Mechanisms of Wood Decay in PNW Snags.

Final Report for Seattle City Light
Wildlife Research Program

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I. Introduction –

Tree cavities provide important habitat for more than one quarter of wildlife in the Pacific Northwest (PNW). Woodpeckers assist in tree cavity formation and are considered keystone species. Their presence in forest systems has well-documented and far-reaching effects on biodiversity and ecosystem health (Lindenmayer et al. 2000, Virkkala et al. 2006, Drever et al. 2008). The list of secondary cavity users in the PNW includes many at-risk species including the northern spotted owl (*Strix occidentalis*) and fisher (*Martes pennant*), in addition to bats, songbirds, swifts, small mammals, cavity-nesting ducks, and raptors (Bunnell et al. 1999); these species use or require tree cavities, but are not capable of excavating cavities themselves.

Despite the importance of tree cavities for PNW wildlife, the mechanisms by which decay fungi colonize trees and how those sites are found by woodpeckers are poorly understood. Recent research in dry coniferous forests of the PNW indicates that wood excavated by woodpeckers at cavities is rare in natural systems. Wood density at woodpecker excavations is <0.23 g/cm^3 and such wood comprises <5% of all standing dead wood in these forests (Lorenz et al. 2015). Ultimately, the formation of cavities is therefore likely brought about by the activities of wood decay fungi that long precede woodpecker excavation (Jackson and Jackson 2004). The mechanisms by which wood decay fungi break down dead wood have not been studied in the PNW with modern methods (e.g., Lindner and Banik 2009, Schilling et al. 2015). In fact, due to methodological limitations, the fungal taxa responsible for wood softening in PNW snags are not known with certainty. Wood decay fungi in this region rarely produce sporocarps for species identification (Lorenz et al. 2015). The genomics techniques required to identify fungi have only been developed in the last few years (Lindner and Banik 2009, Lindahl et al. 2013, Jusino et al. 2014), and have not been applied to examining wood decay fungi in the PNW. Managers therefore lack basic information on which fungal taxa initiate the process of wood softening in PNW snags, as well as management techniques that may influence these processes.

Information on decay agencies and processes in PNW snags could help improve habitat management for wildlife. As noted above, many snags on the landscape contain wood unsuitable for woodpecker cavity excavation, and hence cannot be used by many species of secondary cavity users. Management techniques to increase woodpecker cavity excavations in past studies have met with fairly low success rates. For example, among ~2,000 Douglas-fir snags created by researchers in western Washington and Oregon, fewer than 3% contain evidence of woodpecker nest excavations within ~10 years of snag creation (Bednarz et al. 2013, Walter et al. 2005, Kroll et al. 2012). Foraging holes and cavities of small-bodied excavators (e.g., chestnut-backed chickadee, red-breasted nuthatch) were present in larger numbers in these snags, but importantly these holes are too small to provide habitat for most cavity dependent wildlife (Martin and Eddie 1999, Martin et al. 2004). In contrast, in the first 8-10 years following natural fires in central Washington, research with ~1,000 snags indicates that >16% snags contain woodpecker cavity excavations (Lehmkuhl et al. 2003, Lorenz et al. 2015). In managed forests, researchers created snags either by mechanically topping trees, or inoculating them with a heart rot fungus *Fomitopsis pinicola*. Lacking information on decay agents and processes, in these cases managers may have provided large numbers of snags unsuitable for woodpecker excavations. For instance, inoculation methods may have failed because there is no direct evidence that *Fomitopsis pinicola* is influential in producing wood decay suitable for woodpecker excavations; it is simply a fungal species commonly observed producing sporocarps in these forests.

Overall, past experimentation with snag creation in the PNW has indeed been successful in creating snags, but not necessarily in creating snags that are useable for wildlife. Modern

sequencing methods have the potential to inform these efforts. With modern sequencing methods like high throughput, next generation sequencing (NGS), fungal taxa that occur at woodpecker excavations can be determined accurately and with low bias (e.g., without relying on sporocarps). Recent sequencing studies have found much higher diversity of fungi at woodpecker excavations (e.g., hundreds of fungal taxa) than indicated by sporocarps (Jusino et al. 2015; Jusino et al. 2016). Sequencing could help managers determine if *Fomitopsis pinicola* or other fungal taxa occur at woodpecker excavations, to potentially improve inoculation success. It can also be used to non-destructively track fungal colonization of recent killed trees, to determine if there are management techniques or environmental conditions that are most likely to lead to wood softening.

To explore some of these possibilities, the objectives of our project to identify fungal taxa at woodpecker excavated cavities using NGS. We are studying these processes in the two tree species most commonly used by woodpeckers for cavity excavation in the PNW, Douglas-fir (for forests west of the Pacific Crest) and ponderosa pine (*Pinus ponderosa*; for forests east of the Pacific Crest). Upon completion of the study, we will provide publications and guidance for managers in the form of presentations at workshops and conferences.

II. Methods –

Study area

We conducted this study on the east slopes of the Cascade Range in Yakima and Kittitas Counties, Washington (approximately 46°46′ N, 121°04′ W; and 47°12′ N, 120°27′ W, respectively; Figure 1). Most nests were on U.S. Department of Agriculture, Forest Service land, and with a few nests on Washington Department of Natural Resources lands. Forest composition varied based on aspect, slope, elevation, and longitudinal distance from the Cascade Crest. On most sites ponderosa pine was dominant or co-dominant with Douglas-fir (*Pseudotsuga menziesii*) or grand fir (*Abies grandis*). Other tree species included western larch (*Larix occidentalis*), quaking aspen (*Populus tremuloides*), and black cottonwood (*Populus trichocarpa*).

Locating woodpecker nest cavities

For our first objective of identifying wood decay fungi at woodpecker excavated cavities, we searched for woodpecker nests from May to July 2017. Eleven species of cavity excavators occur on our study sites, but to obtain a large enough sample for statistical analyses we focused on four common species that represent "specialist" and "generalist" habitat guilds. Our specialist species were white-headed (*Picoides albolarvatus*) and black-backed woodpeckers (*P. arcticus*), which are sensitive species or listed species in the region restricted to specialized habitat types (WDFW 2013, Mellen McLean et al. 2013, USFWS 2013, COSWEC 2010). We used hairy woodpeckers (*P. villosus*) and northern flickers (*Colaptes auratus*) as "generalist" species. These two species are widely distributed throughout North America and occur in many habitat types. All four species are considered keystone excavators (Martin et al. 2004, Tarbill et al. 2015). They typically excavate at least one cavity each year for nesting and thus are important for maintaining biodiversity of cavity dependent wildlife in PNW forests.

Vegetation sampling

In August and September 2017 and after the woodpecker nesting season, we returned to nests to measure vegetation characteristics. We restricted vegetation sampling (and fungal

sampling, described below) to nests in ponderosa pine and Douglas-fir, with the exception of one hairy woodpecker nest sampled in a grand fir. For safety reasons, we also restricted our sampling to nests that could be sampled from the ground or from climbing ladders and in which the nest snag appeared stable. We omitted nests from sampling if they were too high (>12 m) to be accessed by ladders (n = 20).

Following the nest season, we measured basic vegetation characteristics at 76 nest sites, including cavity height, snag height, snag species, cavity orientation, and snag diameter at breast height. We also noted cavities and cavity starts on all snags, and the proportion of bark, branches, needles, fine branches, and top missing. We noted whether fungal fruiting bodies (sporocarps) were present on the bole, and whether there were holes characteristic of woodpecker foraging. We measured the torque required to turn an increment borer bit into the snag, which is correlated with wood density (Matsuoka 2000). At a subset of nests that were also included in a study of nest survival (n = 26), we estimated surrounding tree and snag density (0.04 ha plot), shrub cover (0.01 ha plot), and canopy cover (0.01 ha plot). We also measured cavity sill width, cavity horizontal depth, and cavity vertical depth with a ruler.

For making comparisons with unexcavated snags, we paired these 56 nest snags with a randomly selected 'control' snag within 75 m. Control snags matched attributes of nest snags but lacked cavities. We measured all of the aforementioned features at control snags, except cavity width and depth (because control snags lacked cavities). For comparison with nests, we measured wood hardness on control snags at the same height and orientation as its paired cavity. We marked each nest and paired control snag with a tree tag placed in a nearby stump or log and took a series of nine photos at each site. We compared attributes of nest and control snags using paired sample t-tests and considered attributes different if $\alpha < 0.05$.

Remotely sensed data

We overlaid remotely sensed data on management history for each site from three sources: LandTrendr, (Landsat-based detection of Trends in Disturbance and Recovery methods; Kennedy et al. 2010), United States Department of Agriculture Forest Service Activity Tracking System (FACTS) data, and gradient nearest neighbor (GNN) models (Ohmann et al. 2011). LandTrendr uses multiple-year Landsat imagery to categorize forest disturbance based on magnitude and duration, and we considered recent (<10 year), short duration (<1 year), and high magnitude disturbance events from LandTrendr as recent burns, which are an important habitat for most woodpecker species in this study. We used the FACTS database to extract information on the timing of recent thinning and prescribed burn projects for our sites. We used GNN models to provide basic vegetation information for our sites, including canopy cover, basal area of different tree species, and stand age. We used ArcGIS 10 (Environmental Systems Research Institute, Inc., Redlands, CA) to extract these remotely sensed data for woodpecker nests and paired random sites in our study.

Fungal sampling and sequencing

For sequencing of wood decay fungi, we aseptically collected a sample of wood from 76 nest cavities with a sterile sharpened spoon following Jusino et al. (2014). We used the spoon to scrape inside the cavity above the nest chamber, a region also called the cavity dome (Jusino et al. 2014). Second, at 56 nest sites, we used a sterile 9 mm wood drill bit to aseptically collect wood shavings from the bole of each nest snag 2-5 cm above the nest cavity opening. This sample was collected by drilling horizontally into each nest snag with a cordless drill, with the

drill bit parallel to the ground. We collected two wood samples from this region. The first sample contained wood extracted as we drilled from the bark surface to ~3 cm deep. The second sample was collected from wood extracted as we drilled from 3-10 cm deep. We kept these samples separate (and sterilized between samples). Our first sample (0-3 cm deep) simulated sill or cavity entrance wood. Our second sample (3-10 cm deep) represented wood in the nest body region, or cavity chamber wood.

We collected yet a third sample from the bole of 56 control snags paired with 56 nest trees. As noted above, control snags matched attributes of nest snags but lacked cavities. As a result, we could not collect a spoon scraping from control snags. For comparison with nests we sampled wood from control snags at the same height and orientation as its paired nest using the sterile wood drill bit. Spoons and drill bits were sterilized in the field between every sample using a 20% sodium hypochlorite bath, isopropyl spray, and flame sterilization.

We transferred shavings from nest cavities (obtained via spoon scraping) immediately to sterile 1.5 mL tubes containing sterile cell lysis solution (CLS; Lindner and Banik 2009). Tubes were placed on ice until returning to the field station, where they were frozen at -18° C. Shavings from drill samples transferred immediately to sterile 50 mL tubes and placed on ice. Upon returning to the field station each day, drill samples were sterilely transferred to CLS in 1.5 mL tubes (protocols for wood sampling and storage are in Appendix 1). All samples were then maintained at -18° C until DNA extraction, polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region of fungi, and sequencing in September and October 2017.

To identify fungal taxa at nests and paired control snags we used ion torrent semiconductor sequencing with 400 base-pair technology, following the protocols in Palmer and Jusino et al. (2018). We included a custom mock community control in every run to parameterize downstream bioinformatics which were run using AMPtk (http://amptk.readthedocs.io/; Palmer and Jusino et al. 2018). Identification of fungal taxa was carried out following the AMPtk hybrid taxonomy assignment which incorporates the curated UNITE (Kõljalg et al. 2005) database. To compare species composition, we used nonparametric multidimensional scaling (NMDS) in the Vegan package of R (Oksanen et al. 2012) to plot and visualize differences in fungal community composition. We used nonparametric permutational multivariate ANOVA (PERMANOVA) tests (Anderson 2001) to test for significant differences in fungi communities as a function of sampling source (cavity spoon scraping, nest snag bole sample, or control snag bole sample).

III. Results and Discussion –

Locating woodpecker nest cavities

We surveyed approximately 2,314 ha for woodpeckers and located 103 nests (Figure 1). We obtained locations of 12 additional nests from J.M. Kozma, who shared the nest locations from nest searches conducted during concurrent woodpecker research projects in our study areas. The most commonly found species were northern flicker (n = 34 nests) and hairy woodpecker (n = 31 nests), followed by white-headed woodpecker (n = 29 nests) and black-backed woodpecker (n = 21 nests; Figure 1).

Vegetation surveys and remote sensing

Following the woodpecker breeding season, we measured basic vegetation characteristics at a subsample of nest sites. We measured cavity height, snag height, snag species, cavity orientation, and snag diameter at breast height. Forty-six nests were in ponderosa pines, 29 in Douglas-fir, and one in a grand fir. This should not be considered a representative sample of

species used for nesting by woodpeckers because we purposefully omitted some tree species from sampling to simplify the sequencing of fungal taxa. Nevertheless, ponderosa pine and Douglas-fir are frequently used by woodpeckers in this region. In a larger study of tree species used by woodpeckers for nesting in central Washington, Lorenz et al. (2015) reported 77% of nests were in ponderosa pine and Douglas-fir.

We sampled nests in unburned forests, and in forests burned 1 to 15 years prior. The majority of sampled nests were in recent burns (n = 30 in burns 0-5 years post-fire) with fewer sampled nests in burns 11-15 years old (n = 16) and unburned forests (n = 13). Again, this should considered representative of the use of these burns by woodpeckers because our study was not designed to model woodpecker occupancy as a function of burn age. Instead, for our study we aimed to sample nests in burns of all age classes (1-5 years, 6-10 years, 11-15 years, and unburned).

Mean cavity height was 2.6 m (Table 1). Mean diameter of nest snags was 38.6 cm and was similar to control snags ($\bar{x}$ = 38.9 cm; t_{130} = 0.135, P = 0.8932). Two vegetation attributes differed between nest and control sites: tree height and hardness of wood within the tree's interior. Control trees were taller than nest trees (t_{130} = 5.061, P < 0.0001) and contained harder wood 3-10 cm inside the bole (Table 1; t_{111} = 16.472, P < 0.0001).

Fungal sampling and sequencing

We sampled fungi from nest cavities that were excavated and used for nesting by woodpeckers in 2017. We omitted cavities from sampling that had been excavated in prior years. We successfully sequenced fungi from 166 samples, including 64 samples from woodpecker nest cavities, 54 samples from the bole of the nest snags, and 48 samples from the bole of random, non-used snags.

We sequenced 1477 fungal taxa from these samples. Fungal community composition differed between samples taken from woodpecker cavities, the bole of the nest snags, and control snags (Figure 2; r^2 = 0.17, pseudo-F = 16.3, P < 0.0001). Fungal communities were most diverse in cavity samples, with a median of 42 fungal taxa detected per cavity (and 351 total taxa unique to cavity samples), compared to a median of 30 taxa detected in the boles of nest snags (with 160 unique taxa) and 27 detected in the boles of control snags (with 185 unique taxa). These results mirror those from a concurrent sampling effort in central Oregon (Lorenz and Jusino 2017) in which cavities had higher diversity of fungi than samples taken from within the bole of the same snag, and from control snags.

Our results indicate that animals (vertebrates and invertebrates) carry fungal spores into the cavities, either on their bodies, or via fecal deposition, or for the case of specialized invertebrates, via special fungal transport organs. On our Oregon sites, the most prevalent fungal taxa within cavities were ectomycorrhizal symbionts (e.g., truffles and truffle-like fungi; Lorenz and Jusino 2017), which have spores that are often dispersed by small mammals (reviewed in Johnson 1996). These mammals eat the fruiting bodies of truffles and they may carry the fruiting bodies up into the cavities with them or deposit spores in the cavities when they defecate. In this current study in Washington, one of the most common fungi found in cavities was Ophiostomatales sp., and is likely associated with bark and ambrosia beetles. Many Opiostomatales fungi are mutualistically associated with bark and ambrosia beetles and are carried by the beetles in fungal transport organs termed mycangia (Barta 1963). The beetles bore tiny holes in the trees and "plant" their fungi in their excavations, then they lay eggs, and the young beetles feed on the nutritious structures the fungi produce. When the adult beetles leave

the excavations, they carry their fungi with them to the next tree (Huclr and Stenlinski 2017, Harrington 2005, Beaver 1989). Some of these fungi are harmless to forest health, and others have been very detrimental. Due to their internal transcribed spacer (ITS) sequence structure, Ophiostomatales fungi are better detected with different sequencing loci such as the large subunit (LSU), thus it is likely that many fungi in this order went undetected in our study (Harrington et al. 2010, Skelton et al. 2018).

The most commonly occurring fungal taxon detected in the bole of nest snags in this study was *Rhinocladiella atrovirens* (found in 48% of nest bole samples; Table 2). *Rhinocladiella atrovirens* is sometimes classified as a soft rot fungus (Nguyen et al. 2016) and was also detected in control snags (30%). It was widespread and occurred in nests in both ponderosa pine (55%) and Douglas-fir (50%), and in both unburned (58%) and burned snags (52%). The most prevalent Basidomycota fungi sequenced from the bole of woodpecker nest snags were in class Tremellomycetes and Agaricomycetes (both found in 30% of nest bole samples), and these taxa were less common in control snags (19% and 8%, respectively). These taxa were most prevalent in ponderosa pine snags, but occurred in burned and unburned snags. Tremellomycetes are molds often associated with decayed wood and dead and dying trees although it is unknown whether they actively decay wood. The fungal Class Agaricomycetes in contrast, contains the Order Polyporales which includes many well-known wood decay fungi. Unfortunately, many of the fungal taxa detected in our study have not been previously identified. In fact, only an estimated 5-10% of fungi in existence are described, and of those, not all are represented in sequence databases, leaving the taxonomy of many NGS studies somewhat unsatisfactorily defined (Larsen et al. 2017). This is especially true when one is dealing with unstudied substrate types, such as the internal wood of snags, which are not often thoroughly surveyed for fungi.

Overall, our preliminary results illustrate that snags contain diverse communities of fungi, some of which are likely associated with decay, others that are associated with birds and mammals, and still others that are known mutualists with beetles or other arthropods. In our analyses of this dataset over the next year, we hope to gain additional insights into taxa or communities that may be causing or contributing to decay in these snags, and management practices associated with these communities.

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V. Partnerships/Contributors – This report was compiled by T.J. Lorenz, U.S. Forest Service, Pacific Northwest Research Station and M.A. Jusino, Department of Plant Pathology, University of Florida. Sequencing was conducted by M.A. Jusino with support from D.L. Lindner and M.T. Banik at the U.S. Forest Service, Center for Forest Mycology Research, Northern Research Station. The study was designed by T.J. Lorenz and M.A. Jusino. Nest searching was conducted by T.J Lorenz, P.C. Fischer, T.C. Groff, H. Tso, and C. Muller-Schinn. J.M. Kozma kindly shared nest locations from independent research projects on white-headed woodpecker. Fungal sampling and vegetation surveys were conducted by T.J. Lorenz and P.C. Fischer.

VI. Publications – We anticipate completing analyses and preparing a manuscript from this project in fall 2018, and submitting our manuscript to a peer reviewed journal in 2019.

VII. Appendices –

Appendix 1. Protocols and datasheets used for vegetation surveys and fungal sampling for this project central Washington in 2017.

Appendix 2. Project photos from 2017 field sampling.

Table 1. Mean (±standard deviation) of site characteristics for woodpecker nest snags compared to control snags (that lacked woodpecker cavities) in central Washington in 2017.

	Nest snags (n = 76)	Control snags (n = 56)
DBH (cm)	38.6 (±15.8)	38.9 (±14.0)
Cavity height (m)	2.6 (±1.7)	na
Snag height (m)	7.7 (±7.0)*	14.7 (±8.7)*
Percent of char on bole	48.1 (42.1)	32.6 (±36.8)
Cavity wood hardness (newton meters)	1.6 (±1.3)*	9.4 (±3.3)*
Cavity 'sill' wood hardness (newton meters)	5.3 (±3.6)	7.9 (±3.5)
Percent canopy cover	16.0 (±16.2)	
Percent large woody debris cover on ground	14.3 (±11.5)	
Percent small woody debris cover on ground	15.1 (±12.4)	
Percent shrub cover	5.3 (±8.7)	
Number of snags with conks present on bole	2	4
Number of snags with woodpecker forage sign on bole	71	52

* Indicates this characteristic differed at $\alpha < 0.05$ in a paired-sample t-test.

Table 2. The most prevalent fungal taxa (present in at least 25% of samples) sequenced from the bole of nest snags (n = 54), inside woodpecker nest cavities (n = 64), and from the bole of control snags (snags lacking woodpecker excavations; n = 48) in central Washington, in 2017. Note that sample sizes differ from those in Table 1 because some samples could not be successfully sequenced. Also note that not all OTUs have been described or identified to species.

Classification of fungal operational taxonomic units (OTUs)	**Proportion of sites containing OTU**
Samples from bole of nest snags	
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Chaetothyriales,f:Herpotrichiellaceae,g:Rhinocladiella,s:Rhinocladiella atrovirens	0.54
k:Fungi,p:Ascomycota,c:Sordariomycetes,o:Ophiostomatales	0.39
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Eurotiales,f:Trichocomaceae,g:Talaromyces	0.33
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Chaetothyriales,f:Herpotrichiellaceae	0.30
k:Fungi,p:Basidiomycota,c:Tremellomycetes	0.30
k:Fungi,p:Basidiomycota,c:Agaricomycetes,o:Atheliales,f:Atheliaceae,g:Athelia	0.28
k:Fungi,p:Ascomycota,c:Saccharomycetes,o:Saccharomycetales,g:Candida	0.28
k:Fungi,p:Ascomycota,c:Leotiomycetes,o:Helotiales,f:Helotiaceae,g:Collophora	0.28
k:Fungi,p:Ascomycota, c:Leotiomycetes	0.26
Samples from inside woodpecker nest cavities	
k:Fungi,p:Ascomycota,c:Dothideomycetes,o:Capnodiales	0.58
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Chaetothyriales,f:Herpotrichiellaceae,g:Rhinocladiella,s:Rhinocladiella atrovirens	0.48
k:Fungi,p:Ascomycota,c:Dothideomycetes,o:Capnodiales,f:Teratosphaeriaceae,g:Elasticomyces,s:Elasticomyces elasticus	0.34
k:Fungi,p:Ascomycota,c:Sordariomycetes,o:Ophiostomatales	0.30
k:Fungi,p:Ascomycota,c:Dothideomycetes,o:Pleosporales,f:Pleosporaceae	0.30
k:Fungi,p:Ascomycota,c:Leotiomycetes,o:Helotiales,f:Myxotrichaceae,g:Oidiodendron,s:Oidiodendron griseum	0.27
k:Fungi,p:Ascomycota,c:Leotiomycetes,o:Helotiales,f:Helotiaceae,g:Meliniomyces	0.27
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Chaetothyriales,f:Herpotrichiellaceae	0.27
k:Fungi,p:Basidiomycota,c:Agaricomycetes,o:Atheliales,f:Atheliaceae,g:Athelia	0.25
k:Fungi,p:Ascomycota,c:Dothideomycetes,o:Dothideales,f:Dothioraceae,g:Hormonema,s:Hormonema carpetanum	0.25
Samples from bole of control snags	
k:Fungi,p:Ascomycota,c:Eurotiomycetes,o:Chaetothyriales,f:Herpotrichiellaceae,g:Rhinocladiella,s:Rhinocladiella atrovirens	0.40
k:Fungi,p:Ascomycota,c:Leotiomycetes,o:Helotiales,f:Helotiaceae,g:Collophora	0.29

Figure 1. Locations of woodpecker nests located in central Washington in 2017. Red circles indicate white-headed woodpecker nests, yellow circles indicate black-backed woodpecker nests, blue circles indicate hairy woodpecker nests, and brown circles indicate northern flicker nests.

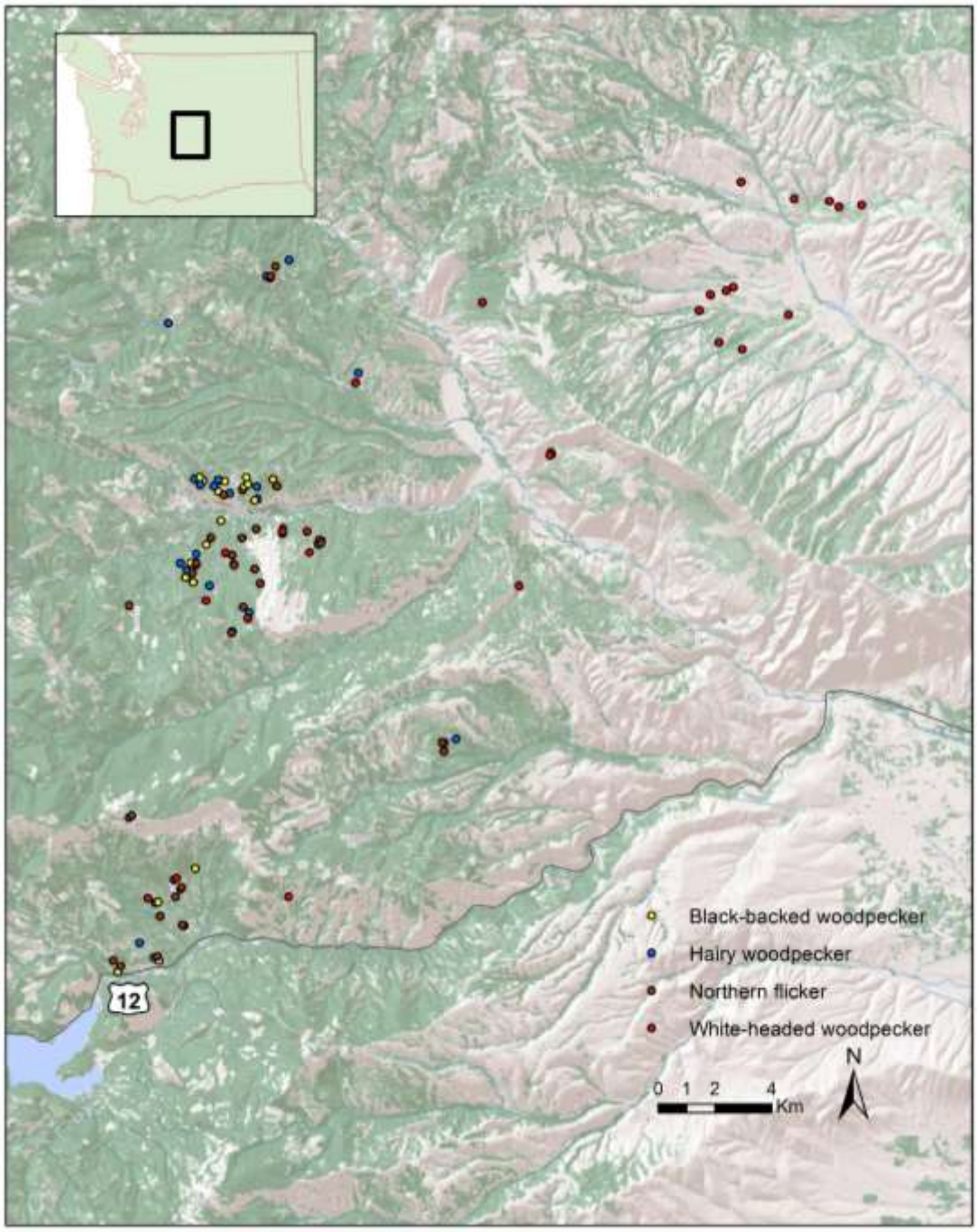
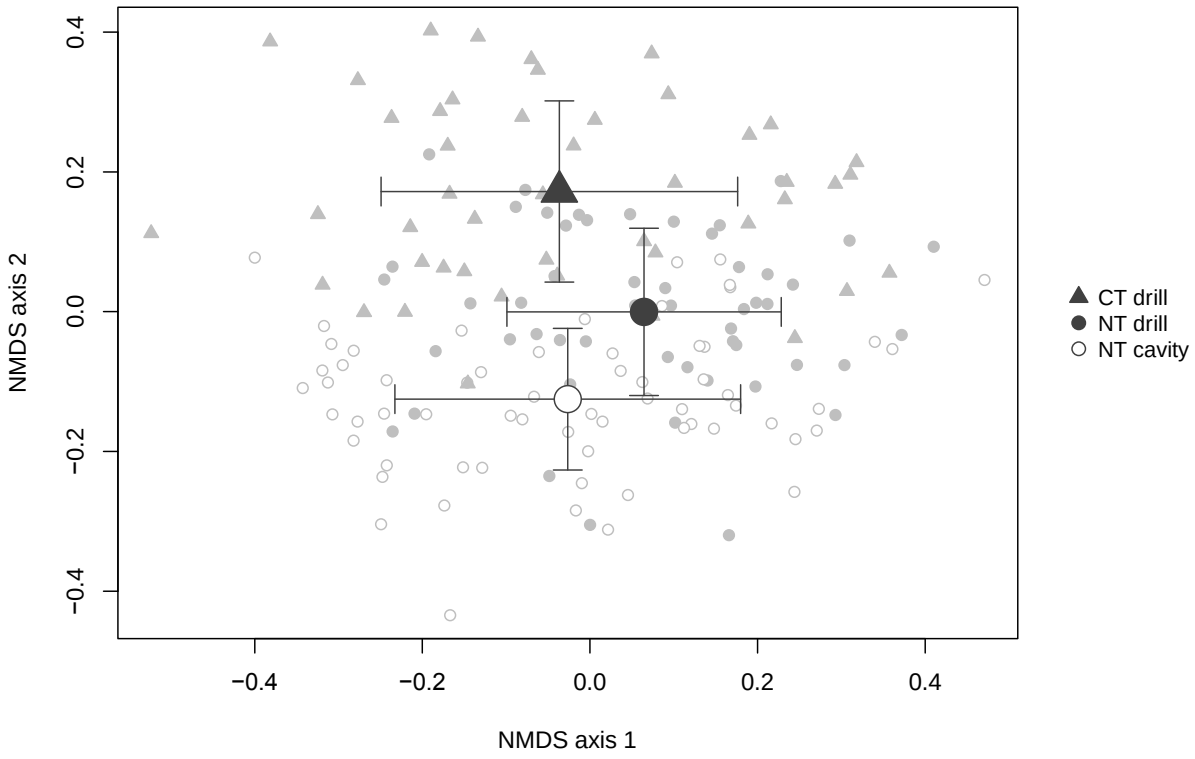


Figure 2. Nonparametric Multidimensional Scaling (NMDS) ordinations of the communities of fungi sequenced from within woodpecker nest tree cavities (NT cavity, *n* = 64), from the bole of woodpecker nest trees (NT drill, *n* = 54), and from control snags not excavated by woodpeckers (CT drill, *n* = 48) in central Washington in 2017. The dots and triangle in the center represent the means of the points on the two NMDS axes, and the bars represent one standard error from the mean.



Appendix 1. Project Sampling and Field Protocols

Field sampling protocol

Navigate to the nest snag/tree.

Record site/tree data and insert tree tag:

1. Record "**General info**" on datasheet:
    a. study area (i.e., Lookout Mtn, Rimrock, Rattlesnake, Nile)
    b. date
    c. study site (for Oregon, this is woodpecker species and number 1-13 on nest card)
    d. woodpecker species that excavate this hole (which is on the nest card)
    e. mark a waypoint at the nest tree and record the waypoint number
    f. GPS unit number being used on this tree
    g. initials of observer collected samples and recorded data.
    h. circle whether this datasheet applies to a nest or random tree – if a random tree, the woodpecker species and study site is the same as for the nest this tree is paired with
2. Put in **tree tag** on a sturdy nearby dead tree or stump, take a waypoint at this tree tag, and note distance and compass bearing to the actual nest or random tree. Do not put tree tag into the tree to be sampled because the nails are not sterile. Record tree tag number which will become the nest id number in all subsequent analyses.
3. Record "**Characteristics of nest/random snag**" on datasheet.
    a. snag species (usually *pipo*)
    b. note whether there are small holes indicative of woodpecker foraging (>80% of trees show woodpecker foraging evidence)
    c. estimate rough proportion of trunk that is blackened from fire, top that is broken, bark missing, needles remaining (usually 0), fine branches missing (branches <2 cm diameter), limbs missing (branches >2 cm diameter)
    d. note whether trees are live or dead (95% are dead); here also note unusual nest substrates like logs, cut stumps, root wad
    e. note whether there are fungal conks visible (which is rare, and usually if there are conks they are small pouch fungus, about 1 inch diameter and round)
    f. count the number of holes including the nest hole – if possible, separately count the number of incomplete cavity starts versus complete cavities. This is usually only possible for cavities that are low enough to reach finger inside (careful not to get bitten by a cavity occupant! I've been bitten by a few squirrels and it is not fun.) Do not stick your finger into the cavity to be sampled because your hands may contaminate the cavity – you will likely inadvertently introduce fungi from your hands into the cavity and we do not want fungi from human hands to be sequenced.
4. Record "**Characteristics of nest cavity**" on datasheet.
    a. *IMPORTANT* - Wait to measure sill width, bark-to-back distance, and cavity depth after you've sampled for fungi with the spoon. We do not want any fungal taxa from your hand getting into the cavity. DO NOT TOUCH the cavity until after you've gotten the spoon scraping, described below.
    b. measure cavity orientation with a compass (stand with back to cavity and point compass in the direction the hole faces)
    c. note obvious signs of damage to the entrance hole, which is rare
    d. note whether there are limbs or old, partially covered tree knots near the cavity entrance. In general, only count knots >2 cm diameter
5. Record "**Trees within 11 m radius**" on datasheet.

a. use the range finder and your compass, and note the number of trees and snags within 11 m radius of the nest/random snag. For each tree, note species, dbh, and whether it is live or dead. For dead broken snags, count only those that reach to 1.4 m height.

6. Record "**Canopy and shrub cover**" and "**Woody debris on ground**" on datasheet:
    a. for canopy cover, follow the directions on the densitometer
    b. for shrub cover, use the range finder to determine approximate boundaries of a circle, 5 m in diameter, centered on the nest tree. For each quadrant, note approximate cover by shrubs (defined as anything with a woody stem less than 1 inch diameter)
7. Take 9 photos
    1. first tree tag,
    2. then ground photos (in this order - north, east, south, west),
    3. then tree photos (north, east, south, west, standing back 10 m)

    If you mess up the photo order, go ahead and delete the messed up photo(s) and start over.

For fungi sample/hardness:

1. **Set up ladder:** Set up the ladder if necessary to the cavity height. Put on harness and other climbing gear if needed. Spread plastic sheet on ground if necessary (if ground is wet or snowy). Place cookie sheet in convenient location to catch isopropyl during flame sterilization.
2. **Sterilely sample inside nest cavity with spoon (nests only):**
    2.1. Clean hands by spraying with isopropyl and rubbing over hands until dry. Put on new, unused, clean gloves. Spray gloved hands with bleach spray or place ~1 tablespoon bleach mixture in hand and rub all over gloves. Wipe on clean paper towel and discard paper towel in trash bag.
    2.2. At first tree every day (or at about every 5 trees) wipe cordless drill housing and torque wrench handle with bleach/isopropyl.
    2.3. Wipe sharpened spoon with clean dry paper towel. Place spoon in bleach bottle containing 20% bleach. Make sure bleach level is as high enough to cover every portion of spoon/handle that will contact cavity. Wait 30 seconds. Then remove and wipe down with clean paper towel, starting at the end where your hand is and wiping down the spoon.
    2.4. Hold spoon over cookie sheet to prevent isopropyl from dropping onto forest floor. Spray spoon thoroughly and generously with isopropyl. While wet with isopropyl, flame sterilize thoroughly with cigar lighter, making sure to hold everything over the cookie sheet to avoid letting isopropyl drop onto forest floor. Make sure spoon is dry with no residual wetness. If necessary, wait a minute for spoon to cool before inserting into cavity (to avoid "cooking" any organisms within the wood, or denaturing dna).
    2.5. At this point, do not touch any part of the sterilized spoon, or allow it to come in contact with anything but air. If it touches the tree, your hand, the ladder, or anything, go back to step 2.3 and re-sterilize.
    2.6. If it is necessary to climb to cavity, steps 2.3 to 2.5 can be completed while on ladder, or on ground if person is adept at climbing tree without using hands.
    2.7. Scrape "dome" of cavity and back of cavity with sterilized spoon. Gently pour shavings into sterile microcentrifuge tube, taking care not to allow any unsterile item to contact inside of tube, or inner lid of tube. Do not allow spoon to touch microcentrifuge tube. Close tube and label with tree tag number, and letter "C" (indicates cavity sample). Label tube on top and side. Close tube and place on ice immediately. Now you can breathe easy and poke your hand in the cavity to measure sill width, bark-to-back distance, and cavity depth – again taking care not to get bitten by any cavity occupants.

3. **Sterilely sample ~2 cm above cavity opening with drill (nests) or at nest height and orientation (random trees)**
    3.1. Clean gloved hands with bleach spray or place ~1 tablespoon bleach mixture in hand and rub all over gloves. Wipe on clean paper towel and discard paper towel in trash bag.
    3.2. Preferably get your assistant/data recorder to vigorously clean drill bit with wire brush. For many trees, this means a good 2 or more minutes of vigorous, hard scrubbing to get off all residue from the previous tree. Scrutinize closely to makes sure there is nothing remaining, using a finger nail if necessary to probe around on edges of the flutes (then put on a new set of gloves and wipe with isopropyl or bleach afterwards). Then wipe off drill bit with a clean (dry) towel.
    3.3. Place drill bit in bleach solution for at least 30 seconds. Make sure bleach level is as high as the deepest place you plan to drill (i.e., make sure the first 15 cm of drill are sitting in the bleach for 30 seconds – sometimes when the bleach level gets low this means tilting the bleach bottle). While waiting, I typically spray gloves again with bleach. Wipe drill bit dry with new and clean paper towel, starting at the end where your hand is, and wiping down the bit.
    3.4. Once bleach has dried, spray drill bit thoroughly with isopropyl and flame sterilize with cigar lighter. Use the same procedure as for the spoon described in item 2.4. Make sure any isopropyl that drips lands on the cookie sheet. After flame sterilization do not touch any part of the sterilized bit, or allow it to come in contact with anything but air. If it touches the tree or your hand, go back to step 3.3 and re-sterilize.
    3.5. Get a sterile funnel tube from within the clean ziplock bag, and place over the sterile drill bit. Begin drilling into the tree about 2 cm above the cavity opening. We are aiming to collect a sample as near the cavity as possible, but above the cavity 'chimney' (dome that birds excavate above their head in the cavity). Be sure to press the funnel tube hard against the tree so that all wood shavings are collected inside the tube. Once you have drilled to ~3 cm, remove drill bit and carefully pour wood shavings into a sterile falcon tube. Label with tree tag number and letter "s", for sill. Place on ice.
    3.6. Repeat steps 3.1 to 3.5, this time drill to about 17 cm deep. Label these wood shavings with "b" for body wood. Place on ice.
4. **Measure wood mass density.**
    4.1. Insert the increment borer into the hole you just drilled. Use the torque wrench to record hardness, counting the number of tick marks on the inch pounds scale. Counting tick-marks avoids potential mistakes people make when trying to count in factors of "5" in the field after a long week of sampling.
5. **Collect a core sample with increment borer.**
    5.1. Attempt to collect a core sample as close to the cavity as possible but without caving in the wood near the cavity opening or the drill hole from steps 3 and 4. Suggest locating this increment core hole at least 2 cm from the cavity opening and the drill hole.
    5.2. Once you have selected a spot for the core, start to turn the increment borer into the bark or wood. Beware that the increment borer can be extremely hard to start in trees with dense, barkless outer wood. Push with your body and get a helper to push with you. Once the drill portion of the increment borer engages in the wood, spinning is relatively easy. Every 2-3 turns, insert the extractor spoon to the tip of the borer to make certain the wood is not jamming.
    5.3. At 3 cm deep, remove all the wood using the extractor and store in a 15 mL centrifuge tube labeled with the tree tag number and "is". Put on ice and out of sunlight.

5.4. Continue step 5.2 until 10 cm and again remove all wood. Place in 15 ml centrifuge tube and label with tree tag number and "ib". Put on ice and out of sunlight.
5.5. Remove the increment borer. Beware that in trees with a hard sill but rotted interior the borer can get stuck and requires a large amount of force to remove.

Protocol for selecting random tree

1 - Select a random orientation

2 - Move out 10 m from the nest snag in this orientation (so we do not sample too close to nests).

3 - Continue walking in the random orientation and select the first snag in this orientation that occurs within 5 m of the invisible line you're walking.

4 - If no suitable snag occurs within 75 m of the nest, return to nest and select a new orientation. Repeat until you find a suitable snag. 75 m is the maximum distance because it represents the approximate radius of a woodpecker nest territory.

5 - Caveats
- snag species must match nest snag species
- dbh must fall between 15.7 and 104.9 cm.
- snag must contain no holes, or cavity starts (foraging holes are ok).
- snag height must be at least as high as the nest, for the corresponding nest (because we will sample random snags at the same height and orientation as the nest).

Protocol for when you return from field

Samples need to be properly stored after getting home from the field. Depending on the number of samples, this procedure takes about 30 minutes.

Equipment:
all tubes containing wood from the day's sampling (50 mL, 15 mL, and 1.5 mL tubes containing wood)
nitrile gloves
20% bleach solution in Nalgene bottle from field, or bleach spray
cookie sheet
isopropyl spray
butane torch
stopwatch
empty 1.5 mL tubes with buffer – one for each 50 mL tube you collected
sampling spoon
cotton cloth/rubber bands/scissors
tube racks
freezer for 1.5 mL tubes containing wood sample

1 – Wash your hands and put on clean nitrile gloves. Clean work space and spray down with bleach spray. Set out large 50 mL falcon tubes from each day's sampling in tube racks. Set cookie sheet on a

clean, clutter-free surface. Set out one 1.5 mL microcentrifuge tube for each 50 mL falcon tube in the little tube rack.

2 – Sterilize sampling spoon like you do in the field: (1) wipe off obvious debris with clean paper towel, (2) set in bleach bath for 30 seconds, (3) remove and spray with isopropyl alcohol, (4) flame sterilize with butane torch. Let spoon cool before proceeding. Do not touch the spoon but carefully put your hand within about an inch of the surface of the spoon – if you can feel heat coming off, wait.

3 – Shake up the first 50 mL falcon tube that you plan to sample so that the wood inside is fairly 'homogenized'. Once spoon is cool carefully open this 50 mL falcon tube. Collect a small amount of sample (roughly ¼ teaspoon) in your spoon. Try to get wood shavings that are representative of the size and type of wood in the tube.

4 – Now holding the spoon (containing a little bit of wood) carefully with one hand (right hand for right handed people), with your left hand pick up one of the 1.5 mL tubes, open very carefully, and gently shake the spoon's contents into the 1.5 mL tube.

5 – Cap the 1.5 mL tube. Cap the 50 mL falcon tube. Now label the 1.5 mL tube with the same exact label as its corresponding 50 mL tube. I recommend looking at the wood inside the 1.5 mL tube to make sure it is fully wet and covered with buffer. Sometimes an airpocket will form at the bottom of the little tubes and the lowest wood is dry. It is important to get this wood wet. You can do this by inverting the microcentrifuge tube and gently tapping it (upside down) against a counter top. Then to store the sample, turn it right side up and tap gently again to get all the sample (now wet, hopefully) back into the bottom of the tube.

6 – Repeat for each 50 mL falcon tube from the field, starting at step 2. **Be certain to thoroughly sterilize spoon in between each and every 50 mL tube.**

7 – When you are finished, put all 1.5 mL tubes in the white cardboard cyrobox that has been stored in the freezer. Put it in the freezer. For 50 mL falcon tubes, open each lid and cover with a small square of cotton cloth. Secure with rubber bands and leave in clutter-free, undisturbed place (but at room temp). Place all 15 mL tubes in the freezer.

Protocol to sterilize funnels

Sterilize funnels after every use (typically at the end of every field day). In the field they get may get coated in wood chips and fine wood powder. This process takes 15-30 minutes, depending on the number of funnels that need to be sterilized

Equipment:
dirty funnels from today's field work
sponge, toothbrush, soap, towel, tap water
nitrile gloves
20% bleach solution (can be reused from day-to-day) in glass bread pan (do not use plastic or metal because bleach will melt plastic and water will cause rust in metal)
cookie sheet
bleach spray

isopropyl spray
stopwatch
oven
new, clean ziplocks

1 – Wash the inside of funnels thoroughly with soap, water, toothbrush and sponge to remove visible debris. Toothbrush is particularly handy for cleaning inside. Rinse well to remove soap residue

2 – Put on gloves. Mix solution of 20% bleach and 80% tap water in a metal or glass bowl (or use bleach from previous day). Soak funnels in 20% bleach solution for 5 minutes. Bleach denatures dna and this step is meant to denature all fungal dna that may be present in funnel tubes from trees sampled that day.

3 – While they are soaking, get a cookie sheet and spray with bleach spray. Let sit bleach soak on cookie sheet while funnels are soaking in bleach. Or you can use isopropyl and flame sterilize cookie sheet – but be careful if you choose the flame approach and make certain loose hair and clothes are tied back and there are no flammable materials nearby. A large empty bathtub works well for flame sterilizing the cookie sheet, with added benefit that water is handy if you get too enthusiastic with your flame.

4 – One by one, take funnels out of bleach and spray with a steady stream of 70% isopropyl alcohol. The goal with this step is to remove all bleach residue, because remaining bleach residue can denature any dna from trees we plan to sample the next day. Do not rinse funnels with tap water because tap water is not dna sterile. Shake excess isopropyl off so funnels are not dripping wet. Set each funnel one-by-one onto now sterile cookie sheet.

5 - Let dry ~5-10 minutes in oven set to low setting (100-150 F). Shake pan a couple times to get funnels to roll and dry on all sides. Do NOT touch funnels with your hand. If you touch them, go back to step 2.

6 – The funnels should now be dna sterile and dry. At this stage, do not touch them or allow them to touch anything unless it has been sterilized. Put on gloves. Spray gloves with isopropyl and let air dry. Carefully gather funnels with your now sterile hands and place all funnels together in new, clean ziplock and seal. Use a clean ziplock every day. Old ziplocks can be used for trash bags and other purposes.

Repeat at the end of every field day and use clean ziplock everyday

Appendix 2. Project Photos.

Appendix 2, Figure 1. White-headed woodpecker at excavated cavity in a burned Douglas-fir snag, in Meeks Table burn, in 2016. This cavity was enlarged by Northern Flicker in 2017 and included in our fungal sampling effort. Photo credit: D. Reiff.

Appendix 2, Figure 2. Black-backed woodpecker drumming above excavated cavity in a burned Douglas-fir snag, in Meeks Table burn, in 2017. This cavity was included in our 2017 fungal sampling effort. Photo credit: T. Lorenz.

Appendix 2, Figure 3. Measuring canopy cover at a random, non-use snag in 2017 in Angel Prescribed burn. Photo credit: P. Fischer.

Appendix 2, Figure 4. Fungal field sampling equipment, including (from left to right) ziploc bag of sterile tubes, increment borer, torque wrench, drill, datasheets, scraping spoon, wood samples within tubes, isopropyl spray (in pink spray bottle), bleach spray, propane torch, cooler for storing samples in field, and on far right bag containing all vegetation measuring tools (clinometer, compass, DBH tape, ruler, densitometer). Photo credit: T. Lorenz.

Appendix 2, Figure 5. Set of five wood samples collected from a typical nest tree, in this case from a Hairy woodpecker nest in a ponderosa pine in Meeks Table burn, including (from left to right) wood shavings from drill from sill wood, wood shavings from drill from body wood, wood shavings from increment borer sample from sill wood, wood shavings from increment borer sample from body wood, and spoon scraping sample from within the woodpecker's excavated cavity (within liquid CLS). All samples were collected using aseptic field techniques and stored in DNA-sterile tubes. Photo credit: T. Lorenz.