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Bacterial and Fungal Midgut Community Dynamics and Transfer Between Mother and Brood in the Asian Longhorned Beetle (*Anoplophora glabripennis*), an Invasive Xylophage

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Abstract
Microbial symbionts play pivotal roles in the ecology and physiology of insects feeding in woody plants. Both eukaryotic and bacterial members occur in these systems where they facilitate digestive and nutrient provisioning. The larval gut of the Asian longhorned beetle (*Anoplophora glabripennis*) is associated with a microbial consortium that fulfills these metabolic roles. While members of the community vary in presence and abundance among individuals from different hosts, *A. glabripennis* is consistently associated with a fungus in the *Fusarium solani* species complex (FSSC). We used amplicon sequencing, taxon-specific PCR, culturing, and imaging to determine how bacterial and fungal communities differ between life stages and possible modes of symbiont transfer. The bacterial and fungal communities of adult guts were more diverse than those from larvae and eggs. The communities of larvae and eggs were more similar to those from oviposition sites than from adult female guts. FSSC isolates were not detected in the reproductive tissues of adult females, but were consistently detected on egg surfaces after oviposition and in frass. These results demonstrate that frass can serve as a vehicle of transmission of a subset for the beetle gut microbiota. Vertically transmitted symbionts are often beneficial to their host, warranting subsequent functional studies.

Keywords Bacterial 16S rRNA · Fungal ITS1 · Asian longhorned beetle · *Fusarium solani* · Vertical transmission · Symbiosis

Introduction

Insects have established relationships with a breadth of microbial associates, many of which can provide important functional benefits [1, 2]. Insect-microbial partnerships can vary considerably, both in terms of the functions they serve [1] and the evolutionary relationships they possess [3, 4]. For several groups of insects, the maintenance of symbionts via vertical, maternal transfer is critical for herbivore success. Maternal transfer of microbiota requires physiological and/or behavioral adaptations to facilitate passage of symbionts and become fixed among individuals and in populations [5]. Maternal microbiota transfer occurs via a diversity of mechanisms, including intracellularly, capsules [6, 7], secretions [8], and excretions [9]. In addition to maternal transfer, there can be environmental acquisition of microbes by insects from food or soil [10, 11], which can have benefits to insect performance and success. Maternal and environmental acquisitions can be intersecting processes, particularly for associations with the insect gut and externally propagated symbionts.

Subcortical phloem and wood feeding occur in several insect orders, and associations with both bacterial and fungal consortia are well known [12–15]. Phloem and wood-feeding insects encounter low nutritive and heavily defended dietary substrates. In these systems, microbial partners can fulfill roles in nutrient provisioning [16–19], toxin metabolism [20–22], and defense against antagonistic microbiota [23–27]. Microbes may inhabit insect gut tissues or be externally propagated in the wood during the excavation of feeding galleries

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[28]. While several studies have investigated bacterial communities of subcortical insects [18, 22, 23, 28–32], the mechanism of transfer of these microbes is unclear in most systems. The filamentous fungal associates in these systems have received more attention in xylophagous insects [13, 14, 33, 34]. Adults of some bark and ambrosia beetles and wood wasp species have specialized structures called mycangia that harbor fungal spores that are inoculated into brood feeding sites [35]. Some bark beetle species lack these structures, but adults carry spores on their cuticle, distributing inoculum as it carves the gallery. When spores are present on the cuticle, the fidelity of the insect-microbe association is lower compared to those adapted to mycangia [36–39]. For other subcortical-feeding insects like cerambycids that only spend the larval and pupal stages inside the host tree, the community dynamics and transfer of both fungal and bacterial members remain unclear.

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae), is an invasive xylophage that attacks and kills healthy trees [40, 41]. *A. glabripennis* has a broad host range that includes species in the genera *Acer*, *Betula*, *Salix*, *Aesculus*, *Ulmus*, and *Populus* [40–42]. Larvae feed solitarily beneath the bark along the phloem–cambium interface in the early instars before boring into and feeding on the heartwood. Adults feed primarily on bark and phloem of 2–3-year-old twigs and leaf petioles.

The *A. glabripennis* larval gut harbors a diversity of bacteria and fungi that have putative roles in nutrient provisioning, lignocellulose metabolism, and allelochemical metabolism [43–47]. Bacterial community composition varies between populations and food sources, but appears to perform similar functional roles [44, 45, 48]. Fungal members of the consortia can also vary, but are usually comprised of yeasts and a filamentous fungus in the *Fusarium solani* species complex (FSSC) [49, 50]. Adult *A. glabripennis* do not contain mycangia-like structures as seen in some bark beetles and wood wasps, nor do they have mycetomes that are present in some cerambycids [51]. In this system, it is unclear how the communities of bacterial and fungal symbionts are acquired, although some bacteria appear to be transferred vertically [52].

In contrast to *A. glabripennis* eggs and larvae that spend the full duration of their development in subcortical tissues, adults feed entirely outside of the tree and do not re-enter the tree to reproduce. *A. glabripennis* females undergo a laborious, multistep egg-laying behavior (Fig. 1) during which she deposits a single egg beneath the bark of the tree. During oviposition behavior, which lasts on average 10–12 min [53], the female chews through the bark of the tree to form an opening to the vascular tissue, and then uses her ovipositor to excavate an egg-laying site (hereafter referred to as an oviposition pit). They deposit secretions and a single egg under the bark and then proceeds to deposit frass into the oviposition pit, spreading both materials over the surface of the exposed wood. This behavior is similar to that of other wood-colonizing cerambycids in the Lamiinae subfamily [54]. We hypothesize that the final step of this behavior transfers and facilitates the colonization of microbiota onto the egg surface, which is subsequently transferred to the larval gut when the newly eclosed larva consumes the chorion of the egg and grazes on the colonized wood in the oviposition pit before boring into the tree.

In this study, we utilized high-throughput 16S and ITS sequencing, taxon-specific PCR, and selective media culturing to assess community and population dynamics of fungal and bacterial communities between different *A. glabripennis* life stages to investigate the stability and potential vertical transfer of the microbial consortia from mother to offspring.

Materials and Methods

Description of Insect Source We used *A. glabripennis* maintained in a colony in the Department of Entomology at the Pennsylvania State University (PSU) for our study. This colony has been maintained for approximately 10 years and has had regular introductions of wild beetles from North America to maintain genetic diversity. The colony is reared in isolation using standard procedures that include two diets suitable for different life stages [43, 55] at 22 °C, ~60% humidity, and an 14:10-h light dark photoperiod. *A. glabripennis* adults are fed on red maple (*Acer rubrum*) twigs for 10–14 days to reach sexual maturity and are provided with red maple bolts for oviposition. Insects from this colony undergo similar mating and feeding behaviors as those observed in the field (Supplemental Video 2). *A. glabripennis* larvae feed in maple bolts up to the second instar before being transferred to artificial diet until pupation [55]. For our study, analyses were conducted using adults that fed exclusively on red maple, and larvae that were harvested from red maple and immediately dissected.

Sampling Scheme for Microbial Community Profiling In order to profile fungal and bacterial communities across the life stages of *A. glabripennis*, we collected eggs, adult female frass, oviposition secretions, and larval and adult male and female midguts ($n = 6$ per sample type). We provided male and female mating pairs with red maple twigs and a bolt of red maple for feeding and oviposition, respectively, and monitored oviposition over a 2-week interval. From the oviposition pits, we collected secretions deposited by females during oviposition, which also contained frass (Fig. 1). Eggs were collected from oviposition pits 2 weeks following oviposition using flame-sterilized forceps. Larvae were collected from oviposition bolts 3 weeks after the first appearance of frass (~6 weeks post-oviposition) and were anesthetized on ice, surface sterilized, and dissected to remove the midgut as described previously [56]. Larvae were early second instars,

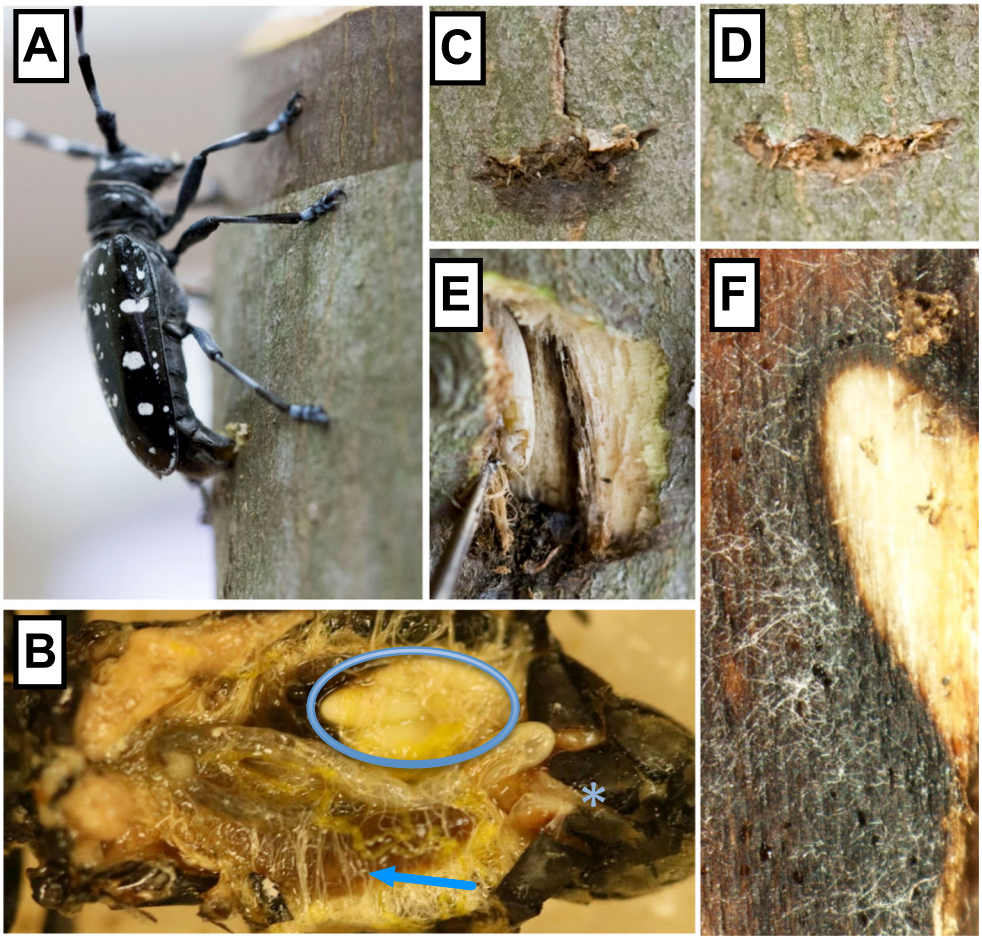

Fig. 1 Oviposition behavior, female gut, and oviposition pits of *A. glabripennis*. After chewing a slit into the bark, the female inserts ovipositor under bark (**a**). Ovipositor is normally tucked in abdomen between gut tissues (**b**). In **b**, the arrow points to anterior midgut, circle indicates ovaries, and the asterisk is at the base of the ovipositor. After laying a single egg, she defecates into oviposition pit (**c**). Oviposition pits are easily distinguished from chew marks not containing eggs (**d**) due to crack in bark and deposition of frass. Deposited frass is not immediately applied to egg tissue, but rather there is ~1-mm distance from where frass is packed and from where the egg is laid (**e**). Hyphae begin to accumulate in the pit ~1 week after oviposition, surrounding and covering the egg and growing on wood (**f**). Video of *A. glabripennis* oviposition is available in online supplement

~1 cm long at the time of dissection, and had a full gut. We separated the male and female pairs into separate jars to collect frass from females only. After the collection of frass, oviposition secretions, and eggs, each adult was dissected to remove the midgut. We selected only midgut tissues since no differences between midgut and hindgut were observed previously [56]. Prior to dissection of gut tissues, adult males and females were anesthetized by holding them at − 20 °C for 3–4 min and then surface sterilizing them. Legs and elytra were removed from the adults and midguts were removed by dorsal dissection. Newly eclosed females ($n = 3$) were also dissected using the same method. After all samples were collected, the tissues were then flash frozen in liquid N_2 and stored at − 80 °C until subsequent processing.

Extraction of DNA DNA was extracted from midgut tissue sections using a ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). Frozen tissues were immediately submerged in the kit's lysis buffer and homogenized with bead beating at 600 rpm for 3 min in a Genogrinder 2000 (SPEX Sampleprep, Metuchen, NJ, USA). All other steps followed manufacturer protocols, except six volumes of fecal DNA binding buffer were added instead of three volumes.

Generation and Sequencing of Fungal and Bacterial Amplicons We generated bacterial and fungal amplicon pools for sequencing using the following Earth Microbiome Project protocols. Primers used for bacterial V4 16S-rRNA amplification were 515F and 806R [57]. Primers used for fungal ITS1 amplification were ITS1f [58] and ITS2 [59]. Amplicons were generated in 20 μL volumes using 5 PRIME Hotstart mastermix (Gaithersburg, MD, USA) containing 1.0 μM of forward and reverse primers and 12.5 ng of template DNA. Reaction conditions for 16S amplification were 94 °C for 3 min, 23 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, followed by a final extension of 72 °C for 10 min. ITS1 amplification followed similar procedures, with an annealing temperature of 55 °C and 35 PCR cycles. Dual indices and Illumina sequencing adapters were added to amplicon pools with eight additional cycles of PCR. Amplicon pools were pooled and sequenced on Illumina MiSeq using 2 × 250-bp paired end reads. Generation of amplicon pools and sequencing of products was completed by AKESOGen Inc. (Norcross, GA, USA).

Processing of Sequencing Data Bacterial and fungal sequencing data were processed and analyzed using mothur v.1.37 [60] and PIPITS v.1.3.3 [61], respectively. 16S-rRNA reads

were analyzed in mothur using recommended workflows [62]. One modification was made to the recommended curation workflow in that the mothur command "pcr.seqs" with pdiffs = 2 was implemented after the command "make.contigs" to remove primer sequences from the reads (Supplemental Methods). Bacterial OTUs were picked at 97% dissimilarity with the opticlust method and used for subsequent analyses. PIPITS was used following recommended pipeline procedures with no modifications. Fungal phylotypes generated by PIPITS were used for subsequent analyses. We used mothur to conduct OTU subsampling (3000 reads) and calculation of diversity metrics.

qPCR of *F. solani* in Guts

We used primers described previously [56] to conduct qPCR to enumerate copies of the FSSC symbiont in the adult female gut, frass, oviposition secretions, and larval guts. Primers targeting *A. glabripennis*'s FSSC translation elongation factor 1 alpha (TEF) gene were used to test for the presence of FSSC. A primer set targeting *A. glabripennis* β-tubulin 2 served as an endogenous control. qPCR was conducted using the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Hercules, CA, USA) under conditions described previously [56]. Each 10 μL reaction was conducted in triplicate and contained 5 μL of iTaq Universal SYBR Green Supermix buffer (Bio-Rad), 200 nM of forward and reverse primer, and 5 ng of template DNA. Copies of FSSC TEF were calculated from a standard curve containing linearized plasmid DNA.

Background Presence of *F. solani* and Culturable Microbes in Maple To determine if FSSC (e.g., endophytic) was present in the wood used to rear beetles prior to oviposition by females, we attempted to detect FSSC in the oviposition logs. We selected five mated pairs of *A. glabripennis* and collected phloem from oviposition pits that contained eggs 1 week after oviposition. Phloem samples immediately adjacent to the pit were also collected. The oviposition pit and adjacent wood samples were freeze dried and DNA was extracted using protocols described above. We amplified TEF using the same primers described above for qPCR, and we also amplified a maple ribulose-biphosphate carboxylase gene to confirm that any negative amplification was not due to inhibitors [63]. End point PCR was conducted using GoTaq master mix (Promega, Madison, WI, USA) in 25 μL volumes containing 750 nM of forward and reverse primers and 40 ng of DNA.

In order to estimate background microbial titers, we randomly selected three females and used dilution plating to compare differences between frass and phloem. We assessed differences in culturable microbes between phloem and female frass by collecting frass immediately upon excretion and carefully carving away phloem from maple bolts. Samples were homogenized in phosphate-buffered saline and serial dilutions were plated on yeast-tryptone medium.

Investigation of Transovarial Presence of FSSC and Bacteria In order to assess the involvement of reproductive tissues in vertical transmission of fungal symbionts, we attempted to culture FSSC from *A. glabripennis* eggs and ovaries. For these experiments, we used Nash-Snyder media, which is selective for FSSC [64]. We collected eggs and ovaries from mated females following dissection, homogenized them, and placed them on growth media. To determine if microbes are transmitted on the egg surface, we removed eggs from oviposition pits and either surface sterilized them by rinsing in 10% Coverage Plus followed by a sterile water rinse, or rinsing them with sterile water only (positive control). Eggs were rolled onto media with sterile forceps and transferred to filter paper on top of 1.5% water agar for hatching. The resultant neonate larvae were then homogenized and plated onto Nash-Snyder media. We also tested for the presence of culturable bacteria associated with the ovaries, eggs at 2 weeks post-oviposition, surface sterilized eggs, and larvae by plating homogenized samples on yeast-tryptone media.

SEM Imaging of Eggs To determine if fungal mycelia were visible on egg surfaces following oviposition, *A. glabripennis* eggs were removed from red maple bolts 2 weeks post-oviposition. Eggs were immediately submerged in 4% paraformaldehyde fixative and incubated at room temperature for 24 h. Each egg was serially dehydrated in ethanol and then critical point dried using a Leica EM CPD300 Critical Point Dryer (Leica Camera, Wetzlar, Germany). Tissues were imaged on a Zeiss SIGMA-VP FESEM (Carl Zeiss AG, Oberkochen, Germany). Sample preparation and imaging were conducted at the PSU Microscopy and Cytometry Facility.

Statistical Analyses To determine if there were differences in microbial communities across life stages and tissue types, we used insect life stage as an explanatory variable and the adult pairing as a random effect in the model. Univariate analysis of variance (ANOVA) statistics were conducted using R v.3.3.1 [65]. Mixed effect models were produced with the package lme4 [66]. Contrasts were made using lsmeans with a Tukey's HSD adjustment. Bray-Curtis dissimilarities were generated using subsampled data incorporating relative abundance of OTUs, and Jaccard dissimilarities were generated using subsampled data incorporating presence-absence. Bray-Curtis dissimilarities are used to evaluate community structure, and Jaccard dissimilarities for community composition. Non-metric multidimensional scaling (nMDS) was conducted in PRIMER-E (v.7.0) using these dissimilarities. A permutation-based multivariate analysis of variance (PERMANOVA) was also conducted in PRIMER-E using 1000 iterations of the model [67]. In order to assess differences in OTUs between

A. glabripennis adults, eggs, and larvae, heat maps were generated using $\log_2$-transformed relative abundances of the most abundant OTUs. The 30 most abundant were selected for the bacterial OTUs, and the 20 most abundant were selected for the fungi. Student's *t* tests were used to compare qPCR results between newly eclosed and mature adult beetles.

Results

Community Composition and Structure Changes Across *A. glabripennis* Life Stages

Bacterial and fungal OTU richness and diversity varied across *A. glabripennis* life stages and sample types (Table 1). Adult female guts, as well as frass and secretions deposited into the oviposition pits, had comparable numbers of bacterial OTUs. Adult male guts had fewer bacterial OTUs than females, while eggs and larvae had the fewest OTUs overall. Female frass and guts of males, females, and larvae had comparable Shannon and inverse Simpson metrics. Oviposition pits and eggs had significantly lower diversity in comparison to adult guts and frass and larval guts. Fungal diversity and richness followed similar trends among tissue types as the bacterial communities (Table 1).

Ordination analyses and PERMANOVA of bacterial and fungal communities indicated large differences between sample types (Fig. 2; Supplemental Table 2). Neither bacterial nor fungal communities (Fig. 2a, c) differed between female guts and frass. The bacterial communities in oviposition secretions also did not differ from those in frass. Larval bacterial community structure was most similar to the adult female guts (Fig. 2a), while the larval fungal community structure was most similar to egg surfaces (Fig. 2c). Bacterial compositions of the eggs and larvae were clearly differentiated from those associated with adults, frass, and oviposition secretions (Fig. 2b). Likewise, the fungal compositions of eggs and larvae were clearly differentiated from the adult beetles and frass (Fig. 2d), while fungal community compositions of oviposition pit secretions were of intermediate similarity to other sample types (Fig. 2d). Adult males and females had similar bacterial and fungal compositions and fungal community structures, but differed significantly in their bacterial community structure (Supplemental Table 3).

At a relatively coarse taxonomic resolution, differences between bacterial and fungal communities across *A. glabripennis* life stages were apparent (Fig. 3). Across all samples, the highest proportion of the bacterial community at the class level was Gammaproteobacteria. Differences in bacterial class designation between life stages were primarily attributed to minor shifts in the relative abundances of Gammaproteobacteria, Alphaproteobacteria, and Betaproteobacteria. Fungal taxonomic differences among sample types were more pronounced compared to the differences observed in bacterial taxonomic composition. In adult female guts, there were more unclassified fungal OTUs and Ascomycetes at the class level compared to other samples, while Saccharomycetes, Eurotiomycetes, and Dothideomycetes comprised between 7.4 and 8.5% of the relative abundance and Sordariomycetes made up less than 1% of the relative abundance. Oviposition pit depositions exhibited a considerable shift in fungal taxonomic composition compared to adult female guts and were dominated by Saccharomycetes (~78%). The relative abundances of Sordariomycetes in both eggs and larval guts were significantly higher (49–55%) compared to the female gut, frass, and oviposition secretions.

Heat maps of fungal and bacterial OTUs between females, eggs, and larvae (Fig. 4) led to the identification of several bacterial and fungal OTUs shared among multiple life stages that serve as strong candidates for vertical transmission. For example, bacterial OTUs 1–6 and 10–14 were detected in the majority of the adults, eggs, and larvae surveyed. Other OTUs were exclusively associated with adult beetles (OTU047, OTU063, and OTU087) while two OTUs were associated exclusively with eggs and larvae. In contrast to the bacterial OTUs, fewer fungal candidates were shared among multiple life stages. Nine fungal OTUs were present in all three stages,

Table 1 Observed OTUs and diversity indices of bacterial and fungal sequences in different *A. glabripennis* life stages

	Male gut	Female gut	Frass	Oviposition pit	Egg	Larval gut	*F* value ($df = 5, 25$)	*p* value
Bacteria								
Number of OTUs	124.2 (23.5) ab	206.8 (32.9) bc	257.0 (42.3) c	202.8 (27.8) c	61.2 (4.0) a	100.2 (12.8) ab	7.70	< 0.01
Shannon	2.5 (0.6) a	3.2 (0.5) a	3.1 (0.5) a	1.9 (0.2) ab	1.6 (0.5) b	3.6 (0.1) a	3.78	0.01
1/Simpson	10.1 (5.0) a	9.3 (2.10) a	13.1 (5.7) a	2.4 (0.3) b	4.7 (2.6) b	21.2 (1.9) a	11.10	0.01
Fungi								
Number of OTUs	40.8 (5.4) ab	45.0 (6.0) ab	59.2 (5.9) a	43.8 (6.5) bc	11.8 (1.7) c	24.5 (2.4) bc	11.15	< 0.01
Shannon	1.9 (0.1) a	1.8 (0.2) a	2.1 (0.2) a	1.6 (0.2) a	0.7 (0.2) b	1.9 (0.3) a	6.69	< 0.01
1/Simpson	4.0 (0.3)	3.7 (0.7)	4.7 (1.1)	3.4 (0.5)	1.7 (0.4)	5.4 (1.4)	2.32	0.07

Numbers represent mean (standard error) and different letters represent statistically significant differences ($p < 0.05$)

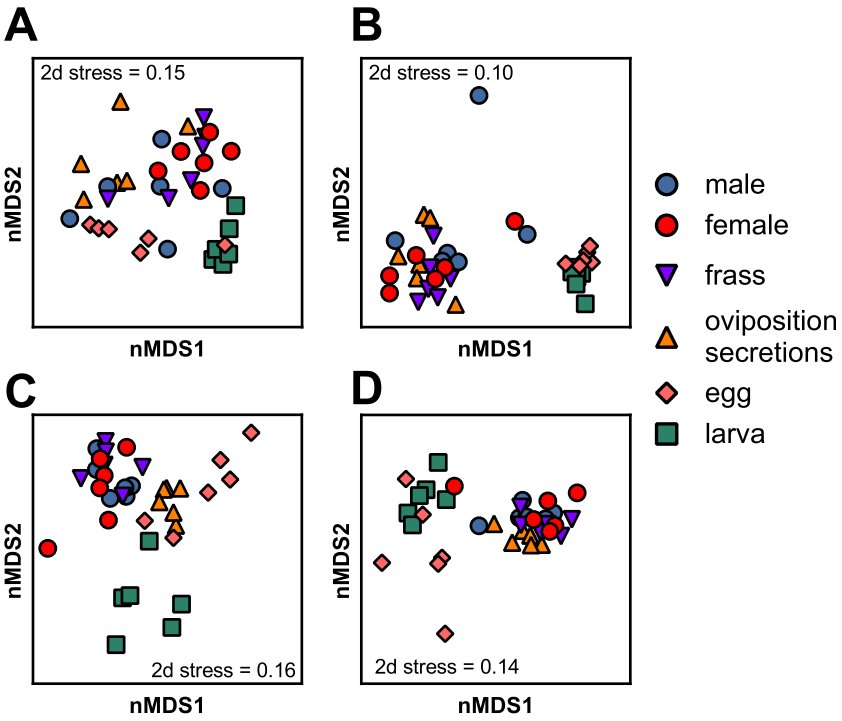

Fig. 2 Nonmetric multidimensional scaling plots of bacterial (**a**, **b**) and fungal (**c**, **d**) communities. **a**, **c** were generated using Bray-Curtis (relative abundance) dissimilarities to assess community structures; **b**, **d** were generated using Jaccard (presence-absence) dissimilarities to assess community composition. Stress values indicate the ease by which the data were collapsed into two dimensions. PERMANOVA and pair-wise comparisons between sample types are illustrated in Supplemental Tables 1 and 2

but their abundances varied both across and within life stages. We did not detect high abundances of OTUs classified as FSSC in the amplicon dataset due to low abundance, so we utilized other taxon-specific methods to evaluate its transfer.

FSSC Isolate Transfer to Offspring

We used a primer set selective for the FSSC to detect this symbiont in tree phloem at the time of egg oviposition and

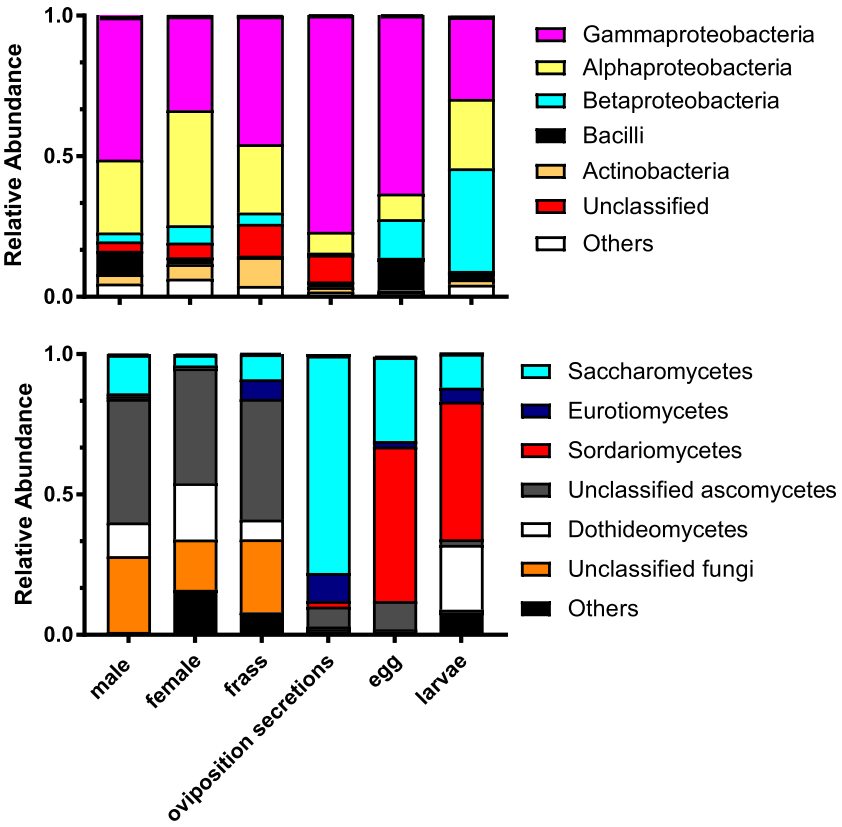

Fig. 3 Relative abundance of taxonomic community at the class level. Top chart includes bacterial members and bottom chart contains fungal members. "Others" are taxa that comprise < 1.0% of the relative abundance

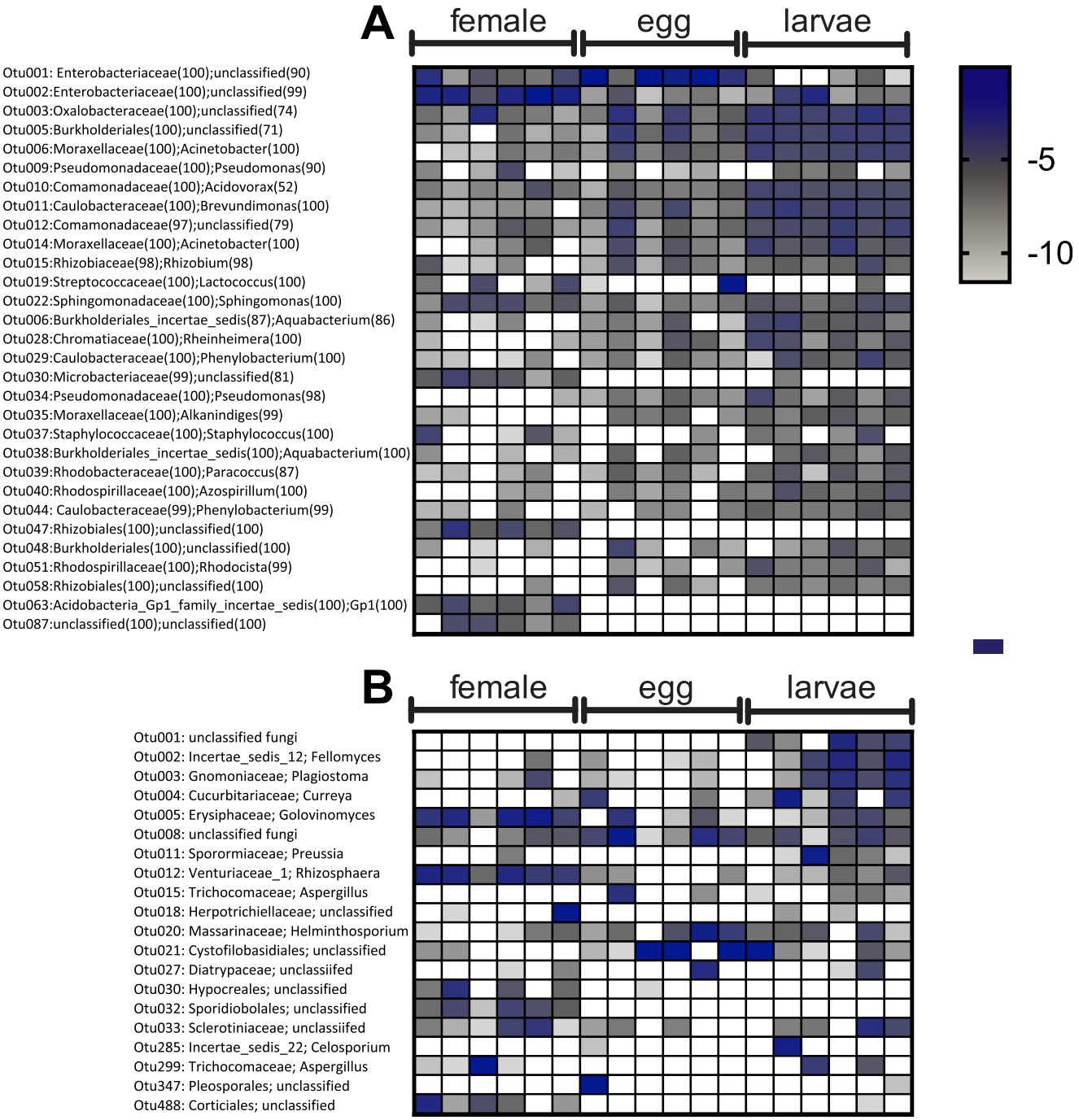

Fig. 4 Heat map of abundant bacterial (**a**) and fungal (**b**) OTUs. OTUs included are the 30 and 20 most abundant. Heat map was generated with $\log_2$-transformed relative abundances. Darker blue cells are indicative of greater abundance. White cells indicate no taxa present

in host insect tissues. FSSC isolates were not detected in red maple phloem tissues that did not contain eggs (0/5) (Fig. 4a). The lack of TEF amplification was not due to poor amplification of the DNA because all tissue samples were positive for the maple housekeeper gene. FSSC isolates were detected in 80% of oviposition pits at 1 week post-oviposition. FSSC isolates were also consistently detected in female adult guts, frass, oviposition pits, and larval guts (Supplemental Fig. 1). In female adult guts, FSSC was present in actively feeding females, as well as those that had recently eclosed from pupae (Fig. 5b). Actively feeding females had significantly higher titers of FSSC-specific copies than those that had not yet undergone plant maturation feeding (Fig. 5).

When eggs were removed from the ovaries prior to oviposition, no FSSC or other microbiota were detected. Plating of dissected female tissues other than from the gut did not yield any microbial growth, suggesting that the symbionts are not delivered via accessory glands or from the oviducts. FSSC was

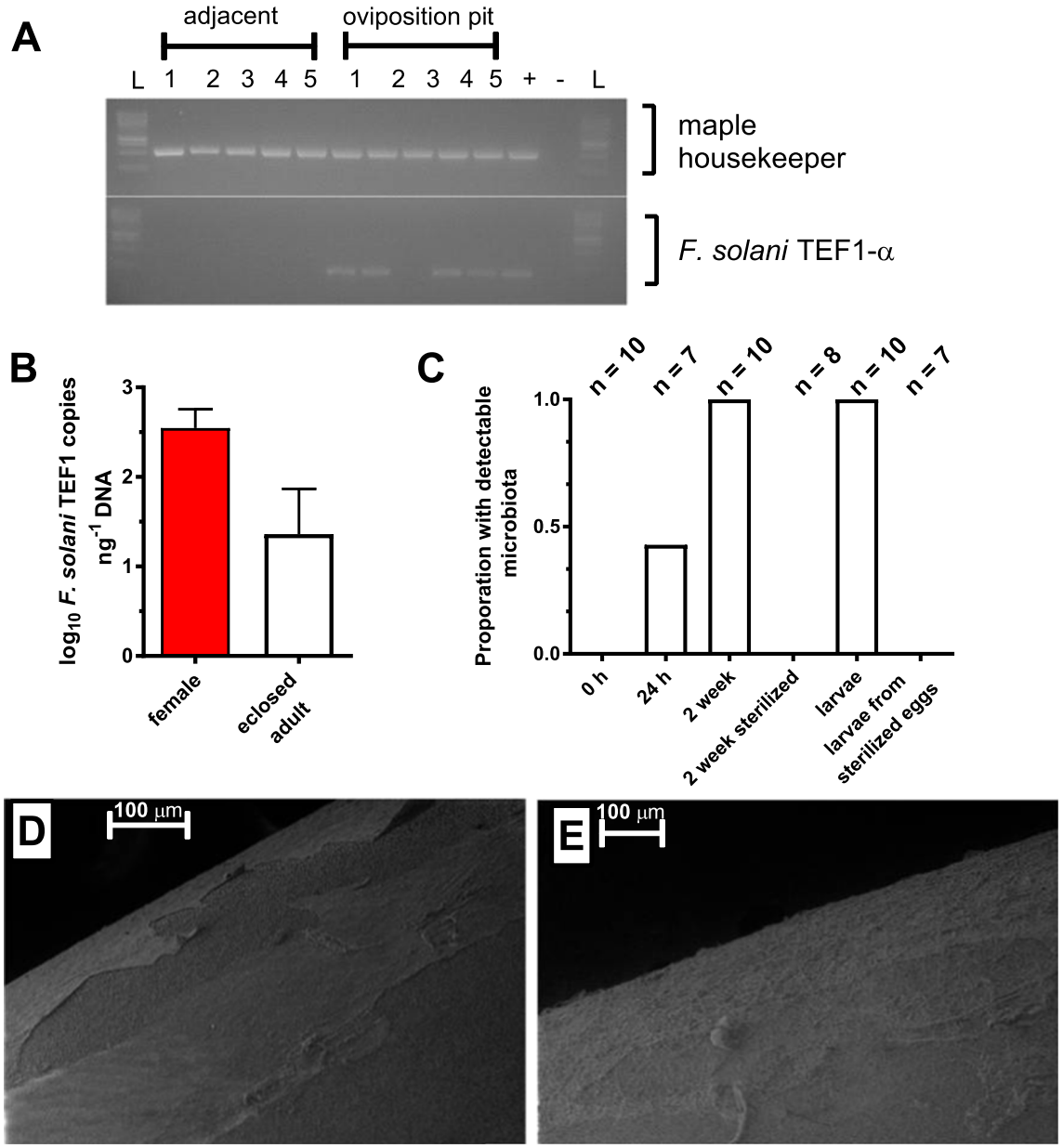

Fig. 5 Dynamics and colonization of eggs and larvae by microbiota. FSSC isolates assessed by endpoint PCR were detected in oviposition pits, but not in immediately adjacent phloem tissue (**a**). FSSC-specific TEF1 was detected in newly eclosed adults, but copy numbers were greater in guts of actively feeding females (**b**). Culturable bacteria and fungi were not detected in female reproductive tissues or immediately after an egg was deposited into wood; FSSC was consistently found in oviposition sites and egg surfaces 2 weeks post-oviposition (**c**). Surface sterilizing eggs removed all culturable microbes, and larvae that hatched from sterilized eggs did not contain microbiota. SEM imaging of eggs immediately after oviposition (**d**) and 2 weeks post-oviposition (**e**)

only detected on the external surface of eggs and was consistently detected 1 week post-oviposition or later (Fig. 5c). SEM imaging indicated a progression of fungal colonization of the egg, with no detectable spores or hyphae when eggs were initially deposited, but near-complete coverage of the egg surface within 2 weeks (Fig. 5d, e). Sterilizing the egg surface produced larvae that did not have any culturable microbiota in their gut compared to water-rinsed controls, suggesting that consumption of the chorion and oviposition pit wood surfaces upon larval emergence facilitates microbial colonization of the gut. The titers of culturable microbes in female frass were more than two orders of magnitude greater compared to that of phloem in the oviposition pit (Supplemental Fig. 2).

Discussion

Our results suggest that there is a transfer of both bacterial and fungal symbionts from mother to offspring. The mechanism of this transfer occurs via frass deposited in oviposition sites,

and the accumulation of symbionts by external growth of these taxa onto the egg chorion and wood surface of the oviposition pit. Our data suggest that there is also environmental acquisition of microbiota by the adults. Adults had greater gut microbial titers compared to that of larvae, suggesting that between life stages, there are both restrictions in colonization as well as expansions. Not all members of the maternal community successfully colonized the guts of the brood, suggesting that some adult microbiota are transients or are not able to colonize the larval gut, colonize the wood, may be outcompeted, or a combination. Adult acquisition of microbes from food and low transfer fidelity of some of the microbial members helps explain some of the variation observed between *A. glabripennis* populations reported in different studies [43–45, 56, 68].

A subset of both bacterial and fungal taxa were detected on egg surfaces and in adult and larval guts, suggesting transfer of a portion of the microbes associated with adults. The female gut, frass, and oviposition secretions had greater bacterial and fungal diversity in comparison to eggs or the larval gut, with eggs being the least diverse. Eggs are deposited under the bark during oviposition and larvae feed exclusively inside the tree, while the oviposition pits are exposed continuously to the external environment. Differences in the microbial community between the oviposition secretions and what colonizes the brood are likely due to differential colonization of microbes in the oviposition pit. Acquisition of environmental microbes may be more variable in egg and larval communities, because, in general, microbial titers in phloem tend to be lower than on bark or foliage (Supplemental Fig. 2) [69].

Differences between female and larval gut microbial communities may be the result of several, non-mutually exclusive processes. First, some microbiota may be more effective colonizers of the oviposition pit and establish in the gut tissues. Conversely, there may be microbiota that are unable to colonize the woody substrate, perhaps due to inhibition from plant defenses. In addition, there is likely competition between bacterial and fungal taxa during egg and larval colonization by some microbes. For example, *Fusarium* spp. commonly produce toxic secondary metabolites that can have anti-bacterial and anti-fungal activities [70–72]. However, some bacterial and fungal OTUs were detected in larvae but not adults (Fig. 4), suggesting that some taxa may be acquired during larval feeding, or lost during pupation.

A. glabripennis-microbe interactions appear to be intermediate in comparison with the general trends observed between two insect-herbivore feeding ecologies. Folivorous insects tend to have variable bacterial gut communities, with ingested microbiota providing a greater diversity of associates than are acquired from vertical transmission [73–76]. This variability contrasts with insects that feed exclusively under bark, which appear to have greater similarities among individuals in their gut bacterial communities [31, 77], indicative of less dynamic communities relative to folivores. Similarly, this variability differs from social wood-feeders whose communities are more consistent due to trophallaxis behaviors that facilitate microbial stability among conspecifics [78, 79]. Folivores and subcortical feeders have different strategies to contend with their substrates, which likely reflect these differences in these microbial associations. Folivores can modulate consumption to accommodate more or less favorable food sources and move between resources to achieve adequate nutrition. In contrast, subcortical feeders cannot move between hosts and generally encounter greater nutritional constraints. *A. glabripennis* encounters different dietary substrates, and therefore nutritional constraints, between life stages which seem to explain these trends.

In addition to ubiquitous associations with bacteria, wood-feeding insects often have associations with eukaryotic partners. Many of the fungal taxa detected in the adult tissues in our study were not present in larvae, suggesting that many of them are transient, or at least do not occupy integral roles in the larval gut. Some of the fungal OTUs detected in adult gut and frass, like *Aspergillus* and *Trichoderma*, are detrimental and/or antagonistic to insects and microbes in bark beetle systems [23, 27], though their roles in *A. glabripennis* are undefined. In contrast, the filamentous fungus FSSC is consistently associated with *A. glabripennis* [45, 50], and yeast-like microbes commonly populate the gut [45]. The frass component of the oviposition behavior, the presence of FSSC in the oviposition pits but not nearby tissues, and the consistent detection of FSSC across studies in space and time suggest that this fungus plays an important role in the life history of *A. glabripennis*. Members of the FSSC are widely distributed, and FSSC mutualisms have been reported in several species of wood-feeding insects [80, 81]. These results are not due to artifacts from our laboratory colony, as FSSC isolates and/or sequences have been detected in multiple independent studies from the field [44, 45, 50, 56]. We did not observe FSSC in any nearby phloem tissue, but did detect it in 80% of oviposition pits. We suspect that there was not 100% detection because of low FSSC biomass in some oviposition pits. To our knowledge, the transmission behavior that ensures transfer of FSSC from mother to offspring occurs throughout the geographic range of the insect. However, the specific members of the FSSC present in a population appear to depend on geographic location (Campbell et al. unpublished data), suggesting that some population-specific assemblages may be present.

Laboratory-reared insects often differ in their gut community composition to those collected in the field [82, 83]. In past studies of *A. glabripennis* feeding on artificial diets in the laboratory, beetles had very simple microbial communities, dominated by Bacilli. Our study shows a level of bacterial diversity similar to some of the studies previously published (Supplemental Table 1), although the makeup can differ

substantially [44, 52]. These differences and the variability observed between laboratory and field populations may arise from maternal feeding and acquisition of environmental bacteria. Supporting this notion is that the females exhibited the greatest diversity of bacteria in all sample types, which could then be passed to offspring. This seems like a relatively stochastic process, but it also appears that different microbes can fulfill similar functions in this system [44, 45]. Notably, there is high variability of bacterial symbionts between different field populations, and this may be partially related to the acquisition and transfer process. The host plant appears to be a second driver of variation of the gut microbial community, which is may be related to which microbes are present, the chemistry of the plant host, and/or their interactions. It is unclear if the fungi follow similar patterns as the bacteria, but warrants further exploration.

A limitation we face in the interpretation of our study involves the maintenance of microbiota throughout *A. glabripennis* development, particularly during adult eclosion. While our study supports a mechanism of microbial transfer from adult to brood, it is unclear how the microbiota is recovered during adult eclosion and what interactions occur between resident bacteria from newly eclosed adults and the newly acquired environmental microbiota from food. We were able to detect FSSC in newly eclosed adult guts, suggesting some maintenance of associates despite feeding on artificial diets. Assessing the microbial dynamics through larval development and pupation in wood are important steps to identify community members that contribute important functional roles.

Transfer to and maintenance of microbiota in subsequent generations can provide insights into the evolutionary ecology of a species, and ultimately highlight the physiological importance of these associates. External growth and accumulation of fungal associates in the oviposition pits may provide a source of nitrogen, sterols, and pre-digested wood for the newly eclosed larva. Assessment of the nutritional and chemical changes in the oviposition pit would provide insights into these potential resources. In contrast to bark and ambrosia beetles (Coleoptera: Curculionidae), fungi are not propagated in the larval galleries of *A. glabripennis* and the only occurrence of visible fungal growth is in the oviposition pit. While putative roles of both bacterial and fungal members in the *A. glabripennis* larval gut have been proposed based on metagenomic and transcriptomic analyses of the gut community [45, 47, 48, 84], quantification of fitness effects by these taxa have not been addressed. Studies manipulating members of the gut microbiota can help identify contributions of different microbial members to nutrient provisioning, metabolic flexibility of bacterial associates, and roles of FSSC in lignocellulose degradation and nutrient acquisition [84, 85]. Together, this information will expand our understanding of how wood feeders utilize microbial partners and contend with challenging substrates.

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