DOI: 10.7589/2016-08-175

Journal of Wildlife Diseases, 53(3), 2017, pp. 482–490

PREVALENCE AND DISTRIBUTION OF *PSEUDOGYMNOASCUS DESTRUCTANS* IN MICHIGAN BATS SUBMITTED FOR RABIES SURVEILLANCE

Samantha L. Darling,[1] Ailam Lim,[1,2] Julie R. Melotti,[3] Daniel J. O'Brien,[3,4] and Steven R. Bolin[1,2]

[1] College of Veterinary Medicine, Michigan State University, 784 Wilson Road, East Lansing, Michigan 48824, USA
[2] Diagnostic Center for Population and Animal Health and Department of Pathobiology and Diagnostic Investigation, 4125 Beaumont Road, East Lansing, Michigan 48824-8104, USA
[3] Wildlife Disease Laboratory, Michigan Department of Natural Resources, 4125 Beaumont Road, Room 250, Lansing, Michigan 48910-8106, USA
[4] Corresponding author (email: obriend@michigan.gov)

ABSTRACT: Since 2006, bat populations in North America have suffered devastating mortality from an emerging disease known as white-nose syndrome (WNS). The causal agent of WNS is the fungus *Pseudogymnoascus destructans*. In April 2014, WNS was discovered in little brown bats (*Myotis lucifugus*) in Michigan, US, and has since been documented in 12 counties. Because current surveillance for WNS focuses primarily on mine-hibernating species in winter, it is subject to geographic, species, and seasonal bias. To investigate species affected and potential associations of gender, seasonal life cycle, and region with *P. destructans* prevalence, 1,040 rabies-negative bats were sampled from May 2014 to May 2015 from animals submitted as part of statewide rabies surveillance. The vast majority (96%) of the sample population consisted of big brown bats (*Eptesicus fuscus*), a noncavernicolous species. Two methods were used to detect *P. destructans*: fluorescence of the muzzle, wing, and tail membranes under ultraviolet light and PCR targeting genomic DNA on wing samples. Only five bats (0.5%), all *M. lucifugus*, were confirmed positive after nucleic acid sequencing of PCR amplicons. No other species were infected. All infected bats were collected from April to May, coinciding with their emergence from hibernation. As *P. destructans* and WNS spread westward, novel surveillance streams may provide a useful tool for wildlife management agencies seeking to detect the fungus where winter hibernacula such as caves and mines are absent or otherwise inaccessible.

Key words: Big brown bat, *Eptesicus fuscus*, little brown bat, *Myotis lucifugus*, *Pseudogymnoascus destructans*, surveillance systems, white-nose syndrome.

INTRODUCTION

White-nose syndrome (WNS) is a disease of bats caused by the psychrophilic fungus *Pseudogymnoascus destructans* (Minnis and Lindner 2013). The disease was first detected in the US in 2006 and by 2012 had resulted in death of over 5.5 million bats (US Fish and Wildlife Service 2012). The fungus attacks the muzzle and wing membranes of hibernating bats and can cause detrimental alterations in physiology and behavior, which can lead to depletion of fat deposits that are critical for survival during hibernation (Brownlee-Bouboulis and Reeder 2013; Cryan et al. 2013; Verant et al. 2014). In addition, the disease can cause extensive damage to wing membranes such as membrane loss and necrosis (Reichard and Kunz 2009; Meteyer et al. 2012). To date, 12 species of bats in the US have proven capable of being infected with *P. destructans*, of which seven manifest diagnostic signs, including the little brown bat (*Myotis lucifugus*; MYLU) and the big brown bat (*Eptesicus fuscus*; EPFU; US Fish and Wildlife Service 2014), which are the most common species of bats in Michigan (Kurta 2008).

In April 2014, the first cases of WNS in Michigan were reported in MYLU located in Alpena County of the Lower Peninsula, and Dickinson and Mackinac counties of the Upper Peninsula. The western Upper Peninsula holds the majority of mines in the state, which are the preferred hibernacula of MYLU residing in Michigan. Currently *P. destructans* has also spread to Alger, Delta, Gogebic, Houghton, Keweenaw, Marquette, and Onto-

nagon counties in the Upper Peninsula and Clare and Manistee counties in the northern Lower Peninsula (US Fish and Wildlife Service 2016).

Surveillance for *P. destructans* and WNS in Michigan has thus far focused on cavernicolous species such as MYLU and the abandoned mines where the vast majority of the state's population hibernates. While logistically efficient, this also has the potential to create bias when determining species, prevalence, and distribution of bats infected with *P. destructans*, because such sites are rare throughout most of the Lower Peninsula. After conducting a 3-yr surveillance study, Winhold and Kurta (2008) found that 81% of bats netted in the southern Lower Michigan were EPFU. The EPFU is classified as a noncavernicolous species (Halsall et al. 2012), for which preferred hibernation sites are buildings (Whitaker and Gummer 2000). The mean ambient temperatures of locations in buildings used for hibernation were between 10 C and 14 C (Whitaker and Gummer 1992; Halsall et al. 2012), which overlaps the optimal temperature range (12.5 C to 15.8 C) for growth of *P. destructans* (Verant et al. 2012). Thus, those hibernacula might also harbor the fungus. Several field studies conducted in other states suggest that EPFU may be resistant to *P. destructans* (Moosman et al. 2013; Frank et al. 2014). Our goals were to obtain a better understanding of the species affected, and the distribution and prevalence of *P. destructans* and WNS in Michigan by testing a surveillance stream that did not rely on winter surveys of mines and caves.

MATERIALS AND METHODS

Specimens

The bat carcasses used in this study were selected from among those submitted to the Michigan Department of Health and Human Services Laboratory from May 2014 to May 2015 as potential human and domestic animal exposures to rabies. For each, the date and county of collection, species, gender, and rabies test results were recorded. All rabies-negative bats were brought to the Michigan Department of Natural Resources Wildlife Disease Laboratory and stored at –20 C until the time of examination.

The bats were allocated into seasonal groups based on Kurta's (2008) description of the general life cycle of a bat in Michigan and date of collection: hibernation/winter (1 November–31 March); emergence from hibernation, and gestation (1 April–30 May); parturition and lactation (31 May to about 15 August); and swarming (breeding) and independence of young (about 16 August–31 October).

Wing fluorescence and tissue sampling

The bat carcasses were thawed and examined for lesions consistent with WNS as described by Reichard and Kunz (2009) under a Luxo magnifier light (model 172537G, Luxo Corporation, Elmford, New York, USA). The muzzle, wing, and tail membranes of each bat were then examined for orange-yellow fluorescence using an ultraviolet (UV) light as described by Turner et al. (2014) for detection of *P. destructans*. Where fluorescence was found, a 5-mm×5-mm piece of tissue was excised. To prevent cross-contamination, each bat was handled with latex gloves and examined over a paper plate, tissue was excised with a razor blade, and a toothpick was used to place the tissue sample into a labeled 1.5-mL microcentrifuge tube. Excepting the tube, all those items were discarded after each bat was processed. Bats with no observed fluorescence had a 5-mm×5-mm piece of tissue cut from the trailing edge of the plagiopatagium. Reichard and Kunz (2009) found that site most prone to membrane loss and necrosis due to WNS, and other studies suggest it is the site most heavily infected with *P. destructans* (Fuller et al. 2011; Meteyer et al. 2011). The right plagiopatagium directly caudal to the right elbow joint was chosen arbitrarily in order to keep the sampling site consistent. All tissue samples were submitted for DNA extraction and PCR assay.

DNA extraction

The sodium dodecyl sulfate (SDS) digestion method described by Goldenberger et al. (1995) was used for extraction of DNA from bat wings, with modifications to accommodate a high volume of samples. Briefly, the 5-mm×5-mm piece of wing in a 1.5-mL microcentrifuge tube was incubated in 80 µL of Proteinase K (IBI Scientific, Peosta, Iowa, USA) digestion buffer for 2 h at 56 C. After digestion, the resulting solution was mixed using a vortexer and pulse centrifuged for 3 s to drive condensate off the lid of the microcentrifuge tube, to avoid contaminating the lab environment when the lid was opened. The tube was then incubated for 10 min at 100 C to

inactivate the Proteinase K, briefly mixed using a vortexer, pulse centrifuged as mentioned earlier, and chilled on ice for 3 min. Finally, 20 μL of polyoxyethylenesorbitan monolaurate (Tween-20, Sigma-Aldrich, St. Louis, Missouri, USA) was added to each tube to neutralize the SDS. Each tube was then mixed until the Tween-20 was homogenized into solution. All tubes were then centrifuged for 3 min at 13,000 × G. The crude DNA from these tubes was used directly for PCR assay. The samples were then stored at –20 C.

Polymerase chain reaction

The PCR primers published by Lorch et al. (2010) were used for amplification of DNA from *P. destructans*. The forward primer, nu-SSI(1506)-184-5′-GD and the reverse primer, nu-5.8S-144-3′-GD were used to target a 625-base pair (bp) segment of the 18S ribosomal RNA gene, the 1506 intron, the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, and the internal transcribed spacer 2 from the *P. destructans* genome. The reagent mixture consisted of 12.5 μL of Go Taq Green PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA), 0.4 μM each of forward and reverse primer, 3 μL of crude DNA, and 8.7 μL of molecular grade water to reach a final volume of 25 μL. The reaction conditions used were 94 C for 4 min; 40 cycles of 94 C for 30 s, 55 C for 30 s, and 72 C for 45 s; and 72 C for 5 min. The PCR amplicons were visualized using UV light after electrophoresis in 1.5% agarose gel containing ethidium bromide stain. Gel images were captured digitally and analyzed. Samples with an amplicon of about 600 bp were considered suspect positive.

Since a crude method of DNA extraction was employed, spurious bands were observed in some lanes of the gels. The samples of DNA showing spurious bands were tested again using a touchdown PCR. The reaction conditions for the touchdown PCR were 94 C for 4 min; 10 cycles of 94 C for 30 s, 30 s of touchdown annealing from 65 C to 56 C (with one cycle at each temperature within that range), and 72 C for 45 s; followed by 30 cycles of 94 C for 30 s, 55 C for 30 s, and 72 C for 45 s; and 72 C for 5 min.

Nucleic acid sequencing and analyses

Suspect positive samples were confirmed by nucleic acid sequencing of the PCR amplicons. The PCR amplicons were excised from agarose gel. DNA was purified using QIAquick Gel Extraction Kits (Qiagen Inc., Valencia, California, USA). Purified DNA was submitted to the Research Technology Support Facility at Michigan State University for nucleic acid sequencing. The nucleic acid sequences were edited using Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and compared to available sequences in the GenBank database using BLAST (National Center for Biotechnology Information 2016). A phylogenetic tree was constructed based on 396-bp sequences using the neighbor-joining method (Saitou and Nei 1987), with 1,000 bootstrap replications. The bootstrap percentages in which the associated taxa clustered together are shown next to the branches (Felsenstein 1985). The distances were computed using the Kimura two-parameter method (Kimura 1980), which involved 14 sites with nucleotide sequence substitutions within the 396-bp segment. Phylogenetic analyses were conducted in MEGA6 (Tamura et al. 2013).

Statistical analysis

We tested for significant variation in *P. destructans* positivity by gender, species, time period of collection and region using a two-tailed Fisher's exact test using Stata 8.1 (Stata Corporation, College Station, Texas, USA). Region was defined by wildlife management regions for 2015: Southeastern Lower Peninsula, Southwestern Lower Peninsula, Northern Lower Peninsula, and the Upper Peninsula (Michigan Department of Natural Resources 2015). Significance was considered attained at $P \leq 0.05$.

RESULTS

Bats were stored frozen for 1–14 mo prior to testing. In total, 1,040 bats were tested (Table 1). Under UV light, three MYLU and two EPFU showed the orange-yellow fluorescence associated with *P. destructans* (Table 2). Nine bats were suspected positive for *P. destructans* based on PCR assay. Of those nine bats, five were MYLU and four were EPFU. Three of the five MYLU and none of the four EPFU had shown fluorescence under UV light. Based on nucleic acid sequencing of the PCR amplicons, five of the nine PCR suspect positives were confirmed positive for *P. destructans*. All five (two males and three females) were MYLU. The derived nucleic acid sequences from all five samples (GenBank no. KY272992–KY272996; Fig. 1) were identical to a nucleic acid sequence derived from a previous Michigan *P. destructans* isolate (labeled MDNR in Fig. 1) and a

TABLE 1. Presence of *Pseudogymnoascus destructans* isolated from bats submitted for rabies surveillance in Michigan, USA, May 2014 through May 2015, by species, sex, season, and region.

	Detection of *P. destructans* (*n* positive/*n* tested)[a]										
	Sex		Sampling period[c,d]				Region[e]				
Species[b,c]	Female	Male	A	B	C	D	SELP	SWLP	NLP	UP	Total
EPFU[f]	0/276	0/564	0/197	0/98	0/345	0/358	0/423	0/438	0/136	0/1	998
MYLU	3/19	2/16	0/1	5/7	0/18	0/9	—	—	4/17	1/18	35
MYSE	0/2	—	—	0/1	—	0/1	—	—	0/1	0/1	2
LANO[g]	0/2	—	0/1	0/1	—	0/1	0/1	0/1	0/1	—	3
LABO	0/1	—	—	—	0/1	—	0/1	—	—	—	1
LACI	—	0/1	—	—	0/1	—	0/1	—	—	—	1

[a] — = none tested.

[b] EPFU = big brown bat (*Eptesicus fuscus*); MYLU = little brown bat (*Myotis lucifugus*); MYSE = northern long-eared bat (*Myotis septentrionalis*); LANO = silver-haired bat (*Lasionycteris noctivagans*); LABO = hoary bat (*Lasiurus borealis*); LACI = eastern red bat (*Lasiurus cinereus*).

[c] Significant ($P<0.01$) differences among strata via two-tailed Fisher's exact test.

[d] A = 1 November–31 March; B = 1 April–30 May; C = 31 May–15 August; D = 16 August–31 October.

[e] SELP = Southeastern Lower Peninsula; SWLP = Southwestern Lower Peninsula; NLP = Northern Lower Peninsula; UP = Upper Peninsula.

[f] Sex was not recorded for 158 animals.

[g] Sex was not recorded for one animal.

published nucleic acid sequence for a *P. destructans* isolate from New York (GenBank no. EU884921). The four remaining PCR suspect positives, all EPFU, harbored *Pseudogymnoascus* species other than *P. destructans*. Two of the derived nucleic acid sequences were similar to a published *Pseudogymnoascus* sp. (GenBank no. KF686753) isolate from Tennessee. The remaining two were similar to each other, but distant from other *Pseudogymnoascus* spp. sequences published to date (Fig. 1).

TABLE 2. Diagnostic results for bats submitted for rabies surveillance in Michigan, USA, May 2014 through May 2015, testing positive on at least one of three tests (fluorescence under ultraviolet light, PCR, or nucleic acid sequencing of PCR amplicons) for *Pseudogymnoascus. destructans*.

Species[a]	Sex[b]	Region[c] (county)	Date of collection	Ultraviolet fluorescence	PCR	Nucleic acid sequencing (GenBank accession no.)
MYLU	M	UP (Houghton)	15 April 2015	+	+	*P. destructans* (KY272992)
MYLU	M	NLP (Alpena)	4 May 2015	+	+	*P. destructans* (KY272993)
MYLU	F	NLP (Grand Traverse)	27 May 2015	+	+	*P. destructans* (KY272994)
EPFU	M	SWLP (Ingham)	27 May 2014	+	−	*Cladosporium* sp.
EPFU	M	NLP (Grand Traverse)	15 August 2014	+	−	Not performed
MYLU	F	NLP (Emmet)	7 May 2015	−	+	*P. destructans* (KY272995)
MYLU	F	NLP (Emmet)	7 May 2015	−	+	*P. destructans* (KY272996)
EPFU	F	NLP (Wexford)	28 May 2014	−	+	*Pseudogymnoascus* sp.
EPFU	M	SWLP (Gratiot)	11 June 2014	−	+	*Pseudogymnoascus* sp.
EPFU	U	SWLP (Gratiot)	25 March 2015	−	+	*Pseudogymnoascus* sp.
EPFU	U	SELP (Washtenaw)	30 March 2015	−	+	*Pseudogymnoascus* sp.

[a] MYLU = little brown bat (*Myotis lucifugus*); EPFU = big brown bat (*Eptesicus fuscus*).

[b] M = male; F = female; U = unknown.

[c] UP = Upper Peninsula; NLP = Northern Lower Peninsula; SWLP = Southwestern Lower Peninsula; SELP = Southeastern Lower Peninsula.

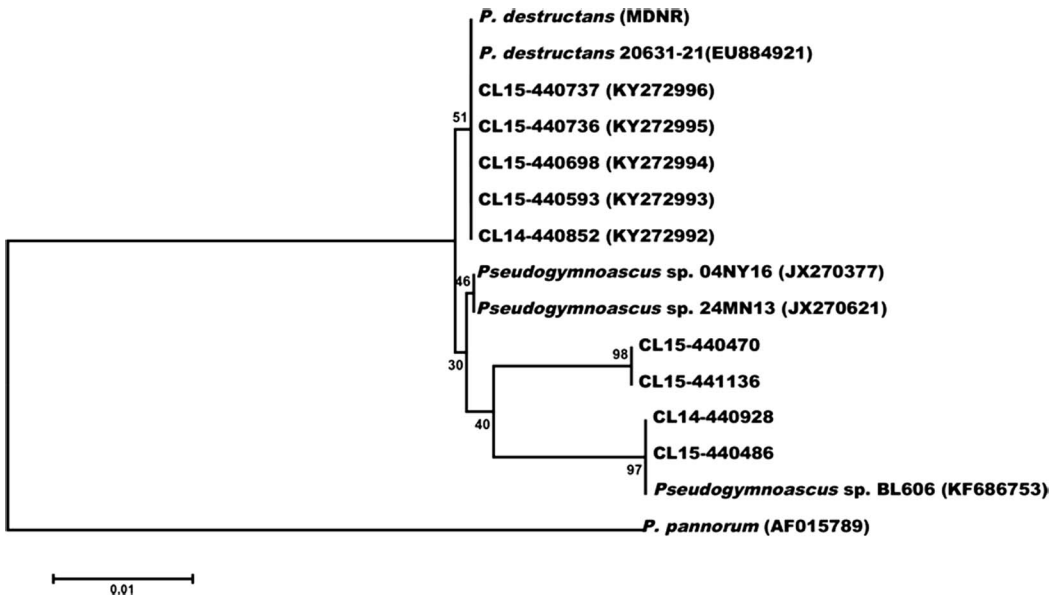

FIGURE 1. Phylogenetic tree showing interrelationships between fungal isolates taken from big brown bats (*Eptesicus fuscus*) and little brown bats (*Myotis lucifugus*) submitted for rabies surveillance in Michigan, USA, May 2014 through May 2015 (with the identification number assigned to it by the Michigan Department of Community Health Laboratory on receipt), and selected isolates of published *Pseudogymnoascus* spp., or *Pseudogymnoascus destructans* (with GenBank accession numbers in parentheses). The analysis was conducted based on 396 base pairs of partial 1506 intron-SSU-ITS-5.8S rRNA sequences, using the neighbor-joining method with 1,000 bootstrap replications in MEGA 6 software (Tamura et al. 2013). The bootstrap percentage is shown next to the branches, and the distance based on the number of nucleotide substitutions per isolate is inferred by the scale.

After statistical analysis, only significant seasonal and species differences were noted (Table 1). While bats of six species were tested, only MYLU were confirmed with *P. destructans* ($P<0.01$). Similarly, although bats were collected in all four seasons of the year, all confirmed *P. destructans* positive bats were collected between 1 April and 30 May 2015 ($P<0.01$; Table 2).

DISCUSSION

Intervention strategies for WNS are still in the preliminary stages (Cornelison et al. 2014), hence, descriptive epidemiologic results can be beneficial to disease managers by describing which species, regions, and habitats are most at risk. To eliminate seasonal bias, we tested all rabies-negative bats that were submitted to the Michigan Department of Health and Human Services over the course of an entire year. The presence of *P. destructans* in a hibernaculum typically leads to the majority of bats becoming infected by the end of the hibernation period (Langwig et al. 2015). Studies have shown that bats that are able to clear *P. destructans* infections generally do so by early summer when prey is abundant and when ambient temperatures exceed the maximum limitations of growth of the fungus (Fuller et al. 2011; Verant et al. 2012). Recovery of energetic reserves from feeding and unfavorable temperatures for fungal growth may contribute to an effective immune response and elimination of the fungus from the bats.

Using fluorescence of the fungus as an initial screening tool, only five of 1,040 bats were identified that might be infected. Both PCR and nucleic acid sequencing confirmed that three of those, all MYLU, were infected with *P. destructans*. All of those bats were collected from April to May, coinciding with

emergence from hibernation. The remaining two bats, EPFU specimens, that showed fluorescence, were not PCR positive for *P. destructans*. A PCR assay targeting the 18S ribosomal RNA gene of fungi was attempted on DNA samples from those two bats, but an amplicon was obtained from only one. The derived nucleic acid sequence was similar to *Cladosporium* spp. The PCR assay for *P. destructans* was also used on samples from all bats that did not fluoresce. Samples from four EPFU and two MYLU specimens were suspect positive, but only samples from the MYLU were confirmed positive for the nucleic acid sequence from *P. destructans*. Nucleic acid sequence analysis showed the four EPFU specimens harbored *Pseudogymnoascus* species other than *P. destructans*. Bat hibernacula in the US are known to harbor *Pseudogymnoascus* species classified in a different clade from *P. destructans* (Minnis and Linder 2013; Reynolds and Barton 2014). While *P. destructans* exhibits saprotrophic characteristics like other *Pseudogymnoascus* species, these characteristics may have attenuated as *P. destructans* evolved pathogenicity (Reynolds and Barton 2014). A single-nucleotide polymorphism has been reported to differentiate *P. destructans* from other *Pseudogymnoascus* species (Shuey et al. 2014). Presence of that single-nucleotide polymorphism was confirmed in our study when we compared the genomic sequences of the *P. destructans* from confirmed positive samples with comparable sequences from samples testing positive for other *Pseudogymnoascus* species.

Predictably, our dataset was regionally biased because 77% of the specimens submitted as potential rabies exposure cases were from the area with the highest human population density, the southern Lower Peninsula (US Census Bureau 2010). Nevertheless, all of the *P. destructans*–positive bats in our study came from the far less densely populated Northern Lower Peninsula and the Upper Peninsula. Those remain the only regions in Michigan where *P. destructans* and WNS have been confirmed to date (US Fish and Wildlife Service 2016), and are also areas where, until recently, MYLU were the most abundant bat species (Kurta 2008; Ingersoll et al. 2016). Consistent with their regional origin, the vast majority of our samples were EPFU (96% of the total), the most abundant species in this area (Winhold and Kurta 2008; Kurta and Smith 2014). Current surveillance methods for WNS and *P. destructans* in Michigan typically focus on winter surveys of mines (Kurta and Smith 2014), and to a considerably lesser extent, caves, thus overlooking the preferred hibernacula of the EPFU, which are buildings (Whitaker and Gummer 1992, 2000; Halsall et al. 2012). Kurta and Smith (2014) demonstrated that EPFU make up only about 0.5% of bat populations that hibernate in Michigan mines. Thus, winter mine surveys provide little information on the prevalence of *P. destructans* and WNS in this species, the second most abundant bat in the state prior to the arrival of WNS (Kurta 2008).

Only five of 1,040 (0.5%) of the total sample tested positive for *P. destructans*, yet five of 35 (14%) samples from MYLU harbored the fungus. None of the other five species included were confirmed positive. Kurta and Smith (2015) found WNS and *P. destructans* more widespread and severe in Michigan than our survey suggests, particularly in MYLU, with nine of the 15 largest hibernacula afflicted. Notably, recent studies incorporating both species-specific fungal loads (Hoyt et al. 2016) and survival models based on physical and behavioral traits (Hayman et al. 2016) suggest that MYLU may struggle to survive the outbreak of WNS. Yet those same studies suggest EPFU may fare better. Our results support previous findings that EPFU appears to be more resistant or less susceptible to infection from *P. destructans* (Moosman et al. 2013; Frank et al. 2014). Our results also suggest that the failure of previous surveys to find high proportions of infected EPFU specimens is not necessarily an artifact of biases in sampling. Some factors that could cause this variation in infection rates among species are differences in size, metabolism, torpor patterns, and hibernation preferences. Upon emerging from hibernation in a New

York mine contaminated with *P. destructans*, EPFU individuals had double the body fat content of those MYLU sampled (Frank et al. 2014). A lower metabolic rate along with a larger body size may aid the EPFU in resisting the effects of *P. destructans* infection (White and Seymour 2003; Hayman et al. 2016). Halsall et al. (2012) reported that EPFU hibernating in buildings can utilize passive rewarming (opportunistically using ambient temperature fluctuations that occur in buildings during winter) to conserve fat stores more efficiently than their cavernicolous counterparts, potentially extending an EPFU's ability to hibernate by several weeks. Moreover, EPFU prefers to hibernate singly, while MYLU prefers to cluster (Raesly and Gates 1987; Whitaker and Gummer 1992). Clustering species have greater transmission rates of *P. destructans* (Langwig et al. 2012). Finally, EPFU prefers less humid areas than MYLU (Raesly and Gates 1987). Because *P. destructans* grows more rapidly in humid conditions, species such as MYLU that prefer to hibernate in such conditions exacerbate their opportunity for exposure to the fungus (Wilder et al. 2011; Langwig et al. 2012).

As *P. destructans* and WNS spread westward, novel surveillance streams may provide a useful tool for wildlife management agencies seeking to detect the fungus where winter hibernacula such as caves and mines are absent or otherwise inaccessible. Yet surveys such as this need not necessarily act as alternatives to surveys of mines and caves, but rather as a complimentary tool for surveillance of species that are poorly represented in those hibernacula. Michigan has been extremely fortunate to have had repeated surveys of bats in mines and caves carried out by the same researchers for two decades (Kurta and Smith 2014). Long before WNS was a compelling issue, unique and critical data on occupancy and habitat characteristics of major hibernacula were being gathered by these surveys. While those surveys have been demanding of the surveyors' time, and entailed some costs to the Michigan Department of Natural Resources, that information has been invaluable for planning and management of bats at both state and regional scales, and could not have been obtained via surveillance streams such as the one used in the current study. They also allow real-time assessment of the disease burden and mortality of specific bat populations. Thus, where resources are available to support them, hibernacula surveys can provide much useful information in addition to just WNS surveillance. However, where resources are scarce and dedicated surveyors and hibernacula are unavailable or inaccessible, surveillance methods such as those used here may provide a cost-effective alternative. This study was carried out as a summer project by a student with laboratory and mentoring support only. Consequently, it was a cost-efficient and sensitive way to carry out *P. destructans* surveillance without necessitating fieldwork, using animals that would otherwise have been disposed of following rabies testing. In the current environment of wildlife agencies finding ways to "do more with less" it serves as a good case study. In addition, it provided a means to extensively survey EPFU, something which could not be accomplished by mine surveys because of the species' regional distribution and hibernation habits.

There were limitations in our study. The Wing Damage Index (Reichard and Kunz 2009) was not used to assess bats because it was unknown whether damage occurred to the bats post mortem as a result of collection or transport. There was also obvious decomposition in many specimens. Consequently, gross evidence of wing damage was not taken into account unless fluorescence was observed concurrently. In the current study, only 60% of the small number of bats confirmed positive for *P. destructans* also fluoresced under UV light. In contrast, Turner et al. (2014) had a 99% success rate diagnosing *P. destructans* in five bat species including EPFU and MYLU, but also cautioned that mild cases of infection could make it difficult to accurately judge whether *P. destructans* was present. Also, while we recognize histopathology as the definitive diagnostic test for WNS (Meteyer et al. 2009), we chose not to use it in our study, because the goal of our study was to

determine the prevalence of the agent *P. destructans* rather than WNS. Finally, these results are based on only a single year's surveillance. Even though over 1,000 bats were tested, as is the case with any cross-sectional study, the possibility remains that these results may not be representative of what would have been found had the study been longitudinal.

By keeping our sample population seasonally and geographically broad, our study made an effort to overcome surveillance biases of previous studies. We also filled surveillance gaps for species-specific infection rates of *P. destructans* as most of our sample population consisted of EPFU. Our study also showed that other species of fungi may fluoresce similarly to *P. destructans* under UV light and that existing PCR assays for *P. destructans* may not be specific to the species level.

ACKNOWLEDGMENTS

Funding for S.L.D. was provided by the Graduate School at Michigan State University and the Merial Veterinary Scholars Program. The authors thank the Michigan Department of Health and Human Services for providing the bats and Allen Kurta, Eastern Michigan University, for determining their sex and species. Comments from the Assistant Editor and two anonymous reviewers significantly improved the manuscript. This work was supported by the Federal Aid in Wildlife Restoration Act under Michigan Pittman-Robertson Project W-147-R.

LITERATURE CITED

Brownlee-Bouboulis SA, Reeder DAM. 2013. White-nose syndrome-affected little brown myotis (*Myotis lucifugus*) increase grooming and other active behaviors during arousals from hibernation. *J Wildl Dis* 49:850–859.

Cornelison CT, Keel MK, Gabriel KT, Barlament CK, Tucker TA, Pierce GE, Crow SA. 2014. A preliminary report on the contact-independent antagonism of *Pseudogymnoascus destructans* by *Rhodococcus rhodochrous* strain DAP96253. *BMC Microbiol* 14:246.

Cryan PM, Meteyer CU, Blehert DS, Lorch JM, Reeder DAM, Turner GG, Webb J, Behr M, Verant M, Russell RE, et al. 2013. Electrolyte depletion in white-nose syndrome bats. *J Wildl Dis* 49:398–402.

Felsenstein J. 1985. Confidence-limits on phylogenies – an approach using the bootstrap. *Evolution* 39:783–791.

Frank CL, Michalski A, McDonough AA, Rahimian M, Rudd RJ, Herzog C. 2014. The resistance of a North American bat species (*Eptesicus fuscus*) to white-nose syndrome (WNS). *PLoS One* 9:e113958.

Fuller NW, Reichard JD, Nabhan ML, Fellows SR, Pepin LC, Kunz TH. 2011. Free-ranging little brown myotis (*Myotis lucifugus*) heal from wing damage associated with white-nose syndrome. *Ecohealth* 8:154–162.

Goldenberger D, Perschil I, Ritzler M, Altwegg M. 1995. A simple universal DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *Genome Res* 4:368–370.

Halsall AL, Boyles JG, Whitaker JO. 2012. Body temperature patterns of big brown bats during winter in a building hibernaculum. *J Mammal* 93:497–503.

Hayman DTS, Pulliam JRC, Marshall JC, Cryan PM, Webb CT. 2016. Environment, host, and fungal traits predict continental-scale white-nose syndrome in bats. *Sci Adv* 2:e1500831.

Hoyt JR, Langwig KE, Sun K, Lu G, Parise KL, Jiang T, Frick WF, Foster JT, Feng J, Kilpatrick AM. 2016. Host persistence or extinction from emerging infectious disease: Insights from white-nose syndrome in endemic and invading regions. *Proc Biol Sci* 283: 20152861.

Ingersoll TE, Sewall BJ, Amelon SK. 2016. Effects of white-nose syndrome on regional population patterns of three hibernating bat species. *Conserv Biol* 30: 1048–1059.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120.

Kurta A. 2008. Bats of Michigan. *Indiana State University Center for North American Bat Research and Conservation, Publication 2*. Terre Haute, Indiana, 72 pp.

Kurta A, Smith SM. 2014. Hibernating bats and abandoned mines in the Upper Peninsula of Michigan. *Northeast Nat* 21:587–605.

Kurta A, Smith SM. 2015. *Exploration of bat hibernacula, population monitoring, and surveillance for white-nose syndrome: An annual report to the Michigan Department of Natural Resources on activities performed in winter 2014–2015*. Department of Biology, Eastern Michigan University, Ypsilanti, Michigan, and S.M. Smith Company, Iron Mountain, Michigan, 27 pp.

Langwig KE, Frick WF, Bried JT, Hicks AC, Kunz TH, Kilpatrick AM. 2012. Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecol Lett* 15:1050–1057.

Langwig KE, Frick WF, Reynolds R, Parise KL, Drees KP, Hoyt JR, Cheng TL, Kunz TH, Foster JT, Kilpatrick AM. 2015. Host and pathogen ecology drive the seasonal dynamics of a fungal disease, white-nose syndrome. *Proc Biol Sci* 282:20142335.

Lorch JM, Gargas A, Meteyer CU, Berlowski-Zier BM, Green DE, Shearn-Bochsler V, Thomas NJ, Blehert DS. 2010. Rapid polymerase chain reaction diagnosis

of white-nose syndrome in bats. *J Vet Diagn Investig* 22:224–230.

Meteyer CU, Barber D, Mandl JN. 2012. Pathology in euthermic bats with white nose syndrome suggests a natural manifestation of immune reconstitution inflammatory syndrome. *Virulence* 3:583–588.

Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, Shearn-Bochsler V, Thomas NJ, Gargas A, Behr MJ. 2009. Histopathologic criteria to confirm white-nose syndrome in bats. *J Vet Diagn Investig* 21:411–414.

Meteyer CU, Valent M, Kashmer J, Buckles EL, Lorch JM, Blehert DS, Lollar A, Berndt D, Wheeler E, White CL, et al. 2011. Recovery of little brown bats (*Myotis lucifugus*) from natural infection with *Geomyces destructans*, white-nose syndrome. *J Wildl Dis* 47:618–626.

Michigan Department of Natural Resources. 2015. *Michigan Department of Natural Resources Wildlife Division management regions and primary offices*. www.michigan.gov/documents/dnr/DNR_Wildlife_offices_map_442532_7.pdf. Accessed July 2015.

Minnis AM, Lindner DL. 2013. Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. *Fungal Biol* 117:638–649.

Moosman PR Jr, Veilleux JP, Pelton GW, Thomas HH. 2013. Changes in capture rates in a community of bats in New Hampshire during the progression of white-nose syndrome. *Northeast Nat* 20:552–558.

National Center for Biotechnology Information. 2016. *Basic local alignment search tool (BLAST)*. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed July 2016.

Raesly RL, Gates JE. 1987. Winter habitat selection by north temperate cave bats. *Am Midl Nat* 118:15–31.

Reichard JD, Kunz TH. 2009. White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (*Myotis lucifugus*). *Acta Chiropterol* 11:457–464.

Reynolds HT, Barton HA. 2014. Comparison of the white-nose syndrome agent *Pseudogymnoascus destructans* to cave-dwelling relatives suggests reduced saprotrophic enzyme activity. *PLoS One* 9:e86437.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.

Shuey MM, Drees KP, Lindner DL, Keim P, Foster JT. 2014. Highly sensitive quantitative PCR for the detection and differentiation of *Pseudogymnoascus destructans* and other *Pseudogymnoascus* species. *Appl Environ Microbiol* 80:1726–1731.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729.

Turner GG, Meteyer CU, Barton H, Gumbs JF, Reeder DAM, Overton B, Bandouchova H, Bartonička T, Martinková N, Pikula J, et al. 2014. Nonlethal screening of bat-wing skin with the use of ultraviolet fluorescence to detect lesions indicative of white-nose. *J Wildl Dis* 50:566–573.

US Census Bureau. 2010. *Estimated population of Michigan regions and statistical areas, 2000–2009*. http://michigan.gov/documents/cgi/cgi_census_Regions0009_329388_7.pdf. Accessed July 2015.

US Fish and Wildlife Service. 2012. *North American bat death toll exceeds 5.5 million from white-nose syndrome*. www.fws.gov/whitenosesyndrome/pdf/wns_mortality_2012_nr_final.pdf. Accessed July 2015.

US Fish and Wildlife Service. 2014. *White-nose syndrome: The devastating disease of hibernating bats in North America*. www.whitenosesyndrome.org/sites/default/files/resources/white-nose_fact_sheet_6-2014_1.pdf. Accessed July 2015.

US Fish and Wildlife Service. 2016. *Where is it now?* www.whitenosesyndrome.org/about/where-is-it-now. Accessed May 2016.

Verant ML, Boyles JG, Waldrep W, Wibbelt G, Blehert DS. 2012. Temperature-dependent growth of *Geomyces destructans*, the fungus that causes bat white-nose syndrome. *PLoS One* 7:e46280.

Verant ML, Meteyer CU, Speakman JR, Cryan PM, Lorch JM, Blehert DS. 2014. White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. *BMC Physiol* 14:10.

Whitaker JO, Gummer SL. 1992. Hibernation of the big brown bat, *Eptesicus fuscus*, in buildings. *J Mammal* 73:312–316.

Whitaker JO Jr, Gummer SL. 2000. Population structure and dynamics of big brown bats (*Eptesicus fuscus*) hibernating in buildings in Indiana. *Am Midl Nat* 143: 389–396.

White CR, Seymour RS. 2003. Mammalian basal metabolic rate is proportional to body mass$^{2/3}$. *Proc Natl Acad Sci U S A* 100:4046–4049.

Wilder AP, Frick WF, Langwig KE, Kunz TH. 2011. Risk factors associated with mortality from white-nose syndrome among hibernating bat colonies. *Biol Lett* 7:950–953.

Winhold L, Kurta A. 2008. Netting surveys for bats in the northeast: Differences associated with habitat, duration of netting, and use of consecutive nights. *Northeast Nat* 15:263–274.

Submitted for publication 2 August 2016.
Accepted 10 December 2016.