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PLANT-MICROBE-ANIMAL INTERACTIONS – ORIGINAL RESEARCH

Nest substrate, more than ant activity, drives fungal pathogen community dissimilarity in seed-dispersing ant nests

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Abstract

Myrmecochory is a widespread mutualism in which plants benefit from seed dispersal services by ants. Ants might also be providing seeds with an additional byproduct benefit via reduced plant pathogen loads in the ant nest environment through their antimicrobial glandular secretions. We investigate this byproduct benefit by identifying fungal communities in ant nests and surrounding environments and quantifying fungal community change (1) through time, (2) between different nest substrates, and (3) as a function of average ant activity levels within nests (based on observed ant activity at nest entrances throughout the summer). We split fungal communities by functional guild to determine seed-dispersing ant-induced changes in the overall fungal community, the animal pathogen fungal community, the plant pathogen fungal community, and the myrmecochore pathogen fungal community. Nest substrate (soil or log) explained much of the variation in fungal community dissimilarity, while substrate occupation (ant nest or control sample) and time had no influence on fungal community composition. Average ant activity had no effect on the community turnover in fungal communities except for the myrmecochore pathogenic fungal community. In this community, higher ant activity throughout the summer resulted in more fluctuation in the pathogenic community in the ant nest. Our results are not consistent with a byproduct benefit framework in myrmecochory, but suggest that nest substrate drives dissimilarity in fungal communities. The influence of nest substrate on fungal communities has important implications for seeds taken into ant nests, as well as ant nest location choice by queens and during nest relocation.

Keywords Myrmecochory · Byproduct benefit · Ant–plant–fungal interactions · Seed dispersal · Plant pathogens

Introduction

Ants are responsible for the seed dispersal of approximately 11,000 plant species worldwide through the mutualistic interaction known as myrmecochory (Lengyel et al. 2010). In this interaction, ants are attracted to the elaiosome, a fleshy seed-coat-derived appendage, on myrmecochore seeds (Rico-Gray and Oliveira 2007). Foraging ants pick up the seed-elaiosome complex (diaspore) after dehiscence and return with it to their nest (Rico-Gray and Oliveira 2007). There, the elaiosome is removed and primarily fed to ant larvae (Fischer et al. 2005). The seed then remains in the nest or is redispersed outside of the nest, sometimes into a refuse pile (Beattie 1985; Canner et al. 2012). Ants receive food in the form of an elaiosome, and plants gain a number of benefits. Hypotheses surrounding plant benefits are supported to a varying degree and include, but are not limited to: escape from distance- and density-dependent mortality agents (Beattie 1985; Giladi 2006), protection from seed predators (Beattie 1985; Giladi 2006), and, perhaps, a nutrient-rich germination site in or near ant nests (Beattie 1985; Giladi 2006). Although recent work has called into question the benefits offered to ants in this mutualism (Warren et al.

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2019), the benefits to plants offered by ant–seed dispersal services are numerous and well documented.

Benefits to plant partners in myrmecochory, however, are not solely due to the act of dispersal away from the maternal plant, but the place of deposition plays a pivotal role in plant growth and development. Ant nests are high in organic matter, potassium, phosphorous, sodium, and nitrogen (Davidson and Morton 1981; Horvitz and Schemske 1986; McGinley et al. 1994; Berg-Binder and Suarez 2012), and myrmecochore germination and seedling emergence is higher in ant nests than surrounding soils (Beattie and Culver 1982; Culver and Beattie 1980). However, ants might influence not only nutrients in the nest, but also microbial communities, which could provide extra benefits to seeds dispersed into the ant nest. Indeed, seed-dispersing ant nests have reduced fungal species richness (Zettler et al. 2002) and have lower plant pathogenic fungal diversity (Tarsa et al. 2018) than nearby non-nest inhabited soil. Thus, ants may be providing an additional benefit to the seeds that they disperse in the form of pathogen deterrence.

Reduced fungal loads in ant nests could be beneficial to seeds and are likely a function of the extensive chemical production of multiple ant glands (as suggested by Tarsa et al. 2018). Ants have a number of glands that secrete chemicals used for a variety of purposes, including trail following behaviors, nestmate recognition, and sanitation (Vander Meer 2012). A few ant glands secrete antimicrobial chemicals, including the venom gland (Tragust et al. 2013), mandibular gland (Brough 1983), and the metapleural gland (Cabrera et al. 2004; Jones et al. 2005; Vieira et al. 2012; Yek and Mueller 2011; Fernandez-Marin et al. 2015). Ants actively apply some of these secretions to themselves, their brood, their nestmates, and the nest material through grooming (Fernandez-Marin et al. 2006; Tragust et al. 2013; Tranter et al. 2013). Ant glandular secretions, notably those from the metapleural gland, have documented negative effects on entomopathogens as well as the growth of other soil-associated microbes, including some plant pathogens (Beattie et al. 1985; Bot et al. 2002). The metapleural gland, which is present in most ant species, is located at the posterior end of the thorax and is the primary gland used in sanitary grooming behaviors (Yek and Mueller 2011). Thus, ant behavior involved with grooming of the metapleural and other glands likely contributes to the reduction of plant pathogens in the ant nest, potentially providing a byproduct benefit (see Sachs et al. 2004) to the seeds in myrmecochory. Although little work in this regard has been conducted on seed-dispersing ants, some characterization has occurred of the chemicals in glandular secretions of the keystone seed-dispersing ant genus in eastern North America, *Aphaenogaster* (Ness et al. 2009). For a number of *Aphaenogaster* species, the chemicals anabasiene and/or anabasine, which have antimicrobial properties (Abdulina et al. 2002; Anthony et al. 2015), are primary components in the venom gland (Wheeler et al. 1981; Attygalle et al. 1998; Leclercq et al. 2001; Lenoir et al. 2011). Thus, fungi in *Aphaenogaster* ant nests, often in soil and decaying woody debris (Lubertazzi 2012), have the potential to be affected by antimicrobial gland secretions.

Ant nests can have reduced plant pathogen fungal species richness (Tarsa et al. 2018) and overall fungal species richness (Zettler et al. 2002), which might be advantageous for the seeds that are dispersed therein. Additional factors (such as the influence of time, different nest substrates, or direct measures of ant activity) could also influence the microbial composition in seed-dispersing ant nests and could be vital for seed germination, growth and long-term health of the plant. Here, we seek to further explore how the presence of seed-dispersing ants and their activity in nests of different substrates might alter microbial communities to potentially benefit dispersed seeds with regard to plant and myrmecochore pathogens. Specifically, we investigate 1) if microbial composition in soil and decaying woody debris substrates is driven by ant presence, substrate type or time and 2) if ant activity levels in the nests alter microbial turnover, particularly plant pathogen loads, which has not yet been studied in the context of spatiotemporal turnover. If a byproduct benefit exists in this mutualism in the form of ant control of plant pathogens in the nest, we would expect 1) microbial community composition to be explained primarily by the presence or absence of ants, regardless of substrate or time and 2) reduced fluctuations in plant pathogenic fungal communities through time with increasing ant activity in the nest.

Materials and methods

Study species

For our microbial investigation of the ant nest, we used *Aphaenogaster* ants. Ants were either *A. picea* or *A. rudis*, which are both in the *Aphaenogaster fulva–rudis–texana* species complex (Umphrey 1996); species in this complex can be difficult to identify morphometrically and sometimes genetic testing is necessary for definitive IDs (Umphrey 1996). Because of the difficulty in distinguishing species, knowledge of ecology and natural history of *Aphaenogaster* ants in the *rudis* group are often compiled rather than separated by species (Lubertazzi 2012). *Aphaenogaster* ants chose nest sites in leaf litter, soil, and decaying woody debris (hereafter referred to as “log” nests). The number of workers per colony ranges from approximately 100 to over 1000 individuals (Lubertazzi 2012). *Aphaenogaster* ants are generalist scavengers and their diet is primarily other invertebrates (Lubertazzi 2012); however, they are also the primary ant dispersers of myrmecochorous seeds in eastern North America (Ness et al. 2009).

Field collections

During the last two weeks of May 2018, we identified 23 total *Aphaenogaster* ant nests at two locations in Montgomery County, Virginia (12 nests (one log nest and 11 soil nests) at Falls Ridge Preserve [37.193452°N, − 80.321685°W] and 11 nests (four log nests and seven soil nests) at Mill Creek Springs Natural Area Preserve [37.16148°N, − 80.343398°W]). To identify nests, we baited for ants with tuna and followed foraging ants back to the nest entrances. We marked nest entrances with flags to ensure nest material collection from the same site on each sample collection day.

During the first week of June and the last week of July in 2018, we returned to each nest to collect nest material samples by scraping soil or decaying woody debris out of the ant nest entrance. Collection of ant nest entrance samples was done with sterile disposable plastic spatulas (smartSpatulas, LevGo). All nest material was placed into a 3.7-mL sterile glass vial. For every ant nest sample, we also collected a paired control sample. If the ant nest sample was from a soil nest, we collected a non-nest soil sample from approximately 1 m away (as in Tarsa et al. 2018); if the ant nest sample was from a log nest, we collected a sample from a non-nest part of the same log at approximately 1 m away from the nest entrance. Samples were transported on ice then stored at − 80 °C.

We also scored ant activity at the nest entrance once a week in June and July with visual observations of foraging workers. Though observations for ant nest entrance activity date back decades (for example, Talbot 1946), we found no universal method for scoring ant activity at the nest entrance, so we scored activity as follows: A score of 3 indicated a very active ant nest, in which ants would swarm the entrance while we were collecting the sample. A score of 2 indicated that an ant was seen near or in the ant nest entrance, but the researchers were met with no defensive behaviors against the taking of the samples. A score of 1 indicated that no ants were seen in the vicinity of the ant nest entrance. This might indicate that the ant nest relocated, or the nest was particularly inactive that day.

Next-generation DNA sequencing

After field collections, 92 samples were prepared for next generation DNA sequencing via Illumina MiSeq. These 92 samples consisted of 23 ant nest samples and 23 paired control samples taken once in June and once in July. These samples were grouped by location (Mill Creek Springs Natural Area Preserve and Falls Ridge Preserve) for statistical analyses to allow for analysis of fungal communities between time points and between ant nest and control samples.

For DNA extractions, we used Qiagen's DNeasy PowerSoil Kit. Extractions were performed following manufacturer's instructions, except for lengthening both 2–8 °C incubation steps from 5 to 30 min. We also allowed the samples to sit in the final solution for 5 min before the final centrifugation. DNA was quantified with a ThermoScientific Nanodrop. Nanodrop readings for samples ranged from 5.5 to 115 ng/μL with an average of approximately 50 ng/μL. All DNA samples were stored at − 20 °C until library preparation.

For library preparation, we followed Illumina's Metagenomic Sequencing Library Preparation Instructions. We used custom mix of primers for the ITS region of fungal rRNA (see Cregger et al. 2018) for the amplification of DNA by polymerase chain reaction (PCR). This mix of primers is better suited to pick up a wide range of fungal taxa that are often missed when universal primers are used (Cregger et al. 2018). The PCR was performed with the following cycles: 95 °C for 3 min, 25 cycles of: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final elongation of 72 °C for 5 min. All PCR products were visualized using gel electrophoresis with 5 μL of product on a 2% agarose gel with ethidium bromide staining. The PCR products were purified with AMPure XP magnetic beads (Agencourt), and cleaned products were individually tagged with different combinations of Nextera XT (Illumina) indexes. Indexes were attached with a second round of PCR with the following cycles: 95 °C for 3 min, 8 cycles of: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and 72 °C for 5 min. Another clean-up with AMPure beads was performed after indexing. Products were normalized after quantification with a BioAnalyzer and pooled. We diluted final library concentrations to 4 pM and 10 pM Phi-X at 20% was spiked in. Samples were loaded on a v3, 600 cycle flowcell, set to read 2 × 275 bases, for Illumina MiSeq in the University of Tennessee, Knoxville, Genomics Core Facility.

Data processing

We processed raw sequences using a Qiime2 pipeline (Online Resource 1). First, sequences were paired and then run through FastQC and MultiQC for quality control. Sequences were trimmed based on a Phred score of greater than 20 and primers were removed. Chimeras were detected and removed. Taxonomy was assigned to the sequences using BLAST and version 8 of the UNITE database (Nilsson et al. 2018). Amplicon Sequencing Variants (ASVs) were assigned taxonomy at 97% matching. Before further processing, we filtered out species that were not identified as fungi and rarefied all fungal data to 22,000 sequences to control for variation in sequencing depth. After rarefication, we had 14,783 identified fungal ASVs.

To assign ecological guilds to each fungal ASV, we uploaded our ASV table to FUNGuild (Nguyen et al. 2016). For analysis of plant and animal pathogens in ant nests and control samples, we filtered the resulting ASV table from FUNGuild by Guild. For animal pathogens, any ASV that was listed as an "Animal Pathogen" for at least one possible guild was included. For plant pathogens, any ASV that was listed as a "Plant Pathogen" for at least one possible guild was included. We also wanted to investigate pathogens that are known to infect myrmecochores. To filter our fungal table by myrmecochore pathogens, we first created a list of myrmecochorous plant genera in eastern North America using the following sources: Beattie and Culver (1981); Lengyel et al. (2010); Schultz (2014). All genera compiled in this list were then entered into the Host Name search in the Fungus-Host Distributions page of the Fungal Database of the Agricultural Research Service of the United States Department of Agriculture (https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm). Any known fungal pathogenic genera were copied from the database and added to a list of myrmecochore pathogens. We searched for those genera in our BLAST identified taxonomic ASV list. All genera that included potential myrmecochore pathogens were included. Myrmecochore list by genus and myrmecochore pathogen list by genus can be found in the supplemental materials (Online Resource 2). Each subset of ASV tables was analyzed in the following way and, from here on, are referred to as: All Fungi (every ASV identified in Kingdom Fungi through BLAST), Animal Pathogens (any ASV identified as a possible animal pathogen by guild in FUNGuild), Plant Pathogens (any ASV identified as a possible plant pathogen by guild in FUNGuild), and Myrmecochore Pathogens (any ASV identified as a pathogen to a myrmecochorous plant through the USDA ARS Fungal Database).

Statistical analyses

Community analyses

All analyses were conducted in R (version 3.6.0). We examined variation in fungal communities (ASV abundances) with distance-based Redundancy Analyses (db-RDAs) with a dissimilarity matrix based on the quantitative generalization of Jaccard dissimilarity (Ružička index). We separately analyzed Falls Ridge Preserve and Mill Creek Springs Natural Area Preserve because Falls Ridge only had a single log nest, which resulted in a singularity if nest substrate was included in a model with both locations. We removed the solo log nest from our datasets. We selected models using forward stepwise selection with the step function in the package "vegan" (version 2.5-5) (Oksanen et al. 2019). Possible predictors included treatment (ant nest or control sample), nest substrate (soil or log nest—only used in Mill Creek Springs datasets), month of sampling (June or July) and all possible two-way interactions. We conducted hypothesis testing on retained predictors via 99,0000 permutations of the data (Legendre and Legendre 2012).

Microbial community turnover

To understand the influence of different explanatory variables on fungal community turnover between ant nest samples and control samples over time, we used Hill Numbers (Hill 1973) (^{q}D) to measure beta diversity of communities in this spatially explicit temporal context. Hill Numbers is a diversity metric that calculates the effective number of species for each sample, and it provides advantages over traditional ecological diversity calculations (Hill 1973; Jost 2006, 2007; Chao et al. 2014). As order q increases, the importance of rare species in diversity calculations decreases (Hill 1973): at $q = 0$, diversity calculations are equivalent to species richness. At $q = 1$, diversity calculations are equivalent to the exponential of Shannon's entropy (H), and at $q = 2$, diversity calculations are equivalent to inverse Simpson's diversity. Here, we conduct analyses from $q = 0$ to $q = 5$.

In R using the package "vegetarian" (version 1.2) (Charney and Record 2012), we calculated pairwise community turnover (T) (Marion et al. 2017) for each ant nest from June to July, and for each control nest from June to July. Thus, T is calculated as: $1\text{-}|{}^{q}D_1\text{-}{}^{q}D_2|$ and represents the expected proportional change in communities in that sample from June to July. A turnover (^{q}T) value of 1 indicates a 100% change in community composition in that sample from June to July, and a ^{q}T value of 0 indicates the sample had identical communities in June and July. Then, we calculated differences in ^{q}T between individual ant nests and their control samples (which we will refer to as $\Delta^{q}T$). Values of $\Delta^{q}T < 0$ indicate that there is more community turnover (or less community compositional similarity) in control samples than in ant nest samples through time. Values of $\Delta^{q}T > 0$ indicate that there is more community turnover in ant nest samples than in control samples through time. Values of $\Delta^{q}T \approx 0$ indicate that the community turnover through time is similar between ant nest samples and control samples.

We separated $\Delta^{q}T$ values by location (Mill Creek or Falls Ridge) and conducted linear models using the "stats" package (version 3.6.0) to test the effect of average ant activity, nest substrate and their interaction (nest substrate and the interaction were only investigated in Mill Creek samples) on values of $\Delta^{q}T$. We again removed the single log nest from Falls Ridge datasets to eliminate singularities. To calculate average ant activity, we averaged ant activity scores from each nest for each week. This average value was used as a proxy for ant activity throughout the myrmecochory fruiting season. Lower ant activity averages (values closer to 1) indicate low ant activity in nests throughout the season, while higher ant activity averages (values closer to 3) indicate high levels of ant activity at the nest entrance throughout the season. We conducted hypothesis testing on the models using ANOVA tests in the "car" package (version 3.0–6) (Fox and Weisberg 2019).

Results

Community analyses

At Mill Creek Springs Natural Area Preserve, for all subsets of data (All Fungi, Animal Pathogens, Plant Pathogens, and Myrmecochore Pathogens), nest substrate was the only predictor chosen for models. This indicates that fungal community variations were most associated with substrate (log vs soil) as opposed to other variables of interest (ant nests/control, time). Likewise, in all subsets, db-RDAs revealed a significant difference in soil and log substrates: All Fungi (Variation Explained = 6.98%, $P < 0.05$; Fig. 1a), Animal Pathogens (Variation Explained =10.12%, $P < 0.05$; Fig. 1b), Plant Pathogens (Variation Explained =7.57%, $P < 0.05$; Fig. 1c), and Myrmecochore Pathogens (Variation Explained =4.37%, $P < 0.05$; Fig. 1d). At Falls Ridge Nature Preserve, for which we did not include substrate as a potential predictor, for all subsets of data (All Fungi, Animal Pathogens, Plant Pathogens, and Myrmecochore Pathogens), model selection chose the intercept-only model with no explanatory variables.

Microbial community turnover

Our estimates of Δ^qT (change in turnover) were largely unaffected by nest substrate, average ant activity, or their interaction for all data subsets (All Fungi, Animal Pathogens, Plant Pathogens, and Myrmecochore Pathogens). For All Fungi at all order q at Mill Creek, we failed to detect an effect of average ant activity, nest substrate, or their interaction on Δ^qT (all Ps > 0.05). Likewise for All Fungi at all order q at Falls Ridge, we failed to detect an effect of average ant activity on Δ^qT (all Ps > 0.05). This pattern repeated for Animal Pathogens and Plant Pathogens for both field sites at all order q (all Ps > 0.05). Additionally, for Myrmecochore Pathogens at all order q at Mill Creek, we failed to detect an effect of average ant activity, nest substrate, or their interaction on Δ^qT (all Ps > 0.05). For Myrmecochore Pathogens at Falls Ridge at $q = 1$, average ant activity was a significant predictor of Δ^1T ($F_{1,9} = 6.60$, $P = 0.03$; Fig. 2). At all other order q for Myrmecochore Pathogens at Falls Ridge, we failed to detect an effect of average ant activity on Δ^qT (all Ps > 0.05).

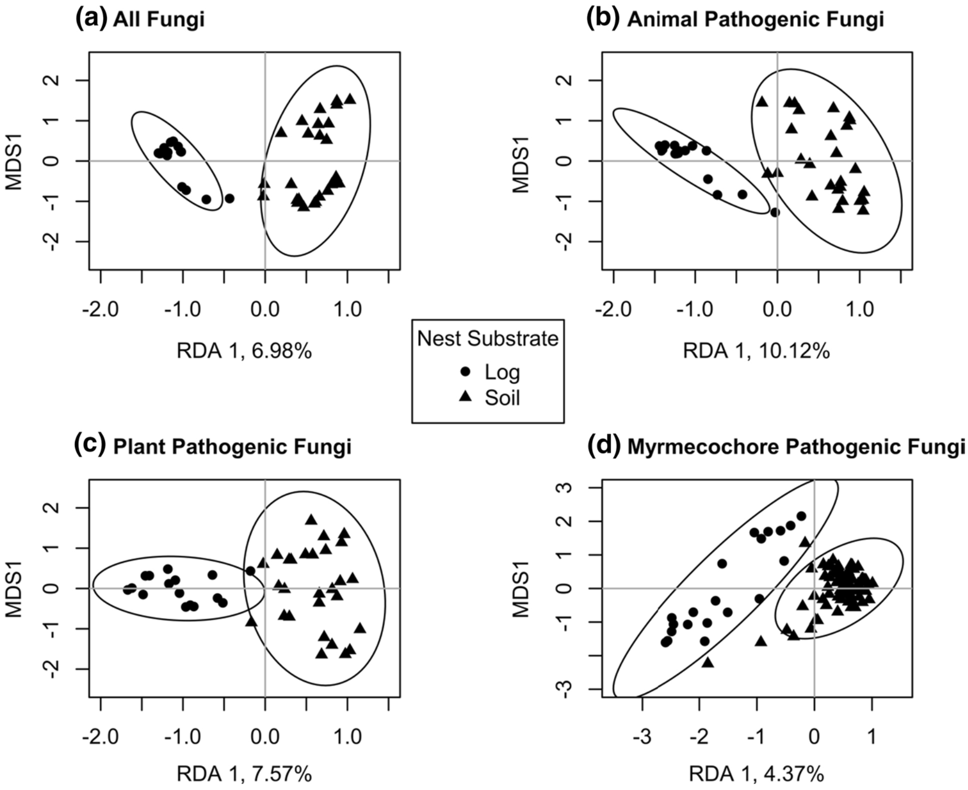

Fig. 1 Multivariate ordination plot of different fungal communities at Mill Creek Springs Natural Area Preserve. Nest substrate is separated into log nests (circles) and soil nests (triangles). Ellipses represent 95% confidence limits. Percentages on the axes represent overall model variation. In all communities, nest substrate is the only significant predictor of the fungal communities ($P < 0.05$)

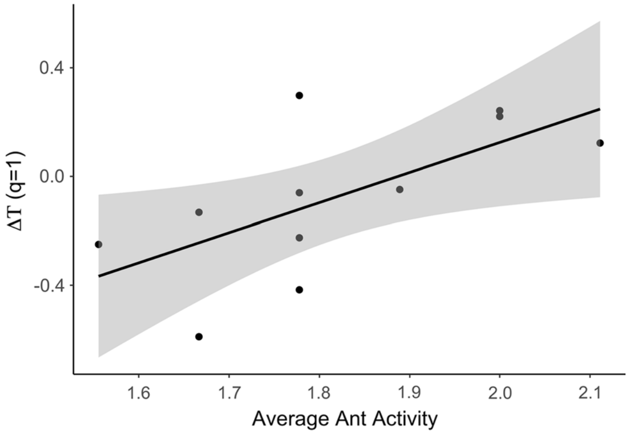

Fig. 2 Relationship between community turnover of ant nest and control samples over time ($\Delta^q T$) and average ant activity scores at *Aphaenogaster* ant nests. The shaded area represents SE. For the myrmecochore pathogenic fungal community at $q=1$ at Falls Ridge Nature Preserve, average ant activity was a significant predictor of $\Delta^1 T$ ($F=6.60$, $df=1$, $P=0.03$; Fig. 2). Lower ant activity in nests resulted in more negative $\Delta^1 T$ values. This corresponded to more turnover in control samples than in ant nest samples. However, when ant activity was high, $\Delta^1 T$ values were above zero, which corresponds to more turnover in ant nest samples than in control samples

Discussion

Our results suggest that nest substrate is an important driver of fungal community diversity in the overall, animal pathogenic, plant pathogenic, and myrmecochore pathogenic fungal communities. Additionally, we found that ant activity has little effect on the turnover in different fungal communities. Indeed, we only saw the influence of ant activity for the myrmecochore fungal pathogenic community at $q=1$ (the exponential of Shannon's entropy (H)). Furthermore, this influence was the opposite of the pattern that would be expected if ants are controlling the fluctuation of myrmecochore pathogens in the ant nest. Although these results are not consistent with evidence for a byproduct benefit in the form of reduced pathogen fluctuations in the ant nest, our results still have implications for the plant partners in this mutualism.

Ant abandonment of the nest during myrmecochore fruiting season could have ramifications for the seeds that remain behind in the nest. Our findings for turnover in the myrmecochore pathogenic fungal community suggest a potential benefit for seeds in ant nests that are low in ant activity, which might correspond to ant abandonment of the nest. We found that reduced ant activity results in a more compositionally similar myrmecochore pathogen community through time in ant nests than in control samples at $q=1$. Stability in these pathogen communities could be beneficial for seeds, even during a short time in the ant nest, as changes in pathogen communities could present new challenges to seeds, especially if these fluctuations involve more virulent pathogens. Stability of myrmecochore pathogens in the ant nest might even provide more advantages for the seeds that are abandoned in the ant nest (specifically in soil nests at one field site). Though Canner et al. (2012) noted that over 90% of seeds dispersed by *Aphaenogaster rudis* are redispersed outside of the nest, they also noted that some seeds (approximately 7% of dispersed seeds) do remain in the nest; our results suggest that seeds that remain or are abandoned in the ant nest might have the advantage of reduced fluctuations in pathogens in certain nest substrates, compared to seeds that are redispersed to non-ant nest locations.

Our results also suggest that nest substrate drives dissimilarity in the fungal community of both ant nests and non-nest samples. Certainly, seeds that are dispersed to and abandoned in newly decaying logs are likely at a disadvantage for successful germinations and subsequent seedling growth and success than seeds that are abandoned in soil substrates. However, seeds dispersed to log nests likely encounter different fungal (particularly different plant pathogenic fungi and myrmecochore pathogenic fungi) communities than seeds dispersed to soil nests, since substrate drives fungal community dissimilarity. Likewise, animal pathogenic fungal community dissimilarity is affected by substrate. This suggests that ant colonies, when beginning or relocating a nest, might encounter different pathogen communities based on the choice to nest in logs or soil. Although little is known about *Aphaenogaster* ant nest location choice, *Formica selysi* queens preferentially and interestingly choose infected nest locations during colony founding (Brutsch et al. 2014). Future work could investigate elements that alter nest location choice for *Aphaenogaster* ants.

FUNGuild classification methods may have influenced our results and may offer explanations as to why our results differed from those found in similar studies. We did not find that ant activity directly influenced turnover in the animal pathogenic fungal community. We expected high ant activity to be associated with reduced animal pathogen fluctuation in the ant nest. We note, though, that FUNGuild output classifies an "animal pathogen" guild and not specifically entomopathogens (insect-infecting pathogens). Ant colonies have social immunity, or a collection of defenses against pathogen takeover of the nest (Meunier 2015). Chemical defenses in ant social immunity include active application of glandular chemical secretions to reduce the growth of ant pathogens (Fernandez-Marin et al. 2006; Yek et al. 2012; Tragust et al. 2013; Tranter et al. 2013). However, *Aphaenogaster* ant colonies abandon nests and move regularly, anywhere from 2–3 times a year (Lubertazzi 2012) to every 21 days (Smallwood and Culver 1997). Though seasonal cues are likely one reason for nest relocation, ants might also move due to parasite loads in the nest (as suggested by Smallwood and Culver 1997) or pathogen takeover of the nest. Perhaps, ants allocate chemical defenses to protection of their nestmates and brood, rather than control of pathogens in nest material; in this case, nest abandonment may take place when entomopathogen loads are too high.

Additionally, there are issues with FUNGuild's general classification of plant pathogens, and this may be why our results do not match those of Tarsa et al. (2018), who conducted their own general classification of phytopathogenic fungi. In particular, FUNGuild simplifies what are far more complex plant-associated fungal relationships that can be context dependent (Schulz and Boyle 2005; Kogel et al. 2006). Endophytic fungi can turn parasitic if the plant is stressed, though in the majority of instances, they remain as beneficial endophytic partners, and fungi can have different classifications when associated with different plant species (Kogel et al. 2006). Because of difficulties with fungal guild classification, we note that the scale at which studies of fungal communities are conducted is important. This was, in part, the motivation behind choosing to separate out myrmecochore pathogens from FUNGuild's classification of plant pathogens. Finally, it is important to note that there is currently no complete pathology for myrmecochorous plants, and our list may not reflect the entire pathogenic community. As our classification of myrmecochore pathogens increases, we can conduct studies with a more refined focus of that pathogenic community.

Although investigations into the microbial community in soil and log ant nests are just beginning, a substantial amount of literature exists on the influence of various factors on the microbial community in decaying woody debris and soil in temperate forests. Fungal community composition in forested ecosystems is affected by a variety of factors, including but not limited to: tree composition (Khlifa et al. 2017), seasonal changes (Voriskova et al. 2014), soil depths (Voriskova et al. 2014), soil pH (Carrino-Kyker et al. 2016), nutrient availability (Carrino-Kyker et al. 2016), and disturbances (Osburn et al. 2019). Even forest management practices can influence fungal community composition and diversity (Goldmann et al. 2015). Thus, effects of ant activity and nest substrate on fungal community composition, as well as the potential influence of fungal communities on ant nest location choice, occur within a complex system. Future work could investigate ant influence on fungal community composition in a more controlled environment, such as in the laboratory or a common garden.

Finally, we acknowledge limitations in our detection power of microbial loads in ant nests due to our choice to sample ant nest entrances rather than underground nest chambers. Indeed, most *Aphaenogaster* ant nests have chambers located anywhere from just below surface level to up to 84 cm below ground (Lubertazzi 2012). Ant nest entrances were chosen as collection points so that we could conduct samples throughout the summer. Taking collections from farther inside the ant nest might destroy nest entrances or disturb ant colonies and trigger nest abandonment. Future work could concentrate on underground chamber sampling (as in Tarsa et al. 2018) rather than incorporating time, especially because we found no effect of time (early or late summer sampling) on overall microbial community composition.

Previous work has determined that plant pathogenic fungal species richness is reduced in ant nest environments compared to surrounding soils (Tarsa et al. 2018), but our work is the first to demonstrate the influence of nest substrate on fungal community composition. Additionally, we did not detect many direct effects of ant activity on different fungal pathogen communities. As suggested by Tarsa et al. (2018), we had speculated that the changes in fungal communities (especially in instances where ant activity was stable or increasing) within the ant nest could result from the chemical secretions from ant glands, which are used in sanitary behaviors to clean the ant nest (Tranter et al. 2013). However, our results suggest that this influence is small. Future work could investigate the effects of *Aphaenogaster* glandular secretions on known myrmecochore plant pathogens. Identification of the primary components of the metapleural glands of *Aphaenogaster* will also be helpful for understanding the impacts of ant glandular chemicals on the plant pathogen community. Investigating what fungal communities are present on myrmecochore seed coats and how that seed coat-associated community changes throughout the dispersal process will also be important for understanding the effects of ant chemicals on plant-associated fungal communities. Thus, our work contributes to the beginning of a better understanding of the benefits offered to plants in myrmecochory, especially when considering byproduct benefits, such as reduction in microbial myrmecochore pathogen loads in the ant nest via ant-associated chemicals. By investigating the dynamics of fungal communities and specific fungal species in myrmecochory, we can contribute to a better understanding of the evolution and persistence of mutualistic seed dispersal.

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Author contribution statement CLL and CK originally formulated the idea and developed methodology. CLL conducted all field and

lab work. JAF generated the statistical framework and JAF and CLL conducted all statistical analyses. CLL wrote the first draft of the manuscript and CK and JAF commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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