Parasites, niche modification and the host microbiome: A field survey of multiple parasites

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Funding information
NSF-USDA joint programme in Ecology and Evolution of Infectious Diseases, Grant/Award Number: 2016-67013-25762 and DEB-1541418; University Cancer Research Fund; Triangle Center for Evolutionary Medicine; The National Science Foundation; University of North Carolina-Chapel Hill (UNC-CH) Graduate School

Abstract
Parasites can affect and be affected by the host's microbiome, with consequences for host susceptibility, parasite transmission, and host and parasite fitness. Yet, two aspects of the relationship between parasite infection and host microbiota remain little understood: the nature of the relationship under field conditions, and how the relationship varies among parasites. To overcome these limitations, we performed a field survey of the within-leaf fungal community in a tall fescue population. We investigated how diversity and composition of the fungal microbiome associate with natural infection by fungal parasites with different feeding strategies. A parasite's feeding strategy affects both parasite requirements of the host environment and parasite impacts on the host environment. We hypothesized that parasites that more strongly modify niches available within a host will be associated with greater changes in microbiome diversity and composition. Parasites with a feeding strategy that creates necrotic tissue to extract resources (necrotrophs) may not only have different niche requirements, but also act as particularly strong niche modifiers. Barcoded amplicon sequencing of the fungal ITS region revealed that leaf segments symptomatic of necrotrophs had lower fungal diversity and distinct composition compared to segments that were asymptomatic or symptomatic of other parasites. There were no clear differences in fungal diversity or composition between leaf segments that were asymptomatic and segments symptomatic of other parasite feeding strategies. Our results motivate future experimental work to test how the relationship between the microbiome and parasite infection is impacted by parasite feeding strategy and highlight the potential importance of parasite traits.

Keywords
biotic interactions, disease ecology, metabarcoding, microbiome, mycobiome, niche modification

1 | INTRODUCTION

The fungi, bacteria and other microbes that comprise an organism's microbiome can interact with parasites in ways that have consequences for host susceptibility, disease severity and parasite transmission (Berg & Koskella, 2018). Yet, while investigations of the association between the microbiome and a single parasite species are becoming increasingly common (Libertucci & Young, 2019), how such associations vary among parasite species is still poorly understood (Aivelo & Norberg, 2018). Variation among parasite species may be explained by their niches, which include both parasite...
requirements of the host environment and parasite effects on the
host environment. Based on the concept of niche modification
(Fukami, 2015; Lewontin, 1983), we develop the hypothesis that
parasites with traits that more strongly modify the environment
within the host can act as niche modifiers and more strongly impact
the microbiome. Parasites with different feeding strategies differ
in their requirements of the host environment, and parasites with
certain feeding strategies may more strongly modify the environ-
ment within the host (Budischak et al., 2018; Glazebrook, 2005).
Thus, these parasites might be associated with differences in the
host microbiome. To begin to confront these ideas with data under
field conditions, we conducted an observational survey of the fungal
microbiome in a grass host and evaluated how microbiome diversity
and composition were associated with natural infections by multiple
co-occurring fungal parasites.

Microbes may interact with parasites by competing for re-
sources, releasing antimicrobial compounds, or eliciting a host
immune response (Bashey, 2015; Graham, 2008; Raaijmakers &
Mazzola, 2012). Such interactions can influence host health, mak-
ing the host more susceptible to, tolerant of or resistant to parasites
(Arnold et al., 2003; Busby et al., 2016; Hayes et al., 2010). The mi-
crobiome is also dynamic, and the introduction of a parasite can lead
to changes in microbiome diversity and composition (Barman et al.,
2008; Jani & Briggs, 2014). The link between parasites and host mi-
crobial diversity varies, with some studies showing no relationship
(Li et al., 2012; Williams et al., 2017), some studies showing a neg-
apitive relationship (Jani & Briggs, 2014; Leung et al., 2018; Mosca
et al., 2016; Wu et al., 2014), and still others showing a positive re-
lationship between parasite infection and within-host microbial di-
versity (Lee et al., 2014). One possible source of this variation among
studies is variation among the parasite species studied. Yet, there is a
lack of studies that examine variation among parasite species in their
associations with host microbiota (but see Aivelo & Norberg, 2018),
perhaps because we have lacked a robust framework for studying
multiple parasites.

A trait-based approach might provide a framework for studying
multiple parasite species (Oliva et al. 2020; Zanne et al., 2019).
As well as being evolutionarily diverse (Weinstein & Kuris, 2016),
parasites vary in traits such as growth rate, generation time and
feeding strategy (Leggett et al., 2017). Different parasite feeding
strategies can stimulate different immune responses in a host and
differently impact host performance (Budischak et al., 2018; Glazebrook,
2005). Parasites infecting plants employ three typi-
cal feeding strategies. Parasites with a biotrophic feeding strat-
ey keep host cells alive to extract resources from them. Parasites
with a necrotrophic feeding strategy kill host cells to access their
resources, creating necrotic tissue while they grow within their
host. Finally, hemibiotrophic parasites infect as biotrophs, then
switch to become necrotrophic. Given that parasites with differ-
et feeding strategies have different requirements to extract re-
sources from the host environment and different impacts on the
host environment, they may also have different associations with
the host microbiome.

When an organism modifies its environment, it can change the
number and types of niches available, and in turn impact the spe-
cies that can reside and colonize within the ecological community
via the process of niche modification (Fukami, 2015; Lewontin,
1983). Within host individuals, symbionts may also act as niche
modifiers by impacting the host environment in such a way that
the host is more or less suitable to new colonizers. We hypoth-
esized that necrotrophic parasites act as niche modifiers for the
microbiome. More specifically, we hypothesized that by producing
necrotic tissue from when they first infect the host, necrotrophic
parasites create new niches within the host for a longer time, and
thus have larger impacts on microbiome richness and composition
than either hemibiotrophic parasites, which only produce necrotic
tissue in later stages of infection, or biotrophic parasites, which
keep host cells alive while extracting resources. Conversely, par-
asites with different feeding strategies may respond differently
to other symbionts that modify the environment within the host.
Because feeding strategy can define how a parasite interacts
with the host environment, a trait-based approach grounded in
parasite feeding strategy might help explain variation in parasite–
microbiome associations.

Few studies have investigated the associations between
host-associated microbiota and parasites under field conditions
(but see Aivelo & Norberg, 2018; Jani & Briggs, 2014; Leung
et al., 2018). The lack of field studies may result from a limited
number of suitable model systems for exploring these questions
in the field. The long-lived nature of some hosts, limited ability
to detect diseases observationally in live hosts in the field, dif-
ficulty of excising infected tissue from animals, and ethical con-
cerns limit the utility of many study systems for field research
(Borer et al., 2011). Foliar fungal parasites are a valuable model
system to investigate microbiome–parasite interactions in field
settings. These parasites are often readily identifiable by exter-
nal, macroscopic symptoms and morphology, which facilitates
observational studies in the field (Arnold et al., 2003; Busby
et al., 2016; Christian et al., 2015). In a rare study, conducted
with inbred mice, in which parasite infection was experimentally
manipulated in both the laboratory and the field, the direction
and magnitude of the relationship between parasite infection
and the host microbiome differed between laboratory and field
conditions (Leung et al., 2018). This finding underlines the im-
portance of investigating parasite–microbiome associations in
the field.

Here, we take a trait-based approach to address variation in
parasite–microbiome associations in the field. We conducted a
molecular survey of the within-leaf fungal community of the grass
tall fescue in a field population infected by three fungal parasites.
These three parasites each employ a different feeding strategy
(biotrophic, hemibiotrophic and necrotrophic). This survey provides
an initial evaluation of whether three parasites, each exhibiting a dif-
f erent feeding strategy, and thus a different hypothesized potential
to act as a niche modifier, have different associations with the host
microbiome.
2 | METHODS

2.1 | Study system

Leaf segments were collected in a grass-dominated field within the Blackwood Division of the Duke Forest Teaching and Research Laboratory in Orange County, North Carolina (35°58′N, 79°5′W). This field was chosen based on proximity to the laboratory (to expedite sample processing), and abundance of tall fescue (Lolium arundinaceum) and its foliar fungal parasites. This study focused on disease symptoms previously identified in another field of the Duke Forest Teaching and Research Laboratory (Halliday et al., 2017, 2018) as caused by parasites with three different parasite feeding strategies (Table 1).

2.2 | Leaf segment collection

Leaf segments were collected over the course of two days in late September 2016 (September 26 and September 30), which is when parasites tend to peak in their abundance in this system (Halliday et al., 2017). Leaf segments were collected at a total of 36 sampling points, spaced every 20 m along six transects; the transects were 100 m long, parallel and spaced 20 m apart (Figure S1).

Only one leaf of each symptom was collected at each point, and therefore leaves with the same symptom were always collected at least 8 m apart. This minimum distance was selected to minimize the effect of spatial autocorrelation on the structure and composition of the microbiome (Arnold et al., 2007; Higgins et al., 2007).

To standardize the relative age of sampled leaves, we always sampled the oldest fully expanded nonsenescing leaf on the tiller. For each leaf, we estimated the percent of leaf area infected with each parasite (infection severity) by visually comparing leaves to reference images of leaves of known infection severity (Halbritter et al., 2020; e.g., Halliday et al., 2019; Mitchell et al., 2002, 2003).

At each of the 36 sampling points, we collected four whole leaves—one leaf with symptoms of a necrotrophic parasite (and no other parasites), one leaf with symptoms of a hemibiotrophic parasite (and no other symptoms), one leaf with symptoms of a biotrophic parasite (and no other parasites) and one asymptomatic leaf. While co-infection is common in this system (Halliday et al., 2017), we avoided collecting co-infected leaves. From the four whole leaves collected at each of the 36 sampling points, we excised seven leaf segments: symptomatic and asymptomatic segments from each leaf infected with one of the three parasites, as well as an asymptomatic segment collected from the one asymptomatic leaf. Thus, we collected a total of 252 leaf segments. All segments were of approximately equal size. The two segments collected from each symptomatic leaf were spaced at least 10 cm apart within the leaf. We stored each leaf segment in an individual plastic bag that was then placed on ice.

Within 4 hr, all segments were processed back in the laboratory. Leaf segments were washed under running deionized water for 30 s to remove fungi that were on the surface of the leaf but not attached.

| Feeding strategy | Parasite | Symptom |
|---|---|---|
| Necrotroph: kills the living cells of a host and then feeds on the dead matter | *Rhizoctonia solani* | ![Rhizoctonia; F. Halliday](image) |
| Biotroph: feeds on living cells of a host, without killing it as part of the infection process | *Puccinia coronata* | ![Puccinia; K. O’Keeffe](image) |
| Hemibiotroph: initially feeds on living host tissue without causing visible symptoms prior to switching to a necrotrophic phase | *Colletotrichum cereale* | ![Colletotrichum; F. Halliday](image) |
to the leaf. Following surface washing, leaf segments were stored in a −80°C freezer.

2.3 | DNA extraction and sequencing

Surface-washed leaf segments were ground under liquid nitrogen with a mortar and pestle and transferred to 96-well plates for DNA extraction. DNA extraction was performed with the DNEasy PowerSoil kit according to the manufacturer’s protocol (Qiagen).

We assayed the fungal communities of tall fescue leaves by sequencing the internal transcribed spacer (ITS) region. The ITS is a region of the nuclear ribosomal RNA cistron that is often used as a DNA barcode for fungi, as it has less intraspecific variation than interspecific variation (Schoch et al., 2012). We amplified the first part of the internal transcribed spacer (ITS1) with a version of the primer set ITS1F and ITS2 modified for parallel sequencing on the Illumina MiSeq platform (Smith & Peay, 2014; White, 1990). Each 25-µl polymerase chain reaction (PCR) had 2.5 µl of 10× PCR Buffer, 3.5 µl MgCl₂, 1 µl ITS1-F, 1 µl ITS2, 0.5 µl dNTPs, 0.63 µl Taq polymerase, 13.12 µl water and 3 µl DNA. The reactions were performed with the following cycle conditions: initial denaturation at 95°C for 1 min followed by 40 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 30 s and a final elongation at 68°C for 7 min. We visualized PCR products using gel electrophoresis, cleaned samples with AMPure beads (Lundberg et al., 2013), and concentration-normalized them (using Qubit Fluorometric Quantitation, Life Technologies). The cleaned amplicons were pooled in one run on an Illumina MiSeq instrument (Illumina) at the UNC High Throughput Sequencing Facility using a paired-end 2 × 250-bp kit. A spike of 10% PhiX was added to the library to increase sample heterogeneity. All raw sequence data are available at https://doi.org/10.5061/dryad.5x69p8d0v.

2.4 | Fungal community analysis

All statistical analyses were performed with leaf segment as the unit of observation, and all analyses were performed in the R environment, version 3.6.0 (R Core Team, 2012). Fungal sequences from the pooled samples were assigned to individual leaf segments (i.e., demultiplexed) using Illumina’s BCL2FASTQ pipeline (version 2.20.0), and sequencing adapters were removed from the fungal ITS sequences using cutadapt (version 1.15, Martin, 2011). Illumina-sequenced amplicon data of microbial communities are often clustered into operational taxonomic units (OTUs) based on a fixed dissimilarity threshold. This clustering reduces the rate at which sequencing errors are misinterpreted as biological variation. The dada2 package in R models and corrects Illumina-sequenced amplicon errors and infers exact amplicon sequence variants (ASVs; herein referred to as taxa); these taxa are biological variants and not sequencing noise (Callahan et al., 2016). This method can resolve biological differences at a high resolution, and the output can be directly compared among studies without the need to reprocess the pooled data. We therefore employed dada2 in this study. Quality control of sequencing reads for each leaf segment consisted of truncating reads at the first quality score of 2 (a quality score of two indicates a portion of the sequence that contains mostly low-quality reads of Q15 or less), and removing any read with ambiguous base calls or greater than two expected errors. Reads shorter than 50 bases after quality trimming were removed.

2.5 | Statistical analyses: Diversity

To compare the diversity of fungal communities among asymptomatic and symptomatic leaves of tall fescue, we quantified Hill’s series of diversity. Hill’s series of diversity (Hill, 1973) comprises three orders (q) of diversity that summarize information about the number and relative abundances of taxa. In Hill’s series, the values of q (0, 1, 2) indicate the relative weight applied to relative abundance (Bent & Forney, 2008). We estimated fungal richness (Hill’s N0, q = 0), the exponent of the Shannon index (Hill’s N1, q = 1), and the inverse of the Simpson index (Hill’s N2, q = 2) (Jost, 2006). Because Shannon entropy and Simpson diversity are less sensitive to the detection of rare taxa than species richness, they each place more weight on abundant taxa. Simpson diversity places even more weight on abundant taxa than Shannon diversity. The unit of each of the numbers within Hill’s series of diversity is the effective number of taxa, allowing comparisons across each value of diversity. Hill’s numbers were calculated with the vegan package (version 2.5.3, Oksanen et al., 2013).

To test whether fungal diversity is associated with symptom type, we used linear mixed models to explain Hill’s N0, N1 and N2. In order to meet assumptions of normality and homoscedasticity of the residuals, we log-transformed diversity. We included symptom type (seven levels: the asymptomatic and symptomatic leaves from leaves with each of the three focal symptoms, plus asymptomatic segments from asymptomatic leaves) as fixed effects. High-throughput sequencing of pooled samples can result in samples that differ in sequencing depth; we accounted for observational bias stemming from this difference by incorporating sequencing depth directly into the models as another fixed effect, following Bálint et al., (2015, 2016). Another common approach to account for observational bias is to normalize the sequence read numbers via rarefaction (Gotelli & Colwell, 2001). However, rarefaction poses multiple analytical and statistical problems, such as loss of power and dependence on arbitrary thresholds. Statistical models can avoid these problems by incorporating sequencing depth into the models as the first explanatory variable, and thereby explicitly account for bias due to sequencing depth. Thus, the explicit incorporation of sequencing depth into models has been robustly tested with simulated microbiome data (Zhang et al., 2017; Luna et al., 2020), and is strongly advocated as an approach to account for differences in library size across samples (Bálint et al., 2016; Nayfach & Pollard, 2016; Weiss et al., 2017). To confirm that this method does not bias our analyses, we also normalized the sequence read numbers via rarefaction and...
found that the inferences remain consistent (Tables S1 and S2). Leaf ID and collection point were included as random effects, with leaf ID nested within collection point. Linear mixed models were assessed in 

emmeans (version 1.3.2, Lenth, 2018) to evaluate the estimated marginal means of diversity indices for each explanatory variable level, adjusted for multiple comparisons (Tukey's honest significant difference [HSD]). After accounting for the variation explained by random effects and sequencing depth, we compared the partial residuals of Hill's numbers among the treatments with Tukey's HSD.

To quantify any changes in within-host microbial diversity as disease caused by necrotrophs progressed, we used disease severity (percent leaf area exhibiting symptoms) as a measure of disease progression. Specifically, we fit three linear mixed models using the 

r package nlme (version 3.1-142, Pinheiro, et al. 2013) with Hill's N1, Hill's N2 or Hill's N3 as the dependent variable and necrotrophic disease severity as an independent variable. As such, only fungal community data from leaf segments symptomatic of necrotrophic parasites were included in these analyses. Hill's N1, Hill's N2 and Hill's N3 were log-transformed to meet assumptions of homoscedasticity and normality. Each model also included the square root of sequencing read numbers obtained for a leaf segment as an independent variable, and collection point as a random effect. Thus, each model had the following form: Hill ~ sqrt(readNumbers) + Severity +1|Collection Point. A clear relationship between disease severity and fungal diversity, in the same direction as the overall association of fungal diversity and symptom type, would suggest that the parasite progressively impacts the fungal microbiome as disease progresses. No relationship between disease severity and fungal diversity, or a relationship in the opposite direction to the overall association of fungal diversity and symptom type, would suggest that any association between the parasite and microbiome is not due to a progressive impact of the parasite on the microbiome.

2.6 | Statistical analyses: Community composition

To test the hypothesis that parasites that modify niches within their host by creating necrotic tissue alter fungal community composition, we tested whether fungal community composition was correlated with symptom type. Bray–Curtis distances were calculated among leaf segments separately and visualized using nonmetric multidimensional scaling (NMDS) implemented in the 

vegan package. The predictors were collection point and symptom type. The adonis function in the 

vegan package was sensitive to the order in which variables are added, so models were run multiple times, varying the order of predictors, to verify important predictors and we report predictors that were significant regardless of order (following Vannette et al., 2016). We further investigated fungal functional composition by assigning fungal ASVs from the rarefied data to trophic mode (saprotroph, symbiotroph or pathotroph, with some taxa characterized by more than one trophic mode) where possible (Nguyen et al., 2016). When taxa could not be placed, they were labelled, "not assigned."

We also investigated whether the homogeneity of the composition of the fungal leaf microbiome varied with symptom type. For each leaf segment, we quantified the distances from each measured Bray–Curtis distance to the centroid of Bray–Curtis distance for that leaf segment’s symptom type. We then compared the dispersion of the measurements within each symptom type across categories using the betadisper function in the 

vegan package.

Within leaves symptomatic of any of the three parasite feeding strategies, we assessed if the symptomatic leaf segments differed from the asymptomatic leaf segments in the relative abundance of each taxon. We report those taxa that differed between symptom types with a false discovery rate (FDR) cutoff of 0.05, as well as their taxonomic placement as determined by 

blastn against the UNITE fungal ITS database (version 7.2 release date, June 28, 2017, Köljalg et al., 2013) and T-BAS (Carbone et al., 2017, 2019) (Supporting Information).

2.7 | Statistical analyses: Interpretation

To improve statistical inference, we report our results using the language of the "statistical clarity concept," instead of emphasizing statistically significant results (Dushoff et al., 2019). This approach puts forward that the results of null hypothesis significance testing are most usefully interpreted as a guide to whether a certain effect can be seen clearly in a particular context.

3 | RESULTS

From the 252 leaf segments, Illumina generated 6,650,600 ITS1 reads. Of these, 4,483,694 reads passed quality filtering. This represents a mean number of reads per leaf segment of 17,792. Using 

dada2, we identified 2,961 unique ASVs (taxa). This represents a mean number of taxa per leaf segment of 70.8 (median of 62). All taxa placed within the kingdom fungi. In total, 12.5% of taxa could not be placed lower than the kingdom fungi. Of the taxa that could be placed lower than the kingdom fungi, 99.2% placed within Ascomycota (1,459) or Basidiomycota (1,110). At the class level, most taxa within Ascomycota were assigned to Dothideomycetes (808) or Sordariomycetes (307), and most taxa within Basidiomycota were assigned to Agaricomycetes (691) or Tremellomycetes (186). The following analyses consider these 2,961 taxa delineated by 

dada2.

3.1 | Diversity

After accounting for variation in sequencing depth, symptom type strongly and clearly predicted variation in all three numbers in Hill's series (ANOVA p < .0001). There were fewer fungal taxa (Hill’s N0)
and there was lower fungal diversity (Hill’s N1 and N2) in leaf segments that exhibited symptoms of necrotrophic parasites compared to leaf segments that were asymptomatic or symptomatic of either hemibiotrophic or biotrophic parasites (Figure 1; Table S3; Tukey’s HSD, \( p < .01 \)). Specifically, in a pairwise comparison of the model estimates, leaf segments symptomatic of the necrotrophic parasite had, on average, 41.8%–55.2% lower Hill’s N0, 68.4%–77.8% lower Hill’s N1, and 58.9%–71.1% lower Hill’s N2 than leaf segments of all other symptom types. In contrast, there were no clear differences in fungal richness or diversity between asymptomatic leaf segments and those symptomatic of hemibiotrophic or biotrophic parasites (\( p > .05 \)). Finally, there were no clear differences in fungal richness or diversity between asymptomatic leaf segments and leaves symptomatic of any type, whether from leaves with any of the parasite symptoms or leaves that were wholly asymptomatic. Thus, the leaf segments symptomatic of a necrotrophic parasite came from leaves where asymptomatic tissue was not detectably different from any other asymptomatic leaves or leaf segments, which suggests that the fungal diversity of the leaves symptomatic of necrotrophic parasites was not lower than that of other leaves prior to infection by necrotrophic parasites.

To investigate the lower richness and diversity of leaf segments symptomatic of necrotrophic parasites, we considered how the diversity metrics as defined in Hill’s series varied with estimated disease severity (per cent leaf area exhibiting symptoms of a given parasite feeding strategy) within the segments symptomatic of necrotrophic parasites. We had predicted that if a necrotrophic parasite decreases the diversity of the fungal microbiome as it grows within its host, fungal diversity would decrease with necrotrophic parasite disease severity. Instead, fungal diversity weakly increased with necrotrophic parasite disease severity (Figure 2; Table S4; Hill’s N0, \( p = .010 \); Hill’s N1, \( p = .043 \); Hill’s N2, \( p = .064 \)). Because these three correlations were not negative, these results suggest that fungal diversity, particularly richness, does not decrease progressively as a necrotrophic parasite spreads through the leaf.

3.2 | Community composition

We analysed variation in community composition using the Bray–Curtis distance metric. The fungal community composition of leaf segments with symptoms of necrotrophic parasites differed from that of leaf segments that were asymptomatic or symptomatic of other parasites (Figure 3; Table S5; PerMANOVA, \( p = .001 \)). In other words, necrotrophic symptoms were associated with not only fewer fungal taxa, but also a different assemblage of fungal taxa compared to the other parasite symptoms. We had predicted that if a necrotrophic parasite alters the assemblage of fungal taxa as it grows within its host, fungal composition would change with estimated disease severity. Within the leaf segments that exhibited necrotrophic symptoms, disease severity did predict fungal community composition, but accounted for a small amount of variation (Figure 4, PerMANOVA, \( R^2 = .08, p = .043 \)). To test if any parasite symptoms were associated with a more homogeneous fungal community, we quantified beta diversity within each
symptom type as the distances to the centroid from all measured Bray–Curtis dissimilarities among leaf segments of that symptom type. There was no effect of symptom type on the homogeneity of fungal community composition (Figure S2; ANOVA, \( p = .693 \)).

Considering fine-scale variation in the fungal microbiome, within leaves symptomatic of any of the three parasite feeding strategies, the symptomatic leaf segments differed from the asymptomatic leaf segments in the relative abundance of multiple fungal genera (Figures S3–S5).

At that fine scale within symptomatic leaves of any parasite feeding strategy, the symptomatic leaf segments differed from the asymptomatic leaf segments not only in fungal taxonomic composition, but also in fungal functional composition. Specifically, the trophic mode of fungi in leaf segments with symptoms of necrotrophic parasites differed from that of fungi in leaf segments that were asymptomatic or symptomatic of other parasites (Figure 5). Of note, there were clearly fewer putative fungal saprotrophs within leaf segments with symptoms of necrotrophic parasites than segments that were asymptomatic or symptomatic of other parasites (Figure S6A; \( p < .001 \) in all pairwise comparisons). These findings were robust to misspecification of trophic mode (Figure S7). A higher number of
Fungal taxa associated with necrotrophic symptoms were not able to be assigned to a trophic mode compared to taxa associated with other symptom types, but this finding has weak statistical support ($p = .09$; Figure S6b).

**4 | DISCUSSION**

This study used a trait-based analysis of multiple co-occurring fungal parasites in a field population of a grass host to evaluate how the host microbiome is associated with different parasite species. Lower microbiome diversity and distinct composition were associated with symptoms of a parasite with a necrotrophic feeding strategy, and not with parasites of other feeding strategies. These results are consistent with a hypothesis based on niche modification: parasites with traits that more strongly impact the host environment and available niches within the host more strongly impact the host microbiome.

In niche modification, a species changes the types of niches available within a site and, consequently, the identities of species that can colonize the community (Fukami, 2015; Lewontin, 1983). Niche modification is closely related to the concepts of niche construction (particularly with respect to evolutionary implications, Odling-Smee et al., 2003) and ecosystem engineering (Jones et al., 1994) and has been documented in numerous communities of free-living organisms (Fukami & Nakajima, 2011; Naiman et al., 2009). Among parasites infecting plant leaves, only parasites with a necrotrophic feeding strategy create necrotic host tissue throughout their entire infection process (Glazebrook, 2005; Suzuki & Sasaki, 2019). We therefore expected the necrotrophic parasite to be a particularly strong niche modifier that impacts the host environment and, consequently, the host fungal community. While our study system included only one parasite species or morphotype per feeding strategy, symptoms of a necrotrophic parasite were associated with fungal communities of lower diversity relative to asymptomatic leaves, and symptoms of two other types of parasite feeding strategies (biotrophs and hemibiotrophs) were not. Our data do not definitively establish the direction of this association, but these results are consistent with our hypotheses based on feeding strategy.

As a survey and not an experimental manipulation, this study does not establish a causative relationship between parasite infection and host microbiota. While we use our results to evaluate a causative hypothesis related to the niche modifying abilities of parasites, our results are correlative. Furthermore, while we take advantage of a naturally occurring host/multiparasite system in which parasites differ in key traits (namely, parasite feeding strategy), this same system was limited to one representative parasite per strategy. Each feeding strategy was not replicated by multiple parasites, which would be required for a more definitive test of the connection between parasite feeding strategy and variation in the host microbiome. Future studies could address these limitations with other approaches, such as longitudinal surveys across different plant/parasite systems in which microbiota are assayed before and after infection.

Despite these limitations, our hypothesis that necrotrophic parasites are particularly strong niche modifiers was supported by analysis of fungal community composition. The composition of the fungal communities of leaf segments exhibiting symptoms of necrotrophic parasites differed from that of asymptomatic leaves, and there was no clear difference in fungal composition between leaf segments exhibiting symptoms of biotrophic or hemibiotrophic parasites and that of asymptomatic leaves. We hypothesized that this
shift in composition resulted from necrotrophic parasite infections making the host environment more suitable for saprobes (Suzuki & Sasaki, 2019). Inconsistent with this hypothesis, the number of putative saprotophhs was lower in leaf segments exhibiting symptoms of necrotrophic parasites than those that were asymptomatic or exhibiting symptoms of other parasites. However, a higher proportion of fungal taxa associated with necrotrophic symptoms was unable to be assigned a trophic mode. Therefore, while the number of putative saprotophhs does not support our hypothesis, the robustness of this specific test was limited by lack of definitive data on trophic mode. On a more general level, the disproportionately high number of uncharacterized fungal taxa in leaf segments exhibiting symptoms of necrotrophic parasites suggests that necrotrophic parasites were associated with fungal communities of distinct functional composition. Given that the region sequenced here, ITS1, cannot reliably place sequences to species (Dobon et al., 2016; Gazis et al., 2011; Lindner et al., 2011; Porras-Alfaro et al., 2014), future work that sequences the entire ITS region and other loci would be required to further characterize both the taxonomic and the functional composition of these communities.

Fungal community composition was only weakly associated with necrotrophic parasite disease severity, and fungal diversity did not have a negative correlation with disease severity. These results suggest that fungal community composition does not change and diversity does not decrease progressively as the parasite grows within a leaf. The parasite may instead disrupt the host environment, and consequently, the fungal microbiome, upon initial infection. Such microbiome disruption upon initial infection is consistent with evidence from at least one other system; in experimental inoculations of frogs with *Batrachochytrium dendrobatidis*, microbiome diversity declined upon infection and had no relationship with pathogen load (Jani & Briggs, 2014).

While we expected necrotophs to act as particularly strong niche modifiers, we expected hemibiotrophicst to modify their environment as well, given that they create necrotic tissue in the latter part of the infection process (Glazerbrook, 2005; Suzuki & Sasaki, 2019). However, we found contrasting results between necrotrophic and hemibiotrophic parasites; the fungal communities of leaf segments exhibiting symptoms of hemibiotrophic parasites had no clear differences in diversity and composition compared to those of asymptomatic leaf segments. While both hemibiotrophs and necrotophs ultimately require killing host cells, they differ in how they initially interact with host tissue. The initial infection by a necrotophic parasite may be the key stage in which diversity of the microbiome declines. This is consistent with a weakly supported positive correlation between disease severity and fungal taxadiversity that we observed, as diversity was lowest when necrotrophic parasite disease severity was low (i.e., change in diversity occurred early in the infection process).

As an alternative to the niche-modification hypothesis, it is possible that parasite–microbiome associations were instead driven by variation in the fungal microbiome that altered host susceptibility to parasite infection (Croswell et al., 2009; Khosravi & Mazmanian, 2013). Specifically, if a less diverse fungal microbiome made leaf sections particularly susceptible to necrotrophic parasites, this could explain why necrotrophic parasites were associated with a less diverse microbiome in symptomatic leaf segments. Additionally, because necrotrophic parasites differ from hemibiotrophic and biotrophic parasites in how they initially interact with host tissue, this could also explain why this association was only observed for the necrotrophic parasite. However, within each leaf with necrotrophic symptoms, fungal diversity was lower and composition was distinct, on average, only in the leaf segment that was symptomatic, and not in the asymptomatic segment of that same leaf, nor in asymptomatic leaves, which suggests that this mechanism is unlikely. To clarify the cause of the parasite–microbiome associations, researchers could survey the microbiome of individuals before and after natural infection by parasites. However, such longitudinal field sampling of individuals is challenging, because assaying the fungal microbiome of leaves is destructive. Alternatively, researchers could experimentally manipulate the microbiome and subsequently monitor for parasite infections, or experimentally manipulate parasite infection then characterize the microbiome (Berg & Koskella, 2018), but such experiments are also challenging.

While we interrogated relationships between parasites and the fungal microbiome, there is growing evidence that bacterial and fungal microbiota associate with different factors (Elhady et al., 2017; Bergelson et al., 2019). For example, while we found that a biotrophic parasite had no relationship with fungal microbiome diversity, recent work investigating the bacterial microbiome of wheat found that leaves infected with a parasite infecting as a biotroph had higher bacterial diversity than uninfected leaves (Seybold et al., 2020). Such differences between the fungal and bacterial microbiome may result from many factors, including their differences in generation times and abundances within a host. For more complete understanding of parasite–microbiome associations, studies that integrate surveys of the bacterial and fungal communities will be essential (Laforest-Lapointe & Arrieta, 2018; Porras-Alfaro & Bayman, 2011).

In investigations of plant, human and other animal diseases, increasing numbers of studies are characterizing how microbial communities associate with specific parasites and progression of disease (Cho & Blaser, 2012; Jani and Briggs, 2014; Jakuschkin et al., 2016; Lebreton et al., 2019). Here, we propose that functional traits can be used to explain variation among parasites in their associations with host microbiota. Trait-based approaches have played an important role in plant functional ecology (Adler et al., 2014; Cadotte, 2017). More recently, Zanne et al., 2019 and Oliva et al., 2020 advocated complementing traditional and genomic approaches to fungal functional ecology and forest pathology with trait-based approaches. Among traits of parasites, they discuss how parasite feeding strategy (what they refer to as nutritional strategy) can help predict how parasites interact with their abiotic and biotic environment. Integrating trait-based predictions with the concept of niche modification, we suggest that parasite feeding strategy may be a key determinant of parasite–microbiome associations. Ultimately, parasite feeding strategy may
be associated with the stability of the microbiome, which can impact disease severity and host health (Coyte et al., 2015).

ACKNOWLEDGEMENTS
We thank two anonymous reviewers and editor, Valerie McKenzie, for feedback on our work. We thank James Umbanhowar for helpful comments. We thank Anita Simha for help collecting leaves, Maggie Wagner and Posy Busby for the primers used, Julie Geyer for help with the DNA extractions, and Betty Wanjiru for help with the PCR amplifications. This work was supported by the NSF-USDA joint programme in Ecology and Evolution of Infectious Diseases (USDA-NIFA AFRI grant 2016-67013-25762) and the University Cancer Research Fund. K.R.O. was supported by graduate research fellowships from the Triangle Center for Evolutionary Medicine and the National Science Foundation, and F.W.H. was supported by a dissertation completion fellowship from the University of North Carolina-Chapel Hill (UNC-CH) Graduate School and the W.C. Coker Fellowship in Botany from UNC-CH biology department. Development of T-BAS 2.1 was supported by the NSF Genealogy of Life (GoLife) Program (DEB-1541418).

AUTHOR CONTRIBUTIONS
K.R.O., F.W.H., C.D.J., I.C., and C.E.M. conceived ideas and designed methodology; K.R.O. and F.W.H. performed field collections and laboratory work; K.R.O. performed analyses and led writing of the manuscript. All authors contributed critically to drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT
All raw sequence data and related metadata are available at https://doi.org/10.5061/dryad.5x69p8d0v.

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