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Heart rot hotel: fungal communities in red-cockaded woodpecker excavations

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ABSTRACT

Tree-cavity excavators such as woodpeckers are ecosystem engineers that have potentially complex but poorly documented associations with wood decay fungi. Fungi facilitate cavity excavation by preparing and modifying excavation sites for cavity excavators. Associations between fungi and endangered red-cockaded woodpeckers (RCWs) are particularly interesting because these are the only birds that specialize in excavating into the heartwood of living pines, a process that takes years to complete. Using molecular methods, we examined fungal communities in complete and incomplete RCW excavations, and non-cavity control trees. In addition to finding a high diversity of fungi, we found three groupings of fungal communities corresponding to the three groups of trees sampled. We show that trees selected for cavity excavation by RCWs are infected by distinct fungal communities, and propose two hypotheses to explain this outcome: the bird facilitation hypothesis and the tree selection hypothesis.

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Introduction

Fungi play important roles in ecosystem processes and functioning. Although general ecological roles of fungal communities can be identified, specific mechanisms are poorly understood because these communities, in particular wood-inhabiting fungal communities, are poorly described taxonomically (Lindner et al., 2006). This is largely because they are "hyper-diverse" (Hawksworth, 2001; Mueller and Schmit, 2007; Blackwell, 2011) and often can be identified only with molecular tools (Peay et al., 2008). Fungi, most notably those that are capable of decaying wood, are habitat modifiers for avian species that excavate cavities into the stems and branches of trees. Identifying the fungi associated with the trees chosen for excavation is imperative to understanding the interactions between cavity excavators and the fungi that

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inhabit excavation sites, especially in apparently sound trees (Jusino et al., in press). Cavity excavating birds (such as woodpeckers) are ecosystem engineers (Jones et al., 1994) and hence the interactions between cavity excavators and fungi are not only important for the excavators, but also for a diverse community of secondary cavity nesters (Blanc and Walters, 2008), and possibly for the persistence of the fungal communities that may develop in excavated cavities.

Possible associations between wood decay fungi and cavity excavating birds have been considered in multiple systems (Conner et al., 1976; Jackson and Jackson, 2004; Witt, 2010; Blanc and Martin, 2012; Cockle et al., 2012; Zahner et al., 2012); however, these perceived associations are often based on visual observations of fungal fruit bodies. Observation of fruit bodies is a poor measure of association because many fungi can inhabit a tree for decades without fruiting (Rayner and Boddy, 1988; Lindner et al., 2011), while others may never fruit at all. Furthermore, frequency of fruit body production is not comparable between species of fungi, given differences in life cycles and hosts (Rayner and Boddy, 1988). Thus, many fungi associated with cavity excavators may be missed in visual fruit body surveys. Given the potential inaccuracy of fruit body surveys (Boddy, 2001; Jusino et al., in press), it is possible there are unseen fungal players that add levels of complexity to the relationships between cavity excavating birds and fungi (Jusino et al., in press). In the absence of more inclusive data, it is not possible to accurately approach questions about how the community composition of fungi in trees affects the excavation process, and thereby broader ecosystem function. Here we look at communities of fungi in living pine trees that have been selected for excavation by federally endangered red-cockaded woodpeckers (*Picoides borealis*) using a recently developed method for detecting fungi in woodpecker excavations in the absence of fruit bodies (Jusino et al., in press).

Red-cockaded woodpeckers (RCWs) are cooperatively breeding, non-migratory birds that live in family groups (Walters et al., 1988) and are endemic to longleaf pine (*Pinus palustris*) forests of the Southeastern United States. RCWs are primary cavity excavators; they excavate cavities through the sapwood and into the heartwood of living pine trees (Ligon, 1970), a trait unique to this species. Within a family group, each bird has its own roost cavity, resulting in several cavity trees per group. RCW groups also maintain a number of incomplete excavations, which are termed cavity starts. The completed cavities and cavity starts belonging to one RCW family group constitute a cluster.

Red-cockaded woodpeckers are considered to be an umbrella species for the conservation of the longleaf pine ecosystem (Costa, 1995). Management for these birds, which includes frequent burning of forest stands and the establishment of multi-aged pine stands, with emphasis placed on conserving older pine trees, helps maintain ecosystem function and benefits other native residents of the longleaf pine ecosystem (Walters, 1991; James et al., 2001). Older pines are needed for the maintenance of RCW populations because only they have sufficient heartwood to house a woodpecker cavity. Furthermore, older pine trees may be more likely to harbour heartwood-infecting fungi, which may reduce the difficulty of cavity excavation.

Longleaf pines are slower growing, longer lived and more resilient to pathogens than most other pine species of the southeastern United States (Clark, 1957). Additionally, longleaf pines have developed a number of adaptations that allow them to flourish in a fire maintained ecosystem. For instance, they spend the first years of their life in a grass-stage, investing heavily in below ground growth, with their meristem protected from the frequent low intensity fires characteristic of the system. Longleaf pines also produce more resin than many other pines, a trait that may protect the trees from pathogens such as fungi. This is also a trait that RCWs appear to exploit: RCWs maintain active resin wells on trees used for roosting and nesting that may prevent predators from accessing cavities. Because longleaf pines are stronger, more resilient trees, RCW cavities in these trees outlast those in other pine species; however, for the same reasons, longleaf pines generally require more time for cavity excavation (Conner and Rudolph, 1995; Harding and Walters, 2004). It has been speculated that wood decay fungi may assist in this process (Conner et al., 1976; Jackson, 1977; Jackson and Jackson, 2004).

Wood decay fungi require access to a woody substratum, typically in the form of an open wound, in order to grow, reproduce and continue their life cycles (Rayner and Boddy, 1988). Living trees have multiple defenses, including bark, which is an effective physical barrier against many pathogens, and functional sapwood, which is a suboptimal environment for many wood decay fungi because it is composed largely of living cells, has a high volume of water, and contains very little oxygen (Boddy and Heilmann-Clausen, 2008). Thus, in living trees, pathways through the sapwood, which are generally only available following a disturbance, are critical for allowing fungi to penetrate into the heartwood. RCWs may provide this disturbance and facilitate colonization of wood-inhabiting fungi by exposing the interior of an otherwise healthy ("apparently sound") tree through the process of excavation; cavity excavators may indirectly help fungi to spread.

Conversely, there is a growing body of evidence that heartwood-infecting wood decay fungi may be present prior to excavation in the trees that woodpeckers select and that these fungi aid in the excavation process (Conner et al., 1976; Jackson and Jackson, 2004; Witt, 2010; Cockle et al., 2012; Zahner et al., 2012). RCWs in particular are thought to preferentially select trees infected with the heart rot fungus *Porodaedalea pini* SE (the recently described Southeastern clade of *P. pini* s.s.; Brazee and Lindner, 2013) for excavation. Cavity excavation by RCWs in longleaf pines can take 10 yr or longer to complete (Harding and Walters, 2004) and once completed, cavities can remain in use by RCWs for decades (Conner and Rudolph, 1995). Excavation time may be decreased in trees infected with heart rot (Conner and O'Halleran, 1987; Rudolph and Conner, 1991; Jackson and Jackson, 2004).

Thus, the presence or absence of certain species of fungi (not necessarily only decay fungi) may be driving the excavation behaviour of RCWs. Therefore, to understand the habitat requirements of these birds, it is important to focus not only on the forest structure, but also the structure of the communities of fungi that colonize the trees in which these birds excavate. To better characterize the relationship these

birds have with fungi, the taxa involved must first be definitively identified, and the dynamics of the fungal community determined. Not only does one need to know which fungi are in trees currently used by the birds, but also which fungi are in trees they could potentially use in the future. It is possible that incomplete RCW excavations are initially colonized by early successional pioneer fungi, which set the stage for later successional fungal species. The communities of fungi associated with complete RCW excavations could represent a "climax" fungal successional community within a living tree. Pioneer or early-arriving fungi may have an effect on later successional species, not just in modifying the environment for them, but also in determining how the community functions (Fukami et al., 2010; Dickie et al., 2012). RCWs may depend on later successional fungal species to soften the wood surrounding excavation sites – this may explain why the excavation process is so temporally expensive. As a first step toward understanding the relationships between fungi and RCWs, we (1) compared the fungi in RCW excavations to those found in similar trees without excavations to determine which fungi, if any, are closely associated with RCW excavations; and (2) examined the fungi associated with complete and incomplete RCW excavations in order to characterize changes over time in the fungal community associated with RCW excavations.

Materials and methods

Field methods

This research was conducted on Marine Corps Base Camp Lejeune (MCBCL), in Onslow County, on the central coast of North Carolina; see Jusino et al. (in press) for a brief description of the study site. The RCW population on MCBCL has been intensively monitored for over 25 yr (starting in 1986) and has grown from 28 groups in 1986 to 99 in 2013. As part of this ongoing larger study, complete RCW cavity trees and RCW cavity starts are documented as they are located on the landscape and examined annually thereafter. RCWs on MCBCL excavate and use cavities in three commonly found species of pine on the base, longleaf pine, loblolly pine (*P. taeda*), and pond pine (*P. serotina*). Essential components of RCW management include cavity provisioning (creating human-made cavities in living pine trees) and frequent prescribed fires (Walters, 2004).

In Sep. 2009, fifteen RCW clusters were selected on MCBCL and all active, complete RCW cavities (i.e., cavities surrounded by active resin wells, which indicates they are currently being used by a RCW; Jackson, 1977) in each cluster were sampled. Wood shavings were scraped from three locations within each cavity using a sterilized sharpened spoon following the protocol in Jusino et al. (in press). This sampling method allowed collection of wood shavings from excavations without causing damage to the tree or the excavation. DNA from samples collected with a sterilized sharpened spoon can be processed molecularly to determine which fungi are present in the wood surrounding an excavation. Cavity starts within the 15 RCW clusters were also sampled. For each cavity start, the excavation was scraped in two locations and the starter aseptically cored approximately 20 cm above the excavation, using a clean increment borer and sterile sample storage techniques. The increment borer was cleaned by scrubbing the outer portions of the borer and extractor with 70 % ethanol and a sterile cloth, then dipping the borer and extractor in 70 % ethanol. After the dip, the inside of the borer was swabbed with a sterile cotton patch affixed to a rifle cleaning rod (that was also dipped in ethanol). The drill-tip was cleaned with a sterile pipe-cleaner. This cleaning procedure was repeated twice prior to coring each tree. The inside of the handle of the increment borer was also cleaned with 70 % ethanol, and only clean borers were stored in the handle. The extractor was flame-sterilized prior to core extraction. The heartwood of these cores was stored in a sterile 15 ml falcon tube; the sapwood portion was sterilely re-inserted into the core site to prevent the artificial introduction of pathogenic organisms. Completed cavities were not cored at cavity height because it is possible to introduce a fissure in the dome of a cavity when coring, which would allow resin to drip into the body of the cavity and cause harm to the cavity occupant(s); this is not the case for cavity starts.

Additionally, within each of the fifteen clusters, four longleaf pine trees were selected with no evidence of RCW activity but with attributes (such as tree diameter at breast height, and tree height) similar to cavity trees. These trees were cored at average cavity height (following the procedure for RCW cavity starts). In Sep. and Oct. 2009, artificial cavity starts (Copeyon, 1990) were aseptically drilled through the sapwood and into the heartwood of each of these trees at average cavity height, mimicking RCW starts. The artificial starts were sampled for fungi in the same manner as RCW-excavated cavity starts (see Jusino et al., in press for sampling locations) to serve as a control group of non-excavated trees to determine if fungal communities in trees selected for excavation by RCWs are distinct from those in non-excavated trees. After sampling, all of the drilled cavity starts were covered with galvanized steel screens with 0.64×0.64 cm openings to prevent RCW access.

For each tree sampled, we recorded tree species, diameter at breast height (DBH), height of the tree (measured by clinometer), resin well activity (quantified by the freshness of the sap in the resin wells that surround the cavity entrance; trees were classified as either active, possibly active or inactive), presence of *P. pini* SE fruit bodies and age of the excavation (determined from JRW's long-term data set on the RCW population at MCBCL). To better assess habitat differences between clusters (sites), ground cover data were collected in three 20 m transects per cluster. For each transect, 20 readings were taken through an ocular tube, and for each reading, the plant in the center of the ocular tube was identified (James and Shugart Jr, 1970). Ground cover variables were calculated as the average percentage of the ground cover composed of the following: *Astrida* sp. (wiregrass), total herbaceous ground cover (including wiregrass), woody-stemmed ground cover and bare ground. The herbaceous variable consisted of *Astrida* sp., other grasses, *Hypericum perforatum* (Saint John's wort), and unidentified non-woody-stemmed species; this variable was not mutually exclusive from the wiregrass variable. The woody-stemmed variable consisted of: bay species, *Ilex* sp. (gallberry), *Liquidambar styraciflua* (American sweetgum), *Pinus*

saplings, *Quercus* saplings, and unidentified woody-stemmed species.

Molecular methods

To identify the fungal species found in the excavations sampled, DNA was extracted and downstream molecular applications were performed on all samples taken from RCW excavations and the non-excavated trees following the protocol described in Jusino et al. (in press). The downstream molecular applications included polymerase chain reactions (PCR) with the Basidiomycota specific primer pair ITS1F and ITS4b-21 (CAGGAGACTTGTACACGGTCC; Jusino et al., in press), followed by cloning and sequencing. ITS4b-21 amplifies fungi in the Hymenochaetoid clade that are often missed with other common Basidiomycota specific primers (Jusino et al., in press). We also performed PCR, cloning and sequencing with an additional primer pair, ITS1F and ITS4 (Gardes and Bruns, 1993). These methods mirrored those used with ITS1F and ITS4b-21 with the exception of the thermocycler settings, which followed those described by Lindner and Banik (2009). All samples with positive PCR products were cloned, and eight randomly-selected clones per sample were sequenced following Jusino et al. (in press). DNA sequences were edited using Sequencher 4.9 and sequence identities were obtained via GenBank BLAST (NCBI), using a 97 % sequence similarity cut-off for species rank. Samples that did not produce a positive PCR product were rerun using a serial dilution series, and any samples that still did not result in a positive PCR product were considered negative for fungal DNA. In addition to running negative controls for each step, our negative DNA extraction controls were processed through every downstream step. Negative DNA extraction controls included all extraction components and steps used for all samples, but did not include a wood sample.

Data analyses

To compare species richness across excavation types, we used taxon accumulation curves generated by the R package, Species (Czederplitz, 2001). To visualize fungal communities in ordination space for both primer pairs, we performed nonparametric multidimensional scaling (NMDS) in the Vegan package of R (Oksanen et al., 2012) using the metaMDS function with the modified Raup-Crick dissimilarity metric described by Chase et al. (2011), calculated by the raupcrick function in Vegan. Though the Jaccard distance measure accommodates presence/absence data and is well suited for data sets that are populated with many zeroes (McCune et al., 2002), the Raup-Crick metric also takes into account variations in the dissimilarities in community composition and is appropriate when comparing communities in the same geographic region (Chase et al., 2011). To test whether individual sample variables (excavation type, species of tree, DBH, height), or site variables (groundcover) were related to fungal community structure, nonparametric permutational multivariate ANOVA (PERMANOVA) tests (Anderson, 2001) were performed by the adonis function in the Vegan package of R (Oksanen et al., 2012). Differences in multivariate dispersion among groups were tested for using the betadisper function in the Vegan package of R (Oksanen et al., 2012).

For the Basidiomycota specific primer pair, ITS1F and ITS4b-21, community analyses were performed on the entire data set as well as on the subset of the data that included only the taxa likely to be associated with wood decay processes (i.e., putative wood decay fungi). To perform the community analyses for the general primer pair ITS1F and ITS4, which captured fungi from the phyla Ascomycota and Basidiomycota, singletons (taxa that were observed only in one tree) were removed from the community data matrix.

The age of the excavation (zero) and the species of tree (longleaf pine) were the same for all of the trees in the control group and thus the effect of the age of an excavation and species of tree on fungal community structure could only be assessed for the RCW-initiated excavations. The excavation age of RCW excavations was 1–24 yr and the tree species in which these excavations were housed included longleaf, loblolly and pond pines. Community analyses were performed to determine the effect of these variables with the subset of the data that included only completed RCW cavities and RCW-initiated starts with both primer pairs.

Results

PCR results

138 trees were sampled, including 36 complete RCW cavities, 42 RCW cavity starts, and 60 control trees. Of these, 89 % (32/36) with complete cavities, 50 % (21/42) with RCW cavity starts and 28 % (17/60) of control trees produced positive PCR band (bands visible following staining with ethidium bromide) with the Basidiomycota specific primer pair, ITS1F and ITS4b-21. Results were similar with the general fungal primer pair, ITS1F and ITS4; of the trees sampled, 86 % (31/36) with complete cavities, 66 % (28/42) with cavity starts and 27 % (16/60) of control trees produced positive PCR bands. All positive samples were cloned and sequenced.

ITS cloning and sequencing results

We identified 53 fungal taxa via cloning of ITS1F and ITS4b-21 PCR products (Supplementary Appendix A) and 94 taxa via cloning of ITS1F and ITS4 PCR products (Supplementary Appendix B). Taxon accumulation curves for both of the primer pairs indicated that the fungal diversity in living pine trees with and without RCW excavations was much greater than the diversity we were able to document (Fig 1). Accumulation curves for individual samples indicate that the majority of the diversity within samples was captured by picking eight randomly-selected clones (Supplementary Appendix C).

Common taxa

Overall, the most common fungi found with the Basidiomycota specific primer pair (ITS1F/4b-21) were *P. pini* SE, an unidentified Exobasidiomycetes species (Exobasidiomycetes sp. 2, which most closely matched an unidentified Exobasidiomycetidae sp. [GenBank accession number DQ682574.1]

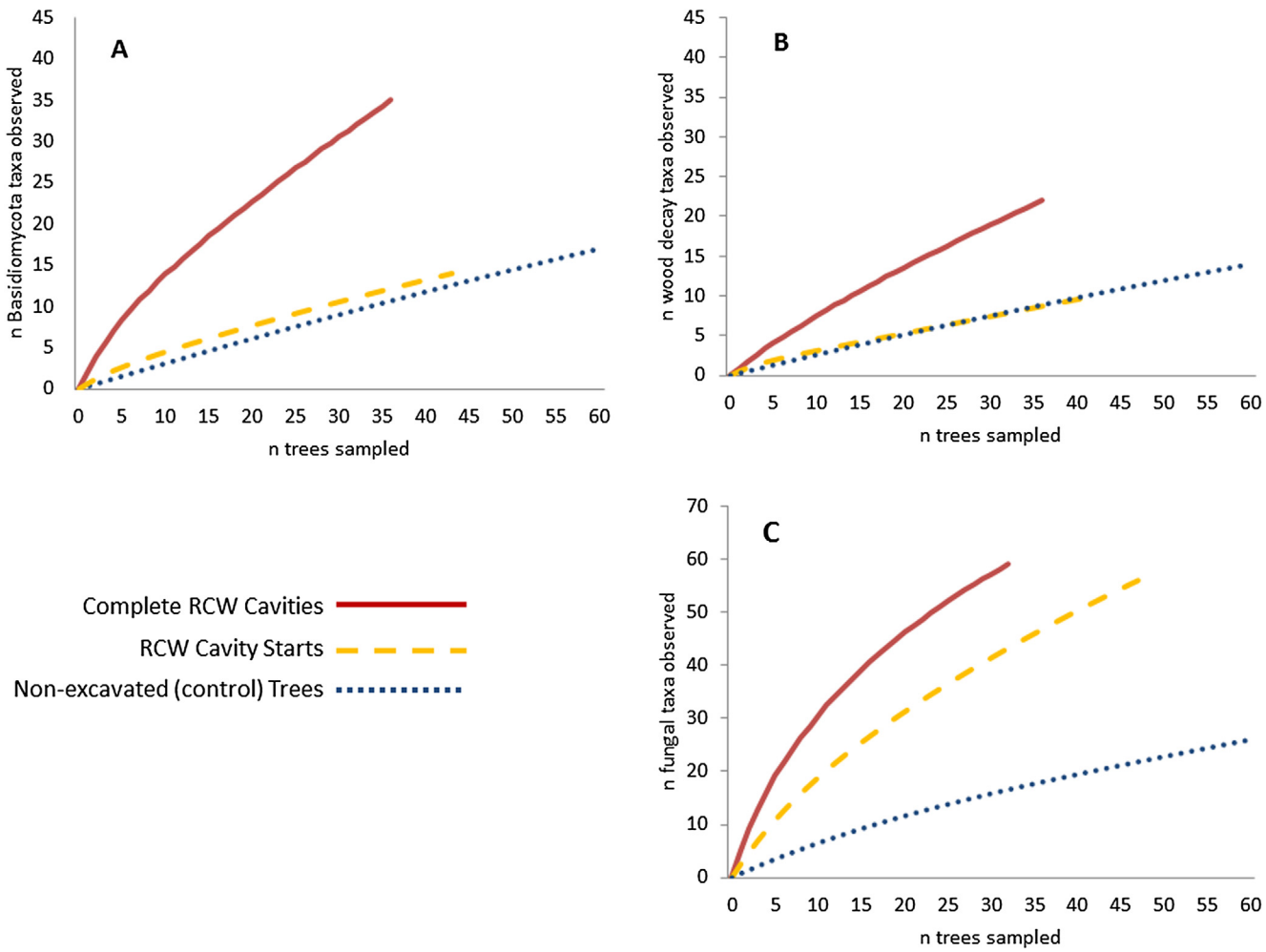

Fig 1 – A) Observed taxon accumulation curves for Basidiomycota, identified with the Basidiomycota specific primer pair, ITS1F and ITS4b-21. Each curve represents the overall Basidiomycota diversity captured in each of the three excavation types sampled. (B) Observed taxon accumulation curves for putative wood decay fungi identified with the Basidiomycota specific primer pair, ITS1F and ITS4b-21. Each curve represents the diversity of putative wood decay fungi in each of the three excavation types. (C) Observed taxon accumulation curves for the general fungal primer pair, ITS1F and ITS4. Each curve represents the overall fungal diversity captured in each of the three excavation types sampled. Note the differences in the scale of the y-axes.

with 96 % similarity), *Acaromyces ingoldii*, and an unidentified *Acaromyces* species (*Acaromyces* sp. 1, which most closely matched *A. ingoldii*). *Porodaedalea pini* SE was found in 23 of the 72 trees that had positive PCR products with ITS1F/4b-21 (Table 1). Exobasidiomycetes sp. 2 was found in 15 of the 72 trees, *A. ingoldii* in 11 and *Acaromyces* sp. 1 in 10. Interestingly, neither of the two *Acaromyces* species nor the Exobasidiomycetes species were found in the control trees. With ITS1F/4b-21, *P. pini* SE dominated the species composition of RCW cavity starts while Exobasidiomycetes sp. 2 was the most

Table 1 – The five most common taxa found with ITS1F/ITS4b-21 in each cavity type, omitting singletons

Complete RCW cavities	*n* trees	RCW cavity starts	*n* trees	Control trees (non-RCW trees)	*n* trees
Exobasidiomycetes sp. 2	13	*Porodaedalea pini* SE	13	*Peniophora incarnata*	2
Acaromyces sp. 1	10	*Acaromyces ingoldii*	3	*Porodaedalea pini* SE	2
Acaromyces ingoldii	8	Exobasidiomycetes sp. 2	2		
Porodaedalea pini SE	8				
Unidentified Basidiomycete 7	4				

common species in complete RCW cavities, followed by *Acaromyces* sp. 1, then *A. ingoldii* and finally, *P. pini* SE (Table 1). 17 taxa were identified in the control trees (non-excavated trees) using ITS1F/4b-21. The most common species were the wood decaying *Peniophora incarnata* and *P. pini* SE, though each of these species was found only in two of the control trees (Table 1).

The most common fungi found using the general fungal specific primer pair, ITS1F and ITS4, were *Sarea resinae* (25 trees), Exobasidiomycetes sp. 2 (23 trees), *Penicillium citreonigrum* (21 trees) and *Toxicocladosporium* sp. 1 (21 trees). With ITS1F/4, Exobasidiomycetes sp. 2 dominated the species composition of complete RCW cavities. *P. citreonigrum* was the most common species in RCW cavity starts and *Fusarium* sp. 1 was the most common fungus in control trees (Supplementary Appendix B).

Decay fungi

Porodaedalea pini SE is known to fruit on living pines in our study site. *Porodaedalea pini* SE was found in 23 trees and was indeed the most common decay fungus found, but also 37 additional decay fungi were identified with ITS1F/4b-21 (Table 2). *Peniophora incarnata*, *Phlebia brevispora*, and *Skeletocutis chrysella* were the second most common decay fungi in our samples; each was found in three trees. 22 taxa of putative decay fungi were identified in complete RCW cavities, 10 in RCW cavity starts and 14 in the control trees. Taxon accumulation curves for decay fungi found with ITS1F and ITS4b-21 (Fig 1B) indicated a higher level of diversity of decay fungi in complete RCW cavities compared to the other two groups of trees (RCW cavity starts and control trees), and that our sampling effort likely captured a larger portion of the diversity of wood decaying fungi in RCW cavity starts and control trees. A number of the unidentified taxa as well as some of the Ascomycota that were cloned may also be associated with the process of wood decay, so the list of putative decay taxa is conservative.

Fungal community analyses

Basidiomycota (ITSIF and ITS4b-21)

Community composition of Basidiomycota was significantly different between the three excavation types (adonis; $r^2 = 0.11$, *pseudo*-F = 4.48, $p < 0.0001$) and the variation between those groups was also different (betadisper; $F = 3.93$, $p = 0.024$). Adonis is sensitive to differences in location and scatter, or dispersion, and betadisper tests only for differences in scatter, thus there may be differences in both, as can be seen in the NMDS visualization (Fig 2). Basidiomycota within complete RCW cavities and RCW cavity starts were much more similar to each other than to those in control trees, and the Basidiomycota communities in the control trees were highly variable (Fig 2A). The DBH of the tree housing the excavation explained some of the differences seen in community composition ($r^2 = 0.05$, *pseudo*-F = 3.76, $p = 0.002$), and there was a weak effect of the percentage of the measured groundcover that consisted of woody-stemmed plants ($r^2 = 0.02$, *pseudo*-F = 1.88, $p = 0.06$). No evidence was found for other site (cluster) effects (percentage herbaceous groundcover, percentage wiregrass, percentage bare ground) or tree height on fungal community composition. Effects of tree age could not be tested for because many older trees with internal decay could not be aged precisely, and tree age cannot be inferred from DBH or height.

Among RCW-initiated excavations, excavation age explained some of the variation in fungal community composition ($r^2 = 0.09$, *pseudo*-F = 5.90, $p < 0.0001$). The excavation type (RCW cavities versus RCW starts) was also significant ($r^2 = 0.08$, *pseudo*-F = 4.52, $p = 0.003$), but there was no variation in dispersion between cavity types ($p = 0.12$). The species of

Table 2 – Likely wood decay fungi found with ITS1F/ITS4b-21, by cavity type

Complete RCW cavities	*n* trees	RCW cavity starts	*n* trees	Control trees (non-RCW trees)	*n* trees
Porodaedalea pini SE	8	*Porodaedalea pini* SE	13	*Peniophora incarnata*	2
Phlebia brevispora	3	Agaricomycetes sp. 1	1	*Porodaedalea pini* SE	2
Coniophora sp. 1	2	Polyporales sp. 1	1	Unidentified Basidiomycete 46	1
Postia sericeomollis	2	Polyporales sp. 4	1	*Athelia arachnoidea*	1
Agaricomycetes sp. 8	1	*Skeletocutis* sp. 1	1	*Ceriporiopsis* sp. 1	1
Agaricomycetes sp. 11	1	*Stereum* sp. 4	1	*Collybia subnuda*	1
Athelia arachnoidea	1	*Trichaptum* sp. 1	1	*Irpex lacteus*	1
Athelliales sp. 2	1	Unidentified Basidiomycete 17	1	*Peniophora* sp. 2	1
Coprinellus sp. 1	1	Unidentified Basidiomycete 42	1	*Polyporus squamosus*	1
Corticiaceae sp. 1	1	Unidentified Basidiomycete 54	1	*Schizophyllum commune*	1
Corticiaceae sp. 3	1			*Skeletocutis* sp. 1	1
Peniophora incarnata	1			*Skeletocutis* sp. 2	1
Peniophora sp. 2	1			*Trichaptum biforme*	1
Polyporales sp. 4	1			*Trichaptum* sp. 2	1
Russulales sp. 1	1				
Serpula himantioides	1				
Skeletocutis sp. 1	1				
Stereum sp. 1	1				
Trametes versicolor	1				
Unidentified Basidiomycete 38	1				
Unidentified Basidiomycete 49	1				
Xeromphalina campanella	1				

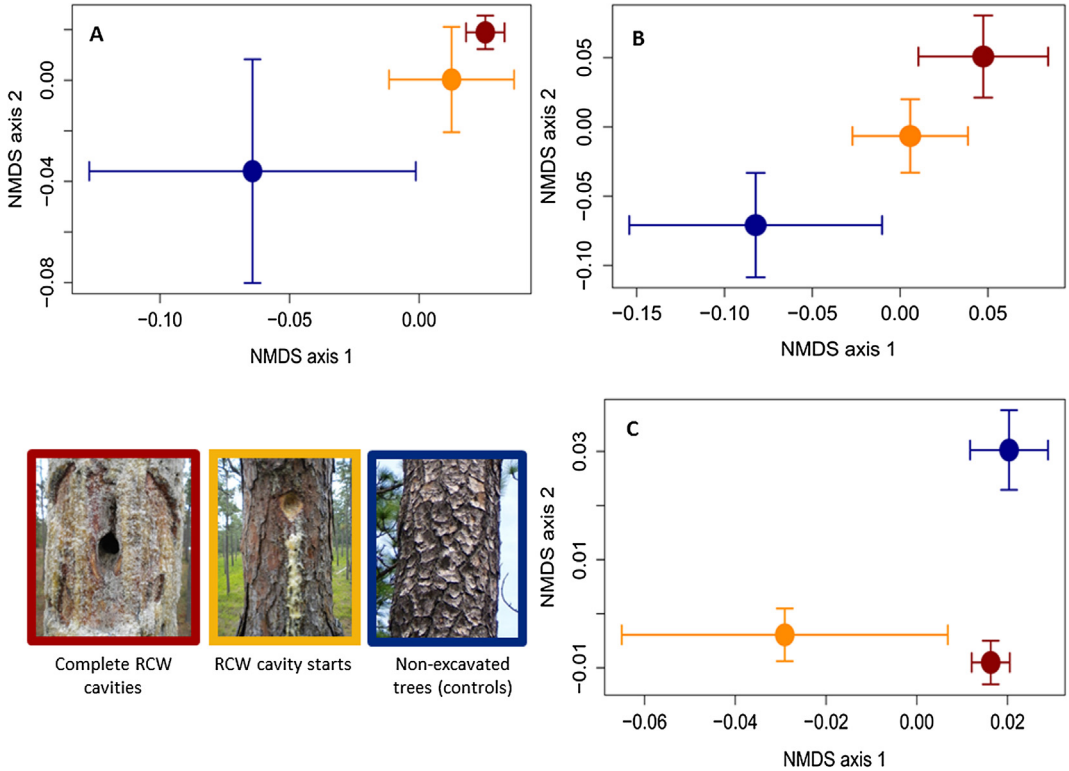

Fig 2 – NMDS plots for fungal communities found in completed RCW cavities (red), RCW cavity starts (yellow), and non-excavated (control) trees (blue). The central dots represent the means of the points on the two NMDS axes, the bars represent one standard error from the mean along both axes. (A) NMDS plot for ITS1F and ITS4b-21, Basidiomycota only, stress = 0.0062, two dimensions, 200 iterations. (B) NMDS plot for the subset of putative wood decay taxa identified with ITS1F and ITS4b-21. Stress = 0.0013, two dimensions, 200 iterations. (C) NMDS plot for ITS1F and ITS4, which includes Ascomycota and Basidiomycota, with singletons removed, stress = 0.0989, two dimensions, 200 iterations.

tree housing the excavation explained some of the differences in community composition in this subset ($r^2 = 0.05$, *pseudo*-F = 3.42, $p = 0.009$), and there was also a dispersion effect ($F = 3.42$, $p = 0.07$).

Decay fungi (ITSIF and ITS4b-21)

Although the communities of putative wood decay fungi in RCW cavity starts and control trees were similar in diversity (Fig 1B), they differed in that the community composition was distinct and much less variable among RCW cavity starts compared to the control trees (Fig 2B). The wood decay community in RCW excavations, especially RCW cavity starts, was dominated by one fungus (*P. pini* SE). The PERMANOVA results confirmed a significant difference in wood decay fungal community composition between excavation types ($r^2 = 0.09$, *pseudo*-F = 2.46, $p = 0.004$), and the betadisper results confirmed that the variation between excavation types was also different ($F = 6.19$, $p = 0.006$). The DBH of the tree housing the excavation was weakly significant and explained some of the differences seen in community composition ($r^2 = 0.035$, *pseudo*-F = 2.00, $p = 0.037$). None of the other variables tested had significant effects on the wood decay communities.

Ascomycota and Basidiomycota (ITSIF and ITS4)

The general communities of Ascomycota and Basidiomycota within RCW cavities and RCW cavity starts also were more similar to each other than to those in control trees (Fig 2C). Community composition of fungi within the three excavation types was significantly different ($r^2 = 0.11$, *pseudo*-F = 4.22, $p < 0.0001$), and there was no dispersion effect ($p = 0.29$). In addition, the percentage of groundcover composed of woody stems ($r^2 = 0.04$, *pseudo*-F = 3.16, $p = 0.008$) explained some of the variation in fungal community composition. The visualization of the NMDS with ITS1F and ITS4 is represented in the abundances of taxa listed in Supplementary Appendix B. Each excavation type was dominated by three or four different taxa, but there was overlap between complete and incomplete RCW cavities.

The effect of excavation age on fungal community composition, with ITS1F and ITS4 using the subset of data from RCW excavations, was significant ($r^2 = 0.05$, *pseudo*-F = 3.16, $p = 0.008$). Excavation type also had a significant effect ($r^2 = 0.04$, *pseudo*-F = 2.53, $p = 0.03$), but tree species did not.

Discussion

Our results have implications for RCW cavity excavation dynamics and help to illustrate the complexity of fungal communities in living trees. To our knowledge, this study is the first to use DNA-based methods to describe fungal communities within the wood surrounding woodpecker

excavations in living trees and to show that there may be a specific community of fungi associated with cavities that have been excavated by birds. Our study is also unique in describing fungal communities within the heartwood of healthy, living pine trees.

We successfully identified fungal species present and fungal community structure in RCW-initiated excavations and in trees without excavations, demonstrating that fungal communities in trees without excavations are highly variable and do not resemble those found in RCW excavations. We have also shown that over 100 fungal species are present in complete and incomplete RCW excavations, in contrast to previous work, which focused on *P. pini*, a decay species that is known to fruit on living longleaf pines. Taxon accumulation curves (Fig 1) indicate that our sampling did not capture all of the diversity present in these trees, and yet even with the high diversity of taxa present, fungal community structure in excavated trees was consistently distinct from that of non-excavated trees. This was seen with both primer pairs that were tested (ITS1F/ITS4b-21, Fig 2A; and ITS1F/ITS4, Fig 2C) and with the putative wood decay fungi (Fig 2B). Cloning does not capture the full diversity within a sample, and it would hence be interesting to compare our cloning results to those obtained with a different method that captures more diversity, such as next-generation amplicon sequencing.

It is important to note that our ITS1F and ITS4 dataset had an abundance of singletons, which masked community level differences. Upon removal of all singletons in the ITS1F and ITS4 data set, we were able to show a clear structuring of fungal communities representative of the structuring we detected in the Basidiomycota and putative wood decay communities. ITS1F and ITS4 is a general fungal primer pair (Gardes and Bruns, 1993), which detects fungi in Ascomycota as well as Basidiomycota; thus many of the taxa we detected with ITS1F and ITS4 may be cosmopolitan fungi. These cosmopolitan fungi are also likely to be pioneer fungi and may help prime the excavation environment for later successional fungal species. The differentiation observed in the fungal communities in trees with RCW excavations versus control trees with ITS1F and ITS4 indicates that specific associations between fungi may give rise to both the Basidiomycota community (detected with ITS1F/4b-21) and the wood decay community associated with RCWs. The cosmopolitan fungi detected with ITS1F and ITS4 may help facilitate the composition of the Basidiomycota decay community and they could also be associated with the process of wood decay.

We sampled active (i.e., in use by a RCW) cavities for our study, and found the number of years a tree housed an excavation was a significant predictor of fungal community structure. However, "inactive" RCW cavities, defined as cavities that are not being used by a RCW, are often utilized by a suite of other species after they are abandoned by RCWs. If we sampled living trees with older, inactive RCW cavities, we might expect to find a fungal community dominated by more advanced decay species. Fungal communities in old, inactive RCW excavations in living trees may represent climax communities of fungal succession in cavities in living trees. These communities would presumably be more characteristic of living trees in decline, with visible signs of decay, and may also be associated with secondary cavity nesters that utilize inactive RCW cavities. Such cavities should be targeted for sampling in future work, in addition to documenting potential shifts in the fungal community after a tree dies. Cavities in living trees may also play an important role in the development of wood-inhabiting fungal communities, and may serve as habitat refugia for some fungi. Tracking fungal community development through time in RCW cavity starts may also provide some insight into fungal community assembly dynamics in healthy, living, non-inoculated trees.

Our data suggest a number of interesting questions, including whether wood decay fungi other than *P. pini* SE are important to RCWs and whether cosmopolitan fungi play a role in the cavity excavation process. For example, *A. ingoldii*, one of the most common fungi we found in complete RCW cavities and RCW cavity starts (Table 1), has been shown to have fatal effects on mites (Gerson et al., 2008) and phytopathogenic fungi (Kushnir et al., 2011). It is possible that *A. ingoldii* attacks mites or other fungi that are detrimental to the birds. If *A. ingoldii* attacks mites, these could be either feather mites that parasitize the birds or mites that prey upon the fungi that aid in the excavation process. Given their predominance, *Acaromyces* fungi could be instrumental in preparing the excavation site for the fungal communities associated with RCW excavations. Indeed, these fungi could help initiate fungal community succession in cavity starts.

Fire plays an important role in the structuring of longleaf pine ecosystems. A well-burned longleaf pine stand is an open park-like savanna, with ground cover dominated with bunchgrasses such as wiregrass or bluestem (*Andropogon* sp.), and containing a diverse community of herbaceous plants (Peet, 2006; Walker and Silletti, 2006). Woody-stemmed plants are correlated with insufficient burning, resulting in poor RCW habitat quality in longleaf pine ecosystems (James et al., 1997, 2001). We found that the percentage of groundcover composed of woody-stemmed plants explained some variation in fungal community structure. Given that all of the trees we sampled were in active RCW clusters, all of which are currently maintained by frequent low intensity burns, it is difficult to determine if the weak relationship to woody stems we saw is a result of differing fire management histories in the RCW clusters we sampled, or some other factor. Changes in fire management regimes could affect fungal communities in a variety of ways. For example, decreases in burn frequency could eventually lead to changes in forest composition, resulting in forested stands composed of pines and hardwoods, with significantly more dead, unburned wood on the forest floor. These conditions could induce changes in fungal habitat availability, making the heartwood of living pine trees a less desirable substratum. This could be tested by comparing our fungal community data to fungal communities in longleaf pines in forest stands on MCBCL that are not frequently burned.

Decay fungi

We identified 22 taxa of putative wood decay fungi in complete RCW cavities, 10 in RCW cavity starts and 14 in control trees (38 overall). The high diversity of decay fungi in our control trees was surprising, given that these were generally healthy, living trees with no visible signs of decay. We

discovered a number of species of wood decay fungi in RCW excavations such as *P. incarnata* and *P. brevisporia* that were not previously documented to be associated with these birds (Table 2). Still, the fungal species with which RCWs have long been thought to have an interesting relationship, *P. pini* SE, was the most prevalent decay fungus found in completed RCW cavities and cavity starts (Table 2). The limited diversity of decay fungi and the abundance of *P. pini* SE in cavity starts indicates that the birds are either (1) selecting trees with a preferred decay community ("tree selection hypothesis"), or (2) selecting trees or sections of trees without any evidence of decay, then subsequently facilitating infection of specific fungi during the excavation process. The birds could facilitate infection either directly, by carrying fungi on their bodies, or indirectly by changing the microhabitat within the tree ("bird facilitation hypothesis").

Tree selection hypothesis

The fungal communities in the trees without excavations (control trees) are highly variable, while the communities in complete RCW cavities and cavity starts are much more consistent (Fig 2). The variation in the fungal communities in trees without excavations lends support to the tree selection hypothesis. The control trees represent the trees available for RCW excavation; all trees in this group had aspects similar to trees excavated by RCWs and were located within active RCW clusters. Thus, in the absence of tree selection, one would expect to find similar levels of fungal diversity and community variation in control trees and recently initiated RCW cavity starts. We did not see evidence of this in our data. The excavation age of RCW-initiated starts influences fungal community composition but the fungal communities in recently initiated RCW starts differ from those in trees without excavations ($r^2 = 0.08$, *pseudo*-F $= 2.45$, $p = 0.008$).

If RCWs are indeed selecting certain trees for excavation, they may do so based on cues associated with the fungi present within a tree. The birds could also select trees for excavation based on cues indicating which fungi are absent from the tree, versus which are present; not all fungi are helpful. Some have speculated that cavity excavators may use fungal fruit bodies as visual cues when selecting excavation sites (Savignac and Machtans, 2006; Witt, 2010; Zahner et al., 2012). However, earlier work showed that RCWs do not use fungal fruit bodies as visual cues for excavation (Rudolph et al., 1995). RCWs could, however, use acoustic and/or olfactory cues to evaluate the suitability of trees for excavation, including the presence of fungi.

Manipulative experiments with fungal volatiles could be conducted to see if RCWs preferentially select trees based on olfactory cues emitted by wood decay fungi such as *P. pini* SE. Acoustic cues would be difficult to manipulate but could be assessed with an instrument that measures the density or the resistance of wood, such as a Resistograph. Resistographs electronically assess the resistance of wood, which is thought to be correlated with decay, but Resistograph data cannot be used to accurately assess the causes of decay (Costello and Quarles, 1999). A recent study that utilized Resistographs to examine the incidence of decay in black woodpecker (*Dryocopus martius*) cavity starts demonstrated that trees selected for excavation by black woodpeckers were more likely to have low wood resistance values indicative of decay than control trees (Zahner et al., 2012). Black woodpecker cavity starts showed evidence of decay, as detected by a Resistograph, 94 % of the time (Zahner et al., 2012), whereas we were able to identify the fungal taxa likely responsible for decay in 45 % of the RCW cavity starts sampled. Like RCWs, black woodpeckers are primary cavity excavators that can take years to finish an excavation and use existing cavities for years (Meyer and Meyer, 2001; Gorman, 2011). Our data are not directly comparable to those of Zahner et al. (2012); still, the black woodpecker study supports the tree selection hypothesis, suggesting it may apply beyond the RCW system.

Bird facilitation hypothesis

An alternative to the tree selection hypothesis is that RCWs directly or indirectly facilitate colonization of particular fungal species, which we term the "bird facilitation hypothesis". We see some support of this hypothesis in the finding that fungal communities in RCW cavity starts are more similar to those in completed cavities than those in control trees. Moreover, there appears to be a successional shift in the fungal community with the RCW cavity starts representing a stage between the control trees and the completed cavities. Further, the communities in RCW cavity starts become more like those in complete cavities with time. Although the role of cavity starts in fungal community development is not yet clear, it seems reasonable to assume that cavity starts can serve as fungal infection courts. The bird facilitation hypothesis could be tested by monitoring fungal community development in human-constructed cavity starts drilled into control trees and comparing fungal communities in starts available for use by RCWs to those to which they are denied access. By tracking changes in fungal communities in the trees that were accessible and inaccessible to RCWs, one could determine if creating the type of wound in a tree that a cavity start represents is sufficient to facilitate a change in the fungal community or whether direct access by RCWs is necessary.

Conclusion

It is clear that *P. pini* SE is an important player in this system, as suggested by previous studies, but our results also demonstrate that there are many other fungi associated with these birds. We cannot yet determine if RCWs are selecting trees with certain types of fungi (tree selection hypothesis) or if they are facilitating fungal colonization via cavity starts (bird facilitation hypothesis).

Though we focused on the excavations of one unique bird species in one ecosystem, it is likely that similar patterns can be seen in both excavated and non-excavated (or "naturally formed") cavities across the world. Research on the fungi associated with tree cavities and cavity excavators in other systems may help ensure the maintenance of biodiversity and could be further applied to retain important ecosystem components.

Finally, like many others before us, we have also effectively demonstrated that there is a hidden level of fungal

biodiversity that is difficult to characterize without DNA-based tools. Without question, we have only just begun to scrape the surface of fungal diversity in RCW excavations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2014.11.002.

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