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TITLE: Distribution and Environmental Persistence of the Causative Agent of White-Nose Syndrome, *Geomyces destructans,* in Bat Hibernacula of the Eastern United States

RUNNING TITLE: Distribution and Persistence of *Geomyces destructans*

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ABSTRACT

White-nose syndrome (WNS) is an emerging disease of hibernating bats caused by the recently described fungus *Geomyces destructans*. First isolated in 2008, the origins of this fungus in North America and its ability to persist in the environment remain undefined. To investigate the correlation between manifestation of WNS and distribution of *G. destructans* in the U.S., we analyzed sediment samples collected from 55 bat hibernacula (caves and mines) both within and outside the known range of WNS using a newly developed real-time PCR assay. *Geomyces destructans* was detected in 17 of 21 sites within the known range of WNS at the time the samples were collected; the fungus was not found in 28 sites beyond the known range of the disease at the time that environmental samples were collected. These data indicate that distribution of *G. destructans* is correlated with disease in hibernating bats and support the hypothesis that the fungus is likely an exotic species in North America. Additionally, we examined whether *G. destructans* persists in infested bat hibernacula when bats are absent. Sediment samples were collected from 14 WNS-positive hibernacula, and the samples were screened for viable fungus using a culture technique. Viable *G. destructans* was cultivated from 7 of the 14 sites sampled during late summer when bats were no longer in hibernation, suggesting the fungus can persist in the environment in the absence of bat hosts for long periods of time.

INTRODUCTION

White-nose syndrome (WNS) is an emerging wildlife disease that by one estimate (see http://www.whitenosesyndrome.org/news/north-american-bat-death-toll-exceeds-55-million-white-nose-syndrome) has killed approximately 5.5 million hibernating bats in North America since its discovery in 2007. The disease results from cutaneous infection by the recently described fungus, *Geomyces destructans* (1,2), and has been implicated in population declines of 72-88% for hibernating bat species inhabiting the northeastern U.S. (3-5). Since 2007, the disease has spread across the eastern U.S. and Canada, threatening the future of North American bat populations (5,6). The sudden emergence and rapid spread of WNS has led to questions regarding the origin of *G. destructans* in North America and how the lifecycle of the fungus allows it to exert such significant impacts on hibernating bat populations.

Laboratory experiments have demonstrated that *G. destructans* is the causative agent of WNS in a North American bat species, eliciting the disease in apparently healthy animals (7,8). In addition, *G. destructans* has been found to occur on hibernating bats throughout most of Europe, but it has not been associated with unusual bat mortality on the European continent (9-12). Together, these findings prompt two hypotheses regarding the origin of *G. destructans* in North America that are consistent with the emergence of a novel infectious disease (13): 1) *Geomyces destructans* is endemic to North America, but a pathogenic strain spontaneously emerged and is spreading across the landscape (8); or 2) *Geomyces destructans* was recently introduced to North America where it is behaving as an exotic pathogen among naïve populations of bats (8,9,11).

Recent research supports the exotic species hypothesis. For example, a European isolate of *G. destructans* has been found to induce lesions diagnostic for WNS and mortality in an experimentally infected North American bat species (8). This demonstrates that a European isolate of the fungus is highly pathogenic to North American bats despite having no apparent effects on wild bat populations of Europe and implicates Europe as the possible source for introduction of *G. destructans* to North America (12). In addition, isolates of *G. destructans* from the eastern U.S. appear to be genetically identical (14), suggesting that *G. destructans* in North America is derived from a single isolate that may have been introduced to this continent.

While evidence is mounting to support the hypothesis that *G. destructans* was introduced to North America, it remains unclear why WNS-related mortality varies between bats of North America and Europe (12) and why some North American species appear to be more vulnerable than others (5). Environmental effects, genetic composition, and behaviors differ among bat species and likely play a role in facilitating infection, disease progression, and mortality, but such factors are difficult to tease apart without a basic understanding of whether presence of *G. destructans* in a hibernaculum correlates with manifestation of WNS in bats. Furthermore, the current assumption that *G. destructans* is limited to areas where WNS has been observed may be biased by the primary means of detecting the fungus through diagnostic analysis of samples derived from sick or dead bats. A previous study addressed these issues by screening sediment samples from bat hibernacula to determine whether *G. destructans* was indeed restricted to areas where

93 WNS has been observed in bats (15). While nucleic acid from *G. destructans* was found
94 to occur at three sites within the known range of WNS and no sites outside the range of
95 WNS, the number of sites sampled was too small given the low detection rate to conclude
96 the distribution of the fungus was correlated to that of the disease. In addition, the
97 method utilized in that study lacked specificity because similar species of *Geomyces*
98 cross-reacted with primers of the conventional PCR-based method (16) and may have
99 masked the presence of *G. destructans* if it was at a low abundance. Screening a greater
100 number of sediment samples from hibernacula using a more specific and sensitive
101 technique, such as a recently-described real-time PCR assay (17), may serve to better
102 determine whether distribution of *G. destructans* is limited to areas where WNS occurs or
103 whether the fungus is more widespread in North America than currently thought.
104
105 The ability to detect *G. destructans* in environmental samples using PCR-based methods
106 could also reveal important information about WNS disease dynamics. Current
107 determination of whether *G. destructans* is present in a hibernaculum usually relies upon
108 first detecting the fungus on sick bats, which makes it difficult to address questions such
109 as: when did the fungus arrive at a given site; how long does it takes for disease to
110 manifest after the arrival of *G. destructans* at a new site; and is disease an inevitable
111 outcome of the fungus' presence in a hibernaculum? Understanding these aspects of the
112 pathogen's interaction with the environment and its host will facilitate disease
113 surveillance of bat hibernacula and potentially enable earlier deployment of
114 interventional strategies to more effectively limit the spread of and reduce mortality
115 caused by WNS.

Most fungi pathogenic to mammals can persist in the environment in the absence of a host (18,19). Given the temperature requirements for growth of *G. destructans* (i.e. it does not grow at or above approximately 20°C [20]), caves and mines have environmental characteristics consistent with potential long-term reservoirs for the fungus as they remain cool throughout the year, even when bats are absent during summer months. While follow-up culture analyses of sediment samples that contained DNA from *G. destructans* from a previous study (15) proved that viable *G. destructans* was present (21), these samples were collected during the hibernation season and may have represented only short-term survival of the fungus after it detached from a bat host. Similarly, *G. destructans* was cultured from the wall of a cave in Estonia where an infected bat had been observed nine days prior (12), again demonstrating only temporary persistence. Thus, the ability of *G. destructans* to survive long-term in the environment in the absence of its bat hosts remains uncertain.

The objectives of this study were to: 1) determine the distribution of *G. destructans* in underground bat hibernacula of eastern North America and examine whether presence of the fungus strictly correlates with occurrence of WNS; and 2) establish whether hibernacula can serve as reservoirs for *G. destructans* during the summer months when bats are largely absent. To address the first objective, we screened sediment samples collected from bat hibernacula across the eastern U.S. for the presence of *G. destructans* using a real-time PCR test (17). To address the second objective, sediment samples collected from WNS-affected hibernacula were screened for viable *G. destructans* using a

139 previously-described culture technique (21) during seasons of both bat hibernation (when
140 bats are present in hibernacula) and activity (when bats are largely absent from
141 hibernacula). By demonstrating the utility of environmental sampling as a non-invasive
142 tool for detecting *G. destructans*, results from these investigations offer the potential to
143 refine WNS surveillance and management.
144
145
146 **MATERIALS AND METHODS**
147
148 **Sample Collection.** Sediment samples used for fungal distribution analysis were
149 collected from the eastern U.S. by volunteers during the winter of 2008-2009. For each
150 sample, clean latex gloves were worn and sterile wooden splints were used to transfer
151 sediment into sterile, labeled sampling bags. A minimum of five samples were collected
152 from the floor of each cave or mine (hereafter referred to as a "site") and immediately
153 shipped on ice to the U.S. Geological Survey – National Wildlife Health Center
154 (Madison, WI) where they were stored at -80°C. The samples included in this study
155 represented a total of 56 sites from 22 states east of the 95th meridian west (95°W
156 longitude), including 8 states within and 14 states outside the known range of WNS at the
157 time the samples were collected (Fig. 1; Table 1). Exact locations of the sampled sites
158 are not provided due to the sensitive nature of bat hibernacula.
159
160 Sediment samples for environmental persistence analysis were collected from 14 bat
161 hibernacula in which bats with WNS had been previously identified. These consisted of

4 sites in New Hampshire, 2 sites in Vermont, 3 sites in Virginia, and 5 sites in West Virginia (see Table 2). Five locations within each site were marked, and samples were serially collected within 30 cm of the markers on three separate occasions: once in February-March 2011 (during the bat hibernation period; hereafter referred to as winter 2010-2011), once in late July-late August 2011 (near the end of the active season and just prior to large congregations of bats returning to the hibernacula; hereafter referred to as summer 2011), and again in October 2011-March 2012 (during the next consecutive hibernation period; hereafter referred to as winter 2011-2012). Exceptions were: samples were not obtained from sites C6, C7, and C8 during the third sampling period (i.e. winter 2011-2012); several sampling markers could not be relocated within sites C7 and C9 during the second visit (i.e. summer 2011), and samples were collected from approximate locations as determined by collectors. Additionally, site C4 flooded in September 2011; the markers were relocated during the winter 2011-2012 visit although 2.5-20 cm of sediment had been deposited on top of the previously sampled sediment.

DNA Extraction and PCR Analysis. DNA was extracted from the sediment samples for the distribution study using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. If more than five samples were collected from a given site, five were chosen at random for inclusion in the study. All extracted DNA samples were stored at -20°C.

Real-time PCR targeting the intergenic spacer (IGS) region of the rRNA gene complex of *G. destructans* was performed on an Applied Biosystems 7500 Fast Real-Time PCR

System (Applied Biosystems, Foster City, CA) as described previously (17). Five microliters of each DNA extraction from sediment (diluted 1:1 and 1:10) were added to each 25 µl PCR reaction. All plates included at least two positive (3.3 pg *G. destructans* genomic DNA [gDNA]) and one negative (water added in place of template) control samples. Individual samples that crossed the cycle threshold (set at 10% of the maximum fluorescence of the positive control sample for each plate [17,22]) within 40 cycles were considered positive for presence of *G. destructans*. Further, a sample was identified as positive if either or both template dilutions (1:1 or 1:10) crossed the cycle threshold as described above; a site was considered positive when at least one sample from that site was PCR-positive.

Sediment often contains humic acid and other substances that can inhibit DNA amplification. Thus, prior to conducting the real-time PCR assay, all samples were screened for PCR inhibition to reduce the chance of false negative results. Conventional PCR targeting the internal transcribed spacer (ITS) region of the rRNA gene was performed with primers ITS4 and ITS5 (23) using GoTaq® Flexi DNA Polymerase (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Five microliters of the 1:10 diluted sediment DNA extraction was used as template. Cycling conditions were as follows: 98°C for 2 min, then 30 cycles of 98°C for 10 sec, 50°C for 30 sec, and 72°C for 1 min, followed by a final extension for 7 min at 72°C. Amplification products were analyzed using an agarose gel. Control reactions containing 33 fg gDNA isolated from pure cultures of *G. destructans* (positive control) or without template added (negative control) were also included. Samples failing to yield bands by

208 the ITS PCR were subsequently spiked with gDNA from *G. destructans* and used as
209 template in a modified version of the real-time PCR assay (17) adapted for use by
210 conventional PCR. Reagents used were as described for the ITS PCR. Five microliters
211 of the 1:10 diluted sediment DNA extraction, 5 µL (containing 33 fg) gDNA, and 1.25
212 µL of each primer used in the real-time PCR were included in the 25 µL total reaction
213 volume. No probe was added. Control reactions as described for the ITS PCR were also
214 included. Cycling conditions were identical to those used for the real-time PCR assay
215 (17). Spiked samples that did not yield amplification products were considered
216 inhibitory. If one or more samples showed evidence of inhibition, the entire site was
217 excluded from the dataset. To ensure the qualitative results of the inhibition screen using
218 conventional PCR were consistent with the more quantitative real-time PCR, a subset of
219 samples was also screened for inhibition on the real-time PCR platform. Single samples
220 from 45 individual sites that tested negative for the presence of *G. destructans* by real-
221 time PCR were randomly selected, spiked with 33 fg of *G. destructans* gDNA, and used
222 as template in the real-time PCR assay. Samples within one C_t value of the positive
223 control well containing 33 fg *G. destructans* gDNA were considered non-inhibitory.
224
225 PCR amplicons from each PCR-positive site were subjected to cloning and sequencing to
226 confirm an exact sequence match to *G. destructans*. PCR products were cloned as
227 described previously (24) and prepared for sequencing according to pre-established
228 methods (15) using primers SP6 (TATTTAGGTGACACTATA) and T7
229 (TAATACGACTCACTATAG), which target pGEM-T (Promega, Madison, Wisconsin)
230 up- and down-stream of the insert. Because of short size of the amplicons (103 bps),

231 blue/white screening of bacterial colonies was not possible, so approximately 8-16
232 random colonies were chosen for screening by PCR amplification, and those yielding
233 amplification products were further characterized by DNA sequencing.
234
235 **Culture Analysis.** For the environmental persistence analysis, approximately 200 mg of
236 each thawed sediment sample was placed into a sterile microcentrifuge tube, suspended
237 in 0.5 mL sterile, deionized water, and serially diluted as previously described (21).
238 Sabouraud dextrose agar plates containing chloramphenicol and gentamycin (BD
239 Diagnostic Systems, Sparks, MD) were inoculated by spreading 150 µL of the 10^{-1}, 10^{-2},
240 and 10^{-3} dilutions onto the medium. Each dilution was plated in duplicate. The plates
241 were incubated at 7°C and checked at 30 days and once weekly thereafter for a total of 60
242 days. Colonies of *G. destructans* were initially identified by examining tape lifts of
243 suspect colonies using a 40X objective to identify characteristic crescent-shaped conidia
244 borne at the end of verticillately branching conidiophores (2). At least one colony of *G.*
245 *destructans* from each site at each time-point, when present, was isolated in pure culture.
246 The ITS region of the rRNA gene of each of these isolates was then sequenced to confirm
247 identification of *G. destructans* using primers ITS1-F and ITS4 (25) and PCR conditions
248 as described previously (21).
249
250 **Data Analysis.** Hibernacula for the fungal distribution analysis were categorized as
251 occurring within one of three zones based on WNS distribution at the time of sample
252 collection: 1) the WNS zone (general geographic area within which the disease had been
253 documented); 2) the buffer zone (general geographic area within which WNS was

documented the year following sample collection); and 3) the outside zone (general geographic area within which WNS was not documented until at least two years after sample collection) (Table 1; Fig. 1). Not all hibernacula falling within the WNS zone had been confirmed to contain bats exhibiting signs of the disease; similarly, not all hibernacula within the buffer zone were confirmed to contain bats with WNS by the following year. For this reason, individual sites were designated as being either diseased sites (WNS documented at the site prior to or at the time the samples were collected), buffer sites (WNS documented one year after the samples were collected), or clean sites (WNS not documented to date or documented more than one year after the samples were collected) based on interviews with individuals from state and federal wildlife agencies (Table 1; Fig. 1).

To test the null hypothesis that distribution of *G. destructans* is not associated with WNS in North America, PCR results (i.e. the number of PCR-positive and PCR-negative bat hibernacula) for sites that occurred within and outside the known range of WNS (WNS and outside zones) at the time samples were collected were compared using Fisher's exact test in SigmaPlot 11.2 (Systat Software, Inc., San Jose, CA). Sites that occurred within the geographic buffer zone were excluded from this analysis because it was equally plausible that *G. destructans* could be present or absent from those sites.

Estimated Probability of Detection. To determine the probability of PCR and culture analysis to detect *G. destructans*, the results of each test were formulated as a binomial variable (1 = *G. destructans* detected; 0 = *G. destructans* not detected). A detection

history for each sampled site was then created as a series of zeros and ones. For example, a detection history of 101 for the environmental persistence analysis indicated that *G. destructans* was detected on the first and last surveys but not the second survey. The probability of detecting the fungus, if present, was then estimated in the following manner. The observed values at site i and replicate (spatial or temporal) t ($y_{i,t}$), were the detection histories, and represent the imperfect observation process uncorrected for the ability of the diagnostic test to detect the fungus. These observations were modeled as Bernoulli trials, where the probability of success (the probability that $y_{i,t}=1$, $p.eff_{i,t}$), was the observed detection at site i and replicate t.

Mathematically, $y_{i,t} \sim Bern(p.eff_{i,t})$, and the observed detections of the fungus were a function of the true infection status of the site (infected/clean) and the probability of the test correctly detecting the fungus if present. Therefore, $p.eff_{i,t} = z_i \times p_{i,t}$; where z represented the true state of the site (infected or clean) and p was the true detection probability. The observations, therefore, were imperfect reflections of the true state due to imperfect detection probabilities. Further, when z was unknown, z was formulated as Bernoulli trials, where probability of success = ψ, the true proportion of sites that were infected (as opposed to the observed). The analysis was conducted in R (R Foundation for Statistical Computing, Vienna, Austria) (26) using the R2WinBUGS library (27) following pre-established methods (28). To aid in convergence, $logit(p_{i,t}) <- -\alpha_1 + \beta_1 X$ was formulated; where X was a matrix of covariate values (similarly, $logit(\psi) <- \alpha_2 + \beta_2 X$). The models for both analyses were checked for convergence using the Gelman-Rubin diagnostics function (gelman.diag) in the CODA package in R (29).

For the PCR analysis, it was assumed the true infection status of buffer sites was unknown and that the true infection status of diseased and clean sites was known. Thus, z was set to 1 for diseased sites and 0 for clean sites. Priors for the estimates of αs and βs were uniform (-5, 5). Ideally, detection and occupancy would have been estimated separately for buffer and infected sites, but there were not enough buffer sites (n=4) for this analysis to converge. Therefore, a pooled occupancy and detection probability was estimated separately for buffer and infected sites.

For the culture analysis it was assumed the true infection status for each site was known as infected, and z was set to 1. Since multiple samples were collected within each cave on 3 different sampling occasions, two models were run, one with an effect of time and one including a random effect for cave. Random effects were formulated as □(μ,τ), where $\tau=1/(\sigma*\sigma)$. Priors on μ were uniform (-5,5); priors on σ were also uniform (0,10).

RESULTS

PCR Analysis. One of the 56 sites tested for the distribution portion of the study exhibited PCR inhibition and was excluded from further analysis. The subset of samples tested for inhibition using real-time PCR yielded identical results to the conventional PCR, indicating the conventional PCR method used to screen samples for inhibition was accurate. Sequences of PCR amplicons from all sediment samples that were PCR-

positive for *G. destructans* were 100% identical to the 103 nucleotide IGS region of the type isolate of *G. destructans* (GenBank accession no. JX415267 [17]).

Distribution and Environmental Detection of *G. destructans*. Nucleic acid from *G. destructans* was detected by real-time PCR in 47 samples collected during the winter of 2008-2009, representing 13 different sites (Table 1). Seven sites that were initially sampled in winter 2008-2009 were re-sampled in winter 2010-2011 as part of the environmental persistence study (see above). Four of these sites (C2, C10, C13, and C14) that were PCR negative for *G. destructans* in winter 2008-2009 were subsequently reanalyzed by real-time PCR using DNA extractions from samples collected during winter 2010-2011 (site designations 23*, 22*, 20*, and 21*, respectively, in Table 1). Bats from one of the four sites (site 20) had been diagnosed with WNS in winter 2008-2009, but bats from the remaining three sites (sites 21, 22, and 23) did not show signs of the disease until the winters of 2009-2010 or 2010-2011. PCR analysis of these samples (collected during winter 2010-2011) showed that DNA from *G. destructans* was present in 9 of 20 samples, representing all four sites.

The occurrence of *G. destructans* as detected in the environmental samples was synonymous with the known range of WNS at the time samples were collected (Fig. 1), with hibernacula within the known range of WNS having significantly higher detection rates for the fungus than hibernacula outside the range of the disease ($p < 0.0001$). Sixteen of the 17 hibernacula in which the fungus was detected were diseased sites; the remaining hibernaculum was a buffer site in which bats with WNS were observed the

following year. Of the 12 WNS-positive hibernacula from which *G. destructans* was detected in winter 2008-2009, five had been designated as WNS-positive in winter 2007-2008, and seven were identified as WNS-positive in winter 2008-2009. Site 6 had not been officially monitored since 1985, but the cave was considered WNS-positive based upon observation of clinical signs in bats suggestive of the disease at the time the samples were collected. The four hibernacula from which *G. destructans* was detected by PCR in winter 2010-2011 included one site designated as harboring WNS-positive bats in winter 2008-2009, two in winter 2009-2010, and one in winter 2010-2011.

Environmental Persistence of *G. destructans*. *Geomyces destructans* was cultured from 27 of the 195 sediment samples collected from bat hibernacula in 2011-2012 with viable fungus detected in 11 of the 14 sites during at least one sampling interval (see Table 2). Seven of the 14 sites were found to harbor viable *G. destructans* in late summer when bats were either absent from the hibernacula or present in only low numbers. Sequences of the rRNA gene ITS regions of isolates from each site were 100% identical to the ITS region of the type isolate of *G. destructans* (GenBank accession no. EU884921 [2]).

Estimated Probability of Detection. All samples from the 36 caves designated as "clean" (i.e. WNS-free) tested negative for *G. destructans*. The remaining 23 samples (representing diseased and buffer sites) were combined into one dataset, and probability of detection for the PCR assay was estimated. Estimated probabilities of detection for a single sample were 0.56 [95% C.I. 0.47-0.67] for diseased sites and 0.11 [95% C.I. 0.02-0.29] for buffer sites. This indicated that with five samples from a given diseased site,

the probability of detecting the fungus was 0.98, and that 4 samples are sufficient to obtain a mean estimated probability of detection >0.95. For buffer sites, the probability of detecting *G. destructans* with 5 samples was 0.44, and at least 26 samples would be needed from each site for the mean estimated probability of detection to be >0.95.

The overall probability of detection using the culture technique was 0.14 [95% C.I. 0.10-0.19]. There was no difference between detection probabilities by time period (0.16 [95% C.I. 0.080-0.25] in winter 2010-2011; 0.13 [95% C.I. 0.062-0.22] in summer 2011; and 0.13 [95% C.I. 0.054-0.23] in winter 2011-2012). However, the random effects model indicated unexplained variation due to the effect of site (i.e. cave/mine). Greater than 20 samples would be needed to have a mean estimated probability of detection ≥0.95.

DISCUSSION

The sudden emergence and spread of WNS in North America has led to speculation that *G. destructans* is an exotic species and may have been recently introduced from Europe (8,9,11). If this hypothesis is valid, the distribution of *G. destructans* would be expected to mirror that of the disease. We screened a total of 295 sediment samples collected from 55 caves and mines in the eastern U.S. using a real-time PCR assay specific for *G. destructans* (17) and detected the fungus in 17 bat hibernacula. All 17 of these sites were situated within the known range of WNS at the time the samples were collected, and *G.*

392 *destructans* was not found to occur outside that area (Fig. 1). Furthermore, the real-time
393 PCR findings paralleled WNS manifestation on a temporal scale. *G. destructans* was not
394 detected in three sites that were unaffected by WNS in the winter of 2008-2009, but the
395 fungus was later detected in sediment samples collected from those same hibernacula
396 subsequent to the appearance of the disease in bats at those sites. These findings suggest
397 that an endemic, less virulent strain of *G. destructans* likely did not occur in eastern
398 North America prior to arrival of WNS and offers further support for the exotic species
399 hypothesis to explain the emergence of *G. destructans* as a novel pathogen in North
400 America (8,13).
401
402 Sixteen of the 17 sites in which *G. destructans* was detected by real-time PCR in this
403 study contained bats showing signs of WNS prior to, or at the time of, sample collection.
404 In the remaining site, WNS was observed the following winter. While these results
405 would seem to suggest that WNS may be an inevitable outcome once *G. destructans* is
406 introduced into a hibernaculum, it is important to interpret these results cautiously
407 because a relatively small number of bat hibernacula were sampled, and all positive sites
408 were located within the same geographic area. Thus, it is unknown how clinical signs of
409 WNS and disease severity may vary as *G. destructan*s spreads to new regions of North
410 America with different environmental conditions and host species. The potential
411 importance of site-specific factors in their relation to WNS may be highlighted by the
412 detection of *G. destructans* in only one of the four buffer sites, which could suggest that
413 different hibernacula have different latency periods between the arrival of *G. destructans*

414 and the manifestation of the disease in bats, or that fungal abundance thresholds that
415 result in the appearance of WNS (i.e. infective doses of the fungus) vary between sites.
416
417 This study represents the first application of a high-throughput PCR technique for
418 directly detecting *G. destructans* in the environment. A previously described PCR assay
419 (16) utilized in a prior study to detect *G. destructans* in the environment lacked
420 specificity and required cloning and sequencing procedures to differentiate DNA of *G.*
421 *destructans* from that of other closely related *Geomyces* spp. common in cave sediment
422 (15,21). Additionally, the real-time PCR assay that targets the Alpha-L-Rhamnosidase
423 gene of *G. destructans* (30) was not tested against environmental samples, but may lack
424 the sensitivity necessary to detect the fungus in sediment given that the Alpha-L-
425 Rhamnosidase gene likely exists at a low copy number within the genome of *G.*
426 *destructans* (17). The work described herein confirms the specificity and sensitivity of a
427 previously developed PCR method that targets the IGS region of *G. destructans* (17) and
428 supports its application for use with environmental samples. Furthermore, the detection
429 of the fungus in a buffer site suggests that PCR screening of sediment samples within
430 caves may allow for early detection of *G. destructans* prior to manifestation of visible
431 signs of disease in bats inhabiting a hibernaculum. The relatively low estimated detection
432 probability for samples collected from buffer sites relative to diseased sites may have
433 been an effect of our inability to estimate infection status and detection probability
434 separately for buffer and infected sites. Thus, we may have underestimated detection
435 probability by overestimating the proportion of buffer sites that were infected.
436 Additionally, the probability of detecting the fungus at buffer sites prior to disease onset

might be enhanced by collecting sediment samples in early fall instead of mid-winter (i.e. months before a hibernaculum might become diseased as opposed to a full year before manifestation of WNS). Also, future work to determine whether certain types of environmental samples or specific locations within caves and mines are more likely to harbor *G. destructans* may further enhance sensitivity of detection.

While using PCR to detect *G. destructans* can provide important information about certain aspects of WNS disease ecology, the method is limited in that it cannot discriminate between viable and non-viable fungus. This is of particular importance in determining what role the environment plays in maintaining infectious populations of *G. destructans*. Detection of live *G. destructans* in 7 of the 14 caves and mines in late summer provides the first evidence that *G. destructans* is capable of surviving in bat hibernacula when bats are either absent or at low densities and that caves and mines serve as likely infection sources when bats return for hibernation in early autumn. However, our ability to culture viable *G. destructans* from sediment samples collected in 2011 from sites C5 and C9 suggests the fungus can survive much longer than a few months in the environment in the absence of a bat host. Specifically, bats had not been observed in one of the sites (C9) for approximately one year prior to sample collection, and site C5 had been sealed such that bats were excluded from the hibernaculum for approximately two years prior to sample collection. Demonstration that sediments from these two mines contained live *G. destructans* one to two years after bats had been extirpated/excluded, indicates the fungus can persist long-term in caves and mines.

460 The culture technique used for this experiment lacked the sensitivity of the molecular
461 detection technique for sites known to be infested with *G. destructans*. Specifically, there
462 was a lack of correlation in detecting viable *G. destructans* across replicate, serially-
463 diluted, and spatially- and temporally-separated samples collected within the same sites.
464 The mean probability of detecting *G. destructans* from contaminated sediment was 0.14
465 with at least 20 samples required from an average site to have a 95% chance of detecting
466 the fungus using the described culture technique. However, detection probabilities varied
467 greatly by site with some sites still not reaching a 50% detection probability with 15
468 samples. Clumping or aggregation of *G. destructans* within sediment, competition or
469 inhibition by other fungi on the artificial culture medium, low abundance of *G.*
470 *destructans* in environmental samples relative to other fungi, differences in abundance of
471 *G. destructans* between sites, and/or differences in abundance between locations within
472 the same site may account for these discrepancies. Whatever the reason, the described
473 culture-based technique is valuable to demonstrate that viable *G. destructans* is present in
474 a tested sample. However, the technique is currently neither suitable for quantifying
475 abundance of *G. destructans* nor for proving the absence of the fungus in environmental
476 samples. Future work focusing on developing a medium that is more selective for *G.*
477 *destructans* may serve to improve the utility of culture-based methods for addressing
478 research questions such as how long the fungus remains viable in different environments,
479 what portions of hibernacula are most conducive to supporting *G. destructans* (including
480 cave ceilings where bats roost and therefore may be most likely to come into contact with
481 the fungus), whether *G. destructans* can propagate (as opposed to simply persist) in

hibernacula without bats, and how abundance of the fungus changes spatially or temporally within sites.

Disease ecology is often represented by a triad that involves interactions between a host, a pathogen, and an environment. To date, research on WNS has focused primarily on bats, *G. destructans*, and interactions between the two. Relatively little information is available regarding the interplay between the pathogen and the environment. This work demonstrates the utility of environmental sampling for enhancing WNS surveillance and furthering research on WNS epidemiology. Specifically, the results of this study show that presence of *G. destructans* in environments where bats hibernate is strongly correlated with disease manifestation; the fungus may be detectable in the environment prior to disease manifestation; and the fungus can persist in the sediment of bat hibernacula for long periods of time in the absence of bat hosts. Additional studies to more fully elucidate the role the environment plays in supporting proliferation of *G. destructans* and facilitating the development and progression of WNS will reveal important factors related to the epidemiology of WNS and may provide information useful in WNS disease management.

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FIGURES AND TABLES

Fig. 1. Map of the eastern U.S. showing the sampling locations described in Table 1. The left halves of the circles depict the disease status of individual bat hibernacula at the time the samples were collected (red = diseased, blue = buffer, yellow = clean). The right halves of the circles represent the PCR results (red = *G. destructans* detected, yellow = *G. destructans* not detected). The solid white line marks the general geographic range of

665 WNS (i.e. WNS zone) at the time of sample collection; the dotted white line marks the
666 general geographic range of WNS during the following winter (i.e. buffer zone) (see
667 http://www.whitenosesyndrome.org/resources/map). *Geomyces destructans* was only
668 detected in bat hibernacula that were situated within the known range of WNS as of the
669 winter of 2008-2009. The PCR results for sites 20, 21, 22, and 23 only represent those
670 from samples collected in the winter of 2008-2009 and not those collected in the winter
671 of 2010-2011.
672

673 Table 1. Bat hibernacula in the eastern U.S. from which sediment samples were collected
674 in the winter of 2008-2009 to test for the presence of *G. destructans* by real-time PCR.
675

Site Designation	State	Geographic Zone	Cave/Mine Status	PCR Result
1	NH	WNS	diseased	+
2	NH	WNS	diseased	-
3	VT	WNS	diseased	-
4	VT	WNS	buffer	+
5	VT	WNS	diseased	+
6	MA	WNS	diseased	+
7	MA	WNS	diseased	+
8	MA	WNS	diseased	+
9	CT	WNS	diseased	+
10	NY	WNS	diseased	+
11	NY	WNS	diseased	+
12	NY	WNS	diseased	+
13	NY	WNS	diseased	+
14	PA	WNS	diseased	+
15	PA	buffer	clean	-
16	PA	buffer	clean	-
17	PA	buffer	clean	-
18	WV	WNS	clean	-
19	WV	WNS	diseased	+
20	WV	WNS	diseased	-
20*	WV	WNS	diseased	+
21	WV	WNS	clean	-
21*	WV	WNS	diseased	+
22	WV	WNS	buffer	-
22*	WV	WNS	diseased	+
23	VA	WNS	buffer	-
23*	VA	WNS	diseased	+
24	VA	WNS	buffer	-
25	VA	buffer	clean	-
26	NC	outside	clean	-
27	NC	outside	clean	-
28	GA	outside	clean	-
29	FL	outside	clean	-
30	FL	outside	clean	-
31	AL	outside	clean	-
32	AL	outside	clean	-
33	AL	outside	clean	-

34	TN	outside	clean	-
35	TN	buffer	clean	-
36	TN	buffer	clean	-
37	KY	outside	clean	-
38	KY	outside	clean	-
39	KY	outside	clean	-
40	KY	outside	clean	-
41	OH	outside	clean	-
42	OH	outside	clean	-
43	OH	outside	clean	-
44	IN	outside	clean	-
45	IN	outside	clean	-
46	IL	outside	clean	-
47	IL	outside	clean	-
48	IL	outside	clean	-
49	AR	outside	clean	-
50	MO	outside	clean	-
51	WI	outside	clean	-
52	WI	outside	clean	-
53	WI	outside	clean	-
54	MI	outside	clean	-
55	MN	outside	clean	-

676

677 * Sites re-sampled in winter 2010-2011; “geographic zone,” “cave/mine status,” and

678 “PCR result” depict results from winter 2010-2011. Sites with the same number, but

679 lacking this symbol represent results from winter 2008-2009 for these same sites.

680 Table 2. Bat hibernacula within the WNS-affected area of the U.S. in which sediment
681 samples were tested for the presence of viable *G. destructans* using a culture technique.

Site Designation	State	Sampling Location	Culture Result		
			Winter 2010-11	Summer 2011	Winter 2011-12
C1	VA		-	+	+
		1	-	-	-
		2	-	+	-
		3	-	-	-
		4	-	-	+
		5	-	-	-
C2	VA		-	-	-
		1	-	-	-
		2	-	-	-
		3	-	-	-
		4	-	-	-
		5	-	-	-
C3	VA		-	-	-
		1	-	-	-
		2	-	-	-
		3	-	-	-
		4	-	-	-
		5	-	-	-
C4	VT		+	+	+
		1	-	+	-
		2	+	-	-
		3	+	-	-
		4	-	-	+
		5	+	+	-
C5	VT		+	-	+
		1	+	-	+
		2	-	-	-
		3	-	-	+
		4	-	-	+
		5	-	-	-
C6	NH		+	+	n/a
		1	+	-	n/a
		2	-	+	n/a
		3	-	-	n/a
		4	-	-	n/a

		5	-	-	n/a
C7	NH		+	-	n/a
		1	-	-	n/a
		2	+	-	n/a
		3	-	-	n/a
		4	-	-	n/a
		5	-	-	n/a
C8	NH		+	+	n/a
		1	-	+	n/a
		2	+	+	n/a
		3	+	-	n/a
		4	-	-	n/a
		5	-	-	n/a
C9	NH		-	+	+
		1	-	-	-
		2	-	-	-
		3	-	-	+
		4	-	+	+
		5	-	-	-
C10	WV		-	-	-
		1	-	-	-
		2	-	-	-
		3	-	-	-
		4	-	-	-
		5	-	-	-
C11	WV		-	+	-
		1	-	+	-
		2	-	-	-
		3	-	-	-
		4	-	-	-
		5	-	-	-
C12	WV		+	-	-
		1	-	-	-
		2	+	-	-
		3	-	-	-
		4	-	-	-
		5	-	-	-
C13	WV		+	-	-
		1	-	-	-
		2	-	-	-
		3	-	-	-
		4	+	-	-

		5	-	-	-
C14	WV		+	+	-
		1	-	-	-
		2	+	-	-
		3	-	-	-
		4	-	+	-
		5	-	-	-

682

683 n/a No sample collected

684