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Project Title: Early Detection of *P. destructans* in New Mexico

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Summary and Significance of Project:

Early detection of *Pseudogymnoascus destructans* (a.k.a. *Pd*) is an important management tool in reducing the spread of this white-nose syndrome (WNS) causing fungus. With WNS having been detected in two neighboring states, New Mexico is now on the leading edge of the spread of WNS. This study aimed to monitor caves across the state of New Mexico, in Spring 2018 and Spring 2019, in order to determine if *P. destructans* has entered New Mexico. Using real-time PCR methods, this study focused on caves/mines that are known hibernacula for either *Corynorhinus townsendii* (COTO) or *Myotis* spp. bats in order to determine if *P. destructans* is present in any of the caves/mines selected as among the most vulnerable to *P. destructans* infection.

Summary of Methods:

Field Methods

Caves and mines were selected in consultation with New Mexico Department of Game and Fish, the Bureau of Land Management, the National Park Service, and New Mexico Abandoned Mine Land Program. Permits and Section 106 compliance letters were obtained from the appropriate (i.e., land management) agency for each cave/mine. A total of 17 caves and one mine were sampled in the spring of 2018 from March through May (Table 1). Due to the urgency of early detection in New Mexico, we chose to sample all of the identified caves in the spring of 2018 and retested a subset of caves in the fall.

The sample sites within the caves were selected based on presence of bats (fresh guano in area) or at pinch points in caves where both humans and bats would have to pass. If guano was present, then it was collected. Figure 1 shows examples of samples and sampling sites. At each sample site, guano and/or soil were collected using sterile techniques. In 2018, approximately 10-30 cc of guano and/or soil were sterilely collected into a sterile 50 cc falcon tube. This tube was designated the primary sample. The sample was thoroughly mixed through agitation and then divided into two additional sterile tubes. The primary sample and one replicate were preserved with sucrose lysis buffer (SLB), which breaks open microbial cells and stabilizes the DNA for long-term preservation. The third tube was left as is with no additional preservative. All tubes were placed on ice or stored at 4°C as soon as possible. They were stored at -80 °C upon returning to the lab. Additionally, at a few locations, swabs of dead bats or other isolated guano samples were collected. In BLM caves 45 and 55 and Cottonwood cave, dead bats were collected in sterile whirl packs. These bats were not counted as samples for this study. These bat samples did not show signs of suspicious fungus, but were collected to send to the USGS National Wildlife Center in Wisconsin for testing.

Table 1: Summary of 2018 Caves, Samples Sites, and Samples.

Cave	County	Number of Samples Sites	Number of Primary samples	Number of Additional Samples (swabs etc.)	Number of Samples Extracted	Number of Samples positive *Pseudogymnoascus destructans*	Ct Value from *P. destructans* Positive Samples
Pinon	Chavez	3	3		3	0	
A'a	Cibola	3	3		3	1	39.05
AJ	Cibola	3	3		3	1	39.17
Bat	Cibola	3	3		3	1	39.92
Brewers	Cibola	3	3		3	1	39.77
Four Windows	Cibola	3	3		3	1	39.82
Hummingbird	Cibola	4	4		4	0	
Junction	Cibola	3	3		3	1	39.09
West	Cibola	2	2		2	0	
BLM CAVE 55	DeBaca	3	3		3	0	
Carlsbad Cavern Bat Cave	Eddy	6	6		6	0	
Carlsbad Cavern LC	Eddy	2	2	1	3	2	39.65, 38.67
Carlsbad Cavern Right Hand Fork	Eddy	2	2		2	0	
Cottonwood	Eddy	4	4		4	3	37.31, 39.92, 39.61
Goat	Eddy	4	4		4	0	
Lake	Eddy	4	4	1	5	0	
Ogle	Eddy	5	5		5	0	
Fort Stanton	Lincoln	6	6		6	1	39.59
BLM Cave 45	Lincoln	5	5	5	10	1	39.82
Nancy Mine	Socorro	4	4		4	2	38.61, 38.61
		Total	72	7	79	15	


A
B
C
CC 180317-23
Site 3 ~
upper Lake


D
E
F
G
Bat Cave Sites

Figure 1: Examples of sampling site, methods, and samples. A. Northup and Hathaway sampling in Fort Stanton Cave. Personal protective equipment such as respirators and gloves were worn while sampling and Hathaway, Strach, and Northup have been certified on respirators by University of New Mexico Safety personnel. Photo by D. Buecher B. Eddie Strach helping to sample in Goat Cave. The photo shows the area sampled, which was several square feet in size. Photo by Kenneth Ingham. C. Large guano pile that was sampled from Carlsbad Cavern. Note the fungus (white) on the guano. Photo by Diana Northup. D. Hathaway scooping individual guano pellets into a sterile falcon tube. Photo by Diana Northup. E. Example of guano with fungus on surface. Photo by Diana Northup. F. Example of sampling site, showing that a single sample could come from several adjacent areas in a cave. Photo by Kenneth Ingham G. Sample that was a mixture of guano and soil/sediment. Soil collected if there was not a lot of guano present in area. Photo by Jennifer Hathaway.

Table 2: Summary of 2019 Caves, Samples Sites, and Samples.

Cave	County	Number of Samples Sites	Number of Primary samples	Number of Samples Extracted	Number of Samples positive *Pseudogymnoascus destructans*
BLM CAVE 55	DeBaca	3	3	3	0
Carlsbad Cavern Bat Cave	Eddy	4	4	4	0
Carlsbad Cavern LC	Eddy	3	3	3	0
Carlsbad Cavern Right Hand Fork	Eddy	2	2	2	0
Ogle	Eddy	4	4	4	0
Cottonwood	Eddy	4	4	4	0
Fort Stanton	Lincoln	5	5	5	0
BLM Cave 45	Lincoln	4	4	4	0
	Total:	29	29	29	

In 2019, one tube of guano was collected from each site, instead of three, due to limitations on time and resources. Samples were again placed on ice/stored at 4°C as soon as possible and stored at -80 °C upon returning to the lab.

A total of 27 people, including several volunteers, helped to collect the samples in this project.

2018 Laboratory Methods

The primary sample was extracted in duplicate using the Qiagen Power Soil Kit following the manufacturer's protocol, with the following modifications: samples were bead beaten for 1.5 minutes at medium speed after the addition of solution C1. Approximately 0.25 g of the sample was used in each extraction and was eluted in 50 *ul* of solution C6. A negative control extraction was also performed to ensure there was no contamination of reagents. This control was exposed to the same conditions and reagents as the samples, but with no sample added.

In order to determine if the extraction was performed cleanly and that fungus was present in the sample, a PCR was performed using universal fungal primers ITS1F and ITS4. This allows for detection of any kind of fungus in the sample. A PCR was also performed on the negative control extraction as quality control of the extraction. If the PCR of the negative control extraction had no band, then we could assume that the extraction was done without contamination.

All samples were then PCR-tested with primers designed to test for the presence of *Pseudogymnoascus* spp*.,* including *Pd* (Lorch et al. 2010). The aim of this step is to identify samples with *Pseudogymnoascus* spp*.* present in them that warrant testing with qPCR. qPCR is a more expensive technique, thus use of the Lorch et al. (2010) primers allows us to target our testing more effectively.

Due to time constraints, samples that were positive for *Pseudogymnoascus* spp*.* were sent to the Foster Lab at Northern Arizona University for qPCR analysis. Initial results indicated that three samples were positive at low levels. In working with the Foster Lab, we refined our DNA extraction protocol, which involved fewer freeze thaw cycles and shorter time between extraction and qPCR. These steps proved to be critical in improving the accuracy of the qPCR analysis. All 74 guano samples were re-extracted under the improved protocol.

Additionally, the third replicate tubes for the *Pseudogymnoascus* spp. positive samples, which did not have sucrose lysis buffer added, were sent to the USGS National Wildlife Center in Wisconsin for testing.

2019 Laboratory Methods

In 2019, only one sample was taken at each location. This was due to time and financial constraints. DNA extractions were performed using the DNeasy PowerLyzer Powersoil Kit with the addition of lyticase at the start of the protocol. Lyticase is an enzyme that helps break up the sturdy cell walls of fungi, allowing for more DNA to be captured in the extraction. Verant et al. (2016) showed this to be a more effective method of DNA extraction for *P. destructans.* Briefly, 300 units of the enzyme lyticase per sample were added to the Power Bead Solution and then filter sterilized. Samples were incubated in the lyticase power bead solution for 30

minutes at 30° C with shaking at 150 rpm. The manufacture's protocol was then followed with a 1.5 min bead beating and a final elution in 50 ul C6. Additionally, a subset of the samples were extracted with the same methods used in 2018 to ensure the new protocol was consistent with results from 2018. Samples were then sent to the Foster lab for qPCR testing, forgoing any initial screening at UNM.

Summary of Results:

In 2018, 79 of the 100 samples for this study were collected and extracted from 18 different caves or mines in six counties across New Mexico. Of these samples, 15 were positive for low levels of *P. destructans.* The positive samples came from 11 caves in 4 counties. qPCR results are reported as Ct values (i.e., the cycle number where the fluorescence level rises above the threshold level). For *P. destructans*, a Ct value of 37 or below is considered to be strongly positive, while Ct values of between 37 and 40 are considered to be low-level detections. All of the Ct values detected in this study in 2018 were between 37 and 40 (Table 1), indicating low levels of *P. destructans* at sites in northwestern and southeastern New Mexico.

The dead bats sent to the USGS National Wildlife Center in Wisconsin did not test positive for *P. destructans.* The duplicate guano samples sent to USGS National Wildlife Center in Wisconsin were not found to be positive for *P. destructans* with the methods used. The differing results are probably due to slight differences in protocols between the two labs.

In 2019, a total of 29 samples were collected from eight caves in three counties. None of the 2019 samples collected for this project have tested positive for *P. destructans*. Based on discussions with Jeff Foster and Katy Parise, we have concluded that the differences in results between 2018 and 2019, with fewer positives in 2019, are not unexpected based on the extensive experience that the Foster lab has with samples collected throughout the western United States. Many of the samples tested over the last few years showed low-level detections that varied from year to year in any given site, with it taking two to three years for a stronger positive detection to show up. Also, many of the sites we sampled (e.g., in El Malpais) have very small deposits of guano, unlike sites in eastern states, and it's possible that our sampling in 2018 by chance hit areas that contained small amounts of *P. destructans*. The El Malpais caves are very cold in comparison to other caves and the fungus will grow slowly, making its detection even more difficult.

Management Implications

We have had extensive communication with Jeff Foster concerning these results and how best to interpret them. Historically, *P. destructans* detection has followed a pattern of low-level environmental detection in the first 1-3 years, followed by low-level detection on bats as well as higher-level environmental detection, and finally wide spread detection (environmental and on bats) and death of bats. However in Texas, the first southwestern state to detect *P. destructans*, the pattern appears to be shifting. Low-level detection has been documented in many locations without it increasing to high levels in the environment or on bats (Foster, personal communication). This may be due to the different hibernating behavior of western bats (more small group, single hibernators) compared to their eastern counterparts. While a single sample from a cave testing positive in the Ct 37 to 40 range is not conclusive enough to say that *P. destructans* is established in New Mexico, it can serve as a forewarning that the

fungus is likely to become established in New Mexico in the next few years. This also strongly justifies agencies in requiring enhanced decontamination procedures, an important control mechanism to prevent/slow further spread of the fungus.

These results suggest that *P. destructans* will be able to become established across several counties in New Mexico. To ensure that best practices and good management are implemented, it is critical to realize that *P. destructans* is likely to already be in New Mexico at low levels, and, with consistent monitoring, we will most likely see the *P. destructans* levels rise over the next several years. Agencies should interpret these results as a call for more monitoring and awareness of *P. destructans* and WNS in New Mexico and the establishment of enhanced decontamination measures.

References:

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