# The fungal communities associated with Red-cockaded Woodpeckers and their excavations: descriptive and experimental evidence of symbiosis

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## Abstract

Cavity-excavating birds, such as woodpeckers, are ecosystem engineers and are often assumed to rely upon wood decay fungi to assist in softening the wood of potential excavation sites. Endangered red-cockaded woodpeckers (*Picoides borealis*; RCWs) are the only birds known to solely excavate through the sapwood and into the heartwood of living pine trees and generally take many years to complete their excavations. These birds may have developed a partnership with wood-inhabiting fungi to facilitate the excavation process. Past attempts to understand the complex relationships between cavity excavators and fungi relied on visual surveys of fruiting bodies, or evidence of decay, resulting in a one bird, one fungus paradigm. Using molecular methods, I investigated the relationships between RCWs and fungi, and found that the relationships between cavity-excavators and fungi involve multiple fungal species and are far more complex than previously imagined. Through a field survey, I showed that RCW excavations contain distinct communities of fungi, and propose two hypotheses to explain this result, (1) RCWs select trees with distinct fungal communities (tree selection hypothesis), or (2) RCWs promote distinct fungal communities via their excavations (bird facilitation hypothesis). By swabbing the birds, I found that RCWs carry fungal communities similar to those found in their completed excavations, demonstrating that RCWs may directly facilitate fungal

dispersal during the excavation process. Through a test of the bird introduction hypothesis which implemented human-made experimental drilled cavity starts (incomplete excavations), half of which were inaccessible to the birds, I showed that RCW accessibility influences fungal community development in excavations. This experimental evidence demonstrates that the relationship between RCWs and fungal communities is a multipartite symbiosis may be mutualistic. Finally, by tracking fungal community development in experimental cavity starts through time, I also demonstrated that the fungal communities found in RCW excavations undergo succession, and that this process is influenced by the birds. The relationships described in this body of work provide the basis for future studies on cavity excavators and fungi, and also have implications for a diverse community of secondary cavity nesters, wood-inhabiting fungi, forest ecology, and the conservation of biodiversity.

## Dedication

This dissertation is dedicated to all of the people, animals,

and fungi that I learned from along the way.

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**Chapter 1. A minimally invasive method for sampling nest and roost cavities for fungi: a novel approach to identify the fungi associated with cavity-nesting birds**

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ABSTRACT

Relationships among cavity-excavating birds, trees, and wood decay fungi pose interesting management challenges and research questions in many systems. Ornithologists need to understand the relationships between cavity-nesting birds and fungi in order to understand the habitat requirements of these birds. Fruiting body surveys are typically used to identify the fungal players in these relationships. This approach enables nondestructive sampling, but vastly underestimates fungal presence and diversity and may miss species of critical importance to cavity-nesting birds; thus new methods for such analyses are necessary. We designed a novel technique to nondestructively sample the wood surrounding excavations, which produces samples that can be processed using DNA-based methods to identify fungi. We tested our method on Red-cockaded Woodpecker (*Picoides borealis*) excavations, half of which were from trees with *Porodaedalea pini* fruiting bodies. Using our new approach, we detected *P. pini* in 90% of the trees with fruiting bodies, but also in 60% of the excavations without fruiting bodies and identified nine additional taxa of wood decay fungi that did not have fruiting bodies present. Our approach offers improved detection of fungi through non-destructive sampling of excavated

cavities and an improved primer specific to the fungal phylum that contains most wood decay fungi (Basidiomycota), thus providing managers and researchers a critical tool to better determine which fungi are important to cavity-nesting birds.

*Keywords*

Cavity-excavating birds and fungi; Cavity-nesting birds and fungi; Basidiomycota specific primer; ITS4b-21; *Phellinus pini*; *Porodaedalea pini*; Red-cockaded Woodpecker (*Picoides borealis*); Wood decay fungi

## Introduction

Primary and secondary cavity-nesting birds rely heavily upon tree cavities. Heartwood infecting fungi help create suitable excavation habitat for cavity-excavating birds by softening the wood surrounding excavation sites (Conner et al. 1976, Jackson and Jackson 2004, Witt 2010, Cockle et al. 2012, Zahner et al. 2012). Fungi also create non-excavated or "naturally formed" cavities which are used by cavity-nesters in many systems (Cornelius et al. 2008, Cockle et al. 2011, Wesołowski 2012). However, very little is known about which fungi actually inhabit the wood surrounding excavated and non-excavated cavities. A better understanding of the fungal inhabitants of excavation sites and sites of naturally occurring cavities is necessary in order to better understand the dynamics of cavity-nesting communities and inform forest management affecting these species.

Some studies have named specific wood decay fungi associated with cavity-excavating birds. For example, Red-cockaded Woodpeckers (*Picoides borealis*) have long been thought to have an association with *Porodaedalea pini* (previously *Phellinus pini*), a fungus that infects the heartwood of the living pine trees in which the birds excavate (Ligon 1970, Conner et al. 1976,

Jackson and Jackson 2004). Cockle et al. (2012) recently suggested that fungi in the family Polyporaceae facilitate cavity excavation in Argentina. Many others have noted that *Phellinus tremulae* - one of the most common causes of decay in live aspen - may be important for cavity-excavating birds in aspen-dominated systems (Hart and Hart 2001, Savignac and Machtans 2006, Witt 2010).

Traditionally, correlations between cavity-excavating birds and wood decay fungi have been based on surveys of external fruiting bodies observed on trees selected by birds for excavation. However, this method is biased to the fungal species fruiting at the time of sampling and thus, a limited fungal community is observable with this method (Blanc and Martin 2012, Cockle et al. 2012). Reliance on visual surveys could cause important fungal species that rarely produce fruiting bodies or produce microscopic or cryptic fruiting bodies to be completely overlooked. Even among those species producing visible fruiting bodies, while the presence of a fruiting body indicates a tree is infected by a fungal species, the absence thereof is not indicative of the absence of fungi. Fungal mycelia can be present within the heartwood of a tree for years, even decades before producing a fruiting body and hence, fruiting body surveys drastically underestimate the prevalence of fungi (Lindner et al. 2011b), as well as the diversity of fungi inhabiting individual trees. Rather, fruiting bodies provide information about which of the fungi present in a tree happen to be fruiting at the time of the survey (Boddy 2001). Furthermore, the presence of a fruiting body on the trunk of a tree may represent a localized fungal infection and does not demonstrate that the entire tree, including the cavity excavation location, has been affected. The columns of decay above and below fruiting bodies are highly variable across host and fungal species (Silverborg 1954). Some heartwood decaying fungi can be responsible for decay in other areas of a tree (e.g. the sapwood) and thus are not necessarily affecting the

excavation site. Similarly, fruiting bodies of fungi that are known as agents of heart rot are not always reliable indicators of the presence of heart rot - for example, *Ganoderma applanatum* can be an agent of both heart and butt rot (Ostry et al. 2011). Thus, fruiting body surveys tell us little about which fungal species are actually in the wood surrounding a cavity that has been excavated.

Reliance on fruiting body surveys to examine the relationships between cavity-nesting birds and wood decay fungi have important management implications in promoting preservation of trees with existing cavities and visible signs of decay. While it is important to preserve trees with existing cavities and visible symptoms of decay, infected trees that do not have visible signs of decay (i.e. apparently sound trees) may be equally or even more important to cavity-nesting birds (Blanc and Martin 2012). For birds like Red-cockaded Woodpeckers, which use their cavities for many years, sometimes decades, living trees without visible signs of decay may be more suitable for excavation than living trees with visible signs of decay because cavities in the former will endure longer. This holds true for dead trees as well. While most cavity excavators complete excavation in dead trees in a relatively short amount of time and only utilize their cavities for one breeding season, it is important that they select trees that are sound and will withstand natural stochastic events; trees with some internal decay but without fruiting bodies fit this description. Furthermore, when a tree with a cavity remains on the landscape for multiple years, it is beneficial to the non-excavating community of cavity users as well as excavating species (Blanc and Walters 2008). Birds often select trees without visible signs of decay for excavation, perhaps purposefully selecting trees that do not have fruiting bodies and thus have not yet entered an advanced stage of decay.

Currently, several methods exist to detect the presence of internal decay in living trees, such as increment coring, destructive sampling (large-scale dissection), the use of Resistographs and sonic and electrical impedance tomography (Brazee et al. 2011). In most cases, these methods only measure the soundness of wood, either visually (coring and destructive sampling) or via resistance (Resistographs). None of these methods allow researchers to determine the identities of the actual agents of decay, and thus like fruiting body surveys, are limited in their utility in documenting fungal communities.

The Red-cockaded Woodpecker, an endangered, cooperatively breeding bird that is an important cavity excavator in the longleaf pine ecosystem of the southeastern United States (Ligon 1970, Walters et al. 1988) may have a particularly interesting relationship with heartwood infecting fungi due to its excavation behavior. Uniquely, Red-cockaded Woodpeckers solely excavate their cavities in the heartwood of living pine trees, and the time to complete this process can range from less than one year to over 15 years (Harding and Walters 2004). These birds have many unique traits, but understanding their excavation behavior is an aspect that continues to be critical to successful management efforts of these endangered birds (Walters 1991).

In an effort to better understand Red-cockaded Woodpecker habitat requirements and help inform forest management decisions based on these requirements, we are interested in studying the communities of fungi found in Red-cockaded Woodpecker excavations, and the role of these fungi in the excavation process. To aid this effort, we designed a tool to collect scrapings from the wood surrounding Red-cockaded Woodpecker cavities in a non-destructive, minimally invasive manner. These samples were processed with molecular methods using a known fungal specific primer (ITS1F; Gardes and Bruns, 1993) and a newly designed reverse primer (ITS4b-21; described herein) specific for fungi in the phylum Basidiomycota, the group

that contains most wood decay species, to determine the presence of fungi within the wood surrounding the cavity. Our new, non-destructive sampling tool, primer, and method not only aid in determining whether or not a tree selected for excavation is decayed, but also allow for the identification of the main agents of decay within the excavation site. This novel approach can be used to help guide future management decisions that focus on the preservation of trees suitable for excavation, not only for Red-cockaded Woodpeckers but also for other avian species in other systems.

## Methods

### *Study Site*

We conducted this research on Marine Corps Base Camp Lejeune (MCBCL), in Onslow County, on the central coastline of North Carolina, USA. This military installation consists of 110,000 acres of land and 26,000 acres of water. Of the pine stands on MCBCL that are not heavily mixed with hardwoods, 64% are dominated by loblolly pine (*Pinus taeda*), 24% by longleaf pine (*Pinus palustris*) and 11% by pond pine (*Pinus serotina*) (Walters 2004). The only heart rot fungus we observed fruiting on living pine trees on MCBCL was *Porodaedalea pini* s.l. (Southeastern clade), hereafter referred to as *P. pini* SE. Fruiting body surveys of this close relative of *Porodaedalea pini* s.s. (see description of *Porodaedalea* sp. 1 in Brazee and Lindner 2013) would suggest that this species is not found in abundance on MCBCL. Over four years of intermittent but intense visual surveying of our study site, we located only 24 *P. pini* SE fruiting bodies in Red-cockaded Woodpecker habitat on MCBCL. Approximately 2% of the trees housing complete or incomplete, human-made (drilled) or Red-cockaded Woodpecker-made excavations had *P. pini* SE fruiting bodies. The proportion of pine trees in similar habitat with *P.*

*pini* SE fruiting bodies and without excavations is less than 1% (M. A. Jusino personal observation).

*Sampling Tool*

In order to collect wood from the interior of Red-cockaded Woodpecker cavities, we designed a specialized tool that can scrape and capture wood shavings from within a cavity without causing destruction of the cavity itself. This tool can be sterilized and easily used at cavity height. Our sampling tool, hereafter called "the sharpened spoon," was designed with ease of sampling in mind. By design the spoon is less dangerous to use for wood collection at cavity height than other tools such as increment borers, chisels or drills. The sharpened spoon enables the collection of wood samples from the interior of cavities or cavity starts and causes much less damage to the tree or the interior of the cavity than drills, chisels, and increment borers.

The prototype of our sampling tool consisted of a 20 gauge stainless steel ½ teaspoon measuring spoon, a 0.33 meter long ¼-20 threaded steel rod and a ¼-20 steel hex nut (Figure 1.1a). We cut the handle of the spoon to a length of 5 centimeters. A 5 centimeter long slot was created in one end of the rod with a grinding wheel affixed to a rotary Dremel tool (model # 8000-05, Robert Bosch Tool Corporation, Mount Prospect, Illinois). The spoon was test fitted into the rod and its width adjusted to allow fitting of the ¼-20 hex nut over the spoon handle. After test fitting, the pieces were disassembled and a sufficient amount of paste flux was applied to the slot within the rod and the spoon handle to allow for a clean solder connection. The pieces were re-assembled and the ¼-20 nut was threaded onto the rod, locking the spoon handle into place. To ensure the pieces would not loosen over time, we soldered the spoon handle into the rod with a flux-core solder and a propane torch. Finally, we sharpened the outer rim and the inside edge of the spoon with a small grinding stone affixed to the Dremel tool and metal files.

Sharpening the inside edge allowed the spoon to scrape the target wood more efficiently (Figure 1.1b). A 0.3 centimeter hole was drilled through the rod opposite the spoon end, allowing a 2.5 centimeter metal key ring to be added for attaching the tool to a belt for field use (Figure 1.2).

*Sample Collection*

We tested our collection tool against two other methods of decay detection, visual fruiting body surveys and core samples at cavity height, using 20 trees on MCBCL with Red-cockaded Woodpecker excavations, 10 trees without fruiting bodies and 10 trees with fruiting bodies (all fruiting bodies were *P. pini* SE). We photographed and aseptically sampled fruiting bodies, but did not collect them because they are very rare on our study site. One of us (MAJ) climbed each selected tree with Swedish climbing ladders, a climbing harness and a tree strap. Once cavity height was reached, the sharpened spoon was flame sterilized with 70% ethanol. We collected samples from two to three locations within the cavity depending on whether the tree housed a fully excavated cavity or a cavity start (i.e. incomplete excavation (Figure 1.3). We flame sterilized the spoon between each sampling location.

The sampling locations within the cavity were determined by how accessible they were with the spoon (Figure 1.3). Sampling location A refers to the entrance tunnel of the cavity, and represents the sapwood of the tree. Location B refers to the dome of the cavity and consists of heartwood. Location C refers to the back wall of the cavity, and consists of heartwood. Location E refers to the front of a cavity start and is sapwood whereas location D refers to the back of a cavity start and is typically heartwood. If a start was not advanced enough to have reached the heartwood, we collected D or E, but not both.

We used the sharpened spoon to scrape wood shavings from each sampling location. Some force was used to effectively scrape the wood and gather the shavings in the bottom of the

spoon. After each scrape, the sharpened spoon was extracted from the cavity and the sample was transferred from the spoon into a sterile 1.5 mL tube. Though each scrape could be considered a separate sample, our data are pooled for this analysis because our goal is to show that the sharpened spoon can be used to identify agents of decay in the wood surrounding Red-cockaded Woodpecker excavations.

Additionally, the cavity starts in our sample were aseptically cored approximately 20 cm above the cavity entrance using a sterilized borer (Figure 1.3 F). The heartwood of these cores was kept; the sapwood portion was re-inserted into the core site to prevent the 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.

*DNA Extraction, PCR, Cloning and Sequencing*

Scraped wood samples and fruiting body samples were stored in 200 µl of filter-sterilized CTAB cell lysis solution (see Lindner and Banik 2009 for solution protocol) and frozen at -80º C. DNA extraction followed the protocol described by Brazee and Lindner (2013). Increment core samples were placed in 30 mL of filter-sterilized CTAB cell lysis solution (Lindner and Banik 2009) and ground using a sterilized immersion hand blender. After the cores were ground, they were frozen at -80º C for at least 24 hours. Subsequent extraction of DNA from cores followed a modified version of the protocol described by Brazee and Lindner (2013). Frozen samples were placed in a 65º C water bath for 3 hours. Then one mL of supernatant was transferred to a 1.5 mL tube and centrifuged at 10,000 rcf for 10 minutes and 100µL of supernatant was transferred to a new strip tube. Negative controls were included for both

extraction protocols, and these extractions served as our negative controls for each downstream step.

We tested standard PCR, cloning and sequencing protocols on *Porodaedalea pini* SE fruiting body DNA using a primer pair that is reportedly specific for fungi in the phylum Basidiomycota, ITS1F and ITS4b (Gardes and Bruns 1993). We had limited success directly sequencing or cloning and sequencing *Porodaedalea pini* SE DNA using this primer pair, even after significant manipulation of variables such as annealing temperature, number of PCR cycles, etc. In order to ensure we successfully extracted *Porodaedalea pini* SE DNA, we performed PCR, cloning and sequencing using the general fungal primer pair ITS1F and ITS4 (Gardes and Bruns 1993), and had no issues obtaining sequences using these primers. Because we wanted to ensure detection of fungi from the phylum Basidiomycota, and especially fungi in the Hymenochaetoid clade, we performed an analysis of mismatches between known sequences from the Hymenochaetoid clade and the primer ITS4b. Based on this analysis, we designed a new primer specific for Basidiomycota, ITS4b-21 (CAGGAGACTTGTACACGGTCC), which is derived from ITS4b (Gardes and Bruns 1993) and which had fewer mismatches for members of the Hymenochaetoid clade. Preliminary testing determined that ITS4b-21 amplifies members of the Hymenochaetoid clade of fungi, including *Porodaedalea pini* SE, that are not amplified by ITS4b (M. A. Jusino and D. L. Lindner personal observation). Many important white-rot fungi belong to the Hymenochaetoid clade, and therefore their detection is vital to studies of fungi associated with cavity excavation.

We performed PCR on all samples taken with the sharpened spoon and the increment borer using ITS1F and ITS4b-21. Our PCR protocol was similar to the protocol for ITS1F and ITS4 used by Lindner and Banik (2009); however, the thermocycler conditions differed (see

below). We diluted the 5x green GoTaq reaction buffer to a 7/6x working concentration and we used 0.0416 units of GoTaq DNA Polymerase per microliter of reaction volume. We also added 0.133 mL of 3% polyvinylpyrrollidone (PVP) per microliter of each PCR reaction. All other PCR component concentrations can be found in Lindner and Banik (2009). Our thermocycler conditions were as follows: initial denaturing at 95º C for 5 minutes; 15 cycles of denaturing at 94º C for 30 seconds, annealing at 55º C for 45 seconds and extension at 72º C for 1 minute; 25 cycles of denaturing at 94º C for 30 seconds, annealing at 52º C for 45 seconds and extension at 72º C for 1 minute; a final extension at 72º C for 10 minutes.

After amplification, we ran the PCR products on a 1.5% agarose gel stained with ethidium bromide (hereafter, gel). Reactions with visible amplification products were cloned and sequenced following the protocol described by Lindner and Banik (2009) and the sequencing protocols described by Lindner et al. (2011a). We did not sequence in both directions; ITS4 was used for all sequencing reactions. We edited our sequences with Sequencher 4.9 (GeneCodes Corp.). Sequences were assigned identities based on BLAST comparisons to GenBank (NCBI) sequences.

## Results

### *PCR Amplification*

Successful PCR amplification was confirmed via gel electrophoresis for 18 of the 20 trees sampled (90%), with varying rates of success achieved by sampling method (scrapes or cores) and presence of fungal fruiting bodies. Specifically, 18 of the 20 trees (90%) sampled with the sharpened spoon (scrapes) and 6 of the 13 (46%) that were cored produced PCR products that could be visualized on a gel. Of the 10 trees that had fruiting bodies, 9 of the 10 (90%) that were

scraped and 5 of the 7 (71%) that were cored produced PCR products that could be visualized on a gel. Of the ten trees without fruiting bodies, 8 of the 10 (80%) that were scraped and 1 of the 6 (17%) that were cored produced PCR products that could be visualized on a gel. None of the negative controls produced PCR products that could be visualized on a gel.

*Cloning and Sequencing Results*

PCR products were ligated and cloned regardless of whether they could be visualized on a gel, although the only samples that produced viable bacterial colonies were those that could be visualized. Our cloning results are summarized in Table 1.1. The most prevalent heartwood infecting fungus we identified in our sample was *P. pini* SE, which we successfully cloned and sequenced from 16 of the 20 (80%) trees that we sampled. We successfully identified *P. pini* SE from spoon scrapes in 16 of the 20 (80%) trees sampled. We also identified 9 other heartwood infecting fungal taxa from spoon scrapes from 8 of the 20 (40%) trees sampled: *Agaricomycetes* sp. 1, *Atheliaceae* sp. 2, *Atheliales* sp. 3, *Peniophora cinerea*, *Peniophora* sp. 4, *Phlebia brevispora*, *Postia sericeomollis*, *Stereum* sp. 4, and *Trametes versicolor* (Table 1.1). *Porodaedalea pini* SE was the only fungus detected from the core samples.

Among trees with *P. pini* SE fruiting bodies (i.e. trees that are known to be infected with a wood decay fungus), we were able to detect *P. pini* SE in 9 of the 10 (90%) trees that were sampled with the sharpened spoon and in 5 of the 7 (71%) core samples. We detected *P. pini* SE in 1 of the 6 (17%) core samples from trees without fruiting bodies. However, when these same trees were scraped, we detected wood decay fungi in 8 out of 10 (80%) trees, including 6 detections of *P.pini* SE. We did not detect any fungi in cores or scrapes from 2 of the 10 (20%) trees without fruiting bodies (Table 1.1).

## Discussion

We have effectively demonstrated that our novel protocol provides a considerable improvement over traditional methods for detecting fungi associated with cavity-excavating and cavity-nesting birds. Our method can be used to identify fungi that would otherwise be undocumented because of the absence of fruiting bodies and the failure of other methods to detect these fungi. When combined with molecular methods and the primer we designed (ITS4b-21), our collection tool can be used to help determine the incidence and identity of decay fungi as well as the other fungal species (lacking fruiting bodies) present within the wood surrounding an excavation site. By identifying the fungi present we can better determine which fungi are associated with cavity excavators and cavity-nesters. This information can be used to inform habitat management for cavity-nesting species.

We found the sharpened spoon to be more useful in detecting fungi than the increment borer in both complete and incomplete Red-cockaded Woodpecker excavations. We also found that molecular methods can be used to detect fungi from both types of samples. However, we detected less Basidiomycota diversity in the cores than in the samples taken with the sharpened spoon. The sharpened spoon helped us capture multiple species of fungi that were not externally apparent and that were not observed with the core samples.

We were able to sample within the actual cavity or cavity start with the spoon and safely sample the heartwood of completed cavities without the risk of creating a fissure in the top of the cavity, which is one potential problem with heartwood cores at cavity height. Creating a fissure in the dome of a cavity would allow resin to leak into the cavity, thus introducing a possible risk to any bird using the cavity. Additionally, samples taken with the sharpened spoon require much less time for collection and processing compared to samples taken with the increment borer. It is

possible for an experienced climber to sample more than sixteen cavities in an eight-hour field day with the sharpened spoon, twice as many as when an increment borer is used. The lab processing time for the cored samples is also a consideration as these samples require a vigorous sterile grinding step prior to DNA extraction. Various grinding methods were tested prior to the immersion blender that was used in this work; the immersion blender was far more efficient than other common sample grinding methods, yet was still laborious compared to the processing of spoon-collected samples.

The cloning and sequencing procedure used in this study does not capture every fungal species present in each sample, but is much more cost effective than pyrosequencing or other similar "next-generation" DNA sequencing methods, especially since one Basidiomycota species (*P. pini* SE) dominated the fungal population. We detected more than one fungal taxon in 12 of the 20 trees we tested and may have detected even more taxa if we had used a more general primer pair capable of capturing more than one fungal phylum, such as ITS1F and ITS4 (Gardes and Bruns 1993). We did not use a more general fungal primer pair for our PCR reactions because we were specifically interested in determining if the samples collected with the sharpened spoon could be used to detect wood decay fungi. Fungi from the phylum Ascomycota are very common and tend to dominate samples (based on preliminary data using general primers; data not shown), and fungi from this phylum are not known to be important contributors to wood decay in living conifers. Wood decay fungi from the phylum Basidiomycota would have been amplified with a more general primer pair, but we increased our chances of successfully detecting such species by using a more specific primer pair. By using our modified primer, ITS4b-21, we were able to detect wood decay fungi in the Hymenochaetoid clade that otherwise would have been missed.

While the presence of a fungal fruiting body indicates that a fungus is present in a tree, it does not indicate that the fungus has spread throughout the entire stem of a tree. Thus, when sampling Red-cockaded Woodpecker excavations in trees with *P. pini* SE fruiting bodies, one should not always expect to find *P. pini* SE in the wood surrounding the cavity. This may explain the instances in which either the sharpened spoon or increment borer failed to detect *P. pini* SE in trees with fruiting bodies. However, that *P. pini* SE was detected in the core sample taken near the cavity in one of these instances, and in the spoon sample but not the core sample in the other two instances, suggests that we did not always detect every heart rot species present in the trees we sampled with our methods. We note that all of these detection failures were associated with incomplete rather than complete excavations. Nevertheless, we have effectively demonstrated that the absence of a fruiting body does not imply the absence of a fungus given that we successfully detected *P. pini* SE from 6 of the 10 (60%) trees without fruiting bodies as well as six other wood decay fungi, from four such trees.

Recently, it has been suggested that primary cavity excavators use fungal fruiting bodies as visual cues to select trees suitable for excavation (Hart and Hart 2001, Witt 2010, Zahner et al. 2012). This hypothesis has only been tested once, by Rudolph et al (1995), who found it to be false for Red-cockaded Woodpeckers. Rudolph et al (1995) attached *Porodaedalea pini* fruiting bodies to 40 trees within 10 active Red-cockaded Woodpecker territories, using 40 other trees within those territories as controls. After three years, none of the trees with fruiting bodies were used by Red-cockaded Woodpeckers, though the birds excavated a cavity start in one control tree.

Some researchers have suggested that primary cavity excavators use acoustic cues to detect decay in a tree (e.g. Zahner et al., 2012). It is possible that some birds assess the density of

wood acoustically, although this would be difficult to test for most cavity excavators. There are multiple other cues and cue combinations that primary cavity excavators can use to determine trees and locations on trees suitable for excavation, including olfactory signals. The use of our new method will help researchers determine if cavity excavators select excavation sites based on the presence or absence of certain fungal species. If they do, the mechanism behind excavation site selection can be tested.

Our results suggest that trees without visible signs of decay may harbor wood-decaying fungi and thus be a potentially important resource for cavity-nesting birds. For researchers, internal detection of rot via acoustic methods is an improvement over visual surveys; however the information gained from these methods is based on the density of wood. One does not learn the identity of important hidden players in a fungal community by simply examining a substrate visually or acoustically. In order to understand whether specific fungi or specific groups of fungi are important to cavity-excavating and cavity-nesting birds, it is necessary to determine which fungal groups are associated with the nest and roost cavities of these birds. Our novel approach allows us to more robustly determine the fungi present in the wood surrounding excavations, a critical first step in resolving the relationships between cavity users and fungi so that forests can be managed effectively for cavity-nesters and cavity excavators.

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**Figure and Table Captions**

Figure 1.1

Our sampling tool is made of a sharpened measuring spoon, a steel rod and a hex nut.

Figure 1.2

An outline of our sampling tool with the components labeled. A indicates the threaded steel rod; B is the key ring hold; C is the five centimeter long slot; D is the hex nut, E is the sharpened measuring spoon.

Figure 1.3

A cross section of a complete cavity on the left, and a cross section of a cavity start on the right. These show approximate sampling locations as well as approximate heartwood and sapwood dimensions. A - E are scrape samples: A & E are in the sapwood while B, C & D are in the heartwood. F is the approximate coring site.

Table 1.1

Whether or not a fruiting body was present on the sampled tree, by type (complete cavity or cavity start), the total number of taxa cloned from the scrape and core samples, and the identities of the wood decay fungi found (in both scrapes and cores). NA denotes not applicable; not all completed cavities used in this analysis were cored.

*These taxa may be associated with processes other than the decay of heartwood.

Figure 1.1: Sampling tool

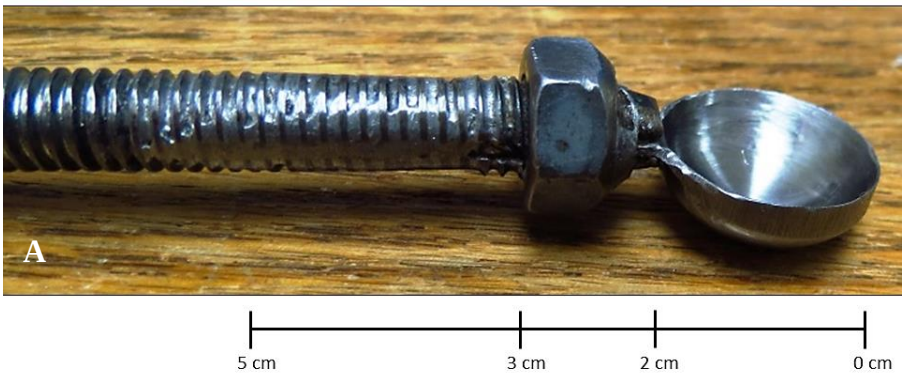


Figure 1.2: An outline of the sampling tool with the components labeled

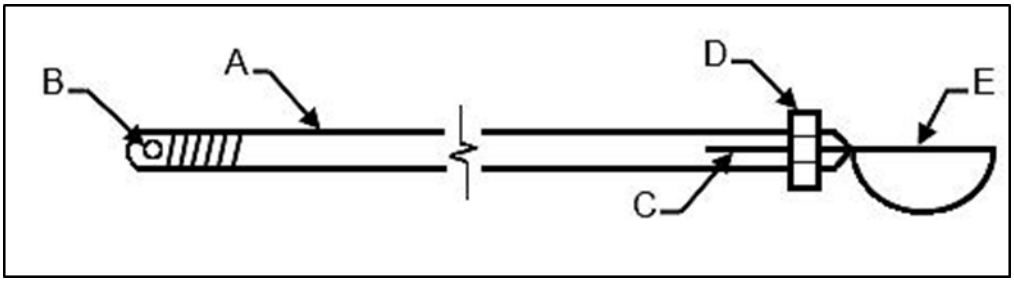

Figure 1.3: Cross sections of a complete cavity and a cavity start

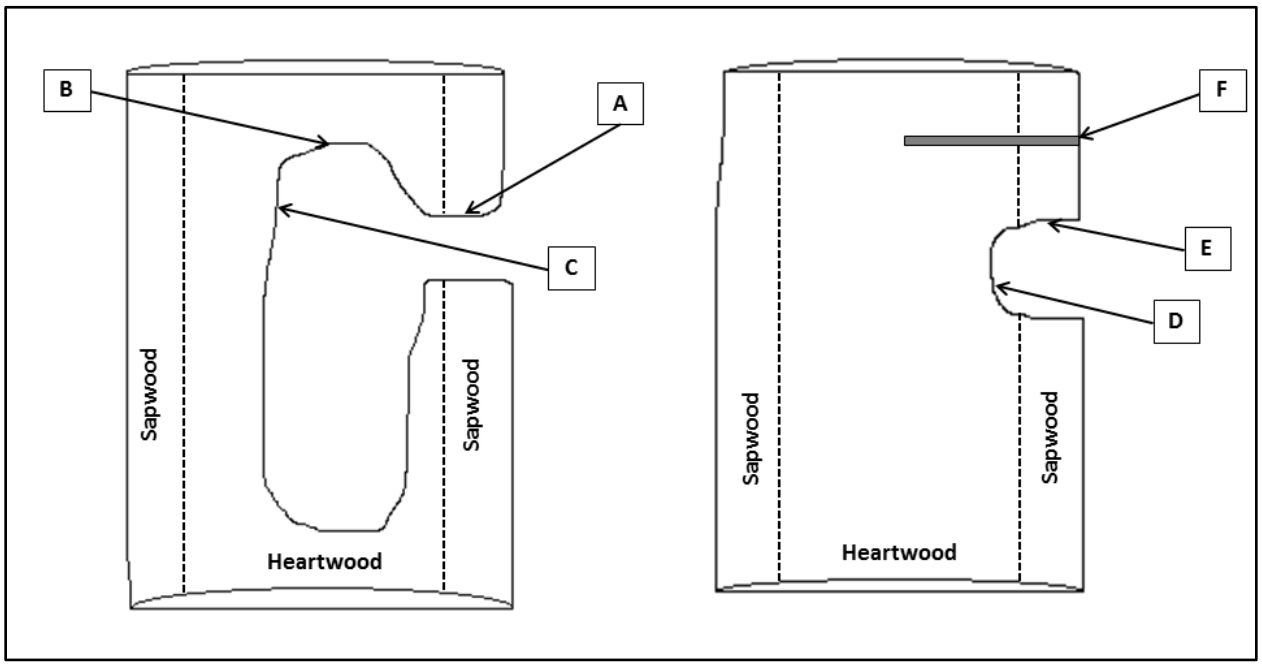

Table 1.1: Fruiting body presence on sampled trees, by excavation type, the total number of taxa cloned from the scrape and core samples, and the identities of the wood decay fungi found

| *P. pini* SE fruiting body present? | Excavation type | n taxa from scrapes | Heartwood infecting fungal taxa from scrapes | Nearest GenBank match (accession number, identity match) | n taxa from core | Heartwood infecting fungal taxa from cores |
|---|---|---|---|---|---|---|
| Yes | Complete | 6 | *Porodaedalea pini* SE<br>*Atheliales* sp. 3* | JX110039.1, 100%<br>JN943909.1, 92% | 1 | *Porodaedalea pini* SE |
| Yes | Complete | 5 | *Agaricomycetes* sp. 1*<br>*Porodaedalea pini* SE<br>*Peniophora* sp. 4 | EF694649.1, 99%<br>JX110039.1, 100%<br>EF672293.1, 99% | NA | |
| Yes | Complete | 3 | *Porodaedalea pini* SE | JX110039.1, 100% | NA | |
| Yes | Complete | 2 | *Porodaedalea pini* SE | JX110039.1, 100% | NA | |
| Yes | Start | 5 | *Porodaedalea pini* SE | JX110039.1, 100% | 1 | *Porodaedalea pini* SE |
| Yes | Start | 2 | *Phlebia brevispora*<br>*Porodaedalea pini* SE | AB084616.1, 99%<br>JX110039.1, 100% | 0 | |
| Yes | Start | 2 | *Porodaedalea pini* SE | JX110039.1, 100% | 1 | *Porodaedalea pini* SE |
| Yes | Start | 2 | *Porodaedalea pini* SE<br>*Trametes versicolor* | JX110039.1, 100%<br>KC176344.1, 99% | 0 | |
| Yes | Start | 1 | *Porodaedalea pini* SE | JX110039.1, 100% | 1 | *Porodaedalea pini* SE |
| Yes | Start | 0 | | | 1 | *Porodaedalea pini* SE |
| No | Complete | 6 | *Phlebia brevispora*<br>*Postia sericeomollis* | AB084616.1, 99%<br>KC585367.1, 98% | NA | |
| No | Complete | 5 | *Phlebia brevispora* | AB084616.1, 99% | NA | |
| No | Complete | 4 | *Atheliaceae* sp. 2<br>*Peniophora cinerea*<br>*Porodaedalea pini* SE<br>*Trametes versicolor* | GU187502.1, 96%<br>GU062269.1, 99%<br>JX110039.1, 100%<br>KC176344.1, 99% | NA | |
| No | Complete | 1 | *Porodaedalea pini* SE | JX110039.1, 100% | NA | |
| No | Start | 2 | *Porodaedalea pini* SE<br>*Stereum* sp. 4 | JX110039.1, 100%<br>JX460856.1, 98% | 0 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| No | Start | 1 | *Porodaedalea pini* SE | JX110039.1, 100% | 0 | |
| No | Start | 1 | *Porodaedalea pini* SE | JX110039.1, 100% | 1 | *Porodaedalea pini* SE |
| No | Start | 1 | *Porodaedalea pini* SE | JX110039.1, 100% | 0 | |
| No | Start | 0 | | | 0 | |
| No | Start | 0 | | | 0 | |

## Chapter 2. 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.

**Keywords:** cavity excavators and fungi; fungal communities in excavations; fungal communities in living trees; red-cockaded woodpecker (*Picoides borealis*); wood decay fungi; woodpeckers and fungi.

## 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 inhabit excavation sites, especially in apparently sound trees (Chapter 1). 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 fruiting bodies. Observation of fruiting 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 fruiting 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 with visual fruiting body surveys. Given the potential inaccuracy of fruiting body surveys (Boddy 2001; Chapter 1), it is possible that there are unseen fungal players that add a level of complexity to the relationships between cavity excavating birds and fungi (Chapter 1). 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 processes, and thereby affect broader level 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 the absence of fruiting bodies (Chapter 1).

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 harbor 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 substrate, 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 spread.

Conversely, there is a growing body of evidence that heartwood-infecting wood decay fungi may be present prior to excavation in the trees 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 *Porodaedalea pini* SE (the recently described Southeastern clade of *Porodaedalea pini* s.s.; Brazee and Lindner 2013) for excavation. Cavity excavation by RCWs in longleaf pines can take ten years 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 behavior 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. In order 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. In order to track fungal community changes over time, one needs a baseline, or a snapshot of which fungi occur in the longleaf pine ecosystem and which, if any, are closely associated with RCW excavations. We examined the fungi associated with complete and incomplete RCW excavations as well as the fungi found in similar trees without 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 Chapter 1 for a brief description of the study site. The RCW population on MCBCL has been intensively monitored for over 25 years (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 (*Pinus taeda*), and pond pine (*Pinus serotina*). Essential components of RCW management include cavity provisioning (creating human-made cavities in living pine trees) and frequent prescribed fires (Walters 2004).

In September 2009, we selected fifteen RCW clusters on MCBCL and sampled all active RCW cavities (i.e., cavity trees with active resin wells, which indicates they are currently being used by a RCW; Jackson, 1977) in each cluster. Wood shavings were scraped from three locations within each cavity using a sterilized sharpened spoon following the protocol in Chapter 1. This sampling method allowed us to collect wood shavings from excavations themselves 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 (Chapter 1). For each cavity start we scraped the excavation in two locations and aseptically cored the starts approximately twenty centimeters 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, we then swabbed the inside of the borer 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, we selected four longleaf pine trees with no evidence of RCW activity but with attributes similar to cavity trees. These trees were cored at average cavity height (following the procedure for RCW cavity starts) and breast height. In September and October 2009, artificial cavity starts (Copeyon 1990) were aseptically drilled into the heartwood of each of these trees at average cavity height. The starts were drilled through the sapwood and into the heartwood to mimic RCW starts. The starts were sampled for fungi in the same manner as RCW cavity starts (see Chapter 1 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 x 0.64 centimeter openings to prevent RCW access.

For each tree sampled, we recorded tree species, diameter at breast height (DBH), height of the tree (attained from a 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 *Porodaedalea pini* SE fruiting 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), we collected ground cover data in three twenty meter transects per cluster. For each transect, we took twenty readings through an ocular tube, and for each reading, we identified the plant in the center of the ocular tube (James and Shugart Jr 1970). Our 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 is 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, we extracted DNA and performed downstream molecular applications following the protocol described in Chapter 1. The downstream molecular applications included polymerase chain reactions (PCR) with the Basidiomycete specific primer pair ITS1F and ITS4b-21 (Chapter 1), followed by cloning and sequencing. 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 the Basidiomycete specific primer pair ITS1F and ITS4b-21 with the exception of the thermocycler settings, which followed those described by Lindner and Banik (2009). In addition to running negative controls for each step, our negative DNA extraction controls were processed through every downstream step. 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 level calls.

*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, we used nonparametric permutational multivariate ANOVA (PERMANOVA) tests (Anderson 2001) performed by the adonis function in the Vegan package of R (Oksanen et al. 2012). For the Basidiomycete specific primer pair, ITS1F and ITS4b-21, we performed community analyses on the entire data set as well as on the subset of the data that included only the likely wood decaying taxa identified with ITS1F and ITS4b-21. In order 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 our 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 ranged from 1 year to 24 years, and the tree species in which these excavations were housed included longleaf, loblolly and pond pines. We performed community analyses 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*

We sampled 138 trees, including 36 complete RCW cavities, 42 RCW cavity starts, and 60 control trees. Of the trees sampled, 89% (32/36) with complete cavities, 50% (21/42) with 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. Our 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 (Appendix A) and 94 taxa via cloning of ITS1F and ITS4 PCR products (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 (Figure 2.1). Accumulation curves for individual samples indicate that most of the diversity in a sample is captured by picking 8 clones (Appendix C).

#### *Common taxa*

Overall, the most common fungi found with the Basidiomycota specific primer pair (ITS1F / 4b-21) were *Porodaedalea pini* SE, an unidentified Exobasidiomycetes species (Exobasidiomycetes sp. 2, which most closely matches an unidentified Exobasidiomycetidae sp. [GenBank accession n DQ682574.1] with 96% similarity), *Acaromyces ingoldii*, and an

unidentified *Acaromyces* species (*Acaromyces sp.* 1, which most closely matches *A. ingoldii*). *Porodaedalea pini* SE was found in 23 of the 72 trees that had positive PCR products with ITS1F / 4b-21 (Table 2.1). 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 common species found in complete RCW cavities, followed by *Acaromyces* sp. 1, then *A. ingoldii* and finally, *P. pini* SE (Table 2.1). We identified 17 taxa in the control trees (non-RCW trees) using ITS1F / 4b-21. The most common species were the wood decaying fungi *Peniophora incarnata* and *Pordaedalea pini* SE, though each of these species only was found in two of the control trees (Table 2.1).

The most common fungi we 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. *Penicillium citreonigrum* was the most common species found in RCW cavity starts and *Fusarium* sp. 1 was the most common fungus found in control trees (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 we found, but we also identified 37 additional decay fungi with ITS1F / 4b-21 (Table 2.2). *Peniophora incarnata*, *Phlebia brevispora*, and *Skeletocutis chrysella* were the second most common decay fungi found in our samples; each of them was found in three trees.

We identified 22 taxa of likely decay fungi 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 (Figure 2.1b) 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 those groups.

*Fungal community analyses*

*Basidiomycota (ITS1F and ITS4b-21)*

Community composition of Basidiomycota fungi was significantly different between the three excavation types ($r^2 = 0.11$, *pseudo*-F = 4.48, $p < 0.0001$). Basidiomycota fungi within complete RCW cavities and cavity starts were much more similar to each other than to those in control trees (Figure 2.2a). 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 we found a weak effect of the percentage of the measured groundcover that consisted of woody-stemmed plants ($r^2 = 0.02$, *pseudo*-F = 1.88, $p = 0.06$). We did not find evidence for other site (cluster) effects (percentage herbaceous groundcover, percentage wiregrass, percentage bare ground) or tree height on fungal community composition. We could not test for effects of tree age 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, $p = 0.003$),

and the species of 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).

*Decay fungi (ITS1F and ITS4b-21)*

Although the communities of likely wood decay fungi found in RCW cavity starts and control trees are similar in diversity compared to those found in RCW cavities (Figure 2.1b), they differ in that the community composition is distinctly different and much less variable among RCW excavations compared to the control trees (Figure 2.2b). The wood decay community in RCW excavations, especially RCW cavity starts is dominated by one fungus (*Porodaedalea pini* SE). The PERMANOVA results confirm a significant difference in wood decay fungal community composition between excavation types ($r^2$ = 0.09, *pseudo*-F = 2.46, p = 0.004). 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 (ITS1F and ITS4)*

The general communities of Ascomycota and Basidiomycota fungi within RCW cavities and cavity starts were also more similar to each other than to those found in control trees (Figure 2.2c). Community composition of fungi found within the three excavation types was significantly different ($r^2$ = 0.11, *pseudo*-F = 4.22, p < 0.0001). 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 Appendix B. We found that each excavation type was dominated by three or four different taxa, but there was overlap between complete and incomplete RCW cavities.

We examined the effect of excavation age on fungal community composition with ITS1F and ITS4 using the subset of data from RCW excavations and again found a significant effect ($r^2$ = 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 *Porodaedalea pini*, a decay species that is known to fruit on living longleaf pines. Taxon accumulation curves (Figure 2.1) 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, Figure 2.2a; and ITS1F / ITS4, Figure 2.2c) and with the likely wood decay fungi (Figure 2.2b). Cloning does not capture the full diversity within a

sample, and it would be interesting to compare our cloning results to results obtained with a different method that captures more diversity, such as next-generation 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 likely 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 such as wood decay fungi. 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 Basidiomycete 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 Basidiomycete decay community and they could also be associated with the process of wood decay. Our NMDS results support this hypothesis (Figure 2.2).

We sampled active (i.e. in use by a RCW) excavations 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 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 who 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.

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, James et al. 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 substrate. 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 likely wood decay fungi in complete RCW cavities, 10 in 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 *Peniophora incarnata* and *Phlebia brevisporia* that were not previously documented to be associated with these birds (Table 2.2). Still, the fungal species with which RCWs have long been thought to have an interesting relationship, *Porodaedalea pini* SE, was the most prevalent decay fungus found in completed RCW cavities and cavity starts (Table 2.2). 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, 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 (Figure 2.2). 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 fruiting bodies as visual cues when selecting excavation sites (Savignac and Machtans 2006, Witt 2010, Zahner et al. 2012). However, past work has shown that RCWs do not use fungal fruiting bodies as visual cues for excavation (Rudolph et al. 1995). They 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 *Porodaedalea 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 and 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 *Porodaedalea 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).

Our data suggest a number of interesting questions, including whether Ascomycota and other cosmopolitan fungi play a role in the cavity excavation process and whether wood decay fungi other than *Porodaedalea pini* SE are important to RCWs. For example, *Acaromyces ingoldii*, one of the most common fungi we found in complete RCW cavities and cavity starts (Table 1.1), has been shown to have fatal effects on mites (Gerson et al. 2008) and phytopathogenic fungi (Kushnir et al. 2011). It is possible that *Acaromyces ingoldii* attacks mites or other fungi that are detrimental to the birds. If *A. ingoldii* is attacking mites, these could be either feather mites that parasitize the birds or mites that prey upon the fungi that aide 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. 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 just begun to scrape the surface of fungal diversity in RCW excavations.

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**Figure and Table Captions**

Figure 2.1

a) Observed taxon accumulation curves for Basidiomycota, identified with the Basidiomycete specific primer pair, ITS1F and ITS4b-21. Each curve represents the overall Basidiomycete diversity captured in each of the three excavation types sampled.

b) Observed taxon accumulation curves for likely wood decay fungi identified with the Basidiomycete specific primer pair, ITS1F and ITS4b-21. Each curve represents the diversity of likely 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.

Figure 2.2

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 likely wood decay taxa identified with ITS1F and ITS4b-21. Stress = 0.00134, two dimensions, 200 iterations.

c) NMDS plot for ITS1F and ITS4, this includes Ascomycota and Basidiomycota, singletons have been removed, stress = 0.0989, two dimensions, 200 iterations.

Table 2.1

Five most common taxa found with ITS1F / ITS4b-21 in each cavity type, omitting singletons

Table 2.2

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

Figure 2.1: Observed taxon accumulation curves for a) Basidiomycota, b) likely wood decay fungi, and c) Ascomycota and Basidiomycota

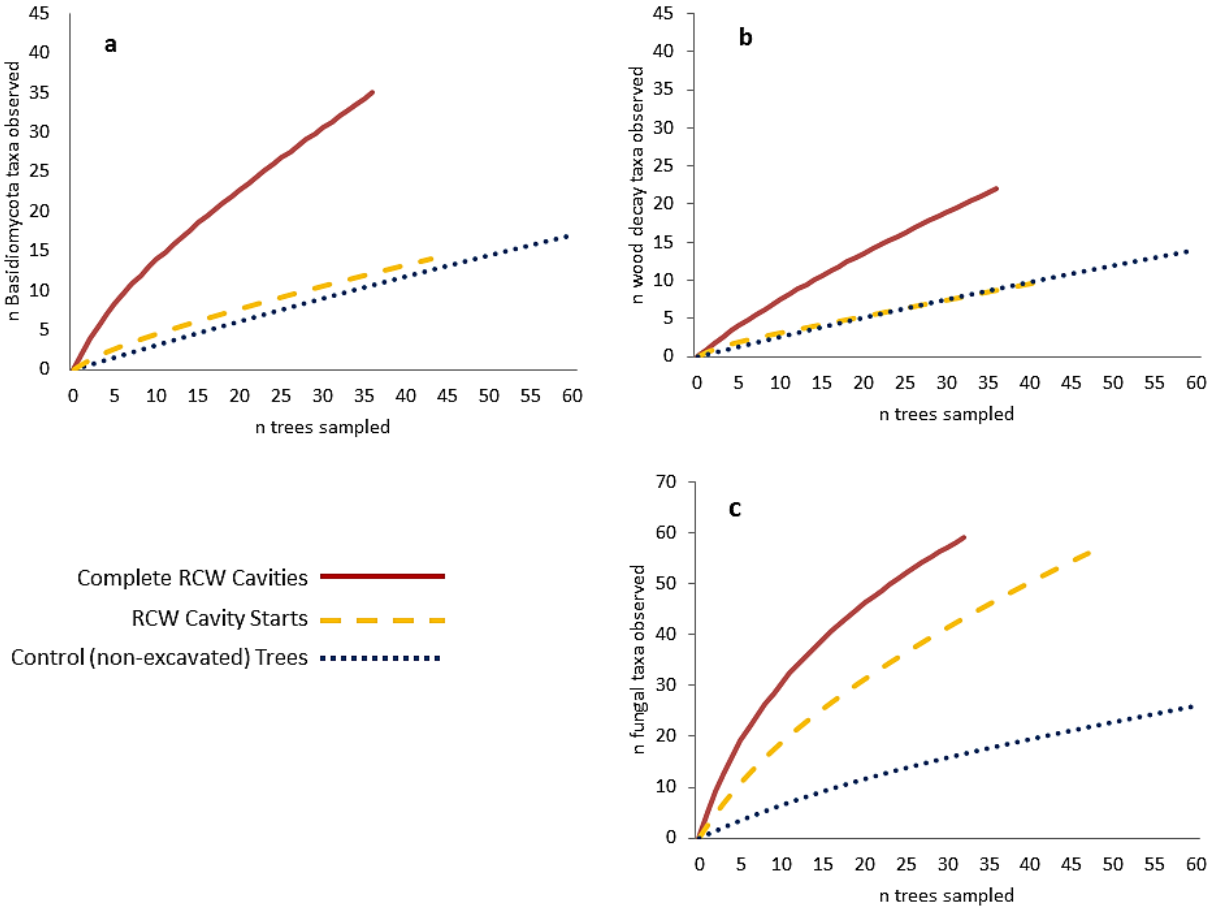

Figure 2.2: NMDS plots for fungal communities found in completed RCW cavities, RCW cavity starts, and non-excavated trees for a) Basidiomycota, b) likely wood decay fungi, and c) Ascomycota and Basidiomycota

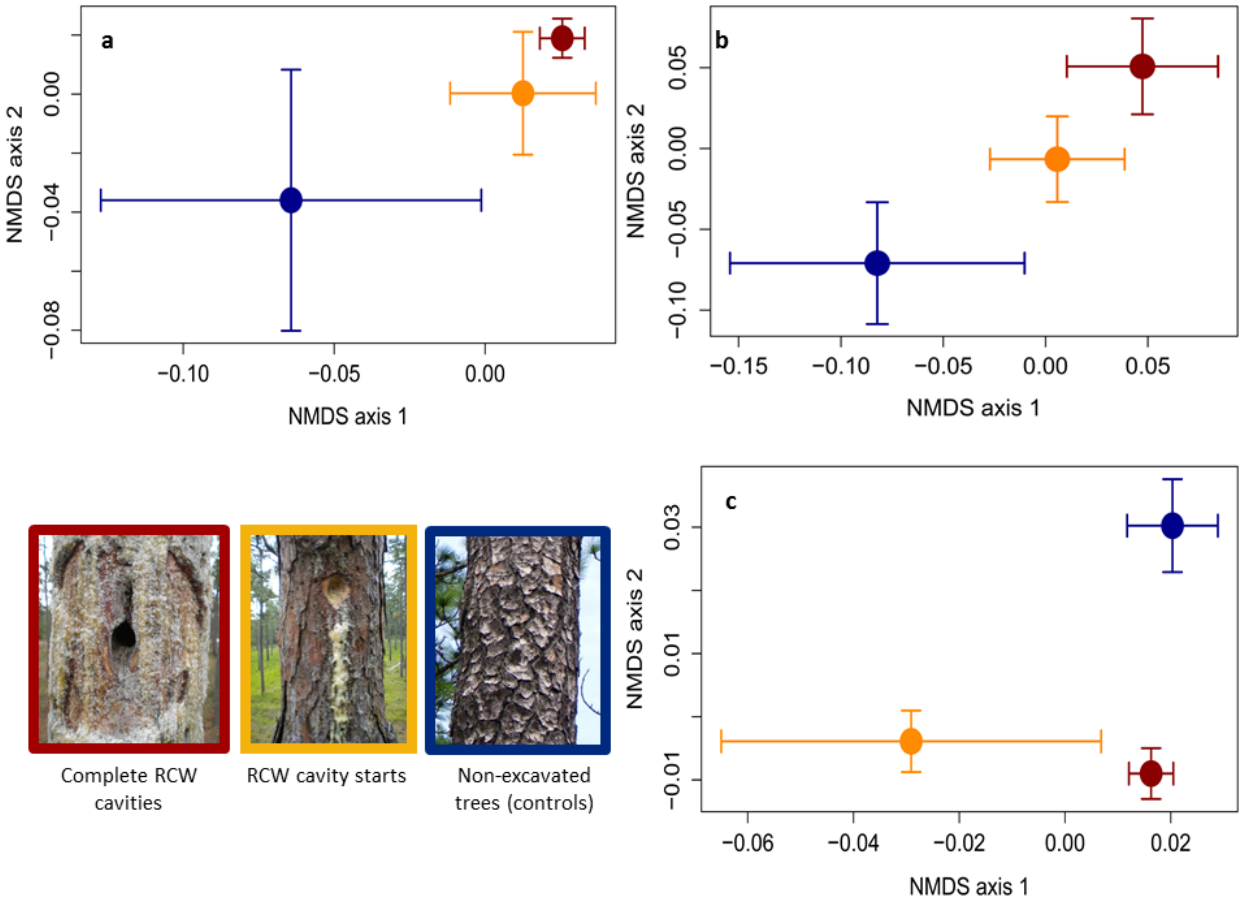

Table 2.1: Five most common taxa found with ITS1F / ITS4b-21 in each cavity type, omitting singletons

| **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 | | | | |

Table 2.2: 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 | |

Appendix A: Basidiomycota taxa identified with ITS1F and ITS4b-21, by excavation type. Numbers indicate the number of trees of each type in which each taxon was found.

| GenBank accession number | Taxon | Closest identified taxon with GenBank BLAST | % Base pair match | % Query coverage | RCW Cavities | RCW Starts | Control Trees | Total number of detect-ions |
|---|---|---|---|---|---|---|---|---|
| KM104040 | *Porodaedalea pini* SE | *Porodaedalea* sp. (JX110039.1) | 100 % | 100 % | 8 | 13 | 2 | 23 |
| KM104130 | Exobasidiomycetes sp. 2 | Exobasidiomycetidae sp. (DQ682574.1) | 100 % | 96 % | 13 | 2 | 0 | 15 |
| KM103937 | *Acaromyces ingoldii* | *Acaromyces ingoldii* (NR_073342.1) | 100 % | 100 % | 8 | 3 | 0 | 11 |
| KM103938 | *Acaromyces* sp. 1 | *Acaromyces ingoldii* (NR_073342.1) | 98 % | 100 % | 10 | 0 | 0 | 10 |
| KM104143 | Unidentified Basidiomycete 7 | *Bullera formosana* (AB118873.1) | 88 % | 70 % | 4 | 1 | 0 | 5 |
| KM104086 | *Trichosporon asahii* | *Trichosporon asahii* (AB369919.1) | 100 % | 99 % | 2 | 1 | 1 | 4 |
| KM104021 | *Peniophora incarnata* | *Peniophora* sp. (KC176330.1) | 99 % | 100 % | 1 | 0 | 2 | 3 |
| KM104030 | *Phlebia brevispora* | *Phlebia brevispora* (AB084616.1) | 99 % | 99 % | 3 | 0 | 0 | 3 |
| KM104058 | *Skeletocutis chrysella* | *Skeletocutis chrysella* (JQ673127.1) | 99 % | 100 % | 1 | 1 | 1 | 3 |
| KM103955 | *Athelia arachnoidea* | *Athelia arachnoidea* (GU187504.1) | 99 % | 100 % | 1 | 0 | 1 | 2 |
| KM103967 | *Coniophora* sp. 1 | *Coniophora prasinoides* (GU187519.1) | 91 % | 100 % | 2 | 0 | 0 | 2 |
| KM104023 | *Peniophora cinerea* | *Peniophora cinerea* | 99 % | 98 % | 1 | 0 | 1 | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | (GU062269.1) | | | | | | |
| KM104036 | *Peniophora* sp. 4 | Polyporales sp. (EF672293.1) | 99 % | 100 % | 1 | 1 | 0 | 2 |
| KM104041 | *Postia sericeomollis* | *Postia sericeomollis* (KC585367.1) | 98 % | 100 % | 2 | 0 | 0 | 2 |
| KM103939 | Agaricomycetes sp. 1 | Polyporales sp. (EF694649.1) | 99 % | 97 % | 0 | 1 | 0 | 1 |
| KM103941 | Agaricomycetes sp. 11 | *Grifola sordulenta* (GU222266.1) | 90 % | 100 % | 1 | 0 | 0 | 1 |
| KM104131 | Agaricomycetes sp. 17 | *Amyloathelia crassiuscula* (DQ144610.1) | 82 % | 91 % | 0 | 1 | 0 | 1 |
| KM103945 | Agaricomycetes sp. 3 | *Entomocorticium* sp. (DQ118417.1) | 97 % | 97 % | 1 | 0 | 0 | 1 |
| KM103957 | Atheliaceae sp. 2 | *Athelia* sp. (GU187502.1) | 96 % | 97 % | 1 | 0 | 0 | 1 |
| KM103960 | *Calvatia* sp. 1 | *Calvatia fragilis* (AJ617493.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM103966 | *Collybia subnuda* | *Collybia subnuda* (U43780.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM103968 | *Coprinellus* sp. 1 | *Coprinellus radians* (AY461815.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104035 | *Crustoderma* sp. 1 | *Crustoderma corneum* (KC585319.1) | 93 % | 100 % | 0 | 1 | 0 | 1 |
| KM103973 | *Cryptococcus* sp. 2 | *Cryptococcus gattii* (JN939462.1) | 93 % | 100 % | 0 | 0 | 1 | 1 |
| KM103969 | *Hyphodontia* sp. 1 | *Hyphodontia* sp. (DQ340319.1) | 99 % | 95 % | 1 | 0 | 0 | 1 |
| KM103970 | *Hypochnicium* sp. 1 | *Hypochnicium punctulatum* (AF429412.1) | 92 % | 90 % | 1 | 0 | 0 | 1 |
| KM104002 | *Irpex lacteus* | *Irpex lacteus* (JX290578.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM103963 | *Mycoacia fuscoatra* | *Mycoacia fuscoatra* | 99 % | 99 % | 0 | 0 | 1 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | (KJ140744.1) | | | | | | |
| KM104010 | Outerspace Basidio 1 | *Exobasidium symploci-japonicae* (AB180351.1) | 95 % | 31 % | 1 | 0 | 0 | 1 |
| KM104047 | *Peniophora* sp. 5 | *Peniophora* sp. (JN198493.1) | 98 % | 95 % | 1 | 0 | 0 | 1 |
| KM104129 | Polyporales sp. 9 | *Trametes versicolor* (JN164975.1) | 85 % | 83 % | 1 | 0 | 0 | 1 |
| KM104039 | *Polyporus squamosus* | *Polyporus squamosus* (AF516589.1) | 100 % | 100 % | 0 | 0 | 1 | 1 |
| KM104054 | *Schizophyllum commune* | *Schizophyllum commune* (JN882337.1) | 99 % | 99 % | 0 | 0 | 1 | 1 |
| KM104083 | *Schizopora* sp. 1 | *Schizopora* sp. (JQ673190.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM104055 | *Serpula himantioides* | *Serpula himantioides* (GU187545.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104139 | *Sistotremastrum* sp. 2 | *Sistotremastrum* sp. (KJ140723.1) | 92 % | 98 % | 0 | 1 | 0 | 1 |
| KM104059 | *Skeletocutis odora* | *Skeletocutis odora* (FJ903307.1) | 100 % | 98 % | 0 | 0 | 1 | 1 |
| KM103948 | Stereaceae sp. 1 | *Acanthophysium lividocaeruleum* (AY618666.1) | 94 % | 96 % | 1 | 0 | 0 | 1 |
| KM104061 | *Stereum complicatum* | *Stereum complicatum* (KJ140563.1) | 99 % | 98 % | 1 | 0 | 0 | 1 |
| KM104063 | *Stereum* sp. 4 | *Stereum* sp. (JX460856.1) | 98 % | 100 % | 0 | 1 | 0 | 1 |
| KM104071 | *Trametes versicolor* | *Trametes versicolor* (KC176344.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104072 | *Trechispora* sp. 1 | *Trechispora alnicola* (DQ411529.1) | 92 % | 99 % | 1 | 0 | 0 | 1 |
| KM104078 | Tremellales sp. 6 | *Hannaella* sp. (JQ753983.1) | 99 % | 94 % | 1 | 0 | 0 | 1 |
| KM104079 | Tremellales sp. 7 | *Bullera psuedoalba* | 100 % | 98 % | 1 | 0 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|
| | | (NR_073231.1) | | | | | | |
| KM104080 | Tremellales sp. 8 | *Tremella foliacea* (AF444431.1) | 94 % | 98 % | 1 | 0 | 0 | 1 |
| KM104082 | *Trichaptum abietinum* | *Trichaptum abietinum* (U63475.1) | 98 % | 100 % | 0 | 1 | 0 | 1 |
| KM104081 | *Trichaptum birforme* | *Trichaptum biforme* (U63473.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM104117 | Unidentified Basidiomycete 17 | *Athelia bombacina* (U85795.1) | 87 % | 98 % | 0 | 1 | 0 | 1 |
| KM104119 | Unidentified Basidiomycete 19 | *Meira geulakonigii* (NR_073343.1) | 93 % | 42 % | 1 | 0 | 0 | 1 |
| KM104127 | Unidentified Basidiomycete 33 | *Acaromyces ingoldii* (NR_073342.1) | 94 % | 75 % | 0 | 1 | 0 | 1 |
| KM104133 | Unidentified Basidiomycete 46 | *Sidera vulgaris* (FN907918.1) | 87 % | 46 % | 0 | 0 | 1 | 1 |
| KM104135 | Unidentified Basidiomycete 49 | *Botryobasidium botryosum* (DQ267124.1) | 90 % | 54 % | 1 | 0 | 0 | 1 |
| KM104154 | *Xeromphalina campanella* | *Xeromphalina campanella* (HQ604741.1) | 98 % | 100 % | 1 | 0 | 0 | 1 |

Appendix B: Fungal taxa identified with ITS1F and ITS4, by excavation type. Numbers indicate the number of trees of each type in which each taxon was found.

| GenBank accession number | Taxon | Closest identified taxon with GenBank BLAST | % Base pair match | % Query coverage | RCW Cavities | RCW Starts | Control Trees | Total number of detect-ions |
|---|---|---|---|---|---|---|---|---|
| KM104048 | *Sarea resinae* | *Sarea resinae* (FJ903329.1) | 96 % | 97 % | 12 | 9 | 3 | 24 |
| KM104130 | Exobasidiomycetes sp. 2 | Exobasidiomycetidae sp. (DQ682574.1) | 100 % | 96 % | 14 | 8 | 1 | 23 |
| KM104014 | *Penicillium citreonigrum* | *Penicillium citreonigrum* (EU497959.1) | 99 % | 100 % | 10 | 10 | 1 | 21 |
| KM104068 | *Toxicocladosporium* sp 1 | Capnodiales sp. (HQ608065.1) | 100 % | 100 % | 11 | 9 | 1 | 21 |
| KM103992 | *Fusarium* sp. 1 | *Fusarium graminearum* (KJ017740.1) | 99 % | 100 % | 2 | 5 | 5 | 12 |
| KM104064 | *Sydowia polyspora* | *Sydowia polyspora* (JN944640.1) | 99 % | 100 % | 3 | 8 | 1 | 12 |
| KM103977 | *Debaryomyces hansenii* | *Debaryomyces hansenii* (KF468214.1) | 100 % | 100 % | 8 | 2 | 0 | 10 |
| KM104013 | *Paecilomyces* sp. 1 | *Paecilomyces formosus* (KC157764.1) | 100 % | 100 % | 8 | 1 | 0 | 9 |
| KM104027 | *Phaeomoniella* sp. 1 | *Phaeomoniella effusa* (JF440607.1) | 99 % | 97 % | 4 | 2 | 3 | 9 |
| KM103952 | *Aspergillus* sp. 2 | *Apsergillus flavus* (JQ781721.1) | 99 % | 100 % | 2 | 2 | 4 | 8 |
| KM104010 | Outerspace Basidiomycete 1 | *Exobasidium symploci-japonicae* (AB180351.1) | 95 % | 31 % | 6 | 1 | 1 | 8 |
| KM104049 | *Sarea* sp. 1 | *Sarea difformis* (FJ903295.1) | 96 % | 99 % | 2 | 4 | 2 | 8 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| KM104040 | *Porodaedalea pini* SE | *Porodaedalea* sp. (JX110039.1) | 100 % | 100 % | 3 | 3 | 1 | 7 |
| KM103951 | *Aspergillus* sp. 1 | *Aspergillus* sp. (FJ755817.1) | 99 % | 100 % | 4 | 2 | 0 | 6 |
| KM104087 | Unidentified Ascomycete 40 | Dothideales sp. (JN835192.1) | 98 % | 85 % | 2 | 4 | 0 | 6 |
| KM103953 | *Aspergillus* sp. 3 | *Aspergillus* sp. (AM901671.1) | 99 % | 100 % | 5 | 0 | 0 | 5 |
| KM103954 | *Aspergillus ustus* | *Aspergillus ustus* (FJ878630.1) | 100 % | 100 % | 0 | 1 | 4 | 5 |
| KM104018 | *Penicillium* sp. 2 | *Penicillium chrysogenum* (KJ185377.1) | 99 % | 100 % | 1 | 0 | 4 | 5 |
| KM104065 | Teratosphaeriaceae sp. 1 | *Catenulostroma germanicum* (EU019253.2) | 95 % | 100 % | 4 | 1 | 0 | 5 |
| KM104090 | Unidentified Ascomycete 10 | *Eremascus albus* (GQ867787.1) | 86 % | 92 % | 3 | 2 | 0 | 5 |
| KM104015 | *Penicillium corylophilum* | *Penicillium corylophilum* (JQ272469.1) | 100 % | 100 % | 0 | 4 | 0 | 4 |
| KM104088 | Unidentified Basidiomycete 61 | Basidiomycota sp. (FJ903338.1) | 91 % | 96 % | 3 | 1 | 0 | 4 |
| KM103958 | *Aureobasidium pullulans* | *Aureobasidium pullulans* (AY225167.1) | 99 % | 100 % | 0 | 3 | 0 | 3 |
| KM103985 | Eurotiomycetidae sp. 1 | *Penicillium* sp. (FJ196612.1) | 90 % | 96 % | 3 | 0 | 0 | 3 |
| KM104029 | *Phialemonium obovatum* | *Phialemonium obovatum* (AB278187.1) | 99 % | 99 % | 2 | 1 | 0 | 3 |
| KM104084 | *Sagenomella griseoviridis* | *Sagenomella griseoviridis* (GQ169320.1) | 99 % | 98 % | 3 | 0 | 0 | 3 |
| KM104050 | *Sarea* sp. 2 | *Sarea* sp. (FJ903326.1) | 97 % | 96 % | 2 | 1 | 0 | 3 |
| KM104051 | *Sarea* sp. 3 | *Sarea difformis* (FJ903295.1) | 96 % | 97 % | 1 | 1 | 1 | 3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| KM104097 | Unidentified Ascomycete 22 | *Moristroma quercinum* (AY254051.1) | 86 % | 91 % | 1 | 2 | 0 | 3 |
| KM104101 | Unidentified Ascomycete 29 | *Candida* sp. (AM410670.1) | 92 % | 92 % | 2 | 1 | 0 | 3 |
| KM104114 | Unidentified Basidiomycete 13 | *Paratritirachium curvibasidium* (KF258724.1) | 85 % | 52 % | 3 | 0 | 0 | 3 |
| KM104151 | Unidentified Basidiomycete 62 | *Paratritirachium curvibasidium* (KF258724.1) | 88 % | 60 % | 3 | 0 | 0 | 3 |
| KM103982 | Capnodiales sp. 3 | *Phaeothecoidea proteae* (EU707898.1) | 92 % | 98 % | 1 | 1 | 0 | 2 |
| KM104102 | Leotiomycetes sp. 1 | Leotiomycetes sp. (JX421713.1) | 98 % | 100 % | 0 | 1 | 1 | 2 |
| KM104146 | Leotiomycetes sp. 2 | *Flagellospora curvula* (KC834045.1) | 85 % | 96 % | 0 | 2 | 0 | 2 |
| KM104007 | *Malassezia japonica* | *Malassezia japonica* (AB105199.1) | 99 % | 100 % | 2 | 0 | 0 | 2 |
| KM104020 | *Penicillium* sp. 12 | *Penicillium glabrum* (JX421718.1) | 99 % | 100 % | 1 | 0 | 1 | 2 |
| KM104025 | *Pestalotiopsis* sp. 1 | *Pestalotiopsis* sp. (HQ608151.1) | 99 % | 99 % | 1 | 0 | 1 | 2 |
| KM104085 | *Trichomonascus* sp. 1 | *Trichomonascus ciferrii* (EF568082.1) | 94 % | 96 % | 2 | 0 | 0 | 2 |
| KM104091 | Unidentified Ascomycete 11 | *Phaeosclera dematioides* (AJ244524.1) | 89 % | 91 % | 1 | 1 | 0 | 2 |
| KM104095 | Unidentified Ascomycete 19 | *Arthrographis pinicola* (NR_077121.1) | 83 % | 89 % | 1 | 1 | 0 | 2 |
| KM104098 | Unidentified Ascomycete 23 | *Phaeosclera dematioides* (AJ244524.1) | 99 % | 92 % | 2 | 0 | 0 | 2 |
| KM104148 | Unidentified Ascomycete 41 | *Cryptosporiopsis* sp. (JN601680.1) | 89 % | 71 % | 0 | 2 | 0 | 2 |
| KM104149 | Unidentified | Ascomycota sp. | 89 % | 62 % | 2 | 0 | 0 | 2 |

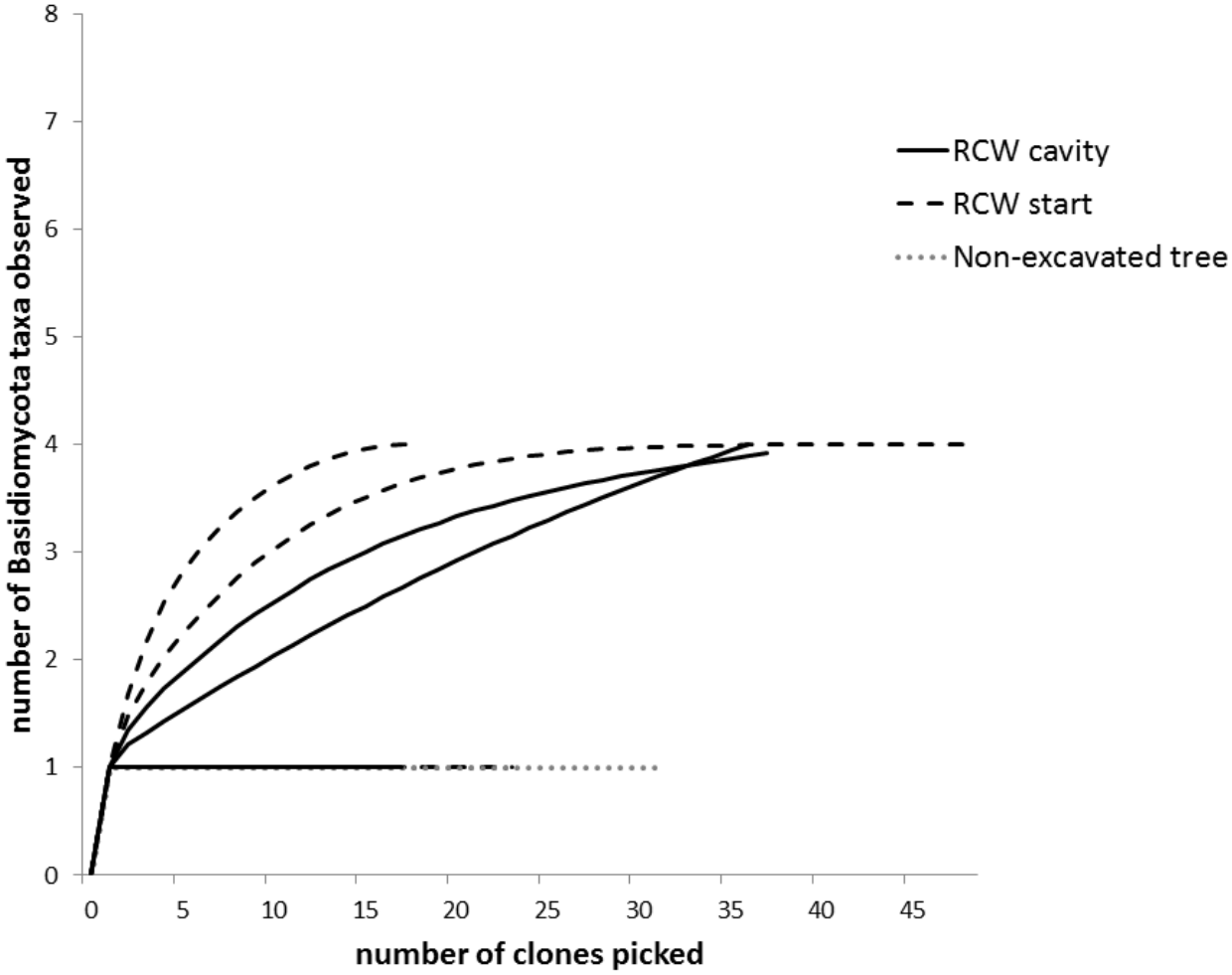
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ascomycete 42 | (JQ272346.1) | | | | | | |
| KM103938 | *Acaromyces* sp. 1 | *Acaromyces ingoldii* (NR_073342.1) | 98 % | 100 % | 0 | 1 | 0 | 1 |
| KM104111 | Agaricomycetes sp. 16 | *Plicaturopsis crispa* (KJ140537.1) | 86 % | 99 % | 0 | 1 | 0 | 1 |
| KM103949 | *Alternaria alternate* | *Alternaria alternata* (KJ082099.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM103983 | *Aureobasidium* sp. 1 | *Aureobasidium pullulans* (KC253970.1) | 99 % | 96 % | 0 | 1 | 0 | 1 |
| KM103961 | *Candida* sp. 1 | *Candida prunicola* (NR_111288.1) | 91 % | 88 % | 0 | 1 | 0 | 1 |
| KM103962 | Capnodiales sp. 1 | *Catenulostroma germanicum* (EU019253.1) | 93 % | 98 % | 1 | 0 | 0 | 1 |
| KM103981 | Capnodiales sp. 2 | *Capnobotryella* sp. (AJ972860.1) | 96 % | 99 % | 0 | 0 | 1 | 1 |
| KM103965 | *Cladosporium perangustum* | *Cladosporium* sp. (KF128859.1) | 99 % | 100 % | 0 | 1 | 0 | 1 |
| KM103967 | *Coniophora* sp. 1 | *Coniophora prasinoides* (GU187519.1) | 91 % | 100 % | 1 | 0 | 0 | 1 |
| KM103975 | *Dacrymyces* sp. 1 | *Dacrymyces* sp. (DQ205684.1) | 99 % | 98 % | 1 | 0 | 0 | 1 |
| KM103978 | *Debaryomyces psuedopolymorphus* | *Debaryomyces psuedopolymorphus* (EF198011.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM103979 | *Debaryomyces* sp. 1 | *Debaryomyces udenii* (NR_077068.1) | 97 % | 99 % | 1 | 0 | 0 | 1 |
| KM104150 | Dothideomycetes sp. 1 | *Staninwardia suttonii* (DQ923535.2) | 88 % | 100 % | 1 | 0 | 0 | 1 |
| KM104093 | Eurotiales sp. 1 | *Aspergillus conicus* (EF652039.1) | 99 % | 91 % | 1 | 0 | 0 | 1 |
| KM103991 | *Fomitopsis pinicola* | *Fomitopsis pinicola* (KC595922.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| KM103995 | *Geosmithia morbida* | *Geosmithia morbida* (KF808301.1) | 98 % | 100 % | 0 | 1 | 0 | 1 |
| KM104005 | *Laetiporus cinncinatus* | *Laetiporus cincinnatus* (AM269786.1) | 99 % | 100 % | 0 | 1 | 0 | 1 |
| KM104006 | *Laetiporus sulphureus* | *Laetiporus sulphureus* (AY835668.1) | 98 % | 99 % | 0 | 1 | 0 | 1 |
| KM104016 | *Penicillium* sp. 10 | *Penicillium* sp. (JX559859.1) | 99 % | 99 % | 1 | 0 | 0 | 1 |
| KM104017 | *Penicillium* sp. 11 | *Penicillium citrinum* (KJ522784.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104019 | *Penicillium* sp. 3 | *Penicillium* sp. (EU076956.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104028 | *Phaeomoniella* sp. 2 | *Phaeomoniella chlamydospora* (HM116754.1) | 91 % | 95 % | 1 | 0 | 0 | 1 |
| KM104030 | *Phlebia brevispora* | *Phlebia brevispora* (AB084616.1) | 99 % | 99 % | 0 | 1 | 0 | 1 |
| KM104043 | *Preussia Africana* | *Preussia* sp. (JN225887.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM104044 | *Rachicladosporium cboliae* | *Rachicladosporium cboliae* (GU214650.1) | 99 % | 100 % | 0 | 0 | 1 | 1 |
| KM104045 | *Rhizopogon psuedoroseolus* | *Rhizopogon psuedoroseolus* (GQ267483.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104046 | *Rhodotorula colostri* | *Rhodotorula colostri* (JN246563.1) | 100 % | 99 % | 0 | 0 | 1 | 1 |
| KM103984 | *Rhodotorula minuta* | *Rhodotorula minuta* (HQ832824.1) | 99 % | 99 % | 1 | 0 | 0 | 1 |
| KM104052 | *Sarea* sp. 4 | *Sarea resinae* (FJ903329.1) | 98 % | 98 % | 0 | 1 | 0 | 1 |
| KM104053 | *Sarea* sp. 5 | *Sarea difformis* (FJ903295.1) | 96 % | 92 % | 1 | 0 | 0 | 1 |
| KM104026 | *Scytalidium* sp. 1 | *Scytalidium* sp. | 93 % | 99 % | 0 | 1 | 0 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | (HQ631037.1) | | | | | | |
| KM104034 | *Skeletocutis* sp. 3 | *Skeletocutis* sp. (JQ673133.1) | 99 % | 100 % | 1 | 0 | 0 | 1 |
| KM104067 | *Thysanophora penicillioides* | *Thysanophora pinicillioides* (AB175247.1) | 99 % | 99 % | 0 | 0 | 1 | 1 |
| KM104089 | Unidentified Ascomycete 1 | Eurotiomycetes sp. (JQ759942.1) | 95 % | 86 % | 0 | 1 | 0 | 1 |
| KM104092 | Unidentified Ascomycete 12 | *Aureobasidium pullulans* (AF423114.1) | 99 % | 63 % | 0 | 1 | 0 | 1 |
| KM104094 | Unidentified Ascomycete 18 | Dothideomycetes sp. (JQ905788.1) | 96 % | 99 % | 1 | 0 | 0 | 1 |
| KM104096 | Unidentified Ascomycete 20 | *Moristroma quercinum* (AY254051.1) | 87 % | 87 % | 0 | 1 | 0 | 1 |
| KM104099 | Unidentified Ascomycete 24 | *Pestalotiopsis* sp. (AB297798.1) | 91 % | 93 % | 0 | 1 | 0 | 1 |
| KM104100 | Unidentified Ascomycete 25 | Dothideomycetes sp. (JQ760724.1) | 90 % | 80 % | 0 | 1 | 0 | 1 |
| KM104103 | Unidentified Ascomycete 30 | *Sarea* sp. (FJ903326.1) | 84 % | 64 % | 0 | 1 | 0 | 1 |
| KM104104 | Unidentified Ascomycete 38 | *Meliniomyces* sp. (JQ711936.1) | 91 % | 97 % | 0 | 1 | 0 | 1 |
| KM104105 | Unidentified Ascomycete 4 | *Sarea resinae* (JX421720.1) | 85 % | 91 % | 1 | 0 | 0 | 1 |
| KM104106 | Unidentified Ascomycete 5 | *Phaeococcomyces eucalypti* (NR_120226.1) | 86 % | 99 % | 1 | 0 | 0 | 1 |
| KM104107 | Unidentified Ascomycete 6 | *Phaeotheca fissurella* (GQ266146.1) | 89 % | 87 % | 0 | 1 | 0 | 1 |
| KM104108 | Unidentified Ascomycete 7 | *Xylomelasma* sp. (FR837913.1) | 90 % | 93 % | 0 | 1 | 0 | 1 |
| KM104109 | Unidentified Ascomycete 8 | *Aulographina pinorum* (GU214622.1) | 87 % | 99 % | 0 | 1 | 0 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| KM104110 | Unidentified Ascomycete 9 | Ascomycete sp. (EF373588.1) | 89 % | 96 % | 1 | 0 | 0 | 1 |
| KM104115 | Unidentified Basidiomycete 14 | Exobasidiomycetidae sp. (DQ682574.1) | 99 % | 86 % | 1 | 0 | 0 | 1 |
| KM104125 | Unidentified Basidiomycete 3 | *Fluvifomes fastuosus* (KJ206286.1) | 93 % | 27 % | 1 | 0 | 0 | 1 |
| KM104153 | *Wallemia* sp. 1 | *Wallemia* sp. (FJ755832.1) | 98 % | 100 % | 0 | 0 | 1 | 1 |

Appendix C: Taxon accumulation curves for individual samples from complete RCW cavities, RCW cavity starts, and non-excavated trees. Note that four samples overlap at the bottom of the graph, 2 non-excavated trees, 1 RCW cavity and 1 RCW cavity start.



## Chapter 3. Pining for fungi: experimental evidence of a symbiosis between red-cockaded woodpeckers and fungal communities

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ABSTRACT

We report the first experimental evidence of a symbiosis between fungi and a primary cavity excavator that facilitates cavity excavation. Interactions between wood-inhabiting fungi and our study species, the red-cockaded woodpecker (*Picoides borealis*; RCW), are particularly interesting because RCWs are the only birds that exclusively excavate into the heartwood of living pines, a process that can take many years to complete. Fungi may facilitate RCW excavation by decreasing cavity excavation time and effort, and RCWs may in turn facilitate the dispersal and colonization of fungi. Our previous work demonstrated that trees containing cavities excavated by RCWs are inhabited by distinct fungal communities, suggesting that the birds either: 1) select trees with distinct fungal communities (tree selection hypothesis), or 2) promote distinct fungal communities via their excavations (bird facilitation hypothesis). We swabbed the beaks, wings and feet of RCWs for fungi and found that RCWs carry wood decay fungi similar to those found in their completed excavations. We then experimentally tested the bird facilitation hypothesis by aseptically drilling cavity starts into non-excavated trees, restricting RCW access to half of these trees, and comparing changes in fungal communities over time. Prior to creating these starts, Basidiomycota fungi were present in 27% of these trees, and the fungal communities were highly variable and did not resemble those in RCW excavations.

When resampled after 26 months, Basidiomycota fungi were present in 75% of these trees, and Basidiomycota prevalence and diversity were similar in the accessible and inaccessible experimental starts. However, the fungal community composition in accessible starts was very different from that in inaccessible starts, but closely resembled that in RCW excavations. RCWs interacted with a whole community of fungal associates, not just a single fungal taxon. Our work provides support for the bird facilitation hypothesis and suggests a mutualistic association between cavity excavators and a community of fungi, with implications for forest ecology, wildlife management and conservation.

## Introduction

Symbioses directly or indirectly influence many levels of ecological organization, from individual organisms to entire ecosystems. While the traditional view of symbiosis was of pairs-wise species interactions, the emerging perspective is that symbioses often involve diverse suites of interacting organisms, and this diversity integral to the outcome of symbiotic interactions. For example, humans have many microbial symbionts on and in the body which are necessary for proper digestion, immunity, and development (Bäckhed et al. 2005, Grice et al. 2009). Coral reefs are hotspots for biodiversity, and their existence is only possible because of a multipartite symbiotic interaction between cnidarians, zooxanthellae algae, bacteria and various other organisms (Rohwer et al. 2002, Hussa and Goodrich-Blair 2013). Thus, understanding these complex interactions is fundamental to understanding how ecosystems function, regardless of the scale.

Woodpeckers are ecosystem engineers whose cavities are used by many species of non-excavating, cavity-nesting birds termed secondary cavity nesters, as well as a variety of mammals, herpetofauna and invertebrates. In forest systems where naturally formed, or non-excavated, cavities are not abundant, cavity excavators are keystone ecosystem engineers. In these cases, cavity excavators help maintain biodiversity by creating nesting and roosting habitat for a diversity of non-excavating cavity users (Martin and Eadie 1999, Blanc and Walters 2008), but cavity excavation is often limited by the availability of trees suitable for excavation. Some excavating species depend on decayed wood in living or dead trees, while others require relatively healthy, living trees with some pockets of decay. Wood decay fungi are also ecosystem engineers (Jones et al. 1996), because they not only facilitate the formation of non-excavated cavities, but also play a critical role by softening the wood of potential excavation sites (Conner et al. 1976, Jackson 1977, Jackson and Jackson 2004, Witt 2010, Cockle et al. 2012, Zahner et al. 2012, Jusino et al. *under revision*), thereby increasing the availability of such sites. Cavity excavators in turn may facilitate fungal dispersal and colonization by carrying a combination of fungal spores and hyphal fragments from existing cavities to future excavation sites (Jackson 1977, Farris et al. 2004, Jusino et al. *under revision*). Thus, the ecosystem function served by excavators may rely upon mutualistic associations with fungi.

Direct links between cavity excavators and fungi have yet to be tested. Birds have long been thought to be capable of fungal spore dispersal (Heald and Studhalter 1913, Warner and French 1970, Farris et al. 2004, Alfonzo et al. 2013, Francesca et al. 2013), and cavity excavators have been speculated as being capable of carrying wood decay fungi, but this has not yet been demonstrated. However, two recent studies provide some evidence that both black woodpeckers (*Dryocopus martius*) and red-cockaded woodpeckers, two species separated by geography and

phylogeny, may facilitate the progression of decay in their excavations (Zahner et al. 2012, Jusino et al. *under revision*).

*Red-cockaded woodpeckers (RCWs)*

Red-cockaded woodpeckers are federally endangered cooperatively breeding birds who live in family groups and are primary excavators in the longleaf pine ecosystem of the southeastern United States, to which they are endemic (Ligon 1970, Walters et al. 1988). Within each RCW family group, every bird requires a RCW-excavated roost cavity in a living pine tree. In addition to the roost cavities, each family group also maintains a number of incomplete excavations in various stages of completion, termed cavity starts. All excavations belonging to one RCW family group constitute a cavity tree cluster. RCWs are the only birds that exclusively excavate through the sapwood and into the heartwood of living pine trees and the time to complete the excavation process can range from less than one year to decades (Harding and Walters 2004). However, once a cavity is complete, it can be used by the woodpeckers for many years, and then afterwards by a diverse community of secondary cavity users. Thus, understanding RCW excavation behavior is critical to management efforts for these endangered birds (Walters 1991) and for the communities their cavities support. RCW cavity excavation is thought to be expedited when wood decay fungi are present within the excavated tree, and RCWs may have a particularly interesting relationship with heartwood infecting fungi due to their unique excavation behavior (Walters 1991).

RCW cavity starts are wounds in the trunks of trees and thus are possible infection courts for fungi that are thought to facilitate excavation first through the sapwood, and then into the heartwood that houses the cavity chamber. Because these birds intermittently visit their cavity starts throughout the excavation process, RCWs may carry fungi from their roost cavities into

their cavity starts throughout the excavation process. In so doing, RCWs would gain decreased excavation time and increased access to a limited resource, that being trees available for relatively fast excavation, and the fungi would gain an additional method of dispersal beyond sporulation. Longleaf pine forests are dominated by living pine trees. Based on surveys of fruiting bodies, which are rarely found on living pine trees in this system (Chapter 2), RCWs were historically thought to seek out trees infected with a particular fungus, *Porodaedalea pini* SE, for excavation. We found in an earlier study that in addition to *P. pini* SE, there are at least 28 other taxa of wood decay fungi associated with RCW excavations (Chapter 2). Our previous work also demonstrated that trees containing RCW excavations are inhabited by distinct fungal communities, suggesting that the birds either select trees pre-colonized by these fungal communities for excavation (tree selection hypothesis), or introduce fungi to trees via their excavations, (bird facilitation hypothesis).

For this study, we conducted a two-part test of the bird facilitation hypothesis, which states that RCWS select excavation sites without any prior evidence of decay, then subsequently facilitate infection of fungi during the excavation process, either directly, by carrying fungi on their bodies, or indirectly by changing the microhabitat within the tree during excavation. First, we conducted a field survey to determine if RCWs carry fungi found in their excavations by externally swabbing the birds and comparing fungal communities found on their bodies to those found in their complete and incomplete excavations. We then conducted a field experiment to test if RCWs facilitate fungal infection in longleaf pine trees during cavity excavation by aseptically drilling cavity starts into non-excavated trees, restricting RCW access to half of these trees, and comparing the change in fungal communities over time in excavations to which RCWs had access and those to which they did not.

## Methodology

### *Field site*

The study was conducted on Marine Corps Base Camp Lejeune (MCBCL), in Onslow County, located on the central coastline of North Carolina. MCBCL is composed of 110,000 acres of land and 26,000 acres of water, including roughly 56,000 acres of pine habitat considered suitable for RCWs (MCBCL 2006). The RCW population on MCBCL has been intensively monitored since 1986 and has grown from a low of 26 groups in 1991 to 99 in 2013. As part of this ongoing study, complete RCW cavities and RCW cavity starts are documented as they are located on the landscape.

### *RCW swabbing*

During 2009 we opportunistically swabbed the beaks, wings and feet of adult RCWs who needed to be captured as part of population monitoring. To swab the birds, we used sterile cotton swabs with one cotton tip removed. After removing one end of the swab, the swabs were autoclaved and flame-sterilized forceps were used to transfer each swab into a sterile 15mL falcon tube.

Capture was accomplished by flushing birds from their roost cavities into a net on the end of an extendable pole. Care was taken not to touch the areas of the bird that were being swabbed; if there was any question of contamination, swabs were discarded. Prior to removing the bird from the net, the individual handling the swabs cleaned their hands with sanitizer, and only the cardboard end of the swab was handled. Once the RCW was in hand, the targeted area was gently wiped with the cotton end of a swab, and after swabbing the swab was placed back into the falcon tube, with the cotton tip in the bottom of the tube and the cardboard end oriented towards the cap, and the tube was sealed. We used this procedure to swab the beak, left wing and

left foot of each bird. After swabbing and additional processing related to population monitoring, the bird was released. We also included three field control swabs, which were handled in exactly the same way, in the same setting, but in the absence of a bird. All swabs were kept on wet ice, and then transferred into a -20º C freezer to await processing.

*Drilling Experiment*

In September 2009, we selected fifteen active RCW clusters on MCBCL that were not in areas with restricted access and contained at least four non-excavated longleaf pine trees with a large enough heartwood diameter to house a RCW cavity. Four non-excavated longleaf pine trees with attributes similar to cavity trees within each of the 15 clusters were selected and aseptically cored at average cavity height, using a clean 4.35 mm diameter, 3-threaded increment borer, following the procedure for RCW cavity starts in Chapter 1. The heartwood of these cores was kept, and the sapwood portion was sterilely re-inserted into the core site to prevent the artificial introduction of pathogenic organisms. In September and October 2009, artificial cavity starts were aseptically drilled into each of these trees (see Chapter 1 for details). The drilled starts tunneled through the sapwood, and into the heartwood. After drilling, all starts were aseptically sampled by collecting wood scrapings in two locations from within the start with a sharpened sample collection device and cored with a clean increment borer approximately 20 cm above the start following the protocol for RCW cavity starts in Chapter 1. Initially, galvanized steel screens with 0.64 x 0.64 centimeter openings were placed over all of the drilled starts in order to prevent injury to birds and other animals from resin leaking into the start. Once the resin stopped flowing copiously (July 2010), two of the four starts in each cluster were unscreened, allowing birds to access them. The drilled starts that remained screened and inaccessible to

RCWs serve as controls, allowing us to specifically test whether woodpeckers facilitate fungal infections. In December 2011, all drilled starts were re-sampled and cored at cavity height.

*DNA extraction, PCR, cloning and sequencing*

DNA was extracted from each drilled start sample following the protocol described by Brazee and Lindner (2013) with the modifications used in Chapter 1. To extract DNA from the swabs, the cotton tips of the swabs were cut off and placed into a 2 mL tube containing 300 µL of 2% hexadecyltrimethylammonium bromide (CTAB) solution, and these tubes were sealed and placed in a -20º C freezer for at least 24 hours. Frozen samples were then placed in a 65º C water bath for 2 hours, the swabs were then removed from their tubes and a 0.5 mL tube with the cap and bottom tip removed were placed on top of the 2 mL tubes, thus creating a small "funnel" that could be used to suspend swab tips in the 2 mL tubes. The swabs were then placed into the modified 0.5 mL funnels, and centrifuged for 15 second intervals at 3000 rcf until at least 290 µL of CTAB was recovered. DNA was extracted from 100 µL of the recovered CTAB following the protocol described by Brazee and Lindner (2013) with the modifications used in Chapter 1.

After DNA extraction, all samples were processed and subjected to PCR amplification, cloning and sequencing of fungal DNA. We performed PCR on the ITS region of the genome with the Basidiomycota specific primer pair ITS1F (Gardes and Bruns 1993) and ITS4b-21 (Chapter 1) following the protocol described in Chapter 1. All samples with PCR products that were visible on a gel were then cloned and sequenced following the protocol described by Lindner and Banik (2009). DNA sequences were edited using Sequencher 4.9 and identifications were obtained via Genbank BLAST (NCBI), using a 97% identity match cutoff for species level identification. In addition to traditional negative controls, and the field swab controls, we

included lab controls that underwent each step of the molecular process (i.e. our lab extraction controls underwent each downstream step).

*Analyses of RCW swabs*

We visualized Basidiomycota communities found on RCWs, in their excavations and in non-excavated trees in ordination space using nonparametric multidimensional scaling (NMDS) performed with the metaMDS function in the Vegan package of R (Oksanen et al. 2012), with the modified Raup-Crick dissimilarity metric (Chase et al. 2011), calculated by the raupcrick function in Vegan. We then statistically compared Basidiomycota community structure on RCW swabs, in complete and incomplete RCW excavations, and in non-excavated trees using nonparametric permutational multivariate ANOVA (PERMANOVA) tests (Anderson 2001) with the adonis function in the Vegan package of R (Oksanen et al. 2012), with the modified Raup-Crick distance metric. We performed these visualizations and tests for all Basidiomycota found and for the subset of the Basidiomycota taxa found that consisted only of known wood decay fungi.

*Analyses of drilling experiment*

The samples taken from each tree were pooled for analysis. We compared the change in Basidiomycota prevalence from pre-drilling to 26 months after drilling (the end of the experiment) using a chi-square analysis in R (chisq.test). We compared the Basidiomycota species richness from pre-drilling to the end of the experiment using species accumulation curves based on rarefaction equations (Hurlbert 1971) generated by the R package, Species (Czederplitz 2001). We compared Basidiomycota prevalence in the RCW accessible and RCW inaccessible experimental drilled starts (at the end of our experiment) using a chi-square analysis and we compared Basidiomycota species richness across these two treatments using the Species package

of R. We followed these same analysis protocols for the subset of the Basidiomycota that we detected that included wood decay fungi.

We then compared Basidiomycota communities in RCW accessible and RCW inaccessible drilled starts at the end of the experiment using PERMANOVA tests, with the modified Raup-Crick distance metric. We also visualized the Basidiomycota communities found in complete RCW cavities, RCW cavity starts, the 60 (non-excavated) experimental trees at the start of the experiment (controls), and the RCW accessible and inaccessible drilled starts using the NMDS ordination procedure described for RCW swabs. PERMANOVA tests were also performed to analyze the fungal community structure between these groups. The community data consisting of known decay fungi for the drilled starts were dominated by singletons and zeroes and community resolution between the two treatment groups could not be consistently resolved. Thus, we analyzed the RCW swabs and the experimental drilled starts separately in order to compare the communities of known wood decay fungi on the swabs to those found in RCW excavations.

## Results

### *RCW swabs*

We swabbed 11 birds and found that RCWs carry a variety of Basidiomycota fungi on their wings, beaks and feet. We produced PCR products for all 11 birds, but did not produce any PCR products or sequences from the swabs used for negative controls in the field or the lab. There was no difference in the fungal communities found on different areas of the birds that were swabbed (PERMANOVA, $p = 0.75$) so all areas per swabbed bird were pooled for all other analyses. We were able to identify 33 taxa of Basidiomycota on RCWs. Consistent with the bird

facilitation hypothesis, 27% (9/33) of the fungi we found on RCWs are also found in RCW excavations and 12% (4/33) were found in trees without excavations (Appendix D). Of the 4 fungal taxa found on RCWs and in trees without excavations, only one (*Irpex lacteus*) was not also found in RCW excavations. 61% (20/33) of the fungi we detected on RCWs were known wood decay fungi, of which 40% (8/20) were also found in RCW excavations. We successfully detected *Porodaedalea pini* SE, a fungus that is thought to be important for RCWs, on one bird out of the eleven we swabbed. We detected other known wood decay fungi on all eleven birds. The communities of Basidiomycota fungi and wood decay fungi found on RCWs are similar to those found in complete RCW cavities, and have some overlap with those found in RCW cavity starts but are compositionally different from those found in non-excavated trees (Figure 3.1). Basidiomycota fungal community composition in non-excavated trees was significantly different from the fungal community composition on RCW swabs, cavities and starts ($r^2 = 0.20$, *pseudo*-F = 6.40, $p < 0.0001$; Figure 3.1a), and this result extended to the subset of known wood decay fungi detected in these groups ($r^2 = 0.18$, *pseudo*-F = 4.69, $p < 0.0001$; Figure 3.1b).

*Drilling experiment*

We sampled our experimental trees upon drilling the cavity starts and found that 27% of them were infected with Basidiomycete fungi; they housed 17 taxa of Basidiomycota, of which 14 were known wood decay taxa (Chapter 2). After 26 months, 75% of the drilled cavity starts were infected with Basidiomycota, and the diversity increased to 46 taxa of Basidiomycota, 30 of which were known wood decay taxa (Appendix D). Seventeen wood decay taxa were detected in RCW accessible drilled starts and 16 were detected in the inaccessible drilled starts, and 3 out of 30 were found in both treatment groups. Prevalence and species richness of Basidiomycota and the subset of Basidiomycota that included wood decay fungi in drilled excavations increased

significantly two years after drilling (Basidiomycota prevalence $x^2 = 26.16$, $p < 0.0001$, species richness $p < 0.0001$, Shannon index $p = 0.038$; wood decay Basidiomycota prevalence $x^2 = 3.8511$, $p = 0.05$, species richness $p < 0.0001$, Shannon index $p = 0.027$ ), but RCW accessibility did not affect fungal richness or prevalence (Basidiomycota prevalence $x^2 = 0.09$, $p = 0.765$, species richness $p = 0.584$, Shannon index $p = 0.265$; wood decay Basidiomycota prevalence $x^2 = 0.27$, $p = 0.60$, species richness $p = 0.862$, Shannon index $p = 0.879$).

*Fungal community structure*

While Basidiomycota fungal species richness and prevalence rates were similar between the treatment groups, the fungal community composition between the two groups was different: RCW accessibility had a significant effect on Basidiomycota fungal community composition ($r^2 = 0.10$, *pseudo*-F = 4.56, $p = 0.002$).

Consistent with the bird facilitation hypothesis, we found significant fungal community structuring between the 5 types of trees ($r^2 = 0.18$, *pseudo*-F = 5.97, $p < 0.0001$; Figure 3.2). The Basidiomycota communities found in the accessible drilled starts at the end of our experiment were compositionally more similar to those found in completed RCW cavities than those found in RCW starts, non-excavated trees and inaccessible drilled starts at the end of our experiment (Figure 3.2). Furthermore, the Basidiomycota communities detected in the accessible drilled starts were more similar to those found in RCW starts than the communities detected in inaccessible drilled starts. The fungal communities present in any type of excavation were compositionally different from the Basidiomycota communities found in non-excavated trees.

## Discussion

We have demonstrated that RCWs carry DNA from a great diversity of fungi, including many wood decay taxa, and have also demonstrated that adult RCWs carry DNA from wood decay taxa and other Basidiomycota that are found in their excavations. Furthermore, the general Basidiomycota community composition detected on RCWs is similar to that found in their excavations (Fig. 3.1a), and this result holds true for wood decay Basidiomycota (Fig. 3.1b). The data from our cavity start experiment suggest that woodpeckers influence fungal colonization and community development in their excavations either directly, by acting as agents of fungal dispersal, or indirectly via habitat modification. Our results support the bird facilitation hypotheses and suggest that RCWs facilitate fungal infection of living trees via transmission during cavity excavation (Fig. 3.2). The fungi in turn may help the birds with excavation by softening the heartwood of the trees in which they excavate.

### *RCW Swabs*

If birds transmit fungi from their roost cavities into their cavity starts, one would also expect the birds themselves to carry fungi found in their roost cavities and cavity starts. The fungal community composition detected on RCWs more closely resembles that of their completed cavities than that of their cavity starts. One explanation for this result is that the birds that were sampled were swabbed immediately after departing their roost cavities. Thus, one would expect most of the fungi found on the birds to also be found in their cavities, and a portion of these fungi should be found in cavity starts.

The most prevalent Basidiomycota taxa detected in RCW cavities and starts, Exobasidiomycetes sp. 2 and *Porodaedalea pini* SE, were also detected on the birds. We only detected *P. pini* SE on one of the eleven birds we swabbed, which could reflect incomplete

sampling. It is also possible that *P. pini* SE and other fungi commonly associated with RCW excavations were not in the cavities these particular birds were using, or that not all birds carry all fungi at all times, or that this species of fungus does not rely much on RCWs for transmission. In addition to *P. pini* SE, we detected eight other wood decay taxa on the birds that also occur in their excavations, as well as twelve other wood decay taxa that we did not detect in their excavations. We also detected many other fungi from the phylum Basidiomycota on the birds whose functions are largely unknown. In addition to carrying fungi found in their excavations, these birds come into contact with many trees on a daily basis and possibly encounter spores in flight or on the invertebrates they consume.

*Experimental cavity starts*

In RCW accessible and inaccessible drilled cavity starts, the prevalence and diversity of Basidiomycota, including fungi from the wood decay group, increased two years after drilling, and while both prevalence and diversity were similar, the fungal communities were different. Thus the experiment demonstrated that excavations that are accessible to RCWs are colonized by different fungal communities than excavations that are inaccessible to RCWs, and the fungal communities in both accessible and inaccessible excavations are different from those in non-excavated trees. RCWs appear to influence fungal colonization and fungal community composition, including wood decay fungi, in living trees in two ways: 1) indirectly, by making a hole in a tree and 2) directly, by carrying fungi from their roost cavities into cavity starts (Figure 3.2).

The fungal communities in accessible starts progress along a fungal community trajectory that is similar to that of RCW cavities while the inaccessible starts take a different path (Figure 3.2). This difference is driven by fungal community composition; the general composition is

different between the two groups and the inaccessible starts have a community dominated by *Acaromyces ingoldii*. This fungus is also present in RCW cavities, RCW starts and in our accessible experimental starts, but is much less prevalent in these cavity types. The Basidiomycota communities in the experimental accessible starts more closely resemble those in completed RCW cavities than those in RCW cavity starts.

There are other fungi besides *A. ingoldii* driving the difference between RCW starts and the experimental starts, and most of these differences may be related to excavation depth which correlates with excavation age in RCW starts. All of our drilled cavity starts were "advanced starts"; these starts tunneled through the sapwood and into the heartwood of the trees, leaving only the cavity chamber unexcavated. Many of the RCW starts sampled were much less advanced and had not yet reached the heartwood of the tree. The process of tunneling through the sapwood is a time limiting step in RCW cavity excavation and thus some of the fungi detected in recently initiated RCW starts may represent early successional species (Chapter 2). We sampled the heartwood of all starts (RCW and experimental) by extracting a core above the start; thus the differences seen in these two groups are not likely due to sampling error. The Basidiomycota communities in RCW starts become more compositionally similar to those in RCW cavities as they become more advanced (Chapter 2). By tunneling through the sapwood and into the heartwood of the experimental starts, we seem to have "artificially advanced" fungal community succession. We further altered the trajectory in inaccessible drilled starts by restricting access to RCWs and other vertebrates (Fig. 3.2).

*Conclusion*

Through a test of the bird facilitation hypothesis, we have provided the first experimental evidence that a primary cavity excavator facilitates fungal colonization of living trees. The

possible relationships between cavity excavators and the fungi that aid excavation have interested researchers for decades, and many have suggested that woodpeckers select trees infected by certain fungi for excavation (Conner et al. 1976, Jackson and Jackson 2004, Witt 2010, Blanc and Martin 2012, Cockle et al. 2012, Zahner et al. 2012). We have demonstrated that the relationships between woodpeckers and fungi are far more complex than previously imagined, and stretch beyond habitat selection by documenting a potentially mutualistic relationship between woodpeckers and multiple species of fungi. Our system is unique in that RCWs solely excavate through the sapwood and into the heartwood of living pine trees, but the possible multipartite keystone mutualism we have described may not be a singular, isolated example. Even excavators who complete cavities very quickly could introduce fungi into trees. Convergent multipartite mutualisms between excavators and fungi might help maintain native biodiversity in forested systems world-wide, and therefore an understanding of these interactions may be vital to conservation efforts for birds, fungi, and all organisms that rely on the tree cavities produced by these ecosystem engineers.

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**Figure captions**

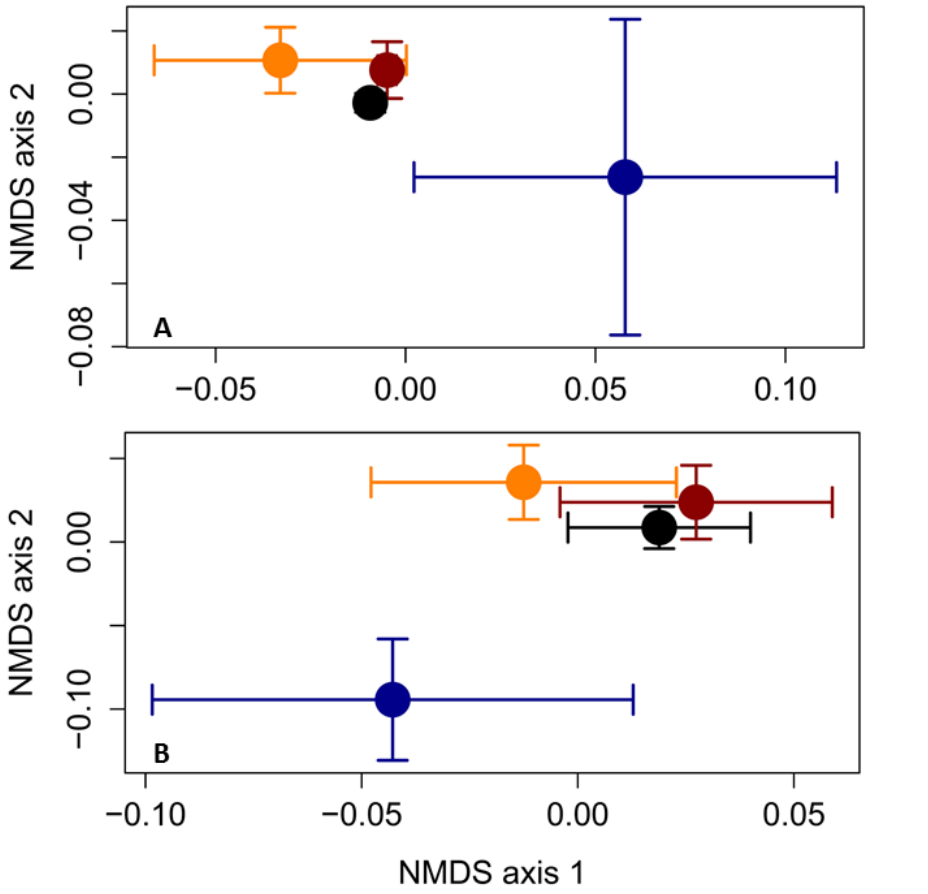
Figure 3.1

NMDS ordinations of the communities of Basidiomycota fungi (A) and a subset of that group that contains the wood decay fungi (B) found on RCWs and in RCW cavities, RCW cavity starts and non-excavated trees. The dots in the center represent the means of the points on the two NMDS axes, and the bars represent one standard error from the mean. A) stress = 0.00687, two dimensions, 200 iterations. B) stress = 0.00387, two dimensions, 200 iterations.

Figure 3.2

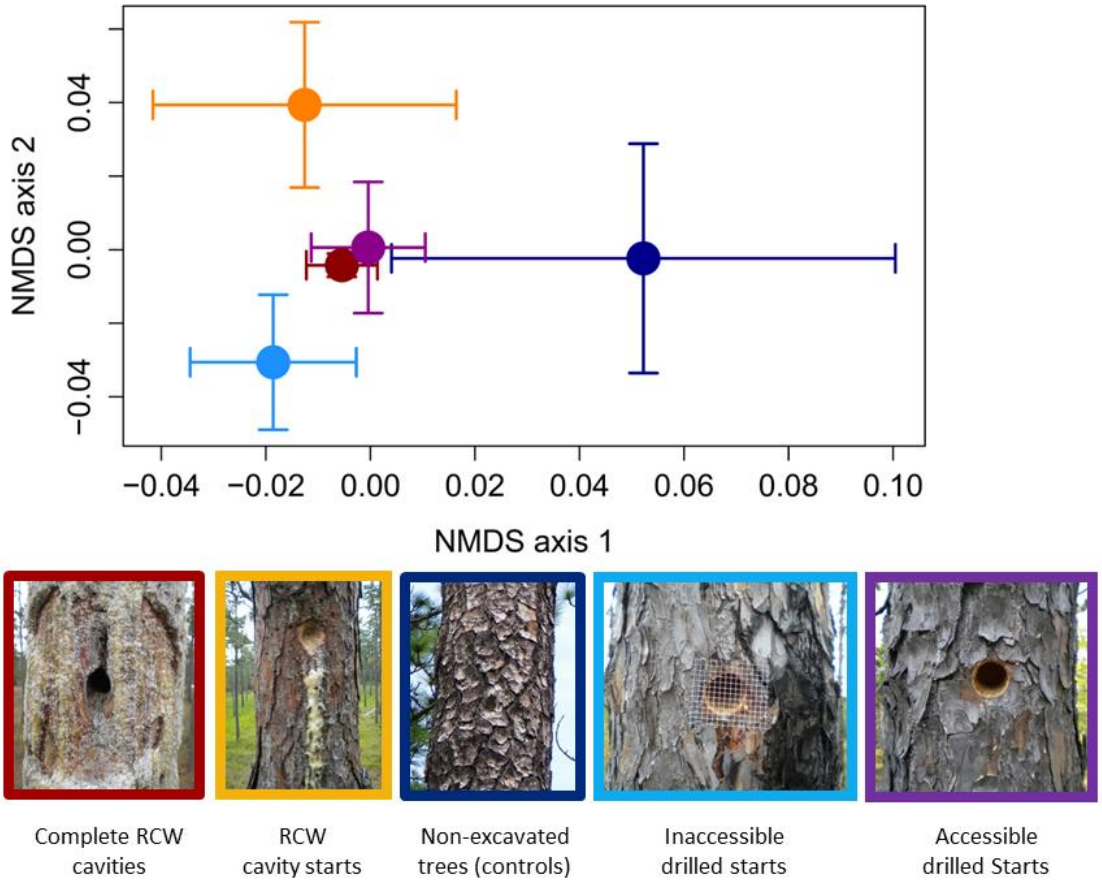
NMDS Ordinations of communities of Basidiomycota fungi found in completed RCW cavities, RCW cavity starts, non-excavated control trees, drilled starts accessible to RCWs and drilled starts inaccessible to RCWs. The dots in the center represent the means of the points on the two NMDS axes, and the bars represent one standard error from the mean. Stress = 0.0062, two dimensions, 200 iterations.

Figure 3.1: NMDS ordinations of the communities of Basidiomycota fungi (A), wood decay fungi (B) found on RCWs and in RCW cavities, RCW cavity starts and non-excavated trees




Complete RCW cavities | RCW cavity starts | Non-excavated trees (controls) | RCW swabs

Figure 3.2: NMDS Ordinations of communities of Basidiomycota fungi found in completed RCW cavities, RCW cavity starts, non-excavated control trees, drilled starts accessible to RCWs and drilled starts inaccessible to RCWs



Appendix D: Basidiomycota taxa identified with ITS1F and ITS4b-21, by sample type. Numbers indicate the number of trees or swabs in which each taxon was found.
WD Indicates wood decay taxa
* Indicates data that were originally reported in Chapter 2.

| GenBank accession number | Taxon | Closest identified taxon with GenBank BLAST (accession number) | % Base pair match | % Query coverage | Inacc-essible drilled starts | Access-ible drilled starts | RCW swabs | Comp-lete RCW cavities* | RCW cavity starts* | Non-excavated trees* | Total detections |
|---|---|---|---|---|---|---|---|---|---|---|---|
| KM103937 | *Acaromyces ingoldii* | *Acaromyces ingoldii* (NR_073342.1) | 100 % | 100 % | 15 | 4 | 0 | 8 | 3 | 0 | 30 |
| KM103938 | *Acaromyces* sp. 1 | *Acaromyces ingoldii* (NR_073342.1) | 98 % | 100 % | 8 | 8 | 0 | 10 | 0 | 0 | 26 |
| KM104040 | *Porodaedalea pini* SE[WD] | *Porodaedalea* sp. (JX110039.1) | 100 % | 100 % | 0 | 2 | 1 | 8 | 13 | 2 | 26 |
| KM104130 | Exobasidiomycetes sp. 2 | Exobasidiomycetidae sp. (DQ682574.1) | 100 % | 96 % | 0 | 1 | 5 | 13 | 2 | 0 | 21 |
| KM103972 | *Cryptococcus* sp. 1 | *Cryptococcus pinus* (NR_111269.1) | 99 % | 94 % | 3 | 8 | 0 | 0 | 0 | 0 | 11 |
| KM103971 | *Cryptococcus neoformans* | *Cryptococcus neoformans* (KC254022.1) | 100 % | 100 % | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| KM103967 | *Coniophora* sp. 1[WD] | *Coniophora prasinoides* (GU187519.1) | 91 % | 100 % | 0 | 0 | 7 | 2 | 0 | 0 | 9 |
| KM104041 | *Postia sericeomollis*[WD] | *Postia sericeomollis* (KC585367.1) | 98 % | 100 % | 0 | 0 | 6 | 2 | 0 | 0 | 8 |
| KM103976 | *Daedaleopsis confragosa*[WD] | *Daedaleopsis confragosa* (KC176348.1) | 99 % | 100 % | 0 | 0 | 7 | 0 | 0 | 0 | 7 |
| KM104071 | *Trametes versicolor*[WD] | *Trametes versicolor* | 99 % | 100 % | 2 | 3 | 1 | 1 | 0 | 0 | 7 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (KC176344.1) | | | | | | | | | |
| KM104143 | Unidentified Basidiomycete 7 | *Bullera formosana* (AB118873.1) | 88 % | 70 % | 1 | 1 | 0 | 4 | 1 | 0 | 7 |
| KM103987 | *Exidia* sp. 1[WD] | *Exidia* sp. (DQ241774.1) | 100 % | 90 % | 1 | 2 | 2 | 0 | 0 | 0 | 5 |
| KM104023 | *Peniophora cinerea*[WD] | *Peniophora cinerea* (GU062269.1) | 99 % | 98 % | 2 | 0 | 1 | 1 | 0 | 1 | 5 |
| KM104061 | *Stereum complicatum*[WD] | *Stereum complicatum* (KJ140563.1) | 99 % | 98 % | 1 | 1 | 2 | 1 | 0 | 0 | 5 |
| KM104021 | *Peniophora incarnata*[WD] | *Peniophora* sp. (KC176330.1) | 99 % | 100 % | 0 | 0 | 1 | 1 | 0 | 2 | 4 |
| KM104086 | *Trichosporon asahii* | *Trichosporon asahii* (AB369919.1) | 100 % | 99 % | 0 | 0 | 0 | 2 | 1 | 1 | 4 |
| KM104030 | *Phlebia brevispora*[WD] | *Phlebia brevispora* (AB084616.1) | 99 % | 99 % | 0 | 0 | 0 | 3 | 0 | 0 | 3 |
| KM104033 | *Pisolithus tinctorius* | *Pisolithus tinctorius* (EU718114.1) | 99 % | 99 % | 1 | 2 | 0 | 0 | 0 | 0 | 3 |
| KM104058 | *Skeletocutis chrysella*[WD] | *Skeletocutis chrysella* (JQ673127.1) | 99 % | 100 % | 0 | 0 | 0 | 1 | 1 | 1 | 3 |
| KM104063 | *Stereum* sp. 4[WD] | *Stereum* sp. (JX460856.1) | 98 % | 100 % | 2 | 0 | 0 | 0 | 1 | 0 | 3 |
| KM104120 | Tremellales sp. 12 | *Cryptococcus* sp. (EF363148.1) | 82 % | 96 % | 1 | 2 | 0 | 0 | 0 | 0 | 3 |
| KM103955 | *Athelia arachnoidea*[WD] | *Athelia arachnoidea* (GU187504.1) | 99 % | 100 % | 0 | 0 | 0 | 1 | 0 | 1 | 2 |
| KM103956 | Atheliaceae sp. 1[WD] | *Amphinema* sp. 7 (JN943925.1) | 96 % | 100 % | 0 | 1 | 1 | 0 | 0 | 0 | 2 |
| KM104112 | *Bjerkandera adusta*[WD] | *Bjerkandera adusta* (KC176334.1) | 99 % | 100 % | 1 | 0 | 1 | 0 | 0 | 0 | 2 |
| KM103973 | *Cryptococcus* sp. 2 | *Cryptococcus gattii* (JN939462.1) | 93 % | 100 % | 0 | 1 | 0 | 0 | 0 | 1 | 2 |
| KM103999 | *Heterobasidion irregulare*[WD] | *Heterobasidion irregulare* | 100 % | 100 % | 1 | 0 | 1 | 0 | 0 | 0 | 2 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (KC492935.1) | | | | | | | | | |
| KM104002 | *Irpex lacteus*[WD] | *Irpex lacteus* (JX290578.1) | 99 % | 100 % | 0 | 0 | 1 | 0 | 0 | 1 | 2 |
| KM104036 | *Peniophora* sp. 4[WD] | Polyporales sp. (EF672293.1) | 99 % | 100 % | 0 | 0 | 0 | 1 | 1 | 0 | 2 |
| KM104032 | *Phlebia tremellosa*[WD] | *Phlebia tremellosa* (AB811854.1) | 99 % | 100 % | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| KM104122 | Polyporales sp. 8[WD] | *Daedaleopsis confragosa* (KJ140587.1) | 82 % | 89 % | 0 | 0 | 2 | 0 | 0 | 0 | 2 |
| KM104055 | *Serpula himantioides*[WD] | *Serpula himantioides* (GU187545.1) | 99 % | 100 % | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| KM104059 | *Skeletocutis odora*[WD] | *Skeletocutis odora* (FJ903307.1) | 100 % | 98 % | 1 | 0 | 0 | 0 | 0 | 1 | 2 |
| KM104073 | *Tremella encephala* | *Tremella encephala* (EU673083.1) | 99 % | 100 % | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| KM104113 | Tremellales sp. 11 | *Cryptococcus* sp. (AY518273.1) | 93 % | 90 % | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| KM104132 | Unidentified Basidiomycete 45 | *Cryptococcus* sp. (GU997163.1) | 88 % | 48 % | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| KM104152 | *Vacellum pretense* | *Vascellum pratense* (DQ112554.1) | 99 % | 95 % | 1 | 0 | 1 | 0 | 0 | 0 | 2 |
| KM103939 | Agaricomycetes sp. 1[WD] | Polyporales sp. (EF694649.1) | 99 % | 97 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM103941 | Agaricomycetes sp. 11[WD] | *Grifola sordulenta* (GU222266.1) | 90 % | 100 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104131 | Agaricomycetes sp. 17[WD] | *Amyloathelia crassiuscula* (DQ144610.1) | 82 % | 91 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM104137 | Agaricomycetes sp. 18[WD] | Agaricomycetes sp. 20KY06 (JX270560.1) | 99 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM103945 | Agaricomycetes sp. 3 | *Entomocorticium* sp. (DQ118417.1) | 97 % | 97% | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM103957 | Atheliaceae sp. 2[WD] | *Athelia* sp. (GU187502.1) | 96 % | 97 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM103947 | Atheliales sp. 3[WD] | *Amphinema* sp. 5 | 86 % | 97 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|
| | | (JN943909.1) | | | | | | | | |
| KM103943 | Boletales sp. 1 | *Boletus pulcherrimus* (EU669376.1) | 88 % | 84 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM103960 | *Calvatia* sp. 1 | *Calvatia fragilis* (AJ617493.1) | 99 % | 100 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM103966 | *Collybia subnuda*[WD] | *Collybia subnuda* (U43780.1) | 99 % | 100 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM103968 | *Coprinellus* sp. 1[WD] | *Coprinellus radians* (AY461815.1) | 99 % | 100 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104035 | *Crustoderma* sp. 1[WD] | *Crustoderma corneum* (KC585319.1) | 93 % | 100 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM103988 | Cryptobasidiaceae sp. 1 | *Clinoconidium* sp. (AB245088.1) | 93 % | 92 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103974 | *Cryptococcus* sp. 5 | *Cryptococcus* sp. (HQ890370.1) | 99 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103942 | Entolomataceae sp. 1 | *Entoloma* sp. (JX029939.1) | 96 % | 100 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM103986 | *Exidia glandulosa*[WD] | *Exidia glandulosa* (AY509555.1) | 98 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM103989 | *Fibroporia radiculosa*[WD] | *Fibroporia radiculosa* (KC585339.1) | 100 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103990 | *Fibulorhizoctonia* sp. 1 | *Fibulorhizoctonia* sp. (AY854062.1) | 92 % | 100 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM103994 | *Ganoderma* sp. 1[WD] | *Ganoderma meredithae* (JQ520191.1) | 99 % | 96 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM103996 | *Gymnopilus* sp. 1[WD] | *Gymnopilus* sp. (AY281019.1) | 95 % | 100 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM103997 | *Gymnopus spongiosus*[WD] | *Gymnopus spongiosus* (AF505784.1) | 99 % | 96 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104000 | *Hyphoderma setigerum*[WD] | *Hyphoderma setigerum* (KJ140750.1) | 99 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103969 | *Hyphodontia* sp. | *Hyphodontia* sp. | 99 % | 95 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1[WD] | (DQ340319.1) | | | | | | | | | |
| KM103970 | *Hypochnicium* sp. 1[WD] | *Hypochnicium punctulatum* (AF429412.1) | 92 % | 90 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104008 | *Mycena clavicularis*[WD] | *Mycena clavicularis* (JF908466.1) | 99 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103963 | *Mycoacia fuscoatra*[WD] | *Mycoacia fuscoatra* (KJ140744.1) | 99 % | 99 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM104009 | *Neolentinus lepideus*[WD] | *Neolentinus lepideus* (AB615456.1) | 98 % | 98 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM104010 | Outerspace Basidio 1 | *Exobasidium symploci-japonicae* (AB180351.1) | 95 % | 31 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104011 | Outerspace Basidio 2 | *Tremella polyporina* (JN053501.1) | 88 % | 56 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104012 | *Oxyporus populinus*[WD] | *Oxyporus populinus* (KJ140633.1) | 99 % | 99 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM104022 | *Peniophora* sp. 1[WD] | *Peniophora pini* (EU118651.1) | 97 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104047 | *Peniophora* sp. 5[WD] | *Peniophora* sp. (JN198493.1) | 98 % | 95 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104031 | *Phlebia radiata*[WD] | *Phlebia radiata* (KF156332.1) | 99 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104037 | *Phlebiella* sp. 1[WD] | *Phlebiella christiansenii* (EU118659.1) | 92 % | 98 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104129 | Polyporales sp. 9[WD] | *Trametes versicolor* (JN164975.1) | 85 % | 83 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104039 | *Polyporus squamosus*[WD] | *Polyporus squamosus* (AF516589.1) | 100 % | 100 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM104054 | *Schizophyllum commune*[WD] | *Schizophyllum commune* | 99 % | 99 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (JN882337.1) | | | | | | | | | |
| KM104083 | *Schizopora* sp. 1[WD] | *Schizopora* sp. (JQ673190.1) | 99 % | 100 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM104144 | Septobasidiaceae sp. 1 | *Septobasidium ramorum* (DQ241450.1) | 87 % | 83 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104056 | *Sistotrema brinkmannii*[WD] | *Sistotrema brinkmannii* (DQ899094.1) | 99 % | 100 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM104139 | *Sistotremastrum* sp. 2[WD] | *Sistotremastrum* sp. (KJ140723.1) | 92 % | 98 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM104057 | *Skeletocutis* sp. 10[WD] | *Skeletocutis diluta* (JF692198.1) | 99 % | 95 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM103948 | Stereaceae sp. 1[WD] | *Acanthophysium lividocaeruleum* (AY618666.1) | 94 % | 96 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104060 | *Stereum sanguinolentum*[WD] | *Stereum sanguinolentum* (KF996533.1) | 98 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM103944 | Strophariaceae sp. 1 | *Psilocybe* sp. (AB968235.1) | 92 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104066 | *Thelephora terrestis* | *Thelephora terrestris* (JQ711777.1) | 98 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104069 | *Trametes conchifer*[WD] | *Trametes conchifer* (JN164988.1) | 99 % | 100 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104070 | *Trametes cubensis*[WD] | *Trametes cubensis* (JN164922.1) | 100 % | 100 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104072 | *Trechispora* sp. 1 | *Trechispora alnicola* (DQ411529.1) | 92 % | 99 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104074 | *Tremella* sp. 1 | *Tremella phaeophysciae* (JN053479.1) | 93 % | 98 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104075 | Tremellales sp. 1 | *Cryptococcus bromeliarum* (EU386359.1) | 99 % | 85 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104076 | Tremellales sp. 10 | *Cryptococcus* sp. (EF363146.1) | 88 % | 96 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| KM104078 | Tremellales sp. 6 | *Hannaella* sp. (JQ753983.1) | 99 % | 94 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104079 | Tremellales sp. 7 | *Bullera psuedoalba* (NR_073231.1) | 100 % | 98 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104080 | Tremellales sp. 8 | *Tremella foliacea* (AF444431.1) | 94 % | 98 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104082 | *Trichaptum abietinum*[WD] | *Trichaptum abietinum* (U63475.1) | 98 % | 100 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM104081 | *Trichaptum birforme*[WD] | *Trichaptum biforme* (U63473.1) | 99 % | 100 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM104142 | *Typhula* sp. 1 | *Typhula* sp. (AF193390.1) | 99 % | 98 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104116 | Unidentified Basidiomycete 15[WD] | *Brevicellicium exile* (HE963779.1) | 91 % | 41 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM104117 | Unidentified Basidiomycete 17[WD] | *Athelia bombacina* (U85795.1) | 87 % | 98 % | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| KM104119 | Unidentified Basidiomycete 19 | *Meira geulakonigii* (NR_073343.1) | 93 % | 42 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104127 | Unidentified Basidiomycete 33 | *Acaromyces ingoldii* (NR_073342.1) | 94 % | 75 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104133 | Unidentified Basidiomycete 46[WD] | *Sidera vulgaris* (FN907918.1) | 87 % | 46 % | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| KM104134 | Unidentified Basidiomycete 48[WD] | *Stereum* sp. (EU009970.1) | 98 % | 66 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| KM104135 | Unidentified Basidiomycete 49[WD] | *Botryobasidium botryosum* (DQ267124.1) | 90 % | 54 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| KM104140 | Unidentified Basidiomycete 57 | Tremellales sp. (EF060778.1) | 91 % | 50 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104141 | Unidentified Basidiomycete 58[WD] | *Coniophora prasinoides* (GU187519.1) | 90 % | 69 % | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| KM104147 | Unidentified Basidiomycete 63[WD] | *Dendrophora albobadia* (AF119522.1) | 99 % | 91 % | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| KM104145 | Unidentified | *Dichostereum* | 92 % | 95 % | 1 | 0 | 0 | 0 | 0 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| | Basidiomycete 9[WD] | *granulosum* (KJ010078.1) | | | | | | | | | |
| KM104154 | *Xeromphalina campanella*[WD] | *Xeromphalina campanella* (HQ604741.1) | 98 % | 100 % | 0 | 0 | 0 | 1 | 0 | 0 | 1 |

## Chapter 4. Succession of fungal communities in living pine trees: a woodpecker facilitated process

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ABSTRACT

Previous work has demonstrated that red-cockaded woodpeckers (*Picoides borealis*) are associated with distinct communities of fungi which may facilitate woodpecker cavity excavation. Fungal communities in other systems, including communities associated with wood decay processes, are known to change in composition and function through time. Temporal changes in fungal communities associated with woodpeckers could have implications for the ability of woodpeckers to efficiently excavate cavities. Here, we used survey data for complete and incomplete red-cockaded woodpecker excavations and long-term monitoring data of cavity age to assess fungal succession in the excavations of red-cockaded woodpeckers. Additionally we assess time series data of fungal communities within experimentally drilled cavities which were manipulated to be woodpecker accessible or inaccessible. We test two hypotheses about fungal community succession in living pine trees, (1) tree excavations undergo directional changes in fungal community composition; and (2) red-cockaded woodpeckers influence fungal community succession in their excavations. Our results show support for both hypotheses. The analyses of the survey data determined that fungal communities in complete and incomplete red-cockaded woodpecker excavations change directionally through time. Experimental time series data demonstrate that red-cockaded woodpeckers may directly influence fungal community succession in their excavations. We relate successional patterns and woodpecker effects to taxon-

specific changes in the fungal communities. Our results show that woodpecker-fungus interactions are more complex than previously assumed and provide evidence for multiple previously undescribed associations between woodpeckers and fungi.

## Introduction

Ecosystems are composed of multiple communities of organisms, some of which are dependent on others. These interdependent relationships are not static, but change over time. In forest systems, for example, the above ground flora that provide habitat structures and primary production for above ground fauna are largely dependent on below ground communities of mycorrhizal fungi. Different mycorrhizal communities appear to be associated with the various successional stages of forests; some of these fungi are generalists, others are specialists, and their dispersal and colonization abilities also vary across a spectrum (Dickie et al. 2013 and citations therein). The succession of the below ground mycorrhizal communities is coupled with the succession of forest systems (Dickie et al. 2013, Peay et al. 2013). Fungi, despite being largely cryptic and easily overlooked in many ecosystems, are critical partners with many organisms and fungal communities undergo succession in concert with their symbiotic partners.

Fungal communities are particularly vital to above ground processes in forests such as wood decay, an important process for the cycling of resources, nutrients, and energy at the ecosystem level (Boddy 2001, Lindner et al. 2011). Fungal communities associated with wood decay also undergo succession, with early arrivers being generally characterized as good dispersers that help "prime" the habitat for later arrivers (Stenlid and Gustafsson 2001). Generally the fungal communities associated with wood decay are studied in fallen logs, or in dead or dying trees. In these habitats there are vast assemblages of arthropods and other

organisms that are dependent on the resources that are made available during the wood decay process, and some organisms have developed specific partnerships with fungi. In some cases, wood-inhabiting beetles have been shown to impact early fungal colonization and subsequent succession in spruce logs (Müller et al. 2002, Strid et al. 2014).

Higher in the canopy, there is another community that may have a partnership with fungal communities associated with decay: vertebrates that utilize tree cavities. In other systems vertebrates have been shown to impact fungal community composition. Examples range from the effects of root grazing herbivores on mycorrhizal fungi (Eom et al. 2001), to the effects that the bark peeling behavior of moose and red deer have on the initial colonization of fungi near the base of trees (Vasiliauskas et al. 1996), which may facilitate the subsequent invasion of arthropods such as bark beetles. Vertebrates that depend on tree cavities for nesting and roosting sites are limited by the availability of cavities, and the formation and excavation of many tree cavities, especially those in living trees, is facilitated by wood decay fungi. Cavity users, especially those that excavate their own cavities, may influence fungal community development in the tree cavities they inhabit. Here we examine this possibility in the recently described (Chapter 2) fungal communities in cavities made by an endangered primary cavity excavator, the red-cockaded woodpecker (*Picoides borealis*; RCW).

Red-cockaded woodpeckers are endemic to the fire-maintained longleaf pine (*Pinus palustris*) ecosystem of the Southeastern United States. These birds are cooperative breeders that live in family groups, and each family group maintains a territory containing a set cluster of complete (cavities) and incomplete (cavity starts) RCW-made excavations. The tree cavity excavation process of RCWs is unique in that they only excavate through the sapwood and into the heartwood of relatively healthy living pine trees, primarily longleaf pine. RCWs can take

many years to excavate a cavity (Harding 1997, Harding and Walters 2004). The estimated average excavation time in longleaf pines across three RCW populations is eight years (Harding 1997), and the fungal communities found in their complete and incomplete excavations differ from those found in non-excavated trees in RCW territories (Chapter 2). These birds could facilitate the formation of fungal communities in their excavations in two ways (Chapter 3). First, simply by excavating cavities they create a wound in the tree that could provide fungi access to the interior of the tree. Second, in accessing their excavations they could transport fungi into the excavation. The fungal communities in human-made excavations that are accessible to RCWs resemble those in completed RCW excavations, but those in identical human-made excavations that are not available to the birds do not (Chapter 3). Furthermore, the fungi found on the birds closely resemble those found in their completed excavations (Chapter 3). These findings suggest that by transporting fungi, RCWs influence the composition of fungal communities in their excavations. Thus, the relationship between red-cockaded woodpeckers and the fungal communities in their excavations may be an example of a multipartite mutualism (Chapter 3), that helps maintain a community of cavity users in at least one ecosystem, the longleaf pine ecosystem.

Fungal community succession is important for late arriving species, such as wood decay fungi, especially in living trees (Stenlid et al. 2008). We suspect that RCWs initiate fungal community succession in living pine trees and then perpetuate it via their excavation behavior. In this study we tracked the change in Basidiomycota communities over time in human-made, incomplete RCW excavations (cavity starts) in living longleaf pine trees, half of which were unavailable to the birds, in order to test the following hypotheses: (1) tree excavations undergo

fungal community succession; and (2) RCWs influence fungal community succession in their excavations.

## Methods

### *Study site and field methods*

This research took place at Marine Corps Base Camp Lejeune (MCBCL) located on the Atlantic coast of North Carolina, USA. See Chapters 1 and 2, and MCBCL (2006) for further information on the study site.

In the fall of 2009, 15 RCW clusters on MCBCL were selected, and all active complete and incomplete RCW excavations were sampled following the protocol in Chapter 1. In each of these 15 clusters, human-made cavity starts were sterilely drilled through the sapwood and into the heartwood of 4 longleaf pine trees that had not been excavated by an RCW, but with aspects similar to trees containing excavations (see Chapters 2 and 3 for details). These starts were sampled following the methods described in Chapter 1 upon drilling and then all starts were screened. In May of 2010, all starts were re-sampled following the same methodology, except that cores were not taken. In July of 2010, after the resin was dry in all of the drilled starts, 2 per cluster were unscreened to allow access to birds, and 2 per cluster remained screened and were thus inaccessible to the birds. The drilled starts were re-sampled a third time in December of 2010, a fourth time in June 2011 and a fifth and final time in December 2011.

### *Molecular methods*

DNA was extracted from each sample following the protocol described by Brazee and Lindner (2013) with the modifications described in Chapter 1. We performed PCR on the

extracted DNA with a primer pair specific to fungi in the phylum Basidiomycota, ITS1F (Gardes and Bruns 1993) and ITS4b-21 (Chapter 1) following the PCR protocol described in Chapter 1. We cloned and sequenced all PCR products that were visible on a gel following the protocol described by Lindner and Banik (2009). Eight clones were picked per sample, and all of the clones from each tree were combined for analysis. We edited DNA sequences using Sequencher 4.9 and DNA sequence identifications were obtained via Genbank BLAST (NCBI), using a 97% identity match cutoff for species level identification.

*Analyses of RCW excavations*

We used a combined approach of multivariate ordination and linear regression to assess successional changes in fungal community composition through the "cavity lifetime" of RCW excavations. In order to make linear response variables describing changes in the composition of Basidiomycota fungal communities in RCW excavations, we ran a principal coordinates analysis (PCOa) of fungal community data from 22 RCW cavity starts and 32 completed RCW cavities using the 'pcoa' function, in the ape package (version 3.0-9) of R, with an analytical Raup-Crick distance matrix (suitable for presence / absence data), calculated using the 'vegdist' function in the vegan package (version 2.0-4) of R. Excavations that were not positive for any Basidiomycota were excluded from analysis to avoid zero sum rows which cause misleading distance calculations. We then used the PCOa axis scores as linear response variables in a generalized linear model (GLM) to quantitatively assess changes in Basidiomycota composition as a function of RCW excavation type (cavity starts or completed cavities) and excavation age. This was done using the 'glm' function of the R base package (version 2.15.1), with Gaussian error distribution. Model fit was assessed by visualizing model fits overlaid with the raw data, Q-Q plots, and predictions/residuals plots (Zuur et al. 2009). We then identified the individual

fungal taxa most strongly related to the first and second PCOa axes using Pearson's correlation coefficients (r) for axis scores and fungal community data.

We developed taxon-specific models for the prevalence of the four most frequently detected Basidiomycota in completed RCW cavities and RCW starts as a function of excavation age and cavity type, *Porodaedalea pini* SE (detected in 21 excavations), Exobasidiomycetes sp. 2 (detected in 15 excavations), *Acaromyces ingoldii* (detected in 11 excavations), and *Acaromyces* sp. 1 (detected in 10 excavations). The next most common taxon was found in 5 excavations and was not included. For these analyses we included excavations for which no Basidiomycota were detected because our question pertained to the prevalence of specific taxa across all trees examined. We modeled taxon-specific prevalence using data from 44 RCW cavity starts and 36 completed RCW cavities using the 'glm' function of the R base package (version 2.15.1), with binomial error distribution (suitable for presence / absence data; (Zuur et al. 2009). For each model, we started with excavation type, excavation age and a type x age interaction as independant variables and sequentially dropped non-significant predictors.

*Analyses of experimental drilled cavity starts*

We assessed the effects of RCW's on fungal infection rates of longleaf pine excavations by comparing Basidiomycota prevalence between RCW accessible (unscreened) and RCW inaccessible (screened) experimental drilled cavity starts through time. We used a generalized linear mixed model fit by the Laplace approximation to compare the Basidiomycota prevalence through time and across treatment groups in the experimental drilled starts. Presence of Basidiomycota was the response variable for the GLMM, treatment group and sampling date and their interaction were fixed effects. Because our experimental design included repeated measures of individual excavations, we included unique tree identifiers as a random effect (random

intercept). We assigned trees to treatment groups for the time points prior to the treatment to ensure non-biased comparisons.

We assessed the effects of RCWs on the temporal dynamics of fungal communities within the experimental drilled starts using ordination and permutational hypothesis tests. We visualized the changes in Basidiomycota communities in the experimental drilled starts through time and across treatments using a PCOa in the ape package of R, based on a Raup-Crick distance metric, calculated using the 'vegdist' function in the vegan package (version 2.0-4) of R. The coordinates for the first two axes were used to generate a visualization of fungal community change through time. We then identified the fungal taxa highly correlated (correlation greater than 50%) to the first two PCOa axes (Pearson's r). We also compared Basidiomycota communities in the experimental drilled starts at the five time points of the experiment using nonparametric permutational multivariate ANOVA (PERMANOVA) tests (Anderson 2001), with the Raup distance metric, in the vegan package of R (Oksanen et al. 2012). Using PERMANOVA tests, we examined the effects of the treatment, the geographic area within the base (comparable to the four cardinal directions), and RCW cluster on the fungal communities found in the experimental starts.

## Results

### *RCW excavations*

Our analysis of RCW-made excavations revealed successional patterns of fungal communities through the lifetime of RCW excavations. The first PCOa axis explained 46% of the survey-wide variation in fungal community composition and was strongly correlated with *Porodaedalea pini* SE (r = 0.93) and Exobasidiomycetes sp. 2 (r = -0.74). The second axis

explained 19% of the variation and was strongly correlated with *Acaromyces ingoldii* (r = 0.79). The excavation age and type were both significant predictor variables for PCOa axis one (excavation age, t value = -3.360, p = 0.001; excavation type, t value = 3.439, p = 0.001; Figure 4.1), but the interaction between excavation age and type was not significant and therefore was dropped during model selection. No significant effects of excavation age or type were found for PCOa axis 2.

The prevalence of several fungal taxa was significantly related to excavation type and age. The prevalence of Exobasidiomycetes sp. 2 increased significantly with cavity age, and it was more prevalent in completed cavities than in cavity starts (excavation age, $z$ = 3.165, p = 0.0015; excavation type, $z$ = -2.836, p = 0.0046; Figure 4.2a). *Acaromyces ingoldii* was also more prevalent in completed cavities than cavity starts (excavation type, $z$ = -2.374, p = 0.0176). Additionally the prevalence of this species increased with age in cavity starts, but not in completed cavities, as evidence by a marginally significant interaction term for cavity age and type (excavation age x type, $z$ = 1.775, p = 0.0759; Figure 4.2b). The prevalence of *Porodaedalea pini* SE was not significantly related to cavity age or excavation type, though it trended towards reduced prevalence with cavity age ($z$ = -1.570, p = 0.12; Figure 4.2c).

*Experimental (drilled) cavity starts*

Similar to our survey of RCW excavations, we observed successional dynamics of fungal communities in the experimental cavity starts. Basidiomycota prevalence increased significantly with time through the duration of our experiment ($z$ = 5.480, p < 0.0001; Figure 4.3). Additionally, prevalence tended to be higher when RCWs were denied access to experimental cavity starts ($z$ = -1.901, p = 0.057; Figure 4.3). The interaction between time and treatment was not significant, and thus the interaction term was dropped out of the GLMM.

In addition to affecting overall prevalence of fungi, exclusion of RCWs from cavity starts also altered Basidiomycota fungal community composition through time. PCOa axis 1 explained 33% of the experiment-wide variation in the Basidiomycota communities of experimental excavations and was strongly correlated with the presence of *Acaromyces ingoldii* (r = -0.84) and *Cryptococcus* sp. 1 (r = 0.64). PCOa axis 2 explained 21% of the variation in the Basidiomycota community and was strongly correlated with the presence of *Cryptococcus* sp. 1 (r = -0.70) and *Acaromyces ingoldii* (r = 0.51). The Basidiomycota communities were compositionally similar to each other when the experimental starts were initially drilled (time point 1), and developed along similar trajectories for the 6 months after drilling, prior to the application of the treatment (time point 2). Visual assessment of the first 2 PCOa axes revealed that Basidiomycota communities were similar among treatment groups prior to unscreening, which occurred between time points 2 and 3. However, fungal community composition of screened and unscreened cavities diverged once RCWs were allowed access to the unscreened treatment group (Figure 4.4).

PERMANOVA revealed that the differences in the fungal communities across drilled excavations at the first time point were not related to treatment group (p = 0.56), geographic area (p = 1.00) or RCW cluster (p = 0.30). At the second sampling date, fungal communities in drilled excavations were significantly different among geographic areas (*pseudo F* = 2.845, $r^2$ = .18, p = 0.002), but not significantly different among treatment groups (p = 0.61) or RCW cluster (p = 0.17). Subsequently, geographic area was not significant for the third, fourth or fifth time points (p = 0.81; 0.36; and 0.31, respectively). For every time point post-treatment (time points 3, 4 and 5), fungal communities differed significantly between treatment groups (time point 3, *pseudo F* = 3.719, $r^2$ = .083, p = 0.013; time point 4, *pseudo F* = 6.277, $r^2$ = .152, p = 0.0006; time point 5, *pseudo*-F = 4.56, $r^2$ = 0.10, p = 0.002). RCW cluster was significant for the third time point

(*pseudo F* = 5.11, $r^2$ = .11, p = 0.007), but not for the fourth or fifth time points (p = 0.11 and 0.83, respectively).

## Discussion

Our experimental and survey data indicate that RCWs alter fungal community dynamics in their excavations. By examining the Basidiomycota communities in complete and incomplete RCW excavations of varying ages, we demonstrated that excavations in living pine trees undergo fungal community succession (Fig. 4.1). Our analyses of the experimental drilled cavity starts showed that these successional dynamics are significantly altered when RCWs are denied access to excavations (Fig. 4.4), suggesting that RCWs may directly influence fungal community succession in their excavations. RCWs can influence fungal community succession during cavity excavation in at least two ways, first, by facilitating fungal dispersal, and second, by mechanically altering the habitat for fungi.

### *Basidiomycota community succession in RCW excavations*

The fungal communities of RCW cavities were significantly different from RCW cavity starts of the same age. These significant differences can be explained by a number of excavation characteristics that may interact with excavation age such as excavation structure. For instance, habitat structure differs between completed excavations and cavity starts. Complete RCW excavations consist of an entrance tunnel that extends from the bark through the sapwood and into the heartwood of a living pine tree; the cavity chamber extends from the entrance tunnel and is housed entirely in the heartwood. In contrast, RCW cavity starts consist of an entrance tunnel that may or may not be advanced enough to enter the intact heartwood. The microhabitat of the tree is altered during excavation, and may be more favorable for fungi that help establish a decay

community during the early excavation stages and favorable to fungi that help maintain the fungal community in the later stages.

Previously, RCWs were thought to seek out trees infected with one fungus, *Porodaedalea pini* SE for excavation (Ligon 1970, Conner et al. 1976, Jackson 1977, Jackson and Jackson 2004). *Porodaedalea pini* SE is relatively common in RCW excavations, but not in non-excavated trees (Chapter 2). However, there are also a number of other fungi, including known wood decay fungi, ubiquitous cosmopolitan fungi, and many fungi whose functions are largely unknown associated with RCW excavations (Chapter 2). Here we show that the prevalence of *P. pini* SE in RCW cavities and starts may decrease with the age of the excavation (Fig 4.2c). Thus, *P. pini* SE may be a relatively early arriving wood decay fungus and could facilitate the development of the complex community of fungi associated with completed RCW cavities. The birds may facilitate its early dispersal either by providing the fungus access to the trees by initiating excavations, and altering the microhabitat during excavation or by carrying spores or hyphal fragments from their existing cavities into their cavity starts (Chapter 2 and Chapter 3). It is also possible that the birds are able to detect *P. pini* SE in living pine trees and choose such trees for excavation (Chapter 2), and subsequently decrease its prevalence during the excavation process either by removing the infected tissue or by bringing in other fungi that out-compete *P. pini* SE.

Despite the differences in microhabitat, the fungal communities in cavity starts become more similar to those in completed cavities as excavation age increases (Chapter 2), and we can see different successional patters in other fungi we examined. For instance, the prevalence of Exobasidiomycetes sp. 2, one of the most common fungi found in completed RCW cavities increases in both RCW cavities and cavity starts with age (Fig 4.2a). Interestingly, the

prevalence of this fungus did not increase in the human-made starts over the 2 year observation period, a pattern also seen in RCW starts 0 to 10 years post-initiation. While the temporal scale is not comparable, the depth of the excavations may be. Exobasidiomycetes sp. 2 may be integral to the maintenance of the fungal communities of complete RCW cavities.

Older cavity starts are more likely to house *Acaromyces ingoldii* than newer starts or completed cavities of any age (Fig 4.2b). The increase in the prevalence of *A. ingoldii* in older cavity starts may be linked to prolonged periods of inactivity during which RCWs do not access the excavation, the high prevalence of A. ingoldii in the RCW inaccessible drilled starts shows some support for this. Other members of the fungal community may also contribute significantly to the increasing similarity of starts to completed cavities with age. It is possible that yet more fungal taxa of importance could be revealed with more intensive sampling and next generation sequencing.

*Basidiomycota community succession in experimental cavity starts*

The changes we observed in Basidiomycota prevalence and community composition in relation to excavation age in the field survey of RCW excavations were also observed in the experimental drilled starts, especially in the early stages. The overall Basidiomycota prevalence in these trees initially increased after drilling, then reached an asymptote. The sharpest increases in prevalence occurred within a year post-drilling, and after the treatment was applied we observed lower Basidiomycota prevalence in the experimental starts that were accessible to RCWs (Fig. 4.3), illustrating that RCWs influence Basidiomycota prevalence through time.

The Basidiomycota communities in the experimental drilled starts also changed through time. The early changes seen between time points 1 and 2 were likely due to dispersal facilitated by wind. After the second time point, when the treatment was applied, the fungal communities in

the starts accessible to the birds did not undergo drastic changes, whereas those in the starts that remained screened did (Fig 4.4). During RCW cavity excavation, excavation through the sapwood is a more rate limiting step than excavation through the heartwood. The birds have to enter the excavation tunnel throughout the excavation process, and if fresh resin is still weeping from the excavation tunnel, the birds will avoid entering the excavation. RCWs can get stuck in the weeping resin while excavating cavity starts and it is possible that their unique excavation behavior has evolved in part to avoid the challenges that fresh resin provides. Thus, it is likely that most RCW cavity starts that have progressed more than a couple of inches into the sapwood are not excavated by RCWs for a period of time while the initial resin dries; the experimental drilled starts mimicked this process. Thus, initial fungal succession is facilitated by RCWs through excavation initiation, but also appears to be dependent on dispersal by wind during periods of RCW inactivity while the resin is weeping.

At the last time point, the fungal communities in drilled starts accessible to RCWs closely resemble those in complete RCW excavations, whereas those in inaccessible starts do not (Chapter 3). The inaccessible starts had a high prevalence of *Acaromyces ingoldii*, a fungus that produces compounds that inhibit the growth of some other fungi (Kushnir et al. 2011) and are toxic to mites (Gerson et al. 2008). It is possible that this fungus facilitated the change in community composition. Multipartite symbioses involving mites, insects and fungi are not uncommon (Ayres 2001, Hofstetter and Moser 2014), and arthropods may very well play a role in the RCW fungal community symbiosis. We show some evidence through the abundance of *Acaromyces ingoldii* that mites might interact with woodpeckers. Excavations that were not accessed by woodpeckers very frequently or not at all have higher prevalence rates of *A. ingoldii*. It is possible that this fungus either hinders the colonization of other fungi associated with fully

excavated cavities or facilitates the colonization of a different community of fungi, similar to that of cavity starts that have not been visited by RCWs for over a year.

The RCW accessible starts do not appear to change drastically over the last three time points when compared to the RCW inaccessible starts, but changes in Basidiomycota community composition in the accessible starts can be observed along PCOa axis 2 (Fig 4.4). This axis is positively correlated with *Acaromyces ingoldii* and negatively correlated with *Crytopcoccus* sp. 1 while the first axis is negatively correlated with *A. ingoldii* and positively correlated with *Crytopcoccus* sp. 1. Most of the changes in the fungal communities of RCW inaccessible starts occur along the first PCOa axis. This suggests that *A. ingoldii* and *Cryptococcus* sp. 1 are most strongly influenced by the presence of RCWs.

Basidiomycota fungal community succession can be observed in complete and incomplete RCW excavations over a 25 year time period. The birds initiate this succession by excavating cavities within the heartwood of healthy, living pine trees, a habitat that is otherwise difficult for fungi to access. The birds may shape succession by carrying fungi into their cavity starts during excavation and the birds may also help maintain a stable Basidiomycota community in their complete cavities. Though the time frame of our experiment was relatively short (2 years), we still observed an altered trajectory of fungal community development by treatment. The initial Basidiomycota colonization that we observed in the drilled starts could represent the early arrivers that help prime the habitat for later arrivers such as wood decay fungi (Stenlid and Gustafsson 2001, Stenlid et al. 2008). We likely captured the start of the habitat priming process but did not allow enough time for the community of late arrivers to become well established.

*Conclusion*

Our experimental and survey data show some evidence of a stable Basidiomycota community associated with RCWs. The experimental time series data from the accessible drilled starts suggest that the Basidiomycota community associated with RCWs represents a stable state, and that an alternative stable state develops in the absence of RCWs. Alternative stable states could depend on immigration history (Boddy 2000, Fukami et al. 2010), and early Basidiomycota dispersal in RCW excavations is dependent on the regional species pool while later dispersal is coupled with the presence of the birds. The change in Basidiomycota communities in the absence of the birds could represent a state shift. This hypothesis could be tested by allowing birds to access all of the experimental drilled starts. If the fungal communities in drilled starts that were previously inaccessible do not change upon RCW access, this may be a good example of a state shift, with alternative stable states.

Just as mycorrhizal community development is linked with successional patterns in forests, the fungal community development in RCW excavations may be linked with the successional patterns of RCW cavity use. Arthropods help initiate fungal colonization and succession in fallen logs and in dead and dying trees (Müller et al. 2002, Strid et al. 2014). Through an exclusion based, non-inoculated field experiment, we have demonstrated that RCWs do the same in the sapwood and heartwood of their excavations in living pine trees. The fungi, in turn may help the birds by assisting in softening the wood in the excavation site.

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**Figure captions**

Figure 4.1

Predicted relationships from a generalized linear model (GLM) fit of fungal community composition (represented as the first principal coordinates axis) as a function of excavation age and excavation type. The solid lines represent the model fit and the dashed lines are 95% confidence envelopes. Completed red-cockade woodpecker cavities are depicted in black, red-cockaded woodpecker cavity starts in gray.

Figure 4.2

Generalized linear model fits of the prevalence of Exobasidiomycetes sp. 2 (A), *Acaromyces ingoldii* (B), and *Porodaedalea pini* SE (C) as a function of excavation age and excavation type. The solid lines represent the model fit and the dashed lines represent 95% confidence envelopes. Completed red-cockaded woodpecker cavities are depicted in black, red-cockaded woodpecker cavity starts in gray.

Figure 4.3

Basidiomycota prevalence rates through time in the experimental drilled starts. The dots in the center represent the mean Basidiomycota prevalence at each time point, and the bars represent the 95% confidence interval. Drilled starts accessible to red-cockaded woodpeckers are represented in grey, those inaccessible to red-cockaded woodpeckers in black. The timeline of the experiment (in months) is on the x-axis, month zero represents the treatment (unscreening), and thus the start of the experiment, the initial drilling occurred 8 months prior to the treatment (-8 months), the first re-sampling occurred 2 months prior to the treatment (-2 months), the third

re-sampling occurred 5 months after the treatment (5 months), and the fourth and fifth occurred 11 and 16 months after the treatment, respectively.

Figure 4.4

Principal coordinates analysis (PCOa) of the communities of Basidiomycota fungi found in red-cockaded woodpecker accessible (gray) and inaccessible (black) experimental drilled starts. The dots in the center represent the means of the points on the two PCOa axes, and the bars represent one standard error from the mean. △represents the first time point, ▽ represents the final time point. The dotted lines trace the path of the fungal communities from time point to time point.

Figure 4.1: Predicted relationships from a generalized linear model (GLM) fit of fungal community composition as a function of excavation age and excavation type

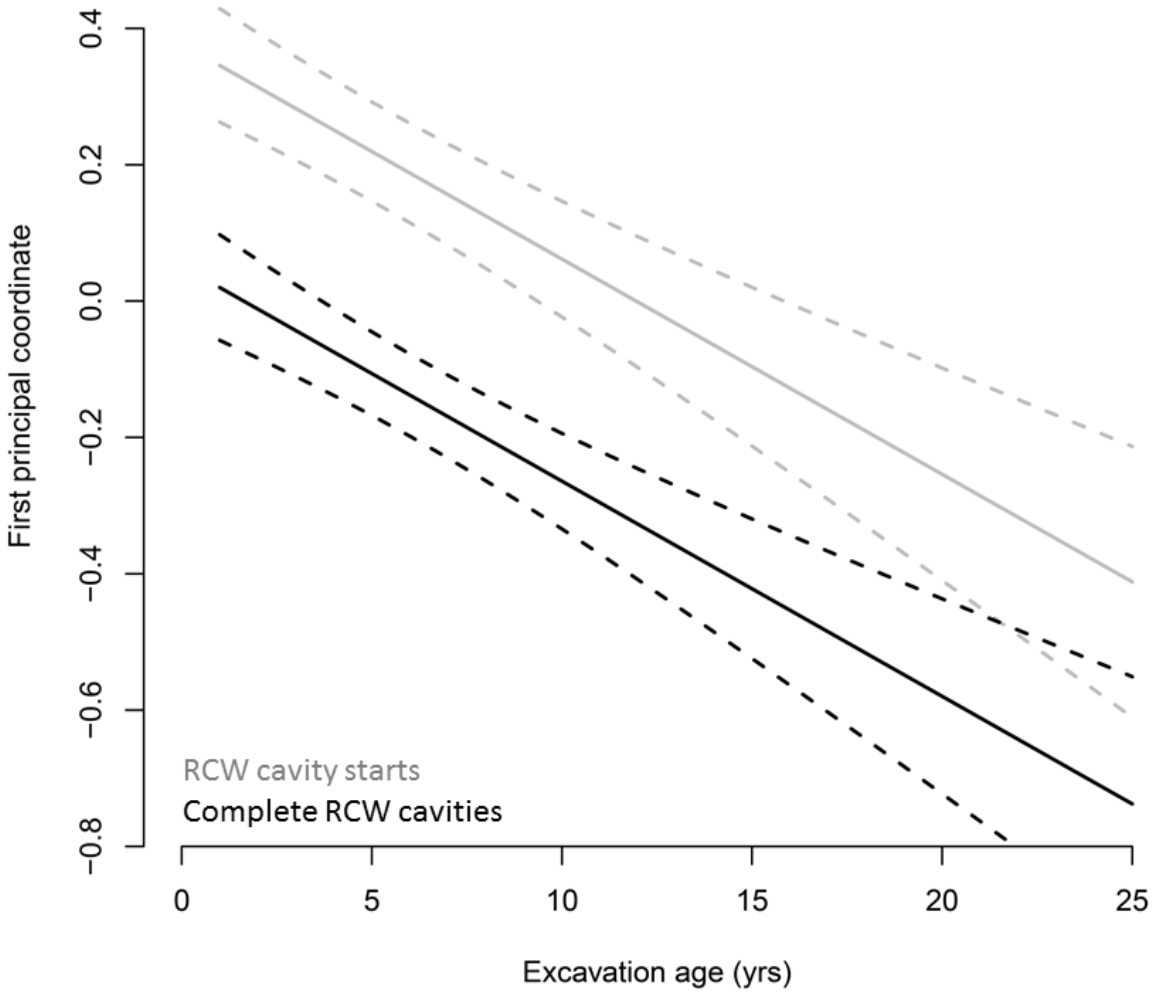


Figure 4.2: GLM fits of the prevalence of 3 fungi as a function of excavation age and type

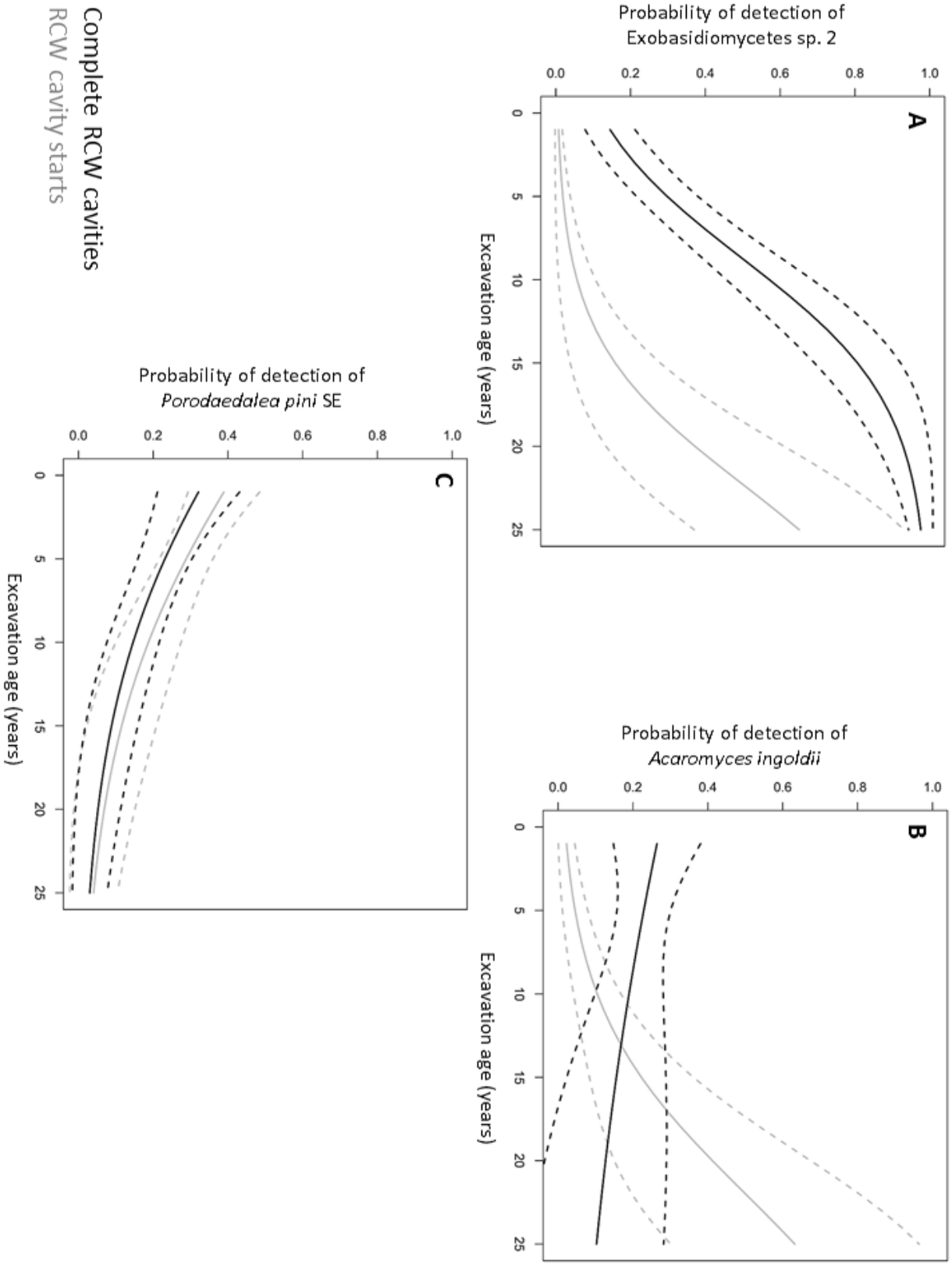


Figure 4.3: Basidiomycota prevalence rates through time in the experimental drilled starts

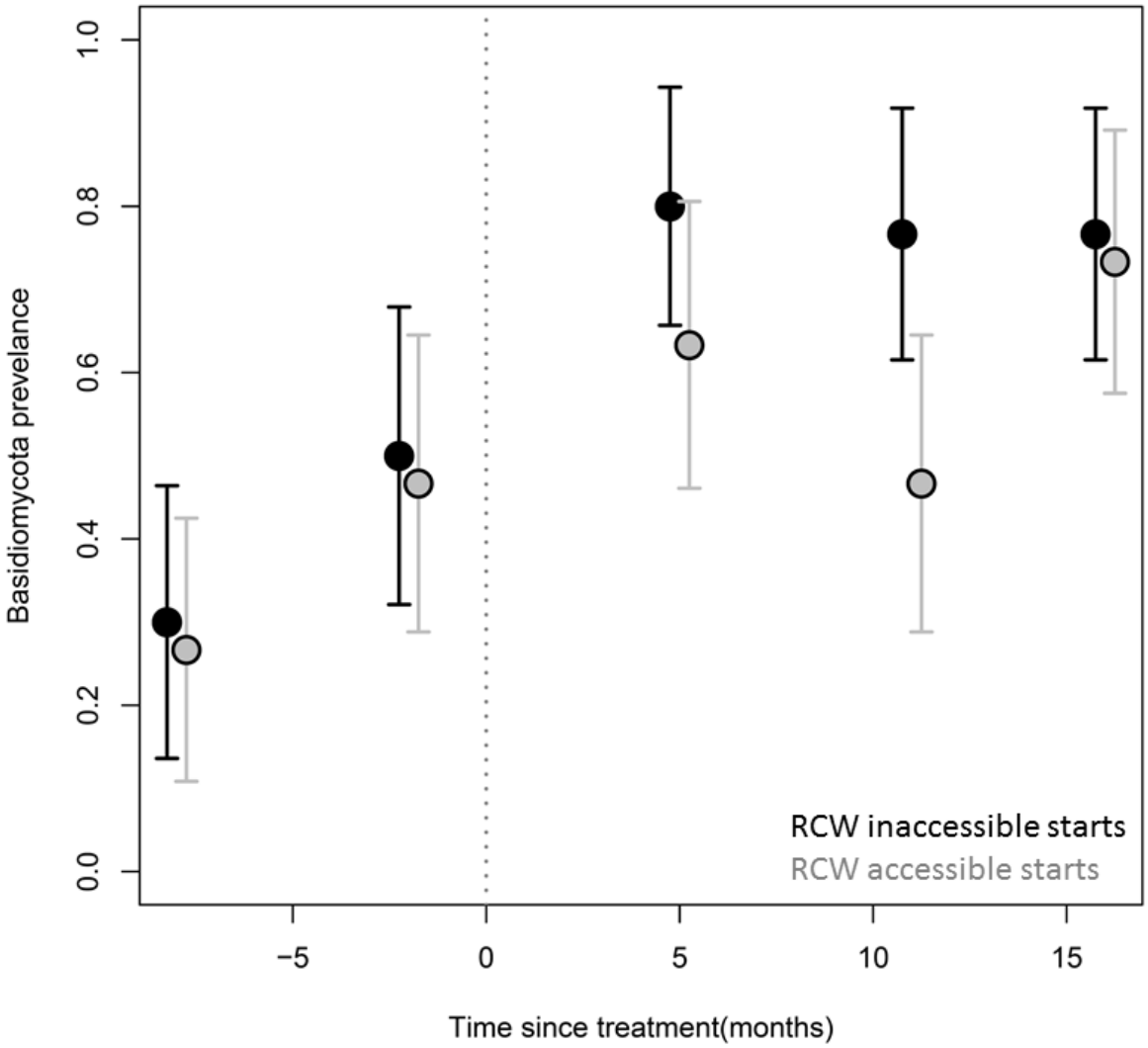
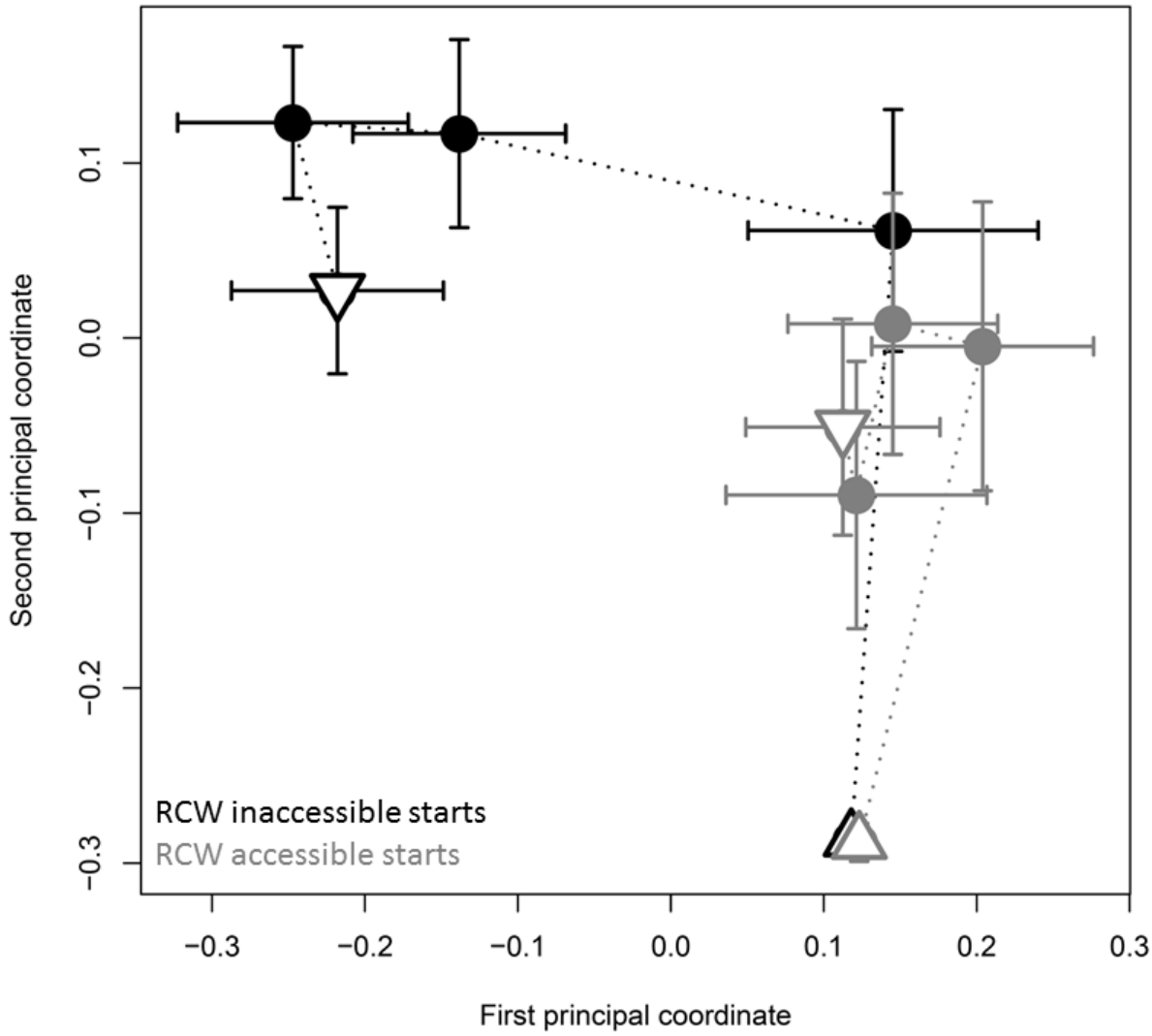


Figure 4.4: Principal coordinates analysis (PCOa) of the communities of Basidiomycota fungi found in red-cockaded woodpecker accessible and inaccessible experimental drilled starts



# Chapter 5. Summary, conclusion, and future directions

Both cavity excavators, such as woodpeckers, and wood decay fungi act as important ecosystem engineers by creating cavities where they are not otherwise abundant for secondary cavity-users and by facilitating natural cavity formation, respectively. However, relationships between cavity excavators and fungi are poorly understood. In certain woodpecker-fungi systems, past studies have generally utilized fruiting body surveys or tools, such as Resistographs, to mechanically detect evidence of decay while focusing on woodpecker excavation site selection. These approaches have led to a "one woodpecker, one fungus" paradigm, but I have demonstrated that complex community interactions occur within tree cavities for red-cockaded woodpeckers, and cavity excavators may generally have multiple fungal associates. The diversity of fungi in tree cavities may help maintain diverse groups of secondary cavity-users in addition to facilitating cavity excavation.

## Summary

In this dissertation, I investigated the interactions between red-cockaded woodpeckers and fungi. Through the development and utilization of molecular techniques that can be used to characterize the fungi that inhabit tree cavities, I demonstrated that visual assessment of decay is not adequate to describe the relationships between cavity-nesters and fungi. Through the collection and analysis of field survey data, I documented the fungal communities found in red-cockaded woodpecker excavations and on the birds themselves, and found them to be similar to the fungi associated with their excavations. I conducted an experiment to determine if the birds facilitate fungal colonization during the cavity excavation process and determined that they do in two ways. First, simply by excavating cavities they create a wound in the tree that could provide fungi access to the interior of the tree. Second, in accessing their excavations they could

transport fungi into the excavation. Finally, I used survey and long-term monitoring data to demonstrate that fungal communities in excavations undergo succession, and through a time-series analysis of experimental data demonstrated that red-cockaded woodpeckers alter the trajectory of fungal community succession.

*Can visual survey methods be used to document the relationships between red-cockaded woodpeckers and fungi?*

I demonstrated that visual fruiting body surveys do not provide an accurate estimate of the prevalence or diversity of fungi in red-cockaded woodpecker excavations. I also provided a novel technique to nondestructively sample the wood surrounding an excavation as well as an improved primer, ITS4b-21, that can be used to amplify fungi in the phylum Basidiomycota (which contains most wood decay fungi). I tested the method described in Chapter 1 on twenty red-cockaded woodpecker excavations, half of which were housed in trees with *Porodaedalea pini* SE fruiting bodies while the other half had no visible signs of decay. With my new approach, I was able to detect *P. pini* SE in 90% of the trees with fruiting bodies and 60% of the excavations that lacked fruiting bodies. I also identified nine additional taxa of wood decay fungi that did not have fruiting bodies present and were previously not known to be associated with red-cockaded woodpecker excavations.

*Which fungal species are associated with red-cockaded woodpecker excavations?*

I used my novel method to conduct a field survey of the fungi in complete and incomplete red-cockaded woodpecker excavations and in non-excavated trees. Using the molecular methods and primer pair described in Chapter 1, in combination with a general fungal primer pair (ITS1F and ITS4; Gardes and Bruns 1993), I was able to document a previously undetected diversity of fungi in red-cockaded woodpecker excavations and in living pine trees

without excavations. Through the use of multivariate analyses, I showed that red-cockaded woodpecker excavations are associated with distinct fungal communities. I developed two hypotheses to explain the results from Chapter 2: (1) the tree selection hypothesis, which states that red-cockaded woodpeckers select trees infected with certain types of fungi for excavation, and (2) the bird facilitation hypothesis, which states that red-cockaded woodpeckers directly and indirectly facilitate fungal colonization via their incomplete excavations.

*Do red-cockaded woodpeckers facilitate the transmission of fungi during cavity excavation?*

Through a two-part test of the bird facilitation hypothesis, I found the first evidence of a multipartite symbiosis and possible mutualism between fungi and a cavity excavator (Chapter 3). By swabbing the beaks, wings and feet of adult red-cockaded woodpeckers, I found that the birds carry fungal communities on their bodies similar to those found in their excavations. In addition, through a test that utilized human-made drilled cavity starts (incomplete excavations) in non-excavated trees, I was able to provide experimental evidence in support of the bird facilitation hypothesis. The tree selection hypothesis is not mutually exclusive and represents the more traditional view of the relationship between cavity excavators and fungi and should also be tested in red-cockaded woodpeckers.

*Do the fungal communities found in red-cockaded woodpecker excavations undergo succession?*

The underlying theme of fungal community succession in red-cockaded woodpecker excavations in Chapters 2 and 3 was developed in Chapter 4. Utilizing data from previous chapters, in combination with time-series data from the experimental drilled cavity starts described in Chapter 3, I provided evidence for fungal community succession in red-cockaded woodpecker excavations. The time-series data on the experimental drilled cavity starts suggest

that the birds directly influence the process of fungal community succession in their excavations. This is the first evidence of woodpecker-mediated fungal community succession.

**Conclusion**

The results described in this dissertation provide strong evidence for a multipartite symbiosis between a cavity excavator and fungi. Red-cockaded woodpeckers have many adaptations that allow them to occupy a prominent niche in the fire-adapted longleaf pine ecosystem. The frequent fires that are characteristic of this ecosystem result in forests characterized by a low density of hardwoods and snags, which are utilized by other cavity excavators, and a high density of fire-adapted conifers. No other cavity excavator relies solely on excavating cavities through the sapwood and into the heartwood of living pine trees, but red-cockaded woodpeckers have managed to capitalize on this resource.

My work demonstrates that the unique excavation behavior of these birds may be coupled with a mutualistic association with fungi. The birds create wounds (cavity starts) on the trunks of living pine trees, thus providing fungi access to sapwood and, eventually, heartwood. The birds may further facilitate fungal development by carrying hyphal fragments and spores into their cavity starts, and through this, and perhaps other means, the birds promote a particular fungal community. As the fungal communities become established, they likely assist the birds by softening the wood surrounding the excavation sites.

The longleaf pine ecosystem only occupies a small portion of its historic range, and much of the remaining habitat is heavily fragmented or degraded (Frost 2006). Red-cockaded woodpecker population growth is tied to cavity availability (Walters 1991), which itself is coupled with the availability of excavation sites, which may in turn be linked with fungi and their ability to colonize the heartwood of living pine trees. A symbiotic relationship with

communities of fungi may be one of the many amazing life history traits that red-cockaded woodpeckers have developed in order to occupy an empty niche. Entire communities of secondary cavity-nesters in the longleaf pine ecosystem rely heavily upon the cavities engineered by red-cockaded woodpeckers (Blanc and Walters 2008). If fungi do indeed assist in preparing excavation sites, the multipartite symbiosis I have described may extend past red-cockaded woodpeckers to the secondary cavity-nester community. Thus, the fungal communities associated with red-cockaded woodpecker excavations may help maintain the biodiversity of the endangered longleaf pine ecosystem.

**Future directions**

I hope that the body of work presented in this dissertation inspires more research on the community ecology of both excavated and non-excavated (or "naturally formed") tree cavities. I focused on one excavator, and though red-cockaded woodpeckers are very unique, it is likely that many other cavity excavators are also associated with communities of fungi, not just the fungi found fruiting on their trees. Research on the fungi associated with cavity excavators in other ecologically sensitive areas may help ensure the maintenance of biodiversity in these regions and could be further applied to retain important ecosystem components. For example, in heavily logged forests, documenting the fungi associated with cavity excavators may help determine if primary cavity excavators can re-inhabit reforested stands on their own, or if cavity addition will be necessary.

Another avenue for future endeavors is to continue where this dissertation left off. The relationship that I described between red-cockaded woodpeckers and fungi is a possible mutualism, but further tests are needed to determine the nature of this symbiotic relationship. The tree selection hypothesis should be tested; Chapter 2 gives more detail on that. The question

of which appeared first in the longleaf pine ecosystem, the woodpeckers or the fungal communities should also be examined in order to explore more mechanistic questions about this symbiosis. The relationships between secondary cavity-users and fungi could open an additional avenue in this research, especially secondary cavity-users of red-cockaded woodpecker cavities. In addition to these broad but very exciting questions, there are still smaller details to be worked out, for instance, work should be done to determine how the fungi in these associations live together and how the microhabitat within the cavities helps or inhibits their growth. The answers to these questions and many other questions like these will help to develop a more comprehensive understanding of the community ecology of cavity-excavators, fungi, and all other inhabitants of tree cavities.

These are just some of the ideas and questions that have resulted from my work that may warrant future research, and ultimately, I hope that this body of work will serve as a "start" to many future studies.

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