DISPERSAL HAZARDS OF PSEUDOGYMNOASCUS DESTRUCTANS BY BATS AND HUMAN ACTIVITY AT HIBERNACULA IN SUMMER

Authors: Ballmann, Anne E., Torkelson, Miranda R., Bohuski, Elizabeth A., Russell, Robin E., and Blehert, David S.

Source: Journal of Wildlife Diseases, 53(4) : 725-735

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/2016-09-206

DOI: 10.7589/2016-09-206 *Journal of Wildlife Diseases*, 53(4), 2017, pp. 725–735

DISPERSAL HAZARDS OF *PSEUDOGYMNOASCUS DESTRUCTANS* BY BATS AND HUMAN ACTIVITY AT HIBERNACULA IN SUMMER

Anne E. Ballmann,[1,3] Miranda R. Torkelson,[1,2] Elizabeth A. Bohuski,[1] Robin E. Russell,[1] and David S. Blehert[1]

[1] US Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA
[2] University of Wisconsin, School of Veterinary Medicine, 2015 Linden Drive, Madison, Wisconsin 53706, USA
[3] Corresponding author (email: aballmann@usgs.gov)

ABSTRACT: Bats occupying hibernacula during summer are exposed to *Pseudogymnoascus destructans* (*Pd*), the causative agent of white-nose syndrome (WNS), and may contribute to its dispersal. Furthermore, equipment and clothing exposed to cave environments are a potential source for human-assisted spread of *Pd*. To explore dispersal hazards for *Pd* during the nonhibernal season, we tested samples that were collected from bats, the environment, and equipment at hibernacula in the eastern US between 18 July–22 August 2012. Study sites included six hibernacula known to harbor bats with *Pd* with varying winter-count impacts from WNS and two hibernacula (control sites) without prior history of WNS. Nucleic acid from *Pd* was detected from wing-skin swabs or guano from 40 of 617 bats (7% prevalence), including males and females of five species at five sites where WNS had previously been confirmed as well as from one control site. Analysis of guano collected during summer demonstrated a higher apparent prevalence of *Pd* among bats (17%, 37/223) than did analysis of wing-skin swabs (1%, 4/617). Viable *Pd* cultured from wing skin (2%, 1/56) and low recapture rates at all sites suggested bats harboring *Pd* during summer could contribute to pathogen dispersal. Additionally, *Pd* DNA was detected on clothing and trapping equipment used inside and near hibernacula, and *Pd* was detected in sediment more readily than in swabs of hibernaculum walls. Statistically significant differences in environmental abundance of *Pd* were not detected among sites, but prevalence of *Pd* differed between sites and among bat species. Overall, bats using hibernacula in summer can harbor *Pd* on their skin and in their guano, and demonstration of *Pd* on clothing, traps, and other equipment used at hibernacula during summertime within the WNS-affected region indicates risk for pathogen dispersal during the nonhibernal season.

Key words: Decontamination, environmental reservoir, guano, human-assisted movement, surveillance, white-nose syndrome, WNS.

INTRODUCTION

White-nose syndrome (WNS) is an emerging fungal disease that has caused high mortality in North American populations of hibernating bats. Since the first detection of the disease in 2007, the causative agent *Pseudogymnoascus destructans* (*Pd*; Lorch et al. 2011) has spread to 33 states (US) and five Canadian provinces (US Fish and Wildlife Service [USFWS] 2017). However, seasonal trends in disease spread and mechanisms by which *Pd* moves across the landscape are poorly understood. Previous research has indicated that detection of *Pd* is highest among hibernating bats during winter. However, *Pd* has also been detected at low prevalence on bats during summer (Dobony et al. 2011; Langwig et al. 2015; Carpenter et al. 2016). While analysis of skin swabs demonstrated superior sensitivity and specificity for detecting *Pd* during early stage infections in hibernating bats (McGuire et al. 2016), reliability of this sampling method for detecting *Pd* in nontorpid bats has not been evaluated.

Vespertilionid bats in North America emerge from underground hibernacula in spring and disperse distances ranging from <50 to 500 km to reach summer habitats that include various above-ground roosts or underground sites that serve as hibernacula for other bats during winter (Fleming and Eby 2003; Altringham 2011). Even species such as gray bats (*Myotis grisescens*) that use underground sites year-round do not typically utilize the same sites for hibernation and active periods (Altringham 2011). Additionally, there

is evidence that some species move between underground sites during summer to conserve energy, reduce parasitism, avoid disturbance, or minimize exposure to unfavorable microclimates (Lewis 1995).

Pseudogymnoascus destructans persists year-round in hibernacula (Lorch et al. 2013), presenting an uncharacterized risk for dissemination by bats that use these underground sites as day or night roosts or as nurseries during summer (Hall and Wilson 1966; Hall and Brenner 1968; Agosta et al. 2005); and people accessing these sites for recreational or research activities also present a potential risk for dispersal of the fungus. Specifically, viable fungal spores picked up from the environment could be carried on the fur or skin of bats and be transmitted to bats at other locations during swarming interactions or live-trapping activities of multiple bats using the same equipment. Additionally, humans may transport viable fungal spores across the landscape and establish environmental reservoirs in previously unexposed hibernacula.

Characterizing the potential for bats and humans to disperse *Pd* during summer will improve our understanding of mechanisms of WNS spread and improve management strategies to reduce risks for dissemination of disease. We evaluated: 1) the potential for bats occupying hibernacula during summer to harbor viable *Pd*; 2) appropriate sampling methods for detecting *Pd* during nonhibernal periods; 3) differences in *Pd* prevalence on bats and relative pathogen abundance in the environment at sites with reduced or stable winter bat counts; and 4) the prospect for fomites such as trapping equipment or clothing used at underground sites during summer to harbor viable *Pd*.

MATERIALS AND METHODS

Study sites

Eight hibernacula (Kentucky, Indiana, Ohio, Tennessee, and Virginia) within the WNS-affected region of the eastern US, with evidence of summer use by bats, were sampled between 18 July–22 August 2012 (Fig. 1). This timeframe allowed sampling of volant pups and adults prior to peak fall swarm, after which bats initiate overwintering behaviors (Briggler and Prather 2003; Norquay et al. 2013). Sites consisted of seven natural caves with single main entrances and one inactive mine complex (Table 1). Bat populations at each hibernaculum were classified as either stable or reduced based on 2012/2013 winter count estimates divided by the average of 2–5 yr of available pre-WNS winter census data (including data from the first year of WNS detection). Hibernacula with >25% decline in winter count estimates since detection of WNS were considered to have reduced populations. Study sites included: three known to harbor hibernating bat populations affected by WNS that previously experienced declines; three known to harbor hibernating bat populations affected by WNS but without documented declines; and two that were visibly free of the disease as of spring 2012 (control sites).

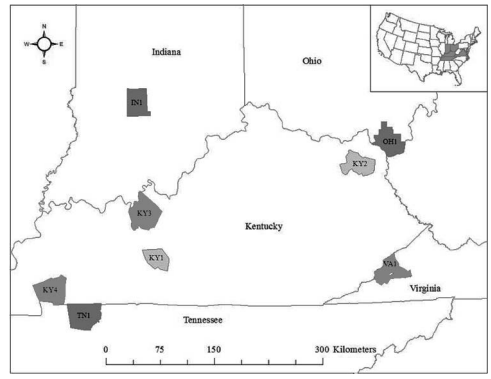

FIGURE 1. The distribution of eight hibernacula to the level of county in the eastern USA where bats and environmental substrates were sampled for *Pseudogymnoascus destructans* between July and August 2012. Control sites with stable winter bat counts and no prior history of white-nose syndrome (WNS) are light gray; sites with stable winter bat counts with known occurrence of WNS are medium gray; and sites with reduced winter bat counts (>25%) with known occurrence of WNS are dark gray.

Bats

All live-animal capture and sampling procedures were conducted in accordance with US Geological Survey, National Wildlife Health Center Institutional Animal Care and Use Experimental Protocol 120524, and other state and federal permits. Decontamination of field equipment followed USFWS guidelines (2012) and individual state requirements. Bats were collected inside hibernacula or trapped while

TABLE 1. Overview of summer hibernacula study sites in the eastern USA and sampling conducted for surveillance of *Pseudogymnoascus destructans* (*Pd*) during July and August 2012 in states with confirmed cases of white-nose syndrome (WNS) or in control sites where *Pd* has not been reported.

Site[a]	Date(s) sampled	WNS status (1st winter of detection)	Winter count[b]	Capture methods	No. trap events	Capture effort[c] (bats/hr)	Species present[d]		No. of bats captured	
							Winter	Summer	Inside	Outside[e]
KY1 (cave)	21–22 August	Control (2012/2013)	Stable	Harp trap, hand	2	20	EPFU	MYLU	12	0
							MYLU	MYSE	13	0
							MYSE	PESU	73	2
							MYSO			
							PESU			
KY2 (cave)	25–26 July	Control (2012/2013)	Stable	Hand	2	54	EPFU	MYLU	36	—
							MYLU	MYSO	48	—
							MYSE	PESU	5	—
							MYSO			
							PESU			
KY3 (cave)	23–24 July	WNS+ (2011/2012)	Stable	Harp trap	2	22	MYGR	MYGR	2	0
							MYLE	MYLE	2	2
							MYLU	MYLU	30	32
							MYSE	MYSE	3	0
							MYSO	MYSO	28	23
							PESU	PESU	36	5
KY4 (cave)	15 August	WNS+ (2010/2011)	Stable	Harp trap	1	86	EPFU	MYGR	85	10
							MYLU	MYLU	21	0
							MYSE	MYSE	1	0
							MYSO	PESU	8	0
							PESU			
VA1 (cave)	9 August, 13 August	WNS+ (2011/2012)	Stable	Mist net	2	7	EPFU	EPFU	3	2
							MYLE	MYLE	9	2
							MYLU	MYLU	4	3
							MYSO	MYSE	7	17
							PESU	PESU	26	20
TN1 (cave)	6–7 August, 14 August	WNS+ (2010/2011)	Reduced	Mist net	3	3	EPFU	EPFU	4	14
							MYLU	LABO	0	2
							MYSE	MYLU	14	12
							PESU	MYSE	2	0
								PESU	28	25
IN1 (cave)	18–20 July	WNS+ (2010/2011)	Reduced	Harp trap (modified), hand	2	9	EPFU	MYLU	2	—
							MYLU	MYSO	41	—
							MYSO	PESU	2	—
							PESU			
OH1 (mine)	31 July–2 August	WNS+ (2010/2011)	Reduced	Harp trap, mist net	3	5	EPFU	EPFU	1	4
							MYLU	MYLU	25	4
							MYSE	MYSE	38	31
							MYSO	MYSO	1	0
							PESU	PESU	7	24
Total									617	234

[a] KY = Kentucky; VA = Virginia; TN = Tennessee; IN = Indiana; OH = Ohio.

[b] Explanation of winter bat count appears in the main text (Materials and Methods).

[c] Denotes total number of bats captured/total number of trapping hours (all methods) at the study site.

[d] EPFU = *Eptesicus fuscus*; MYLU = *Myotis lucifugus*; MYSE = *Myotis septentrionalis*; MYSO = *Myotis sodalis*; PESU = *Perimyotis subflavus*; MYGR = *Myotis grisescens*; MYLE = *Myotis leibii*.

[e] — = not applicable.

exiting a site during evening emergence. Harp traps, mist nets, or hand-capture techniques were used, as appropriate, for no more than three trapping events to achieve a target sample size of 45 to 115 individuals per site. This allowed for detection of at least one *Pd*-positive bat given an estimated prevalence of *Pd* in summer between 2–5% (90% confidence interval). Collaborating state and federal partners provided state-dedicated trapping equipment at each site. Traps were placed at or near the main entrance of each hibernaculum with additional barriers to prevent bats from escaping around the trap(s) and to allow directional differentiation of captures. To minimize cross-contamination among captured individuals, bats were quickly removed from traps using a new pair of nitrile gloves for each animal. At one site (IN1), the harp trap was modified by removing the catch bag, and bats were hand-captured as they slid down the trap strings. Bats were held in individual, single-use bags (cloth or paper) until processing. Guano was collected opportunistically from holding bags into a sterile vial containing a single silica gel desiccant bead (Dry & Dry®, SilicaGel Factory, Brea, California, USA). Basic demographic and morphometric data were recorded from sampled bats using standard techniques (see Supplementary Material Table S1). Wing membranes were assessed for damage (Reichard and Kunz 2009) and inspected under ultraviolet (UV) light (51 bulb LED UV [385 nm] flashlight with filter, LED Wholesalers, Hayward, California, USA) for fluorescence suggestive of WNS (Turner et al. 2014). Nonthreatened or nonendangered species of bats with suspicious fluorescence were euthanized and stored at –20 C for diagnostic evaluation; nonlethal wing biopsy samples (3 mm) of similarly fluorescing areas of skin from threatened or endangered species were collected into 10% neutral buffered formalin and stored at room temperature for histopathologic evaluation.

A single, sterile swab (PurFlock® Nylon Flocked Micro Ultrafine Tipped Swab, Puritan Medical Products Company LLC, Guilford, Massachusetts, USA) was moistened in a sterile vial containing 500 µL diethylpyrocarbonate-treated, DNAase- and RNAase-free, sterile water (Fisher Scientific, Pittsburgh, Pennsylvania, USA), rolled across the entire dorsal surface area of one wing from each bat, and returned to the vial. To prevent resampling, prior to release all bats were marked on the fur at mid-dorsum with temporary, nontoxic paint (All-Weather Paintstik® Livestock marker, LA-CO Industries, Inc., Elk Grove Village, Illinois, USA).

Environmental substrates

Paired environmental samples consisting of a swab of wall or ceiling substrate and floor sediment ("soil") sampled underneath the swab were collected at eight locations inside each hibernaculum. Samples were collected from areas where bats were known to roost during the winter, where bats were observed roosting during summer sampling, and where there was fresh guano suggestive of recent roosting activity. To minimize disturbance, samples were either collected in the evening, after the majority of bats had emerged from the hibernaculum, or the following day after trapping efforts at a site were completed. A 15.3-cm diameter circular area of the wall or ceiling was sampled using a sterile swab (Fisherbrand® polyester-tipped applicator, Fisher Scientific) moistened in 500 µL water as described. Sediment from an equivalent surface area on the floor was collected and placed into a sterile bag (Whirl-Pak®, Nasco, Fort Atkinson, Wisconsin, USA) using a clean plastic spoon. To avoid cross-contamination, nitrile gloves were changed after collecting each sample.

Research equipment

Prior to cleaning and decontamination at the conclusion of each trapping session, various pieces of equipment that had been in direct or indirect contact with bats or hibernaculum substrates were sampled to test for presence of *Pd*. Swabs from a minimum of five items per study site were collected. A single, sterile swab (Fisherbrand polyester-tipped applicator, Fisher Scientific) moistened in 500 µL water as described earlier was rolled several times across variably sized areas of clothing, traps, or processing equipment and placed into a sterile vial. Mist nets were swabbed over the entire surface area. Different parts of harp traps were swabbed separately to distinguish areas that might harbor *Pd*.

All samples were stored on ice while in the field and transferred to a –20 C freezer within 6 h of collection. Samples were held at –20 C for up to 5 wk, after which they were stored at –80 C until processed.

Molecular methods

Nucleic acid from swabs of bat wings, hibernaculum walls, and equipment, as well as from guano and sediment, was extracted using commercial kits with modifications described by Verant et al. (2016). Real-time PCR targeting the ribosomal RNA gene region intergenic spacer of *Pd* (Muller et al. 2013) was conducted with an ABI7500 Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Califor-

nia, USA) and commercial master mix (QuantiFast 2x Real-Time PCR kit, Qiagen Inc., Valencia, California, USA). A standard curve was run in conjunction with all environmental samples to verify efficiency of the PCR across multiple reactions and to compare relative abundance of *Pd* DNA among the sites. Based on previous optimization, the threshold for identifying a PCR-positive reaction was set at 4% of maximum fluorescence (Verant et al. 2016), and an extrapolated cycle threshold (Ct) value of $\geq$40 was considered equivocal for the presence of *Pd*.

Fungal cultures were performed on PCR-positive wing swabs (n=3) to assess the viability of *Pd* on bats sampled during summer. Culture analyses were also performed on all equipment samples (n=53) and on a subset of PCR-negative bat wing swabs (n=53) from two study sites (KY3, KY4) known to harbor bats with WNS during winter. Sabouraud dextrose agar plates containing chloramphenicol and gentamycin (BD Diagnostic Systems, Sparks, Maryland, USA) were inoculated with 200 µL of sample. Fungal cultures were incubated at 7 C and examined every 7–10 d for up to 8 wk to assess fungal growth. Identity of fungal isolates resembling *Pd* was confirmed by microscopic evaluation (Gargas et al. 2009).

Data analysis

We evaluated the effects of site characteristics (i.e., hibernacula with stable winter bat counts and hibernacula with reduced winter bat counts) and sample type (soil or wall swab) on PCR results using linear regression in a Bayesian framework with R2OpenBUGS (Sturtz et al. 2005) in R (R Core Team 2013). We modeled Ct values as a function of covariates using linear regression with a normally distributed random-effect intercept term (where site was the random effect). Priors for mean intercept and slopes were normally distributed with mean=0 and variance of 10,000 (or 1/variance=0.0001); priors for SD of the random effect were uniformly distributed from 0 to 10. For Ct values of 40, the maximum number of PCR cycles, we modeled observations as a truncated normal with a minimum value of 40. This formulation allowed for estimated Ct values greater than 40. Detections of *Pd* on bats or in environmental samples were also modeled in a Bayesian framework as a series of Bernoulli trials using a logistic link where the observed value was 1 when *Pd* was detected and 0 when *Pd* was not detected. Priors for intercept and coefficient terms in the logistic regression were normally distributed with a mean of 0 and variance of 10,000 (or 1/variance=0.0001). Effects of site, sample type (soil, wall, or bat), and species of bat on detection of *Pd* were estimated. The big brown bat (*Eptesicus fuscus*) and eastern small-footed bat (*Myotis leibii*) were excluded from the analyses because <25 specimens were sampled.

RESULTS

Samples were collected from 617 bats of seven species captured inside eight hibernacula; 234 additional bats were trapped outside the study sites (Table 1). The diversity of bat species present inside each site during summer was less than or equivalent to that recorded historically during winter surveys. Little brown bats (*Myotis lucifugus*) and tricolored bats (*Perimyotis subflavus*), two species commonly diagnosed with WNS, were captured at all summer study sites. Only five recaptures (<1%, 5/744) were detected among the seven study sites where entrance trapping was conducted. Overall, males significantly outnumbered females by more than four to one (χ^2=184.9, P<0.001) at all sites (see Supplementary Material Table S1), including KY4 which contained a maternity colony of gray bats. Species-specific body mass indices were similar among all study sites (see Supplementary Material Table S1).

No bats had visible evidence of a fungal skin infection during summer sampling. Nearly all bats examined (98%, 603/617) had little to no evidence of wing damage and none exhibited severe damage. Three individuals (two tricolored bats and one Indiana bat [*Myotis sodalis*]) at two sites (KY2 and KY3) exhibited fluorescence on their wings suggestive of WNS when examined under UV light. For all three bats, wing swabs were negative by PCR for *Pd*, and skin samples lacked lesions of WNS by histopathology analysis.

In all, *Pd* was detected on 40 bats and environmental samples from seven of eight study sites (Table 2 and Supplementary Material Table S1). At one site (VA1) known to harbor bats with WNS, the fungus was detected from environmental substrates only. At one control site (KY1) a single hand-captured, juvenile, male, tricolored bat tested PCR-positive for *Pd* on its wing, representing the first known detection of *Pd* at this location,

TABLE 2. Detection of *Pseudogymnoascus destructans* (*Pd*) by PCR or fungal culture at hibernacula sites in the eastern USA occupied by bats during summer (July–August 2012).

	No. of samples										
	Bats					Environment					
	Wing		Guano			Wall		Soil		Equipment	
Site[a]	Total	*Pd*+[b]	Total	*Pd*+	Total	Total	*Pd*+	Total	*Pd*+	Total	*Pd*+
KY1	98	1	2	0	1	8	0	8	0	7	0
KY2	89	0	72	0	0	8	0	8	0	5	0
KY3	101	2	41	22	24	8	2	8	8	7	1
KY4	115	1*	31	4	4	8	1	8	4	5	0
VA1	49	0	27	0	0	8	1	8	5	5	0
TN1	48	0	12	1	1	8	3	8	4	6	0
IN1	45	0	10	5	5	8	5	8	8	5	1
OH1	72	0	28	5	5	8	1	8	8	13	1
Totals	617	4	223	37	40	64	13	64	37	53	3

[a] KY = Kentucky; VA = Virginia; TN = Tennessee; IN = Indiana; OH = Ohio.

[b] Pd+ = positive for *Pd* by PCR or culture.

* *Pd* detected by fungal culture only.

and WNS was confirmed in bats at both control sites the following winter (2012/2013). Guano accounted for 93% (37/40) of all *Pd* detections in bats sampled during summertime. Comparatively, *Pd* prevalence on bats during summer was 17% (37/223) based on analysis of guano and <1% (4/617) for wing swabs. While *Pd* was identified in guano from five species of bats (little brown, Indiana, gray, eastern small-footed, and tricolored), the fungus was detected from only two species (little brown and tricolored) on wing membranes (see Supplementary Material Table S1). *Pseudogymnoascus destructans* was detected on bats of both sexes (33 males, 7 females) and age classes (28 adults, 12 juveniles). Additionally, *Pd* was cultured (indicating viability) from wing skin and detected in guano from a postlactating little brown bat collected on 15 August 2012. Prevalence of *Pd* on bats during summer ranged from 0–24% among study sites where WNS had been previously confirmed in winter bat populations (Fig. 2A). Species composition varied considerably by site (Table 1). After controlling for species, bats sampled from sites with reduced winter counts were less likely to test *Pd*-positive (odds ratio=0.39 [95% Bayesian credible interval, 0.15–0.88]) than were bats sampled from sites with stable counts (Fig. 2B).

A total of 128 environmental samples (soil and wall-substrate swabs) collected at hibernacula during summer were analyzed for presence and relative abundance of *Pd*. *Pseudogymnoascus destructans* was not detected in samples collected inside the two control sites whereas *Pd* was found in samples from all six sites where WNS occurred in the overwintering bat populations (Table 2). No statistically significant differences in relative abundance of *Pd* in environmental samples were detected between WNS-positive sites with stable winter bat counts compared to those with reduced winter counts. However, relative abundance of *Pd* detected in soil was significantly greater than that detected from equivalently sized areas of hibernaculum wall (Fig. 2C), as evidenced by PCR amplification of *Pd* occurring in 10.3 fewer cycles (95% Bayesian credible interval, 6.6–14.4) in soil compared to wall-based samples.

Equipment from three WNS-impacted sites, including harp trap strings (KY3), a harp trap catch bag (OH1), and a backpack (IN1) tested PCR-positive for *Pd* (Tables 2, 3).

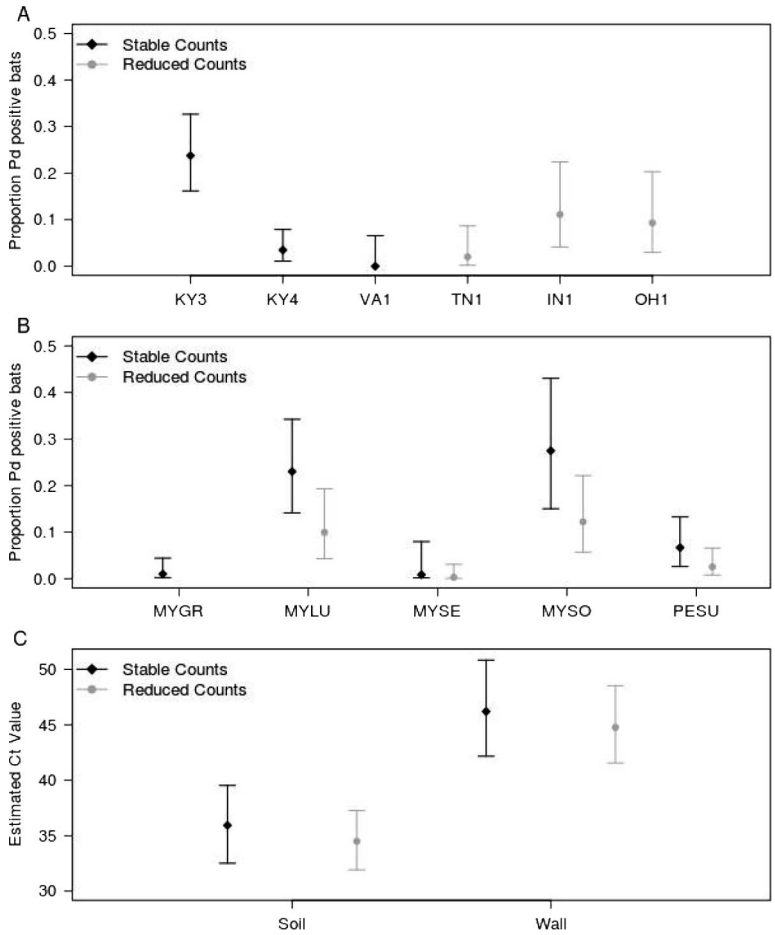

FIGURE 2. Results of PCR- and culture-based surveillance for *Pseudogymnoascus destructans* (*Pd*) in bats and the environment at white-nose syndrome (WNS)-positive hibernacula in the eastern USA that were used as summer roosts between July and August, 2012. In all graphs, data-points from WNS-positive hibernacula with stable winter bat counts are black; data-points from WNS-positive hibernacula with reduced winter bat counts (>25%) are gray. Bars indicate 95% Bayesian credible intervals. A) Proportion of bats using WNS-positive hibernacula in summer that demonstrated exposure to *Pd* (KY=Kentucky; VA=Virginia; TN=Tennessee; IN=Indiana; OH=Ohio). B) Prevalence of *Pd* exposure by species at WNS-positive hibernacula. (MYGR=*Myotis grisescens*; MYLU=*Myotis lucifugus;* MYSE=*Myotis septentrionalis*; MYSO=*Myotis sodalis*; PESU=*Perimyotis subflavus*). C) Estimated PCR cycle threshold (Ct) values of different environmental sample types collected at WNS-positive hibernacula. "Soil" refers to floor sediment and "wall" to swabs of hibernaculum wall or ceiling substrates. Relative fungal abundance for each specimen type is inversely proportional to the Ct value.

Equivocal PCR results suggested that additional equipment used at site KY3 may have been contaminated by *Pd* at levels below the detection limit of the assay. Although *Pd* was not detected on mist nets, the number of nets sampled (n=5) was small compared to harp traps (n=24). Viable *Pd* was not detected by fungal culture analysis from any equipment samples.

DISCUSSION

We demonstrated that bats occupying hibernacula contaminated with *Pd* during summer face ongoing risk of exposure to the

TABLE 3. Detection of *Pseudogymnoascus destructans* by PCR on equipment surfaces following research activities conducted during summer (July–August 2012) at hibernacula sites in the eastern USA occupied by bats.

Category	Item	No. positive/ no. sampled
Trap equipment	Harp trap	3[a]/24
	Mist net	0/5
Clothing-personnel	Boots	0/4
	Hair	0/1
	Handling gloves	0/2
	Headlamp	0/3
	Outer clothing	0/4
	Backpack	2[a]/4
Processing equipment	Calipers	0/1
	Camera	0/2
	Data sheet	0/1
	Plastic cooler	0/1
	Platform scale	0/1

[a] Includes an equivocal PCR result obtained from one item.

pathogen. Prevalence of *Pd* on bats, based on analysis of skin swabs, declines substantially following winter hibernation and spring emergence (Langwig et al. 2015), and mortality from WNS has not been reported in free-ranging bats after late May (US Geological Survey 2016). However, environmental persistence of viable *Pd* at hibernacula that have harbored infected bats indicates that these sites serve as pathogenic reservoirs for bats that use these sites during nonhibernal periods (Lorch et al. 2013). Although exposure to *Pd* does not result in disease during summertime, our results demonstrate that bats can carry viable fungus on their skin during summer and thus facilitate movement of the pathogen. Our finding extends the surveillance period for *Pd* reported by Carpenter et al. (2016), particularly when sampling efforts are focused around hibernacula used by WNS-susceptible bat species during summer.

We conducted sampling prior to the peak fall swarm in late August–September (Hall and Brenner 1968; Agosta et al. 2005) to reduce the likelihood that sampled bats would overwinter at study sites (Norquay et al. 2013). Low observed recapture rates were consistent with low summer night-roost fidelity as reported by Agosta et al. (2005) and suggested that frequent movement of bats using underground roosts in WNS-affected areas during summer could contribute to movement of *Pd* across the landscape. While sites analyzed for this study were biased towards hibernacula used by bats during summer, detection of *Pd* on a bat captured in mid-August inside a control site (KY1) that was previously thought to be free of the pathogen further supports the potential for bat-mediated movement of *Pd* outside of the hibernation season.

The bias that we observed for males among bats occupying caves and mines during summer was similar to that reported by others (Hall and Brenner 1968; Brack 1985). Males and nonreproductive females tolerate lower roosting temperatures and more-frequent torpor bouts during summer because of reduced energy requirements compared to reproductive females (Altringham 2011). Males also tend to forage over larger areas and may roost at various underground sites within their summer home range to reduced competition for resources (Agosta et al. 2005), which could account for low recapture rates. Differences in summer roosting behaviors and higher prevalence of *Pd* in males compared to reproductive females (Langwig et al. 2015) suggest males may play an important role in *Pd* dispersal during summertime.

To optimize detection during summer, surveillance for *Pd* on bats should include collection and analysis of fresh guano in addition to wing-skin swabs. Specifically, *Pd* was detected in the guano of 37 bats sampled at five of six study sites with a history of WNS whereas *Pd* was only detected on wing skin of four bats at three sites. Although skin swabs demonstrated superior detectability of early *Pd* infections in hibernating bats compared to other diagnostic methods (McGuire et al. 2016), they failed to detect *Pd* in 97% (36/37) of samples collected from active bats with *Pd* in their guano. The relative abundance of *Pd* on wing skin in summer was low (Ct value range: 39.43–39.99). Possible explanations for

our poor detection rate from skin swabs may have been the dilution of samples in water to ensure sufficient volume for inoculation of culture plates or that *Pd* abundance on bats is lower in summer and therefore it is more difficult to detect. It is also possible that in summer, bats groom the majority of *Pd* from their wing surface, thus preventing detection by analysis of skin swabs. However, because *Pd* was not always detected in guano of bats with *Pd*-positive wing swabs, and because guano collection can be more challenging than obtaining a skin swab, we recommend collecting both sample types when conducting *Pd* surveillance in nonhibernal bats.

Our results further suggest that guano may serve as an important route for dispersal of *Pd* during summer months. Accumulation of *Pd* in guano likely results from bats ingesting the fungus from body surfaces during grooming, and WNS-positive bats are known to groom more frequently than do uninfected bats (Brownlee-Bouboulis and Reeder 2013). Furthermore, gastrointestinal transit studies in various species of insectivorous bats demonstrate nearly complete passage of food items within 24 h (Lukens et al. 1971; Buchler 1975; Stalinski 1994; Roswag et al. 2012). Therefore, detection of *Pd* in fresh guano collected from bats captured at evening emergence suggested recent ingestion of the fungus. Due to low sample volumes, we only analyzed guano by PCR, thus the viability of *Pd* in guano remains unclear. If *Pd* has the ability to survive gastrointestinal transit, bat movements between hibernacula during summer and fall could contribute substantially to dispersal of *Pd* on the landscape. However, further study is required to determine the reliability of guano as a surveillance strategy for *Pd* on bats sampled away from potential reservoir sites.

The high probability of detecting *Pd* in guano may also contribute to the higher probability of detecting *Pd* in soil compared to wall or ceiling substrates, as both guano and spores shed from the skin of infected bats are likely to accumulate on hibernaculum floors. This indicates that sediment from areas underneath where bats roost should be targeted when conducting surveillance for *Pd* in the absence of accessible bats or when disturbance is inappropriate.

We hypothesized that sites with populations of hibernating bats that had declined would exhibit higher prevalences of *Pd* on bats and greater abundances of *Pd* in environmental samples during summer compared to sites with stable counts of hibernating bats. While summer exposure of bats to *Pd* was detected at all sites with reduced winter bat counts, the majority of *Pd*-positive bats (70%) in summer were found at WNS-positive sites with stable winter counts. This was primarily due, however, to the high prevalence of *Pd* predominantly among little brown and Indiana bats at one study site (KY3). In contrast, there was no statistical difference in relative abundance of *Pd* detected in environmental samples from study sites that differed in winter bat counts. Once introduced into an underground environment, *Pd* is capable of growth and amplification in the absence of bats (Reynolds et al. 2015). Thus, abundance of the fungus in a hibernaculum environment may be multifactorial in addition to impacts of WNS on the resident bat population.

We also demonstrated that screening bat wings with long-wave UV light during mid- to late-summer can result in false-positive indications of *Pd* infection. The pattern of fluorescence occasionally seen on bats during summer was not associated with WNS and did not resemble the widespread, miliary pattern typically seen on wing membranes of bats with WNS during winter (Turner et al. 2014). Presence of *Pd* on bats during summer likely represented superficial colonization, which is not identifiable by examination under UV light. Therefore, use of this screening technique should be limited to examination of bats during periods of winter hibernation or spring emergence.

Finally, we demonstrated that equipment used at hibernacula where WNS occurs can become contaminated with *Pd* during summer months. Human activities have been hypothesized to contribute to the introduction and spread of *Pd* in the US (Leopardi et al. 2015). We identified *Pd* both on equipment used exclusively outside of hibernacula entrances

and on equipment used inside of hibernacula during summer, a period of low *Pd*-prevalence on bats. Although we did not demonstrate the presence of viable fungus on equipment, this finding was not unexpected given the high PCR Ct values (>38), signifying low abundance of fungus, that were obtained from samples of equipment. Spores of other fungal pathogens such as *Aspergillus*, *Microsporum*, and *Trichophyton* are known to maintain long-term viability in association with fomites (Kramer et al. 2006). Thus, routine decontamination of equipment, including mist nets and harp traps, following use during summer is warranted, particularly in areas where risk for geographic expansion of *Pd* is the greatest.

Findings from this study support several important considerations regarding the movement of *Pd* across the landscape. The foremost is that bats occupying hibernacula during summer can harbor *Pd* on their skin and in their guano, creating the potential for broad dispersal of this fungal pathogen during the period of fall mating swarms. Furthermore, we provided evidence for potential human-mediated dispersal of *Pd* on equipment and clothing following trapping of bats and other cave-associated activities during summer months when prevalence of WNS in bats is low. This work highlights the importance of selecting appropriate sampling methods for surveillance of *Pd* on bats during summer and expands our knowledge of dispersal hazards of *Pd* by bats and humans during the nonhibernal season.

ACKNOWLEDGMENTS

Funding for this project was from the USFWS (IAA 4500036148) and the US Geological Survey. We thank Carol Meteyer and Daniel Schenkman who evaluated histopathology, Nathan Ramsay for his GIS skills, and Jeff Lorch whose review improved our manuscript. We also thank the many state, federal, university, and nonprofit agency personnel who assisted with sample collections for this project: Scott Johnson, Tim Shier, Cassie Hudson (Indiana Department of Natural Resources); Brooke Hines, Jim Barnard (Kentucky Department of Fish and Wildlife); Melissa Moser, Travis Clime (Ohio Department of Natural Resources); Terry Hopkins, Josh Campbell (Tennessee Wildlife Resources Agency); Cory Holliday (The Nature Conservancy); Rick Reynolds, Johnny Wills (Virginia Game and Inland Fisheries); Lori Pruitt, Andy King, Rich Geboy, Mike Armstrong, Keith Lott, Dave Henry, Levi Miller, Rebecca Miller (USFWS); Katrina Schultes, Lois Boggs, April Almond, Ben Reed, Pat Mercer, Jeff Metcalf, Chandler Bryant, Sarah Bowen (US Forest Service); Rickard Toomey, Steve Thomas, Stella James, Laura Shultz (National Park Service); Luke Dodd, Mike Lacki, Zach Couch (University of Kentucky); Michelle Verant (University of Wisconsin, Madison); Murray State University students; and volunteers MJ Woixcom, Wally Smith, Philip Shelton. Use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US government. Data collected for this study are available at GS ScienceBase (http://dx.doi.org/10.5066/F78P5XRX).

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2016-09-206.

LITERATURE CITED

Agosta SJ, Morton D, Marsh BD, Kuhn KM. 2005. Nightly, seasonal, and yearly patterns of bat activity at night roosts in the central Appalachians. *J Mammal* 86:1210–1219.

Altringham JD. 2011. *Bats: From evolution to conservation*, 2nd Ed. Oxford University Press, Inc., New York, New York, 324 pp.

Brack V. 1985. The foraging ecology of some bats in Indiana. *Proc Indiana Acad Sci* 94:231–237.

Briggler JT, Prather JW. 2003. Seasonal use and selection of caves by the eastern pipistrelle bat (*Pipistrellus subflavus*). *Am Midl Nat* 149:406–412.

Brownlee-Bouboulis SA, Reeder DM. 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.

Buchler ER. 1975. Food transit time in *Myotis lucifugus* Chiroptera: Vespertilionidae. *J Mammal* 56:252–255.

Carpenter GM, Wilcox EV, Bernard RF, Stiver WH. 2016. Detection of *Pseudogymnoascus destructans* on free-flying male bat captured during summer in the southeastern USA. *J Wildl Dis* 52:922–926.

Dobony CA, Hicks AC, Langwig KE, Von Linden RI, Okoniewski JC, Rainbolt RE. 2011. Little brown myotis persist despite exposure to white-nose syndrome. *J Fish Wildl Manage* 2:190–195.

Fleming TH, Eby P. 2003. Ecology of bat migration. In: *Bat ecology*, Kunz TH, Fenton MB, editors. University of Chicago Press, Chicago, Illinois, pp. 156–208.

Gargas A, Trest MT, Christensen M, Volk TJ, Blehert DS. 2009. *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. *Mycotaxon* 108:147–154.

Hall JS, Brenner FJ. 1968. Summer netting of bats at a cave in Pennsylvania. *J Mammal* 49:779–781.

Hall JS, Wilson N. 1966. Seasonal populations and movements of the gray bat in the Kentucky area. *Am Midl Nat* 75:317–324.

Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 6:130.

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* 282:201442335.

Leopardi S, Blake D, Puechmaille SJ. 2015. White-nose syndrome fungus introduced from Europe to North America. *Curr Biol* 25:R217–R219.

Lewis SE. 1995. Roost fidelity of bats: A review. *J Mammal* 76:481–496.

Lorch JM, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, Hicks AC, Ballmann AE, Coleman JTH, Redell DN, Reeder DM, et al. 2011. Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. *Nature* 480:376–378.

Lorch JM, Muller LK, Russell RE, O'Connor M, Linder DL, Blehert DS. 2013. Distribution and environmental persistence of the causative agent of white-nose syndrome, *Geomyces destructans*, in bat hibernacula of the eastern United States. *Appl Environ Microbiol* 79:1293–1301.

Lukens MM, Van Eps J, Davis WH. 1971. Transit time of food through the digestive tract of the bat, *Eptesicus fuscus*. *Exp Med Surg* 29:25–28.

McGuire LP, Turner JM, Warnecke L, McGregor G, Bollinger TK, Misra V, Foster JT, Frick WF, Kilpatrick AM, Willis CKR. 2016. White-nose syndrome disease severity and a comparison of diagnostic methods. *Ecohealth* 13:60–71.

Muller LK, Lorch JM, Linder DL, O'Connor M, Gargas A, Blehert DS. 2013. Bat white-nose syndrome: A real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*. *Mycologia* 105:253–259.

Norquay KJO, Martinez-Nunez F, Dubois JE, Monson KM, Willis CKR. 2013. Long-distance movements of little brown bats (*Myotis lucifugus*). *J Mammal* 94: 506–515.

R Core Team. 2013. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/. Accessed December 2015.

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, Ingersoll T, Barton HA. 2015. Modeling the environmental growth of *Pseudogymnoascus destructans* and its impact on the white-nose syndrome epidemic. *J Wildl Dis* 51:318–331.

Roswag A, Becker NI, Encarnação JA. 2012. Inter- and intraspecific comparisons of retention time in insectivorous bat species (Vespertilionidae). *J Zool* 288:85–92.

Stalinski J. 1994. Digestion and food passage rate in the insectivorous bat *Myotis myotis*. *Acta Theriol* 39:1–11.

Sturtz S, Ligges U, Gelman A. 2005. R2WinBUGS: A package for running WinBUGS from R. *J Stat Softw* 12:1–16.

Turner GG, Meteyer CG, Barton H, Gumbs JF, Reeder DM, Overton B, Bandouchova H, Bartoniĉka T, Martínková 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 syndrome. *J Wildl Dis* 50:566–573.

Verant ML, Bohuski EA, Lorch JM, Blehert DS. 2016. Optimized methods for total nucleic acid extraction and quantitation of the bat white-nose syndrome fungus, *Pseudogymnoascus destructans*, from swab and environmental samples. *J Vet Diagn Invest* 28: 110–118.

USFWS (US Fish and Wildlife Service). 2012. *National white-nose syndrome decontamination protocol–Version 06.25.2012*. https://www.whitenosesyndrome.org/sites/default/files/resource/national_wns_revise_final_6.25.12.pdf. Accessed January 2017.

USFWS. 2017. *Where is it now?* US Fish and Wildlife Service. https://www.whitenosesyndrome.org/about/where-is-it-now. Accessed June 2017.

US Geological Survey. 2016. *Wildlife health information sharing partnership event reporting system (WHISPers) on-line database*. http:/www.nwhc.usgs.gov/whispers/. Accessed July 2016.

Submitted for publication 26 August 2016.
Accepted 30 March 2017.