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White-nose Syndrome Pathogen *Pseudogymnoascus destructans* Detected in Migratory Tree-roosting Bats

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ABSTRACT: White-nose syndrome (WNS) is an emerging fungal epizootic disease that has caused large-scale mortality in several species of North American bats. The fungus that causes WNS, *Pseudogymnoascus destructans* (*Pd*), has also been detected in bat species without diagnostic signs of WNS. Although these species could play a role in WNS spread, understanding of the spatial and temporal extents of *Pd* occurrence on WNS-resistant species is limited. This study evaluated the presence of *Pd* on 272 individuals of three species of migratory tree-roosting bats: hoary (*Lasiurus cinereus*), eastern red (*Lasiurus borealis*), and silver-haired (*Lasionycteris noctivagans*) bats, obtained opportunistically during summer and autumn from throughout much of their ranges in North America. We also compared tissue sampling protocols (i.e., tissue swabbing, fur swabbing, and DNA extraction of excised wing tissue). We detected *Pd* on three eastern red bats from Illinois and Ohio, US, one silver-haired bat from West Virginia, US, and one hoary bat from New York, US, all via DNA extracted from wing tissue of carcasses. These results document the first publicly reported detections of *Pd* on a hoary bat and on migratory bats during the autumn migratory period, and demonstrate the potential for using carcasses salvaged at wind-energy facilities to monitor for *Pd*.

Key words: Carcass, emerging infectious disease, *Lasionycteris noctivagans*, *Lasiurus borealis*, *Lasiurus cinereus*, *Pseudogymnoascus destructans*, surveillance, white-nose syndrome.

White-nose syndrome (WNS) is an epizootic disease caused by the fungus *Pseudogymnoascus destructans* (*Pd*) that has led to severe declines in the populations of several North American bat species (Hoyt et al. 2021). Although *Pd* loads peak in affected species during the winter, *Pd* may persist on some WNS-affected species into summer (Carpenter et al. 2016; Huebschman et al. 2019). Besides the 12 bat species affected by WNS, six species without diagnostic signs of WNS have tested positive for *Pd*, including two migratory tree-roosting species that are infrequently found in winter hibernation sites: eastern red (*Lasiurus borealis*) and silver-haired (*Lasionycteris noctivagans*) bats (Bernard et al. 2015; Huebschman et al. 2019). However, previous studies indicating that tree-roosting species can harbor *Pd* were not specifically focused on such species and thus had small sample sizes (the former detected *Pd* on 1/3 silver-haired bats and 2/6 eastern red bats, and the latter detected *Pd* on 1/14 eastern red bats). Thus, understanding of *Pd* pervasiveness on tree-roosting species remains limited.

We aimed to test for the presence of *Pd* on three migratory tree-roosting bat species: eastern red, silver-haired, and hoary (*Lasiurus cinereus*) bats from throughout North America (Table 1). If sufficient numbers of *Pd*-positive individuals existed in our data set, a secondary aim was to provide an initial comparison of detection rates tissue sampling protocols: fungal DNA extracted from wing tissue, swabbing muzzle and forearm, and swabbing feet and tail membranes (uropatagia). We predicted that a small proportion of tree-roosting bats sampled would test positive for *Pd*, and that sampling of salvaged carcasses might allow for detection of *Pd*. Samples were opportunistically obtained during the course of collections for other projects (Pylant et al. 2016; Sovic et al. 2016; Nelson et al. 2018; Campbell et al. 2020) during 2003–18. We

TABLE 1. Individuals of hoary (*Lasiurus cinereus*), eastern red (*Lasiurus borealis*), and silver-haired (*Lasionycteris noctivagans*) bats sampled, by state (all USA) and range of years and month of year sampled.

Bat species	Source state	Year	Month	*n*
Eastern red	Delaware	2015	7	3
	Illinois	2015	7–8	8
	Indiana	2009–14	7–10	30
	Maryland	2011–17	6–10	36
	New York	2010	N/A[a]	1
	Ohio	2014–16	5–6	2
	West Virginia	2011–15	9–10	15
Hoary	California	2016	6	1
	Illinois	2015–18	8	11
	Indiana	2009–14	4–10	23
	Maryland	2003–17	7–10	3
	Nevada	2013	10	1
	New York	2008–09	8	16
	Ohio	2014	6–8	5
	Pennsylvania	2013	6–9	9
	West Virginia	2011–16	5–10	6
Silver-haired	Idaho	2012–15	6–10	54
	Illinois	2018	8	1
	Indiana	2009–14	4–10	21
	Maryland	2015	5	2
	New York	2009–10	6–8	11
	Ohio	2014–16	6–8	3
	Rhode Island	2014–18	8	2
	West Virginia	2015	9–10	8

[a] Sampling metadata was not available.

conducted DNA extraction from swabs following Verant et al. (2016), with modifications described in the Supplementary Material.

Traditional muzzle-and-forearm swabbing was performed on 38 live bats captured by mist net and harp trap during foraging (n=36), swarm (n=1), and emergence surveys (n=1) near hibernacula entrances as part of ongoing monitoring efforts (for example, Nagel and Gates 2017). For a few individuals, feet and uropatagium were also swabbed, because those body parts may be the most likely to encounter and retain *Pd* from cave substrate (Fig. 1). Carcasses of 234 individuals were collected during postconstruction monitoring surveys at 25 wind-energy facilities throughout the continental US between May and September (Table 1), with one additional carcass obtained via wild salvage in a residential area. We used only individuals that were identified to species and thus in relatively fresh, rather than highly degraded, condition. Because these samples were collected opportunistically, they were not necessarily obtained using a standardized or uniform protocol. However, *Pd* spores are highly resilient (Hoyt et al. 2015) and probably persist in a viable state on carcasses well beyond when they are collected in the field. Before extraction of DNA from tissue, whole carcasses were kept in individual bags and already-excised tissue in 95% ethanol; all tissue was stored at –80 C. Approximately 35 mm^2 of wing tissue was excised from the lower plagiopatagium of carcasses. We extracted DNA from tissue using DNeasy Blood & Tissue Kits (Qiagen Inc., Valencia, California, USA). We followed the kit protocol, with the addition of a 6-min centrifuge step following tissue digestion to remove fur and pigments. When whole carcasses were available, we also swabbed the muzzle and forearm.

Samples were tested for *Pd* DNA using a quantitative PCR assay (Verant et al. 2016; Supplementary Material) at the University of Maryland Center for Environmental Science. Consistent with previous studies, we considered a sample positive for *Pd* DNA if exponential amplification of fluorescent intensity above background occurred within 40 quantification cycles (Cq; Bernard et al. 2015; Carpenter et al. 2016; Huebschman et al. 2019). Samples with one or more positive results were rerun in duplicate on a second plate for a total of four runs. We considered an individual *Pd* positive when a Cq value was determined for at least one of the four replicates. Detections on one of multiple replicates per sample are common when *Pd* quantities are near the assay's detection limit (Bernard et al. 2015; Carpenter et al. 2016; Huebschman et al. 2019).

We performed quantitative PCR pathogen detection on a total of 322 samples representing 272 individuals (119 samples from 95 individual eastern red bats, 76 samples from 75 individual hoary bats, and 127 samples from 102 individual silver-haired bats; Table

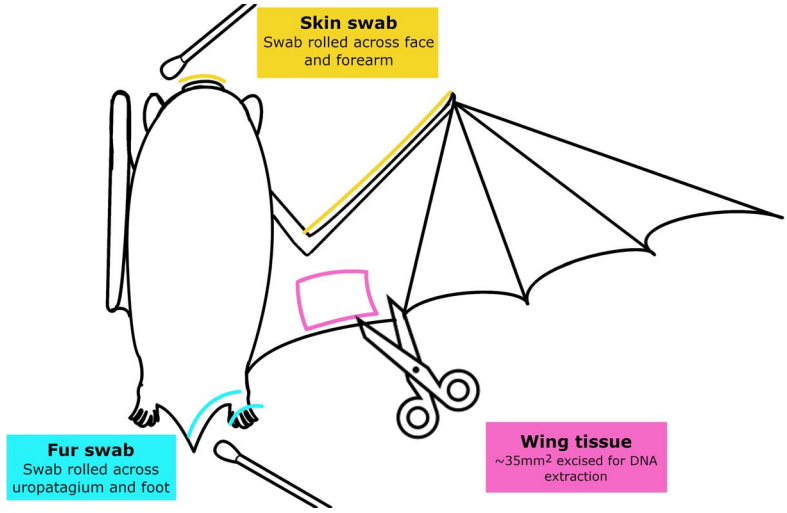

FIGURE 1. Three methods were applied to sample bat tissue for *Pseudogymnoascus destructans* (*Pd*) DNA: the standard swabbing protocol for detecting *Pd* on the white-nose syndrome-affected areas of muzzle and forearm (yellow or pale gray), a modified swabbing protocol of the foot and uropatagium (blue or dark gray), and a section of tissue excised for DNA extraction from the plagiopatagium of bat carcasses (pink or midgray). Swabs were conducted by rolling a purified-water moistened sterile swab three times along the regions of targeted tissue.

2). Five bats had Cq values indicating exponential amplification (Cq range = 31.7–37.0) and thus were considered positive for *Pd*: one hoary (from New York, US), three eastern red (from Illinois and Ohio, US), and one silver-haired bat (from West Virginia, US, Table 3). These positive numbers represent approximately 1%, 3%, and 1%, respectively, of individuals of these species that we tested. All positive samples were from DNA extracted from wing tissue from turbine-killed individuals; none of the *Pd*-positive individuals had skin or fur swab samples available. Each positive sample was collected after *Pd* had been documented in the state where they were collected (White-nose Syndrome Response Team 2019).

This study represents the first publicly available report of detection of *Pd* on a hoary bat and additional detections of *Pd* on eastern red and silver-haired bats. Additionally, it represents the first publicly reported detection of *Pd* on a silver-haired bat outside of the winter, and of *Pd* on tree-roosting bats collected in Illinois, Ohio, New York, and West Virginia. Previous studies documented *Pd* in eastern red and silver-haired bats in Tennessee and Wisconsin (Bernard et al.

TABLE 2. Numbers of hoary (*Lasiurus cinereus*), eastern red (*Lasiurus borealis*), and silver-haired (*Lasionycteris noctivagans*) bats from North America sampled by each method (tissue taken from the wing, and swabs of various body sites). Note that individuals sampled by multiple methods (e.g., tissue and muzzle/forearm swab) correspond with multiple samples.

	Carcass salvage (n=234)		Live caught (n=38)				
Species	Tissue only	Tissue and muzzle/forearm swab	Muzzle/forearm and foot/uropatagium swabs	Muzzle/forearm swab only	Foot/uropatagium swab only	Total individuals sampled	Total samples taken
Eastern red	60		24	8	3	95	119
Hoary	74		1			75	76
Silver-haired	77	23	2			102	127

TABLE 3. Result details for hoary (*Lasiurus cinereus*), eastern red (*Lasiurus borealis*), and silver-haired (*Lasionycteris noctivagans*) bats from North America testing positive for *Pseudogymnoascus destructans* by quantitative PCR. Sampling protocol was by tissue only. Quantification cycle (Cq) results are presented from greatest to smallest; results of NA indicate that amplification was not detected.

Bat species	Source state (USA)	Sample date	Cq results
Eastern red	Illinois	11 August 2015	36.9, NA, NA, NA
	Illinois	14 August 2015	36.1, 37.0, NA, NA
	Ohio	30 June 2014	35.1, 36.9, 37.0, NA
Hoary	New York	22 August 2009	31.7, 32.9, 35.8, 37.0
Silver-haired	West Virginia	16 September 2015	33.9, 35.7, NA, NA

2015; Huebschman et al. 2019), bringing the number of states observed to four for eastern red, two for silver-haired, and one for hoary bats. We detected *Pd* on bats sampled during the early summer (June) and late summer and autumn (August and September), suggesting that these WNS-resistant species might have detectable fungal loads before and during their migratory period (Cryan 2003). However, in conjunction with previous results (Bernard et al. 2015; Carpenter et al. 2016; Huebschman et al. 2019), our results suggest that the rates at which these tree-roosting species carry *Pd* is relatively low.

Because of the opportunistic nature of some sampling, we cannot completely rule out the possibility of cross-contamination by *Pd* of carcasses processed before shipment to our laboratory. However, to the best of our knowledge, carcasses were widely distributed on the landscape, and handled and stored individually. Furthermore, potential cross-contamination of bat tissue by *Pd* via handling and processing is unlikely to be dramatically higher than that of bats captured in the same mist net or harp trap, protocols widely used as part of WNS monitoring efforts (Ballmann et al. 2017; USGS-NWHC 2020).

Sampling protocol may play an important role in the detectability of *Pd*, especially in WNS-resistant species with presumably low *Pd* levels. Although all *Pd*-positive samples in our study were obtained from carcasses sampled with DNA extraction only (Table 2), the small number of positive detections in our data set and the lack of swab samples from the individuals that tested positive limit our ability to assess whether DNA extraction of the relatively large sections of bat tissue we used has a differential *Pd* detection rate than other methods. Extracting all DNA from a relatively large section of tissue probably maximizes the chances of detecting *Pd* beyond skin swabbing and wing biopsy (Janicki et al. 2015). Given the general availability of salvaged bat carcasses of the three species evaluated here (Arnett and Baerwald 2013), our results suggest that such carcasses might be useful for *Pd* monitoring.

The mechanisms by which hoary, eastern red, and silver-haired bats might be occasionally exposed to *Pd* outside of winter remain unclear. Although silver-haired bats sometimes hibernate in *Pd*-positive hibernacula, they are rarely found roosting there during the summer or during their autumn migrations (Kunz 1982; Barclay et al. 1988). Hoary and eastern red bats are almost exclusively tree-roosting species and are thought to enter hibernacula very rarely (Shump and Shump 1982a, b). One possible exposure mechanism is interspecific interactions, such as multispecies swarming and aggression behaviors (Myers 1960; Brokaw et al. 2016; Neubaum and Siemers 2021). Resistance to WNS and propensity for long-distance dispersal and seasonal migration (Cryan et al. 2014) suggest that WNS-resistant migratory bats could be capable of acting as vectors to spread WNS across large geographic scales (Maher et al. 2012; see also Escobar et al. 2014). Future studies could explore potential mechanisms of *Pd* exposure, persistence, and possible transmission by these species.

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SUPPLEMENTARY MATERIAL

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