded onto a 1-1.5%, wt/vol agarose gel made with 0.5-1% Tris-borate-EDTA buffer (Amresco, Solon, OH, USA). All products were purified with ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA, USA) and submitted to either Eurofins MWG Operon (Huntsville, AL, USA) or the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL, USA) for Sanger sequencing. Chromatograms were assembled and inspected in MEGA 6 (Tamura et al. 2013) or Geneious (Geneious version 9.0.5, Biomatters, Auckland, New Zealand).

Mycangial studies

Ten female *Ambrosiophilus atratus* were assessed for the presence of mycangia. Five *A. atratus* were extracted on August 27, 2015 from an expansive multigenerational gallery inside a recently killed *Ailanthus* stem on the Evansdale campus of West Virginia University in Morgantown, West Virginia. Beetles were preserved in 95% ethanol and stored at 4°C prior to sectioning. A further five *A. atratus* were trap-caught females from the cryopreserved beetle depository in the Hulcr lab. These latter individuals represented individuals from several populations in North Carolina, all collected throughout 2013 from ethanol baited funnel traps near Alligator River (Dare and Tyrrell Counties), preserved in cryotubes with 95% ethanol and stored at -80°C. To confirm the presence of mycangia, we specifically targeted beetle heads. This was based on the exclusivity and dominance of a single fungus from female heads from culture-dependent studies, the phylogenetic position of *Ambrosiophilus* among other Xyleborini with confirmed preoral mycangia located just behind the mandibles in the head (Storer et al. 2015), and a previous confirmation of preoral mycangia from the sister genus, *Ambrosiodmus*

(Li et al. 2015). Heads were prepared as previously described by Li et al. (2015). Aseptically removed heads were oriented with the most anterior end facing downward to permit transverse sectioning and simultaneous bilateral visualization of the paired mycangia. A Microm HM 325 rotary microtome (Walldorf, Germany) was used to cut 10 µm transverse sections. Selected slides confirmed by immediate viewing were then dried at 60°C for 24 h, double-stained with Harris-hematoxylin and eosin-phloxine, and examined and photographed using a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville, New York) equipped with a Nikon Digital Sight DS-Ri1 high-resolution microscope camera and Nikon NIS-Elements BR 3.2 imaging software.

Oogenesis and oviposition by non-dispersed female progeny

The mixed brood generations and overlapping life stages throughout a single expansive gallery system suggested that progeny females were participating in egg laying. To test whether newly hatched females participate in reproduction in their natal gallery before dispersal, we examined females sampled from an active *Ambrosiophilus* colony on dead *Ailanthus altissima* in West Virginia. Twenty-four females were randomly chosen from the gallery and sorted into four stages of development (Fig. 2): orange (freshly eclosed), light brown (slightly more sclerotized), nearly black (sclerotized, but still obviously a new generation), and dark (unable to distinguish the old and new generation). All females of the first three stages were dissected and scored for the presence of follicular epithelium production and/or fully developed eggs as an indicator of active egg development. To test whether egg presence in pre-dispersal females is unique to *Ambrosiophilus* rather than a standard feature of pre-emergent ambrosia beetles, we also analyzed similar pre-dispersal specimens of *Euwallacea validus*. *E. validus* is a beetle of similar

size that often occurs on similar host trees to *A. atratus*, but its ecology is more representative of other ambrosia beetles as only a single generation is produced on a given tree. We collected 48 females (double sampling effort) from their natal galleries, sorted them into the same age classes, and dissected them for follicular epithelium production and/or the presence of eggs.

Decay tests

Fungal isolates used in wood decay and tannic acid tests To assess the wood decomposing properties of *Ambrosiophilus* symbionts, two representative isolates (593-A and 593-G) recovered from fungus colonized galleries and from a surface disinfested head of *A. atratus*, respectively, were chosen for a series of experiments including decay, hardness, and enzymatic tests. To examine the unique niche of these ambrosial fungi, ascomycete symbionts of another ambrosia beetle, *Euwallacea validus*, namely *Fusarium* sp. AF-4 and *Raffaelea subfusca* (Kasson et al. 2013), were also included for comparisons (Table 1). *Euwallacea validus* is also common on *Ailanthus* killed by *Verticillium* wilt (Kasson et al. 2015) and was found co-occurring with *A. atratus* in this study. *Pleurotus ostreatus* and *Schizophyllum commune* both served as references in the wood decay assay as both are well-studied wood decay fungi and their lignin decomposing abilities have recently been resolved (Mansur et al. 2003, Ohm et al. 2010, Riley et al. 2014). Both species have also been reported from *Ailanthus* (Farr and Rossman 2016), which suggests this is a suitable substrate to evaluate their wood decay abilities. In addition to all the fungal strains used in the decay tests, twelve additional strains were included in the tannic acid agar test, which represent well-characterized brown- and white-rot fungi and close allies of known decay fungi but with unknown affinity (Table 1).

Soil block tests were conducted using French square bottles as described by Worrall et al. (1997) except for the following details. Sapwood of *Ailanthus altissima* was used for tester blocks and had the following dimensions, 2.2 cm x 2.1 cm x 0.8 cm, while sapwood of tulip-poplar (*Liriodendron tulipifera* L.) was used for feeder blocks and had the following dimensions, 3.7 cm x 2.9 cm x 0.3 cm. French square bottles (192 mL volume) were filled $\frac{3}{4}$ of the way full (about 150 mL) with 100% vermiculite. Approximately 70-80 ml of water were added to adequately moisten the vermiculite, after which tulip-poplar feeder blocks were placed on top of the moistened substrate, lids placed back on, and autoclaved for 45 min on liquid setting. After cooling for several days, sterile feeder blocks were inoculated with the assigned fungus by placing 5 mm diameter fully-colonized mycelial plugs on each of two transverse faces to permit rapid colonization via the vascular tissues and incubated at 27°C. A total of ten feeder blocks were inoculated per treatment and five feeder blocks inoculated with sterile agar served as negative controls.

After 18-21 d of colonization of tulip-poplar feeder blocks, *Ailanthus* tester blocks that had been oven dried (60°C for 48 h) and weighed were placed aseptically, one per bottle, atop the pre-colonized feeder blocks to permit rapid colonization of tester blocks. Caps were threaded $\frac{3}{4}$ of the way on to permit gas exchange. After 12 weeks, bottles were removed from the dark and colonized tester blocks were removed, rinsed of mycelial growth, oven dried (60°C for 96 h), weighed, and recorded. To calculate how much mass was lost as the fungus converted the carbon in the wood to carbon dioxide, dry weight (12 weeks post-inoculation) was subtracted from pre-inoculation oven dry weight and then divided by the pre-inoculation oven dry weight. Means were tallied by treatment and standard error calculated.

Hardness test Tests to measure changes in resistance of wood to denting, wear, and breakage (and, indirectly, ease of colonization and gallery formation as well as vulnerability of stem to breakage) were conducted using a modified hardness test as outlined in ASTM D143-14, Standard Test Methods for Small Clear Specimens of Timber (ASTM 2014). Following the completion of the wood decay assay, hardness testing was conducted on all blocks across all treatments. This test served to evaluate potential reduction in wood material strength of *Ailanthus* tester blocks subjected to various fungal treatments in relation to each other and a negative control. Due to the size limitations and configuration of the tester blocks (2.2 cm x 2.1 cm x 0.8 cm), one test on wood hardness on the radial face was performed. Additionally, given the thickness of the tester blocks, a modified hardness test was employed that utilized a smaller 7.874 mm hardened steel ball, as compared to the standard ball outlined in ASTM D413-14. To determine hardness, an MTS (Eden Prairie, MN USA) Universal Test Machine (UTM) was used to insert the steel ball into the radial face of the tester blocks at a rate of 6 mm min⁻¹. Hardness was measured as the load (N, newtons) recorded when the steel ball had penetrated 3.302 mm into the tester block. To minimize any influence of the support surface on the load values, a 25.4 mm soft maple (*Acer rubrum* L.) block was used as the base supporting the tester block. Additionally, the distance of penetration (3.302 mm) where the crushing/compaction of the wood would not influence the load value was determined through preliminary testing. To calculate loss in radial surface hardness, hardness of oven dried *Ailanthus* tester blocks (12 weeks post-inoculation) was subtracted from mean hardness of ten

oven dried non-treated tester blocks and then divided by the mean hardness of the non-treated blocks. Means were tallied by treatment and standard error calculated.

Enzyme tests Bavendamm reactions have been used diagnostically since the 1920's for detecting secreted polyphenol oxidases by white rot fungi (Bavendamm 1928, Davidson et al. 1938, Boidin 1951, and Nobles 1965) as a means of assessing their ability to decompose lignin (Ander and Eriksson 1976). A positive Bavendamm reaction results in the production of a "corona" or brown diffusion zone of varying intensity and size relative to fungal mat growth and species tested on 0.5% tannic and/or gallic acid agar but is always negative for brown rot fungi (Davidson et al. 1938) and sometimes positive for soft rot fungi (Worrall et al. 1997).

Tannic acid agar (TAA) was prepared by adding 5 g of tannic acid dissolved in 150 ml of hot autoclaved water to 850 ml of 1.5% malt extract agar (Tuite 1969). In addition to all the fungal strains used in the decay and hardness tests, twelve strains were added and represented well-characterized brown and white rot fungi as well as members of the Polyporales and other fungi with unknown affinity (Table 1). Observations on the intensity of reaction and growth of mycelium were made after 7 d. Plates with limited or no growth and/or no evidence of diffusion after 7 d were retained for an additional 7 d, after which colony growth and discoloration were reassessed. A single TAA plate received one inoculation and was replicated across three individual plates for all treatments with the exception of the *Ambrosiophilus* symbionts, *Laetiporus sulphureus* and *Piptoporus betulinus* (negative controls), *Junghuhnia nitida*, *Irpex lacteus*, and *Trametes versicolor* (references), which were replicated six times per

isolate. Isolates that did not grow after 7 d on TAA were also plated on PDA to confirm the viability of the original parent cultures, 7-10 d after the initial inoculum plugs were cut and placed on TAA. Colonies were evaluated for their total growth on TAA, as well as the appearance and size of the brown diffusion zone relative to the outermost edge of mycelial growth, and the time required for its development (Davidson et al. 1938).

Results

Sampling and isolation of fungi from host beetles and galleries

Overall results of the culture-dependent studies revealed one dominant fungus, molecularly confirmed as *Flavodon ambrosius* (see below) from preoral mycangia (see mycangial studies). *Flavodon ambrosius* was recovered from the heads of 21 of 29 females and 1 of 3 males with a mean count of 736 CFUs (80%) for individual females and 62 CFUs (32%) for individual males (Fig. 3). In contrast to its dominance in heads, this fungus was never recovered from pronotal (males = 3, females = 24) or abdominal (males = 3, females = 24) macerates from these same beetles (data not shown). In females, two different morphotypes of *F. ambrosius* were recovered. The first morphotype (#1), similar to *F. ambrosius* described from *Ambrosiodmus lecontei* (Li et al. 2015, Simmons et al. 2016), exhibited rapid colony growth with white hyphae of two distinct diameters. Mature colonies produced arthrospores originating from both hyphal types as well as a previously undocumented creamy light green mass of hyphae at the center of the colony comprising thin generative hyphae and a high number of oily droplets giving the center a light green appearance, which resembled the fungal growth present throughout the

gallery tunnels (Fig. 1). Crystals with octahedral morphology, possibly calcium oxalate, were noted from all examined cultures grown on GYE amended with streptomycin sulfate and tetracycline. This filamentous morphotype was recovered from only five of 29 female beetles, four of which yielded 10 CFUs and represented 0.29% – 2.5% of the total CFUs recovered from those individuals (data not shown). Total CFUs recovered from the fifth female head were mostly of this filamentous morphotype comprising 700 CFUs or 68% of all CFUs recovered from this beetle. This filamentous morphotype also dominated serial dilution platings of gallery scrapings and excised wood sections. Furthermore, this filamentous morphotype was recovered well beyond the boundaries of the gallery, extending more than 1.5 m vertically in both directions from the edge of the active gallery and was recovered five months after the gallery had been excised. The second morphotype (#2) of *F. ambrosius* has not been previously reported and was characterized by extremely slow growing white globular masses comprising germinating and budding arthrospores (Fig. 4). This morphotype was distinctly more common from females. It was recovered from 21 of 29 beetle heads and represented the only morphotype recovered from males. The average incidence of CFUs of *F. ambrosius* morphotype #2 was 757 colonies or 77% across all female beetles and ranged from 220-1800 colonies or 21-100% of total CFUs recovered from individual female beetles.

A second fungus, molecularly identified as *Yamadazyma mexicana* (see below), was commonly recovered from heads of female and males as well as from larvae and pupae, and represented >96% of all CFUs from the latter two life stages (624 and 1880 CFUs, respectively). This Saccharomycetales yeast also dominated serial dilution platings of macerated male and female beetle pronotum (males = 2/3, females = 13/24) and abdomen (males = 3/3, females =

18/24) sections (data not shown). *Penicillium* sp. was recovered from 100% of larva and pupae, albeit at low levels (<2% CFUs for both groups). *Pseudocosmospora* sp. was another occasional yet notable fungus identified from beetles and gallery scrapings (data not shown).

Molecular identification

GenBank BLASTN results of ITS sequences from 30 isolates identified putatively as the fungal symbiont in this study were identical. Two isolates (593G and 593A) utilized throughout this paper (deposited as Genbank accessions **KU695252** and **KU695251**, respectively) were 99% identical to *Flavodon ambrosius* Hulcr 6855 and 6853 (**KR119073** and **KR119072**) recovered from *Ambrosiodmus lecontei*. One nucleotide located at position 574 had co-dominant Adenine and Guanine peaks that could not be resolved in all examined sequences whereas position 574 within the sequences of *Flavodon ambrosius* Hulcr 6855 and 6853 had Guanine. Otherwise, the sequences were identical, even between the two morphotypes. LSU sequences (deposited as **KU695254** and **KU695253**) were 100% identical to *Flavodon ambrosius* Hulcr 6855 and 6853 (**KR119076** and **KR119075**). Sequences from nine representative yeast isolates sampled across all life stages and common from both female and male adults and dominant from larvae and pupae (Fig. 2) were 98% identical to two *Yamadazyma mexicana* sequences (**AB365477** and **EU343839**). An ITS sequence from a third fungal morphotype recovered from both gallery scrapings and from female heads was 93% identical to a *Pseudocosmospora metajoca* sequence (**KC291745**). For the wood decay and tannic acid tests, the identities of all fungal isolates listed were confirmed using ITS (data not shown). An unknown fungal contaminant recovered from

several of the wood decay bottles was 99% identical to *Paecilomyces variotii* (JX231002)(see decay tests below).

Mycangial studies

Serial transverse sections from the heads of a majority of female *A. atratus* confirmed that the preoral mycangial cavity at the base of the mandibles was packed with fungus in 2 of 5 beetles extracted from a log in West Virginia and in 4 of 5 trap-caught beetles from North Carolina. Despite optimal orientation, the cavity was remarkably empty in some of the log-extracted beetles, which corroborated observations from other ambrosia beetle species suggesting that the mycangium and its contents are not developed in callow adults, and develop fully during the dispersal phase (Li et al., in press). One remaining beetle head from North Carolina could not be visualized on account of sub-optimal orientation during paraffin embedding. Mycangia from both trap caught and log extracted beetles contained tightly packed globose arthroconidia (Fig. 4).

Oogenesis and Egg laying by non-dispersed female progeny

Numerous independent field observations in Florida and West Virginia suggest that *Ambrosiophilus* engages in extensive cooperative tunneling and a communal social structure. Four features set *Ambrosiophilus* colonies apart from most other ambrosia beetles: 1) Multiple (up to sixteen observed) independent *Ambrosiophilus* females initiate their galleries very close to one another, often in clusters, while leaving the rest of the dead tree unoccupied. 2) The

tunnel systems can add up to significant lengths, between 1.6 and 2.2 m in total. 3) Gallery systems in the xylem are frequently interconnected, allowing males and females to enter tunnels of other families' tunnels. 4) Numerous eggs, larvae, and pupae were observed interspersed throughout the gallery systems, even in places to which the parental generation did not have access (tunnels filled with F1 generation).

Dissections of females demonstrated that follicular epithelium development and egg laying commences as early as in the light brown stage of maturation, and continues throughout the process of maturation, even before dispersal (Fig. 2, 5A). Four out of 19 (26%) of non-black females contained fully developed eggs. In contrast, none of the dissected *E. validus* contained any eggs, despite double the sampling effort (Fig. 5A).

Decay tests

Results of the 12-week decay assay revealed that *F. ambrosius* out-performed all other fungi in decaying *Ailanthus* blocks, including the white rot fungus *Pleurotus ostreatus*. Mean percent weight loss was 34% and 28% for *F. ambrosius* isolates 593-G and 593-A, respectively, compared to a 26% weight loss with *P. ostreatus*, 5% loss for *Schizophyllum commune*, 4% for *Raffaelea subfusca*, 3% for *Paecilomyces variotii* (see explanation below) and 2% for *Fusarium* sp. AF-4 (Fig. 6). Wood colonized by *F. ambrosius* appeared discolored and mottled, indicative of decay (Fig. 7). All Basidiomycota tested, with the exception of *Schizophyllum commune*, caused significantly more weight loss than Ascomycota beetle symbionts (Fig. 6). Colonization by *Raffaelea subfusca* resulted in significant staining of the tester blocks (Fig. 7), similar to the staining seen in the galleries of *E. validus* inside *Ailanthus*. Number of usable tester blocks was

reduced by 50% for both *Raffaelea subfusca* and *Fusarium* sp. AF-4 inoculated tester blocks and for 20% for negative controls as a result of contamination by fungi apparently residing, albeit at low levels, inside the oven dried wood. The contaminant was consistent from all contaminated blocks, and was identified as *Paecilomyces variotii* (teleomorph *Byssoschlamys spectabilis*) based on morphological and molecular data. *Byssoschlamys spectabilis* is a fast-growing thermotolerant fungus that is able to grow at low oxygen concentrations and survive heat treatments (Houbraken et al. 2008).

To assess the potential influence of the *P. variotii* contamination on the wood decay results, we tested for antagonistic interactions between *P. variotii* and the target fungal strains. Each *P. variotii*-target strain pair was co-cultured in eight replicates on GYE. Results showed no inhibition towards any of the fungal treatments when challenged with *P. variotii*. Furthermore, within 5 d of inoculation, *F. ambrosius* had overgrown *P. variotii*, with other decay fungi, *Schizophyllum commune* and *Pleurotus ostreatus* showing overgrowth of *P. variotii* by 11 days post-inoculation.

Hardness test Hardness tests performed on the radial faces of the tester blocks revealed significant loss of hardness for *Ailanthus* tester blocks treated with either isolate of *F. ambrosius* (64% and 56% reduction in hardness) or *P. ostreatus* (51% reduction) compared to the negative control (Fig. 6). Maximum load at 3.302 mm penetration required for *F. ambrosius* was 916 N (593-G) and 1,130 N (593-A) compared to 1,259 N for *P. ostreatus* and 2,571 N for the negative control. *Ailanthus* tester blocks treated with *Fusarium* sp. AF-4, *R. subfusca*, and *S. commune* as well as blocks naturally infested with *Paecilomyces variotii* all showed a

significantly lower reduction in radial face hardness compared to *F. ambrosius* and *P. ostreatus*. Compared to the negative control, *Fusarium* sp. AF-4, *R. subfusca*, *S. commune*, and *Paecilomyces variotii* all showed a significantly higher reduction in radial face hardness with mean losses of 27%, 21%, 20%, and 18%, respectively. However, these losses were markedly greater when compared to their corresponding weight losses of 2%, 4%, 5%, and 3% (Fig. 6). Maximum load values at 3.302 mm penetration were 1,864 N, 2,042 N, and 2,060 N, respectively. Wide ranges of hardness across each of these three treatments resulted in large standard errors and, therefore, were not significantly different to the negative control. The hardness test results, however, provided relative loss of material properties related to wood decay (Fig. 6).

Enzyme tests Polyphenol oxidases were detected in 15 of 20 tested fungal isolates including both isolates of our target fungus, *F. ambrosius*, as evidenced by discoloration of TAA after one week. Isolates in which no discoloration was detected in any of the replicates included *Gloeoporus pannocinctus* (decay status unknown), *Laetiporus sulphureus* and *Piptoporus betulinus* (both known brown rot fungi), *Raffaelea subfusca* (an Ascomycotan symbiont of *Euwallacea validus*) and *Paecilomyces variotii*. *Gloeoporus pannocinctus* BL042 and 4 of 6 replicates of *Junghuhnia nitida* BL528 were exceptional in that no growth was observed from either the inoculation source (GYE agar plug) or on TAA after 1 week (Table 1), indicative of a toxic effect. The two remaining replicates of *Junghuhnia nitida* BL528 as well as all three replicates of *Trametopsis cervina* BL492 exhibited growth that was restricted to the inoculation source (GYE agar plug). The viability of all four of these isolates were subsequently confirmed

by plating three replicates of identical sized fungal plugs from the same retained parent cultures on nutrient rich non-selective GYE. Enzymatic activity on TAA varied across some treatments for both *Ceriporiopsis gilvescens* BL174 and *Junghuhnia nitida* BL528, which had 2 of 3 replicates and 1 of 6 replicates with diffusible zones present, respectively. In the absence of growth, the reaction was always negative.

Due to the slow growth in the aforementioned isolates and previous reports of polyphenol oxidase activity occurring in selected fungi between 7-14 d post-inoculation, all cultures with no evidence of diffusion were re-examined at 14 d post-inoculation. Surprisingly, three of three *Fusarium* sp. AF-4 replicates had limited diffusion zones extending beyond the inoculation plug as did the remaining *Ceriporiopsis gilvescens* BL174 replicate that was negative seven days post-inoculation (Table 1). Mean fungal mat diameter varied across treatments with *Phlebiopsis flavidoalba* BL311 and *Trametes versicolor*, and both isolates of *Flavodon ambrosius* exhibiting the fastest mean growth (Table 1). There seemed to be no relationship between growth and mean diffusion as several isolates that were confined to the inoculation plug exhibited substantial diffusion zones.

Discussion

We show that *Ambrosiophilus atratus* carries a symbiotic fungus, *Flavodon ambrosius* that is capable of decomposing lignocellulose and can provide nutrition even in the presence of other wood decay fungi, long after tree death. Such a long-lasting and competitive fungal mutualist provides an extended availability of resources for *Ambrosiophilus*, which can sustain overlapping generations in a single tree and develop a more complex social structure not seen in a majority of ambrosia beetles. Repeated field observations suggest that both

Ambrosiophilus and the closely related *Ambrosiodmus* live in large, multi-family, interconnected galleries containing hundreds to thousands of individuals facilitated by *Flavodon ambrosius* and can be found throughout the invaded regions: Eastern US, from Florida to Michigan, and in Italy (Faccoli et al. 2009). Such social structure exceeds the complexity seen in nearly all other ambrosia beetles with the exception of one eusocial platypodine (Kent and Simpson 1992) and *Xyleborinus saxesenii* under favorable conditions (Biedermann and Taborsky 2011). However, the combination of communal social structure, overlap of generations, and a fungal symbiont that facilitates the development of such communities, has not yet been reported for any other ambrosia beetle.

It is likely that the production of lignocellulolytic enzymes into the wood provides additional benefits, such as softening the substrate, breakdown of cellulose into simpler sugars, and the support for additional mycobiota, including yeasts. Other saproxylic (decay- and/or dead wood-dependent) Coleoptera, including minute tree-fungus beetles (Ciidae), rove beetles (Staphylinidae) and stag beetles (Lucanidae), also readily colonize decomposing wood in advanced stages of decay, capitalizing on the long-term availability of these substrates and associated Polyporales that inhabit and degrade them (Araya 1993, Midtgaard et al. 1998, Jonsell et al. 2001, Johansson et al. 2006). Nonetheless, beetles confined to living inside basidiocarps are not likely facilitating their spread nor enhancing the longevity of these decay fungi, but are instead depleting a finite resource. In contrast, the symbiotic relationship between *A. atratus* and *F. ambrosius* rests on the colonization of freshly dead trees followed by widespread invasion into wood around the galleries through active excavation combined with enzyme production.

The presence of egg-laying progeny in *A. atratus* and their absence in *E. validus* conforms to the hypothesis of a trade-off between dispersal and reproduction. New *A. atratus* females can afford to forfeit dispersal and invest into eggs because of their long-lived mutualist, while immature *E. validus* do not appear to invest any resources into reproduction until after dispersal. Oviposition delayed until after a new gallery and new fungus garden establishment has been reported in other ambrosia beetles besides *E. validus* (two species of *Xylosandrus*; French and Roeper 1972, Weber and McPherson 1983). In most ambrosia beetles, the fungal growth present in newly established galleries provides the necessary nutrition or is a necessary stimulus for egg production.

Several Asian species within the genus *Ambrosiophilus* engage in another interspecific interaction – fungus stealing (myco-cleptism; Hulcr and Cognato, 2010). Instead of making their galleries in uninhabited wood, these parasitic *Ambrosiophilus* species search for galleries established by the much larger ambrosia beetles in the genus *Beaverium* and excavate their galleries immediately next to the existing tunnels. The fungus established by *Beaverium* spp. therefore immediately grows in the gallery of *Ambrosiophilus* spp. Whether or not *Beaverium* also introduces *Flavodon* into the wood should be explored (Cognato et al. 2011).

Flavodon ambrosius exhibits many of the properties of other well-known aggressive white rot fungi in the Polyporales including *Trametes versicolor*, *Phlebia tremullosa*, and *Bjerkandera adusta*. These fungi are characterized by their ability to cause significant weight loss in decay tests (Worrall et al. 1997) and strong polyphenol oxidase reactions on tannic acid agar with diffusion zones extending beyond the margin of mycelial growth (Davidson et al. 1938). Whether these reactions result specifically from laccases, tyrosinase, peroxidase or other

enzymes cannot be resolved solely using Bavendamm reactions. However, Worrall (1997) indicated that many of the white rot fungi with Bavendamm-positive reactions are also laccase and peroxidase positive, and infrequently tyrosinase positive, indicating a suite of laccase and peroxidase enzymes acting synergistically to decompose lignin in many of these fungi. All true white rot fungi including *Trametes versicolor* and *Pleurotus ostreatus*, as well as *Schizophyllum commune*, which is an intermediate between white-rot and brown-rot species, are known to possess dozens of genes related to lignin-decomposing GMC oxidoreductases, which are completely absent in brown rot fungi, and likely present in *Flavodon* (Riley et al. 2014). In addition, true white rot fungi have numerous genes related to the production of Class II peroxidases, copper radical oxidases, and laccases, which are significantly reduced or absent in *S. commune* (Riley et al. 2014). Many of these white rot fungi, including *F. ambrosius*, are also able to quickly grow on TAA, which probably indicates these fungi can quickly detoxify tannic acid and extract nutrients. Such characteristics are ideal for fungal symbionts in that they can quickly colonize the substrate making it unsuitable for slower-growing primary and secondary saprotrophs.

Unlike their white rot counterparts, brown rot fungi cannot decompose polyphenol tannic acid despite their ability to cause significant weight loss in wood decay tests (Davidson et al. 1938, Worrall et al. 1997). Similarly, many Ascomycota, including *R. subfusca*, an Ophiostomatoid symbiont of *E. validus* used in the present study, lack the enzymatic machinery necessary to breakdown polyphenol tannic acid. Indeed, previous studies of the wood decay properties of *Ophiostoma* indicate that this group of fungi lacks most enzymes needed to degrade lignin (Worrall et al. 1997). In contrast, *Fusarium* sp. AF-4, another Ascomycota

symbiont of *E. validus* exhibited polyphenol oxidase activity, albeit only after 14 d post-inoculation. These results conform to previous studies by Norris (1980), which demonstrated that a *Fusarium* symbiont (AF-9, see Kasson et al. 2013) of *Xyleborus ferrugineus* used lignin as a sole carbon source.

To the best of our knowledge, this is the first study to examine the impact of decay fungi on resistance of wood to denting, wear, and breakage, which can be used to better understand the role these fungi likely play in aiding rapid gallery expansion. The hardness test indicated *Flavodon*-treated wood experienced a >50% reduction in hardness. *Ailanthus* tester blocks treated with *Fusarium* sp. AF-4, *Raffaelea subfusca*, and *Schizophyllum commune* all showed a significantly lower reductions in radial face hardness.

In less than a decade, the research on the ambrosia symbiosis has intensified and led to the discoveries of several new associations, including beetles with novel ecological strategies and fungi that are new to science and possess surprising metabolic capabilities. In this work, we demonstrate that an association with an evolutionarily novel mutualist may have cascading effect on the entire system, including the evolution of a more complex social structure. The vast majority of ambrosia beetle species remain unstudied as do their symbionts, and these fungi promise to yield novel mechanisms for turning recalcitrant biomaterials into animal nutrition and facilitating the evolution of complex ecological and social strategies.

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Figures

Fig. 1: Gallery with associated fungus of *Ambrosiophilus atratus* excised from a recently killed *Ailanthus* in Morgantown, West Virginia, USA. A, gallery showing fungus filled (white arrow) with adults, pupae (black arrow) and larvae; B, pure culture of fungus on GYE recovered from gallery scraping; C-E, Microscopic details of fungus showing monomitic hyphae (c), oil droplets (d), and arthroconidia originating from each of the hyphal size classes (e). Scale bars are as follows: A, 1 cm; A detail, 3 mm; B, 25µm; C, 10 µm; D, 25µm; E, 10 µm.

Fig. 2: Classification of *Ambrosiophilus atratus* development: orange (freshly eclosed), light brown (slightly more sclerotized), nearly black (sclerotized, but still obviously a new generation), and dark (unable to distinguish the old and new generation). The first three stages were evaluated for egg-production. Scale bar, 1mm.

Fig. 3: Mean occurrence of fungi from the heads of log extracted female and male *A. atratus* as well as whole body platings of *A. atratus* larvae and pupae recovered from colonized *Ailanthus* in West Virginia.

Fig. 4: *Ambrosiophilus atratus* excised from a recently killed tree and the fungi that dominated their mycangia. A, gallery showing adult male and female *A. atratus*. White arrow points to general location of mycangium in female beetle; B, 100X cross section of *A. atratus* mycangium

with globose arthrospores; C-E, fungal morphotype associated with serial dilution platings from macerated heads of female *A. atratus*. C, typical serial dilution plate after 10 days on GYE; D is area of detail indicated by white box in C; E, Microscopic examination of white globose fungal structures in C-D showing creamy masses of germinating arthrospores of various shapes and sizes. Scale bars are as follows: A, 1mm; B, 25µm; C, 25 mm; D, 0.5 mm; E, 25µm.

Fig. 5. Follicular epithelium production and egg production in unemerged progeny females of *Ambrosiophilus atratus* and *Euwallacea validus*. A) Proportions of females with fully developed eggs inside their ovaries (black) and females without eggs (grey). Only non-black females were sampled to avoid confusion with the parental generation. B) Both ovaries (four ovarioles) from a light brown immature female of *Ambrosiophilus atratus* with egg development (left) and without egg development (right) sampled from their natal galleries. On the left figure, egg production is underway, with follicular epithelium forming on the left, and a fully developed egg on the right.

Fig. 6. Comparisons of mean percent weight loss and hardness loss across fungal treatments 12-weeks post-inoculation. Bars represent standard error. Two types of negative controls were used for these studies. The decay assay, which measured weight loss, used sterile agar inoculated blocks subjected to identical moisture, light, and temperature conditions for 12 weeks, whereas the negative controls for the hardness test included 10 un-inoculated oven dried tester *Ailanthus* blocks cut from the same parent stock as the fungus treated blocks.

Fig. 7. Post-inoculation comparisons of fungus treated *Ailanthus* tester blocks. Fungal treatments are as follows: A-B, *Flavodon ambrosius* (593G and 593A); C, *Fusarium* sp. AF-4 (MB14); D, *Raffaelea subfusca* (WVF3), E, *Pleurotus ostreatus*, and; F, Negative control, and G, *Paecilomyces variotii*.

Fig. 8. Post-inoculation comparisons of diffusion zones resulting from polyphenol oxidase activity on fungus-inoculated tannic acid agar plates. Fungal treatments are as follows: A, *Flavodon ambrosius* (593G); B, *Schizophyllum commune*; C, *Pleurotus ostreatus*; D, *Trametes versicolor*; E, *Phanerochaete raduloides*; F, *Fusarium* sp. AF-4; G, *Raffaelea subfusca*, and; H, *Laetiporus sulphureus*. Photos of F-H were taken at 14 days post-inoculation on account of slow growth.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1. Incidence of diffusion, mean diameter growth, and mean diffusion zone of various white rot fungi on tannic acid agar

Fungal ID, stain # [decay class]	Substrate, State	Incidence of diffusion
<i>Phlebiopsis flavidoalba</i> BL311	Tulip-poplar log, VA	3/3
<i>Trametes versicolor</i> [W]	KLCC*	6/6
<u>Flavodon ambrosius</u> 593G	<i>Ambrosiophilus atratus</i> head, WV	6/6
<u>Flavodon ambrosius</u> 593A	<i>Ambrosiophilus atratus</i> gallery, WV	6/6
<u>Schizophyllum commune</u> [I]	KLCC*	3/3
<i>Irpex lacteus</i> BL523 [W]	<i>Brachycybe lecontii</i> , TN	6/6
<i>Bjerkandera adusta</i> BL305 [W]	Tulip-poplar log, WV	3/3
<u>Pleurotus ostreatus</u> [W]	KLCC*	3/3
<i>Phlebia subserialis</i> BL054	Tulip-poplar log, VA	3/3
<i>Ceriporia</i> sp. BL045	Birch log, VA	3/3
<i>Phanerochaete raduloides</i> BL709	<i>Brachycybe lecontii</i> , SC	3/3
<i>Trametopsis cervina</i> BL492	<i>Brachycybe lecontii</i> , TN	3/3
<i>Ceriporiopsis gilvescens</i> BL174	Hickory log, VA	2/3 (1/3 [#])
<i>Junghuhnia nitida</i> BL528	<i>Brachycybe lecontii</i> , TN	1/6
<u>Fusarium</u> sp. AF-4 (MB14)	<i>Euwallacea validus</i> head	3/3 [#]
<i>Laetiporus sulphureus</i> [B]	Oak stump, WV	0/6
<i>Piptoporus betulinus</i> [B]	Birch snag, WV	0/6
<u>Raffaelea subfusca</u> (WVF3)	<i>Euwallacea validus</i> head	0/3
<i>Gloeoporus pannocinctus</i> BL042	<i>Brachycybe lecontii</i> , VA	0/3
<u>Paecilomyces variotii</u> 8-4	Oven-dried <i>Ailanthus</i> wood	0/3

Bold and underlined isolates denotes their use in wood decay and hardness tests in addition

[] Decay classes based on previous studies include [W] white rot, [B] brown rot, or [I] intermed

* Isolates from the Kasson Lab Culture Collection (KLCC) but whose provenance is unknown.

Observations and measurements taken at 14 days post-inoculation.

^ Normal growth on 3 replicates on Glucose Yeast Extract Agar (GYE).