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Fungi That Live Within Animals: Application of Cell Cytometry to Examine Fungal Colonization of Ambrosia Beetle (*Xyleborus* sp.) Mycangia

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Abstract: Ambrosia beetles bore into trees, excavating galleries where they farm fungi as their sole source of nutrition. These mutualistic fungi typically do not cause significant damage to host trees, however, since their invasion into the U.S. around the turn of the 21st century, the beetle *Xyleborus glabratus* has vectored its mutualist fungal partner, *Harringtonia lauricola*, which has turned out to be a devastating plant pathogen resulting in the deaths of over 500 million trees. Here, we show differences in mycangial colonization of the indigenous *X. affinis* ambrosia beetle by *H. lauricola*, and the native fungal species, *H. aguacate* and *Raffaelea arxii*. While *X. affinis* was a good host for *H. lauricola*, the related ambrosia beetle, *X. ferrugineus*, was only marginally colonized by *H. lauricola*. *X. affinis* beetles neither fed on, nor were their mycangia colonized by the distantly related fungus, *Magnaporthe oryzae*. Mycangial colonization was affected by the nutritional state of the fungus. A novel method for direct quantification of mycangial contents based on cell cytometry was developed and validated. The method was used to confirm mycangial colonization and to demonstrate alternating fungal partner switching, which showed significant variation and dynamic turnover. The two *X. affinis* pre-oral mycangial pouches were visualized using fluorescent and light microscopic imaging, which revealed that newly emerged pupae that were not fed a partner fungus displayed uncolonized mycangia, whereas beetles fed *H. lauricola* contained largely single-celled fungal cells within 6 h post-feeding with little to no hyphae. Mixed populations of fungal cells could be seen in the mycangia of beetles as a consequence of alternating colonization. DAPI nuclear counter-staining revealed insect cells surrounding the mycangia. These data highlight both variation and specificity in ambrosia beetle-fungal pairings and provide a facile method for direct quantification of mycangial contents.

Keywords: fungal mutualism; ambrosia beetles; *Xyleborus*; mycangia; cell cytometry; *Harringtonia* and *Raffaelea* sp.

1. INTRODUCTION

Fungal mutualisms with animals have existed for over 300 million years, with significant ecological and evolutionary consequences. As exemplified by diverse lineages of beetles (ambrosia beetles), which have independently "domesticated" equally diverse lineages of obligate fungal partners as their major, if not sole, sources of nutrition [1–3]. Thus, the term "ambrosia beetle", refers to a polyphyletic group of tree- and wood-boring weevils (*Curculionidae: Platypodinae* and *Scolytinae*) which have convergently evolved fungal "farming" with similarly diverse lineages of fungi (e.g., select members within the *Hypocreaceae, Ophiostomataceae, Polyporales, and Ceratocystidaceae*). Ambrosia beetles excavate galleries in host tree sapwood, where they cultivate their fungal partners [4]. In some instances, the beetle-fungus partnership is apparently specific whereas for others, the beetle can associate with a variety of closely related fungal partner species, sometimes having more than one partner at the same time [5–9]. Xyleborini ambrosia beetles associate with a consortium that

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can include facultative bacteria and yeasts, but typically have at least one major obligate filamentous fungal partner.

Most ambrosia beetle-fungal pairings do not result in the death of host trees, however, there are several instances of ambrosia fungi, vectored by their beetle partner, acting as phytopathogens, particularly those that are invasive [2,10]. Around the turn of the 21st century, an invasive beetle to the southeastern United States, *Xyleborus glabratus*, harboring its fungal symbiont, since named *Harringtonia lauricola*, has led to an epidemic disease of lauraceous plants termed laurel wilt that has killed hundreds of millions of redbay, swamp bay, sassafras trees, and now threatens the avocado industry in the United States [11–13]. *H. lauricola* has been shown to be the causative agent of laurel wilt and its potential spread westwards to California and south to central and South America is of significant concern. *H. lauricola* has now been isolated from environmental samples of indigenous *Xyleborus* beetles and the ability of these beetles to maintain *H. lauricola* as their major symbiont confirmed in the laboratory [11,14,15]. The mechanism of plant pathogenicity remains obscure, but ambrosia beetles inoculate the wood substrate of gallery walls with fungal spores during excavation, growing nutritionally-enriched cells for beetle consumption [16,17]. Fungal cells are thought to proliferate in the plant xylem, causing damage and potentially a series of hypersensitive responses which are ultimately lethal to the plant.

The beetle inoculum is maintained in specialized fungal transport structures termed mycangia, which sustain the mutualism between microbe and host into new environments and across beetle generations [18,19]. However, due to the independent instances of fungal domestication by different groups of ambrosia beetles, there is a wide diversity of mycangial shapes, sizes, locations on the insect body, as well as distribution between the sexes [20,21]. Complicating matters, in some instances, clearly defined mycangia have not been found [22]. *Xyleborus* (female) ambrosia beetles have twin pre-oral mycangia located within the head, just beneath the mandibles [15]. Structural studies of preoral paired mycangia from several other genera of beetles including *Euwallacea*, *Ambrosiophilus*, *Ambrosiodmus*, and *Premnobius* have been performed [6,20,23–25]. Micro-CT scanning across the life stages of *Euwallacea validus*, revealed the absence of mycangia in larvae and early-stage pupae, while these structures could be detected in late-stage pupae and adult females [20]. *Xyleborus* beetles can harbor fungal species now separated into the *Raffaelea*, *Harringtonia*, and *Dryadomyces* genera (all previously characterized as *Raffaelea*). These different species can co-occur within the same beetle/beetle colony, indicating flexibility with regards to the fungal partner association [8,26,27].

More recently, the pre-oral mycangia of *Xyleborus* beetles were probed using fungal strains transformed to express reporter (eGFP and RFP)-marker genes [15]. These data showed that colonization follows a characteristic time course and was stable to starvation. Transmission electron microscopy showed a dimorphic shift in the growth of the fungus in the mycangia, although the characteristics of these cells remain obscure. Here we show that although *X. affinis* is a good host for *H. lauricola*, the related beetle species, *X. ferrugineus* is apparently colonized to a much lower extent by this fungus. In addition, differences in the colonization pattern of *X. affinis* mycangia was seen between, *H. lauricola*, *H. aguacate* and *Raffaelea arxii*. To better examine mycangial contents, cell image cytometry was used for direct quantification of mycangial contents. Both CFU counting and cell cytometry was used to examine beetle responses to a series of switching experiments of its fungal partner. Microscopic imaging was used to examine morphological features of the mycangia with surrounding insect cells visualized. These data expand our understanding and ability to probe the physiology and cellular mechanisms by which the symbiotic association within the mycangia functions.

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2. EXPERIMENTAL PROCEDURES

2.1. Insect Rearing and Fungal Strains and Culture Conditions

Xyleborus affinis and *Xyleborus ferrugineus* beetles were collected by light trapping in Gainesville Florida during spring and summer seasons (2021-2023). Following sunset, a white sheet was stretched between two posts and bright white light was shone on it to attract insects. Ethanol was also used as an attractant by splashing it directly onto the sheet, or by hanging a 50 mL tube in front of the sheet containing cotton balls soaked in 100% ethanol. Ambrosia beetles attracted to the sheet were collected into vials and identified in the laboratory using a dissecting microscope. Colonies of *X. affinis* and *X. ferrugineus* were maintained under laboratory conditions and aposymbiotic beetles were reared for colonization experiments as previously described [15]. Briefly, sawdust agar medium was made by mixing 60 g wood flour, 15 g coarse sweetgum sawdust, 20 g agar, 10 g sucrose, 5 g corn starch, 5 g casein, 5 g yeast extract, 1 g Wesson salt mixture, and 2.5 mL wheat germ oil into 500 mL of water. Following autoclaving and cooling, 350 mg streptomycin and 10 mg tetracycline were suspended in 5 mL 95% ethanol and added to the mixture. Roughly 15-20 mL of this mixture was added to 50 mL tubes and allowed to dry for at least seven days prior to initiating colonies. Three days before initiating colonies, a square of agar containing actively growing *H. lauricola* was cut out of a petri dish and placed into media tubes to inoculate them. The surface of the sawdust media in culture tubes was also scratched with a sterile probe at this time to create a rough surface that was easier for beetles to begin burrowing through. To initiate colonies, 3-15 female beetles and 1-3 male beetles were added to tubes and the tubes were maintained with caps loosely tightened for 25-30 days in the dark at 23-25°C. Following this incubation period, sawdust medium was removed from colony tubes and carefully dissected to remove adult beetles, pupae, and larvae. Adult beetles were used to initiate new colony tubes while pupae and larvae were separated, surface sterilized with 70% ethanol and three washes with sterile distilled water and maintained on sterile moistened filtered paper until adult beetles emerged to generate aposymbiotic beetles for colonization assays.

Fungal strains of *Harringtonia lauricola* (RL4), and *H. aguacate* (PL1004), *Raffaelea arxii* (CBS273.70) were maintained as glycerol stocks at -80°C and routinely grown and maintained on potato dextrose agar/broth (PDA/PDB), Sabouraud dextrose agar (SDA), and/or Czapek-Dox agar (CZA) as indicated. *Magnaporthe oryzae* (KV1) was kindly provided by Dr. Jessie Fernandez for use in colonization experiments and was maintained in her lab at the University of Florida. Plates were incubated in the dark at 25°C for 7-21 d. For colonization assays, 96-well plates containing 100 µL PDA in each well were inoculated using sterile toothpicks by scraping fungal spores from mycelia on an actively growing plate and stabbing it into each well. Plates were then grown as above for 5-7 days prior to introducing beetles into wells. For colonization assays testing growth from different media types, 100 µL of each medium was pipetted into wells of a 96-well plate. *H. lauricola* spores were collected into water and the concentration was adjusted to $1x10^8$ spores/mL and 1 µL of this solution was spotted onto wells containing each media type. These cultures were then grown for 7 d as above prior to introducing beetles into the wells.

2.2. Experimental Mycangia Colonization, Determination of Colony Forming Units (CFUs), and Cell Cytometry Assays

Aposymbiotic female beetles were experimentally colonized with desired strains and species of fungi by placing them into individual wells of 96-well plates containing the desired medium and previously inoculated as above. Beetles were allowed to feed on fungal cultures for the desired amount of time, and plates were stored in the dark at 25°C. Plates were periodically examined to ensure that beetles were not stuck on their backs or to the walls of wells and were able to freely feed on the fungal cultures. Following this feeding period, beetles were removed from wells and, using two sterile syringe needles, their head (containing mycangia) was removed from the body and placed into a sterile 1.5 mL tube. Individual heads in tubes were then surface sterilized by washing with 70% ethanol for two minutes followed by three water washes for one minute each. After this last wash, heads were resuspended in 200 µL of water and a sterile glass bead was added to the tube. Beetle

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heads were then macerated in a bead beater (MP Fast Prep 24, MP Biomedicals, Solon, OH) at 4m/s for one minute. Following bead beating, the solution containing the macerated beetle head was diluted 5-fold into a final volume of 200µL of water and 50µL of this solution was plated in triplicate onto 60mm petri dishes containing PDA amended with 200 µg/mL cycloheximide and 100 µg/mL streptomycin. For experiments involving culturing of *Magnaporthe oryzae*, cycloheximide was not included in the media formulation as the fungus was susceptible to this selection agent. CFU plates were incubated in the dark at 25°C until countable colonies appeared (5-7 days) at which point colonies were manually counted for each plate and the average of the three triplicates was used as the CFU count for that beetle.

For cell cytometry analysis, heads of colonized beetles were removed, and surface sterilized as above before being fixed briefly in a 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS). PFA was then removed, and heads were washed three times in water, suspended in 200µL of water and macerated by bead beating. This undiluted solution was then pipetted into individual wells of a 96-well plate. Plates were left on the benchtop for 20-30 minutes to allow fungal cells to settle to the bottom of wells. Plates were then loaded into a Keyence BZ-X800 fluorescence microscope (Keyence Corporation of America, Itasca, IL) with image cytometry hardware for analysis. The microscope was programmed to autofocus using the brightfield channel every capture with 25% light intensity and 20% aperture with a 1/2500 second exposure time before switching to the RFP and GFP channels with 10% laser intensity and 1 second exposure time. Images of quadrants covering the entire area of the wells were captured to collect images of all cells present in the well. The compilation of images was then processed using the Keyence BZX-800 Analyzer software (Keyence Corporation of America, Itasca, IL) to yield a quantity of fungal cells per well representative of the quantity of fungal cells contained in the mycangia of each beetle.

2.3. Microscopy

To obtain microscope images of fungal cells within *X. affinis* mycangia, aposymbiotic beetles were colonized using GFP-expressing *H. lauricola* as above, embedded in Optimal Cutting Temperature (OCT) mounting medium (Sakura Finetek USA, Torrance, CA), and frozen in a bath of isopentane cooled in liquid nitrogen. Frozen blocks were then sectioned using a Leica 3050S cryostat (Leica, Wetzlar, Germany) to a thickness of 5-20µm. Sections were then collected directly onto microscope slides, followed by fixation in 4% PFA for 20 minutes and three washes in sterile distilled water for three minutes each to remove PFA and OCT. Slides were then dried and mounted in Vectashield hardset mounting medium containing DAPI and phalloidin (Vector laboratories, Plain City, OH) for fluorescent staining of nuclei and actin filaments, respectively. Mounted sections were then visualized using a Keyence BZX-800 fluorescence microscope at 40x, 60x, and 100x objective magnification using brightfield, TRITC, GFP, and DAPI fluorescent channels. For fluorescent images, Z stacks were collected over a range encompassing the signal and assembled into a full focus image in the Keyence BZX-800 Analyzer software while for brightfield photos, single images were taken and overlayed with full-focus fluorescent images.

2.4. Data Analysis

All violin and box and whisker plots were generated in R (R core team, 2024, https://www.R-project.org/) using the GGplot2 package [28]. Datapoints plotted on these graphs consisted of the averages of three technical replicates for CFU experiments and the total cell counts per beetle from image cytometry experiments. All statistical analyses were performed in R using: dplyr (Wickham et al, 2023, dplyr: A Grammar of Data Manipulation, R package version 1.1.4, https://CRAN.R-project.org/package=dplyr), car (Fox and Weisberg, 2019, An R Companion to Applied Regression 3rd edition. Sage, Thousand Oaks CA., https://socialsciences.mcmaster.ca/jfox/Books/Companion/, and/or FSA (Ogle et al, 2025, FSA: Simple Fisheries Stock Assessment Methods, R package version 0.9.6, https://CRAN.R-project.org/package=FSA). To test the normality of distribution within sample groups, the Shapiro-Wilk test was used. To determine the homogeneity of variances across groups, Levene's test was used. For the non-parametric comparison of two groups, the Wilcoxon rank-sum

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test was used. For non-parametric comparison of multiple groups, the Kruskal-Wallis test was used with Post Hoc pairwise comparisons made using the Dunn test with Bonferroni correction. For pairwise comparisons of normally distributed samples, t-tests were used to compare between groups. For multiple comparisons of normally distributed data ANOVA tests were used with the Tukey HSD post hoc test.

3. RESULTS

3.1. Mycangial Colonization Is Dependent Upon Beetle and Fungal Partner Species

Newly emerged aposymbiotic *X. affinis* adults were fed different ambrosia beetle fungal partners including *Harringtonia lauricola*, *H. aguacate*, and *Raffaelea arxii*, or the non-ambrosia beetle fungus, *Magnaporthe oryzae*, a well-known plant fungal pathogen, over a time course up to 7 d (Figures 1 and 2). These data indicated robust colonization by *R. arxii* and *H. lauricola* over the entire time, although some differences in the temporal dynamics between the two fungal partners were noted. As previously described [15], (the invasive, non-native to the US) *H. lauricola* showed rapid colonization of indigenous (to the US) *X. affinis* mycangia within 1 h of feeding, with a slight increase within the 12-24 h time period, followed by a gradual decrease to a lower steady state within the 96-168 h (4-7 d) time period examined, with a wide sample variation seen. The native ambrosia beetle species, *R. arxii* showed a slightly more gradual colonization curve (1-24 h, peaking at 24 h), which was then maintained (from 24-120 h), only gradually decreasing at the later time points (144-168 h, 6-7 d). In contrast, (the native) *H. aguacate* showed very poor colonization of *X. affinis* mycangia, with little to no colonization until 24 h, at which time point, a notable wide variation in fungal cell counts recovered was seen, after which levels dropped back to very low colonization (72 h and beyond). To validate specificity of mycangial colonization, a *gfp*-expressing strain of the non-ambrosia beetle fungus but plant pathogen, *Magnaporthe orzyae*, was fed to newly emerged aposymbiotic *X. affinis* adults (Fig. 2A-C). Whereas colonization by *H. lauricola* and accompanying fungal CFU recovery was apparent for the positive control, no fungal counts were recovered after *M. orzyae* feeding, although bacterial colonies were recovered for the latter and not the former. Sections from beetle heads as well as gut were examined by fluorescence microscopy with RFP (corresponding to *H. lauricola*) but not GFP (*M. orzyae*) evident in both mycangial pouches and the gut, which for the latter showed no signal aside from autofluorescence of insect structures (Fig. 2D-G). As indicated, we have previously shown [15], and confirmed here, that *H. lauricola* can effectively colonize the mycangia of (the native) *X. affinis* beetles. To determine whether this phenomenon could be extended more broadly to other (native) *Xyleborus* species, we examined *H. lauricola* colonization of *X. ferrugineus* (Fig. 3). These data showed little to no mycangial colonization of *X. ferrugineus* over the 7 d time course examined.

3.2. Mycangial Colonization Is Affected by the Nutritional State of the Partner Fungus and Long-Term Switching of Partner Fungi Can Occur

To examine effects of the nutritional state of the fungal partner on efficiency of mycangial colonization, *H. lauricola* was grown on four different media: PDA (dextrose, medium nutrition), CZA (minimal media + sucrose, low nutrition), SDA (rich media), and SDA + yeast extract (enhanced rich media) before newly emerged aposymbiotic beetles (*X. affinis*) were allowed to feed for 12 and 24 h (Fig. 4). Mycangial colonization of fungal cells grown on PDA or CZA showed robust colonization at both 12 and 24 h (albeit with significant variation), but with mean levels significantly ($P < 0.001$) higher than *H. lauricola* grown on SDA or SDA+Y which remained low.

We have previously shown that mycangial occupancy is dynamic and shows turnover under conditions of continuous feeding when the host fungal partner is switched during the initial 3 d of colonization [15]. However, these experiments did not examine turnover once the mycangia had been fully colonized, *i.e.* at time points > 3 d, nor at time points in which a stable mycangial occupancy had occurred (> 24 h), nor after a series of sequential switching. As before, to track fungal cell switching in the mycangia, we used two *H. lauricola* reporter strains, namely, *H. lauricola*^{GFP} and *H. lauricola*^{RFP}. To examine the dynamic nature of the mycangia over long term occupancy, newly emerged *X. affinis*

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beetles were first exposed to/allowed to feed on *H. lauricola*^{GFP} for 24 h, after which the beetles were transferred to wells containing *H. lauricola*^{RFP} for 24, then back to the *H. lauricola*^{GFP} for 24 h (3 d), with the sequential switching continued for 4, 5, and 6 d (Fig. 4). Beetles fed the GFP-expressing strain (for 24 h), then the RFP-expressing strain (24 h), then back to the GFP-expressing strain (24 h), showed high level of GFP-cells (1867+/-370), with low levels of the RFP (220+/-77). Beetles fed GFP-RFP-GFP-RFP, retained a moderate level of the GFP cells (894+/-126), with RFP overtaking (1971+/-294). Beetles fed GFP-RFP-GFP-RFP-GFP, showed a dramatic decrease in RFP (110+/-48), with high levels of GFP cells (1873+/-257). Beetles fed GFP-RFP-GFP-RFP-GFP-RFP, again retained moderate to high levels of GFP cells (1616+/-278), with most beetles showing only low RFP cell levels (1045+/-538), although significant variation was seen.

3.3. Application of Cell Cytometry to Quantify Mycangial Content and Imaging of the Mycangia

To develop a more medium-throughput assay that did not rely on plating of mycangial extract and waiting for fungal colonies to be apparent (3-4 d) and then counting (CFU approach), we applied cell cytometry to directly visualize and count cells in the mycangia. Using the *H. lauricola*^{RFP} and *H. lauricola*^{GFP} reporter strains, we first confirmed that fluorescent fungal cells could be visualized and counted in multi-well plates as detailed in the Methods section. These experiments indicated concentration dependent fungal cell cytometry counting that showed >95% correspondence to direct cell counting using a hemocytometer and by CFU plating (data not shown). As the cell cytometry coupled imaging of samples with cell counting, both the qualitative images and quantitative cell counts are obtained (Fig. 4). To validate the method, a time course of *H. lauricola* occupancy of newly emerged *X. affinis* beetles was performed (Fig. 4B). These data were in good agreement with that collected via CFU counting.

Similar to experiments described above, the cell cytometry assay was used to examine the dynamic nature of the mycangia turnover. Newly emerged *X. affinis* beetles were first exposed to/allowed to feed on *H. lauricola*^{GFP} for 12 h and then fed on RFP for 12 (Fig. 6A, leftmost data). To extend the "switching" assay, aposymbiotic beetles were first fed GFP for 24 h, then RFP, with the sequential switching continued for 4, 5, and 6 d. Beetles fed the GFP-expressing strain for 12 h then the RFP-expressing strain for 12 h showed almost complete switching to the RFP strain (1014+/-217 RFP, 34+/-6 GFP). In contrast, beetles fed GFP (for 24 h), then RFP (24 h) showed low to moderate levels of the GFP cells (289+/-60), with high levels of RFP cells (2966+/-586). Beetles fed GFP-RFP-GFP, showed moderately higher levels of GFP cells (986+/-266) as compared to RFP (157+/-44). Beetles fed GFP-RFP-GFP-RFP, retained only low levels of the GFP cells (107+/-15), with RFP overtaking (2655+/-482). Beetles fed GFP-RFP-GFP-RFP-GFP, showed a dramatic decrease in RFP (157+/-26), with high levels of GFP cells (1269+/-342). Beetles fed GFP-RFP-GFP-RFP-GFP-RFP, retained low levels of GFP cells (439+/-53), with high RFP cell levels (2687+/-435). Fluorescent cell cytometry imaging of the microtiter plates containing the mycangial extracts allowed for direct visualization of beetles fed in the switching assays, with both GFP- and RFP-expressing cells apparent (Fig. 6 B).

To better define the mycangial organ, sections of uncolonized aposymbiotic beetles and those fed *H. lauricola*^{GFP} for 12 h were examined using via brightfield and fluorescent microscopy with samples counterstained with the nuclear dye DAPI (Fig. 7A-D). Empty mycangial organs were visible in uncolonized beetles, with green autofluorescence of surrounding mandibular structures. Brightfield and (GFP) fluorescent images of colonized mycangia revealed fungal cells within the two mycangial organs. Insect cells as seen by DAPI staining could be seen around the contours of the mycangia as well as throughout the head. Fungal cells within the mycangia could be seen within 6 h of feeding (Fig. 7E), and mixed populations of *H. lauricola*^{RFP} and *H. lauricola*^{GFP} could be seen for cells fed *H. lauricola*^{GFP} for 12 h and then switched to *H. lauricola*^{RFP} for 12 h (Fig. 7F).

4. DISCUSSION

The nature and diversity of mutualistic associations between fungi and animals remains largely understudied. The term "ambrosia" beetle does not refer to a monophyletic group but represents a lifestyle of (mostly) obligate relationships between divergent lineages of beetles, with equally

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divergent fungal partners [3,10,29]. Similarly, "mycangia" represent the different "organs", that can vary greatly in structure, location on/within the insect body, and mechanisms of development and selection, evolved by the beetles to house and transport their respective fungal partners [18,30,31]. Characterization of the contents of mycangia has typically relied on isolation of fungal colonies on plates, and the application of metagenomic techniques for characterization, identification, monitoring, and/or phylogenetic reconstruction of the fungal partners of specific beetle species [6,32–34]. More recently, a systematic characterization of the dynamics of mycangial colonization demonstrating host switching from the invasive (to the US) beetle species *X. glabratus* which carried with it the (invasive) laurel wilt fungal pathogen, *H. lauricola* to *X. affinis* beetles has been reported [15]. These data also demonstrated that *H. lauricola* colonization of *X. affinis* is stable, maintained even during starvation, and shows rapid turnover within the early period of mycangial colonization (up to 3 d). These, and other data reported for *H. lauricola* isolated from environmental samples and laboratory reared *Xyleborus* beetles (*X. affinis*, *X. bispinatus*, *X. volvulus*, and *X. glabratus*) have all relied on plating of mycangial contents and CFU calculations [14,35–37]. However, CFU counting could involve systematic biases since: (i) fungal cells may grow as hyphae (or some other multicellular form) and therefore CFU counts would underestimate actual fungal biomass, and (ii) CFU counts would only capture viable cells, with the potential of the existence of a pool of non-viable cells in the mycangia. In addition, CFU counting can be time consuming (requires waiting for the colonies to grow), and factors such as dilutions and replicates can lead to errors. To overcome these issues, we applied a simple cell cytometry approach to examining mycangial contents. In this case, after tissue disruption, the entire (or aliquots) of the mycangia can be added to microtiter plates, and the total number of cells counted by cytometry without the need for plating. The method is applicable to medium throughput in that samples can be put into (96-well) microtiter plates and imaged. By using cells expressing a fluorescent reporter (GFP or RFP), the method becomes even more powerful in that identification and discrimination of the fungal cells from insect or other tissues/cells is rendered facile. In addition, using strains bearing different reporter tags, direct visualization and counting of such mixed communities becomes simple. Results from the cell cytometry approach were in good agreement with CFU counting, although some differences were noted. In general, the cell cytometry method yielded a larger range of counts at the higher end, *i.e.*, data points with >10,000 cells counted as compared to the CFU method. Since the cell cytometry method is a direct quantification, this suggests that CFU counting may underestimate contents at the higher end of cell numbers. This is likely due to dilution and colony counting (and subsequent back calculations).

Using both CFU counting and the cell cytometry methods, and our two reporter strains (*H. lauricola*^{RFP} and *H. lauricola*^{GFP}) we sought to address how long-term occupancy (24 h), followed by switching to another strain (for 24), and then continuing this "switching" over a 7-d time course affects mycangial contents. These data confirmed that even such long-term colonization and switching resulted in dynamic turnover. In each case, the general trend was that mycangial contents followed introduction onto "fresh" fungal cells, albeit some of the fungal cells from the previous feeding period always remained. Both CFU and cell cytometry data were in good agreement, except for the last switching time point (7 d) in which the CFU data showed significant retention of the previous fed (in this case *H. lauricola*^{GFP} strain after switching to the *H. lauricola*^{RFP} strain). In contrast the cell cytometry showed very low levels of the *H. lauricola*^{GFP} which was mainly replaced by *H. lauricola*^{RFP} cells. The most likely explanation may be a combination of higher error in the CFU coupled to the large variation seen in the data in general. With respect to variation in the data, we have shown all our data points (as jitter in violin plots) to better illustrate this issue. Based upon these and our previous data [15], we now increasingly consider this to be an intrinsic aspect of mycangial colonization. Reasons for this variation can include: (i) an aspect of the dynamic turnover, *i.e.*, as a result of cells entering and leaving the mycangia, (ii) difference in the nutritional state of the beetle which may affect feeding and/or mycangial occupancy, and/or (iii) differences in the nutritional state of the fungus.

With respect to the nutritional state of the fungus, we examined mycangial colonization as a function of growth of the fungus on different media. Our standard media (PDA) can be considered a

"medium level" nutritional substrate for fungal growth and showed robust colonization of the mycangia. Growth on CZA (minimal media containing sucrose as the sole source of carbon) showed similar results. Intriguingly, growth on rich media (SDA and SDA + yeast extract) resulted in poor mycangial colonization. To the best of our knowledge this is the first report indicating a link between the (nutritional) status of the fungus and colonization of the mycangia. It is unclear why fungal cells grown on "rich" media would result in poor mycangial colonization, but two main factors could account for these results. In rich media, there would be significantly more hyphae and mycelium which, may be poor candidates for sequestering into the mycangia. Second, the tree gallery (where the fungus is grown) is likely a nutritionally poor substrate more similar to PDA/CZA and hence may favor the production of cells more amenable for mycangial colonization. Our data also show significant difference in colonization by different *Xyleborus* partner fungi. For *X. affinis*, both the (invasive) *H. lauricola* and the (indigenous) *R. arxii* were good mycangial colonizers. Intriguingly, the (indigenous) *H. aguacate* was apparently a poor partner for *X. affinis*. The reasons for this are unclear and an additional wider survey of fungal partners can help shed light on this issue. To confirm specificity of the relationship, we tested the non-ambrosia fungus, but plant pathogen, *M. oryzae*, and demonstrate that this fungus is neither eaten nor able to colonize the mycangia. These data also indicate that ambrosia beetles are not likely to vector any non-ambrosia fungi (plant pathogens). We further show that the related (indigenous) *X. ferrugineus* ambrosia beetle does not appear to be a good host for *H. lauricola*. Again, these data suggest mechanisms of specificity that remain obscure and indicate the need for examining wider pairings between ambrosia beetles and their fungal partners.

Fungal-animal mutualisms are important ecological, evolutionary, and even pathogenic systems that remain understudied. The ambrosia beetle-fungal partner symbioses is a unique model that can provide insights into the dynamics of co-evolution and its consequences on host and partner development. Significant questions remain regarding how specificity in the mycangia is achieved, the nature of the fungal-host interactions, *i.e.*, potential for nutrient exchange, and the mechanism by which (the pre-oral) mycangia in *Xyleborus* beetles function, i.e. how do the fungal cells enter and exit? Our data provide a framework for further exploring such questions via quantitative and cellular approaches. Coupling these efforts for genetic investigations will help undercover the physiological and molecular determinants that underlie such fungal-animal mutualisms.

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