White-Nose Syndrome in 2015: A review of current knowledge and potential management strategies of the lethal disease of North American bats

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Introduction

White-nose syndrome (WNS) is a disease of insectivorous hibernating bats in eastern North America that is rapidly becoming one of the most destructive wildlife diseases to emerge in recent history. Named for the characteristic white fungus found on the nose, ears, and wing membranes of infected bats, WNS has caused over six million deaths in North America since its emergence in 2006 (Meteyer *et al.* 2009). The causative agent has been identified as the psychrophilic (cold-loving) pathogenic fungus *Pseudogymnoascus* (formerly *Geomyces*) *destructans* (*Pd*) (Lorch *et al.* 2011; Alves *et al.* 2014). WNS is the first identified highly pathogenic disease to target bat populations in large numbers and is the first invasive cutaneous ascomycosis to be reported in mammals (Ehlman *et al.* 2013; Turner *et al.* 2014).

WNS was first discovered in the winter of 2006-2007 at Howes Cave in Albany, New York, and has spread rapidly across the eastern United States and Canada, over 1,200 miles from its epicenter (Figure 1: whitenosesyndrome.org; Alves *et al.* 2014).

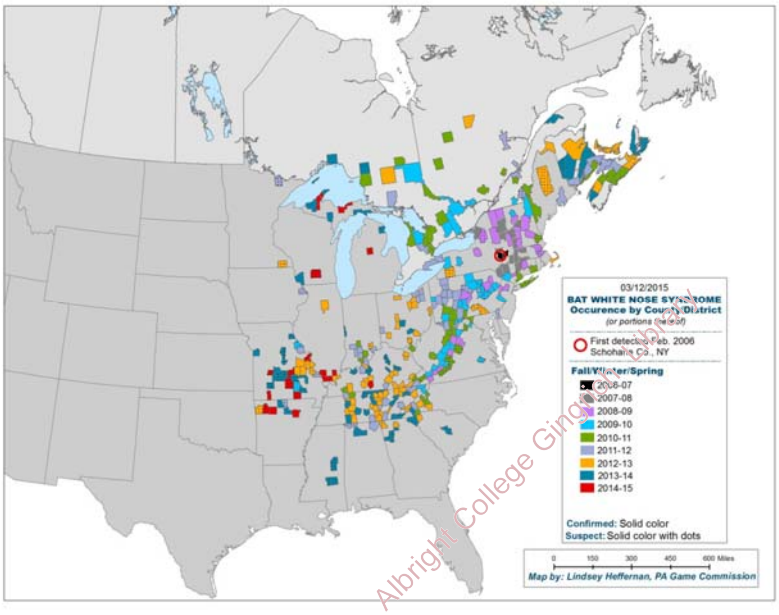

Figure 1. Map of current spread of WNS in North America (as of March 12, 2015) showing progression of the disease over time and status ("confirmed" or "suspect"). The presence of *Pd* has been confirmed in 28 states and 5 Canadian provinces (https://www.whitenosesyndrome.org/about/where-is-it-now).

To date, seven species of North American bats have been identified with diagnostic symptoms of WNS, including the big brown bat (*Eptesicus fuscus)*, eastern small-footed bat (*Myotis leibii*), gray bat (*Myotis grisescens*), Indiana bat (*Myotis sodalis*), little brown bat (*Myotis lucifugus*), northern long-eared bat (*Myotis septentrionalis*) and tricolored bat (*Perimyotis subflavus*). Several North American species have been identified with presence of

Pd but no diagnostic signs have yet been documented: eastern red bat (*Lasiurus borealis*), southeastern bat (*Myotis austroriparius*), silver-haired bat (*Lasionycteris noctivagans*), Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), and the Virginia big-eared bat (*Corynorhinus townsendii virginianus*) (whitenosesyndrome.org).

In North America, *Pd* has been isolated only from the bodies of bats exhibiting clinical signs of WNS or in the soils of environments where WNS mortality has occurred. Thus, it is believed that transmission of the disease occurs through direct bat-to-bat contact or indirectly through contact with fungal spores in the environment (Zukal *et al.* 2014). Patterns of disease spread suggest that *Pd* is either an exotic-invasive species recently introduced to North America, a native species that only recently became pathogenic to bats, or a co-factor of an unknown primary mortality agent (Flory *et al.* 2012). However, current consensus is that *Pd* is an invasive species introduced from Europe.

Infectious diseases occur when a pathogen affects a susceptible host population in favorable environmental conditions. For WNS, the pathogen is the fungus *Pd*, the hosts are hibernating insectivorous bats, and the environments are hibernacula – cold, humid, underground sites in which hibernating bats spend the winter (Blehert 2012). Once a bat is exposed to *Pd*, countless factors can influence disease progression. The concept of the disease triangle represents the relationships of different factors through the interactive process of a susceptible host, a virulent pathogen, and a favorable environment (Figure 2). The triangle provides a useful framework for management since different strategies target different components of the triangle. In particular, this concept is useful for analysis of plant and animal diseases in natural habitats, where environmental conditions may affect pathogen survival and exposure of the host to the pathogen (Knudsen *et al*. 2013).

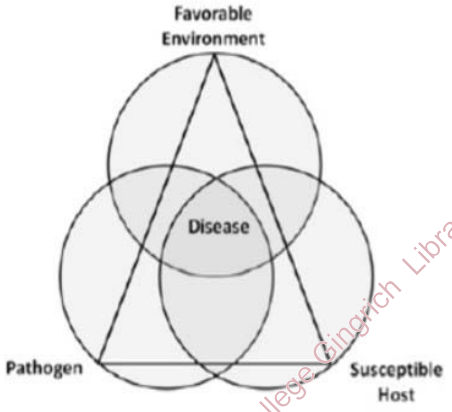

Figure 2. The disease triangle, showing interrelationships between a host, pathogen, and favorable environment (Turner *et al.* 2011).

Emergent and infectious wildlife diseases like WNS present challenges to conservation because of their rapid, unforeseen onset and high rates of mortality. Because understanding of emergent diseases is lacking in the initial states, there is potential for global spread and large numbers of deaths while scientists struggle to gather information and create and implement management strategies, as is the case with WNS (Puechmaille *et al.* 2011a). It is critical to gain as much understanding as possible about WNS in order to establish successful methods of combating the disease.

The Fungus

Fungi are heterotrophs that obtain carbon for growth from dead or decaying organic material or from parasitic relationships with plants, animals, and other fungi (Figure 3). Some parasitic fungi are opportunistic pathogens, which are only able to infect hosts that are physiologically or immunologically compromised. When infecting animals, fungi affect a host in one of three ways: 1) by directly invading host tissue (mycosis); 2) by causing allergic reactions that lead to host development of hypersensitivity to fungal pathogens; and 3) through ingestion of toxic fungal metabolites (mycotoxins). Although the exact ecological role of *Pd* is yet to be identified, it appears that mycosis is the primary mode of action (Knudsen *et al.* 2013).

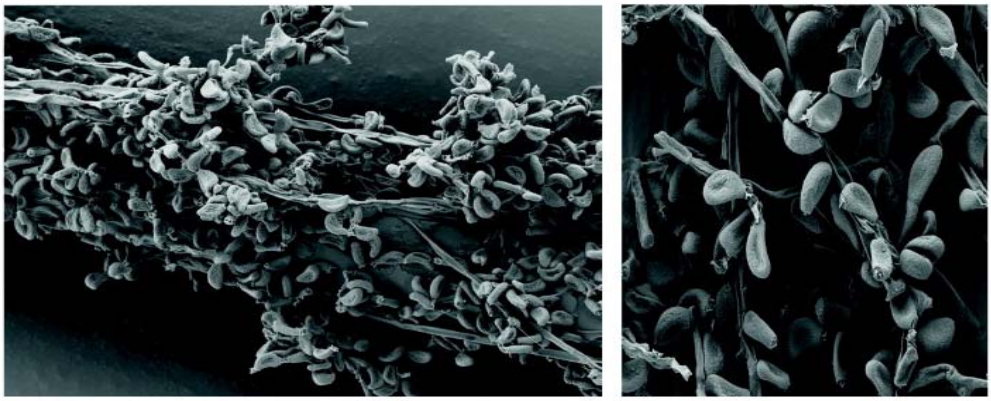

Figure 3. Scanning electron micrograph showing (left) a hair of *M. myotis* colonized with *Pd*, and (right) conidia (spores) of *Pd* (Puechmaille *et al.* 2011).

Originally, WNS was thought to be caused by the fungus *Geomyces destructans*, but Minnis and Lindner (2013) reclassified the fungus as *Pseudogymnoascus destructans*, a member of an extremely diverse genus of fungi. Like many plant pathogens, *Pd* is an ascomycete fungus. Ascomycete plant pathogens penetrate the plant cuticle and produce distinct subsurface hyphae that release virulent chemical products. *Pd* may behave similarly to fungal pathogens of plants; after penetration of the epidermis, hyphae might secrete proteins, metabolic products, and enzymes that contribute to tissue erosion in bats (Turner *et al.* 2014).

Fungi in the genus *Pseudogymnoascus* are slow-growing, utilize nitrogen, are tolerant of alkaline conditions, and can saprobically utilize different complex carbon sources in caves (Raudabaugh and Miller 2013). Optimal temperatures for growth of *Pd* are between 12.5°C and 15.8°C, with an upper critical range between 19.0°C and 19.8°C (Verant *et al.* 2012). The fungus is not species, genus, or family specific in its host (Zukal *et al.* 2014).

Lindner *et al.* (2011) provided the first analysis of the environmental occurrence of the fungus responsible for WNS. The study isolated *Pd* in soil samples from bat hibernacula in the eastern United States using PCR testing, establishing the environment as a possible reservoir for the fungus. Lindner *et al.* suggested that the occurrence of *Pd* in soil from hibernacula environments indicates that, if the fungus is viable, it is possible that humans or other animals that enter the infected sites can spread the fungus to other unaffected areas. Given this possibility, more research is needed to determine the role of infected environments in transmission of *Pd* and the spread of WNS in North America.

Given that *Pd* can persist in cave sediments even in the absence of bats, Reynolds and Barton (2014) compared the function of several enzymes that could be beneficial for *Pd* in saprotrophic (feeding by absorbing dead or decaying matter) or pathogenic context. Enzymes

such as lipases, hemolysins, ureas, chitinases, and cellulases are required for growth on major components of cave ecosystems and may support the saprotrophic growth and pathogenic lifestyle of *Pd* (Reynolds and Barton 2014). Cave environments are limited in carbon and energy due to lack of sunlight to drive photosynthesis, therefore fungal substrates available are in the form of soil detritus, organic waste of trogloxenes (animals that spend only a portion of their life cycles in caves), and bats with their chitin- and urea-rich guano (Reynolds and Barton 2014). *Pd* found in caves can produce enzymes to utilize these organic substances by functioning as generalist decomposers, suggesting that the pathogen has an environmental origin. It is still unknown how or why *Pd* moved from a cave soil substrate into a bat host (Reynolds and Barton 2014).

Characteristics of affected species

Bats comprise one-fifth of all mammal diversity on the planet. Of the 45 species that are found in the United States, more than half hibernate in caves, mines, and/or deep rock crevices. Most bats hibernate collectively, with aggregations in the thousands, and they vary geographically and among species in hibernation behavior, roosting, sociality, clustering in hibernacula, and regional migration (Knudsen *et al.* 2013).

Social behavior and group formations of bats differ between species and sexes as well as among seasons. In the spring, females travel to maternity colonies to reproduce, and males tend to spend the spring and summer seasons away from females, roosting alone or within small groups at cooler locations. In autumn, males and females reunite for the mating season, and during the winter both sexes hibernate together (Foley *et al.* 2010). Females store sperm until the following spring when fertilization takes place (Harvey *et al.*1999). Bat species have high annual adult survival and low fecundity (females typically produce one offspring per year), which result in modest population growth rates (Foley *et al.* 2010).

Adult females enter hibernation with greater fat stores, which they consume more slowly than males or juveniles, making females more likely to survive the winter with WNS. However, WNS females may not be able to energetically support reproduction after emerging from hibernation. Male survivors are more likely to be fungus vectors because their summer roosts tend to be in colder locations where the fungus may survive. Additionally, males participate in larger-scale migrations that make them more likely to spread the pathogen (Jonasson and Willis 2011).

Bats must accumulate enough fat stores to survive hibernation lengths of 6-8 months (Flory *et al.* 2012). To cope with food shortages and adverse temperatures during the winter, bats enter a state of decreased physiological activity called torpor, which is characterized by reduced metabolic rates and drop in body temperature to reduce expenditure of precious energy reserves (Ehlman *et al.* 2013). Throughout hibernation, long bouts of torpor are interspersed with short periods of arousal (Verant *et al.* 2012).When in torpor, hibernating bats reduce their metabolic rates by 96-98% and downregulate immune responses, which do not return to normal functioning until basal metabolic rates and body temperature increase to euthermic levels (such as during arousal). The physiological conditions of torpor may make hibernating bats more susceptible to infection by *Pd* (Meteyer *et al.* 2009) because mammalian endothermy is an effective defense against fungal invasion. During hibernation, bats regulate body temperature to below-normal euthermic temperatures of 30-40°C, which may partially nullify the defense of endothermy. Body temperature during torpor decreases to within a few degrees of the ambient temperature in hibernacula, which typically ranges from 3-15°C (Flory *et al.* 2012). Body temperatures of

hibernating bats are typically 2-15°C, a range that coincides with optimal growth temperatures of *Pd* (Knudsen *et al.* 2013). A decrease in body temperature and immune system suppression combined with the cold, dark, and damp environments of hibernacula provide favorable conditions for the growth of the cold-loving fungus (Flory *et al.* 2012).

Euthermic arousal periods are 1% of the total time budget during hibernation; however, 80-90% of fat reserves are consumed during these arousals. Alterations in torpor patterns may contribute to increased mortality due to premature depletion of fat stores (Reeder *et al.* 2012). Severity of infection may be correlated with an increase in arousal frequency and/or length of arousal, meaning that bats in the later stages of disease progression are more likely to die before the end of the winter season due to lack of sufficient metabolic reserves (Reeder *et al.* 2012).

Clustering during hibernation is common in North American bat species in order to take advantage of collective body heat and thus reduce the amount of energy expended to maintain individual body temperature. Greater clustering of bats with WNS may increase the rate of bat-to-bat transmission of *Pd*, but clustering behavior is not indicative of infected bats (Zukal *et al.* 2014). It is interesting to note that reduced clustering has been observed in many species of European bats, a behavior that is speculated to be a co-evolutionary adaptation to the fungus (Ehlman *et al.* 2013). This difference in hibernation behavior might be a factor in the severity of WNS in North American populations while their European counterparts are comparatively less affected.

The volant (capable of flight) lifestyle of bats leads to high rates of infection transmission and spread (Foley *et al.* 2010). Infection of the wings that allow for this unique mammal lifestyle can prove fatal if normal functioning is impaired. Normal wing structure and function are critical for flight, feeding, predator avoidance, and physiological homeostasis, and thus are critical for bat survival (Fuller *et al.* 2011) Wing membranes are thin and highly vascularized to facilitate passive cutaneous gas exchange, maintain water balance by preventing evaporative water loss, regulate blood pressure, and support thermoregulation (Blehert 2012). In addition to investigating the role of wing physiology with WNS, there are ongoing studies of wing membrane microfauna and the potential roles they may have in differential survival among bat species (Turner *et al.* 2011).

Symptomology of disease

WNS is named for the characteristic white fungus that grows on the epithelial tissue of the nose, ears, and wing membranes of infected bats (Figure 4). *Pd* penetrates the skin and invades the dermal layers, an ability that makes the fungus particularly lethal by causing tissue infarction and necrosis (Figure 5) (Ehlman *et al.* 2013). Hyphae of the fungus invade and fill epidermal glands and then penetrate underlying tissues and capillary beds, causing erosion of tissue (Fuller *et al.* 2011). Severity of these symptoms ranges from cup-like intraepidermal colonies with erosions to extreme ulceration of skin and deep penetration by fungal hyphae into underlying connective tissue. On the muzzle, fungal hyphae fill hair follicles and sebaceous and apocrine glands with penetration of the underlying connective tissue as well (Wibbelt *et al.* 2013).

Figure 4. Tri-colored bat with visible signs of WNS on muzzle, ears, and wings. (https://www.whitenosesyndrome.org/resources/image-gallery).

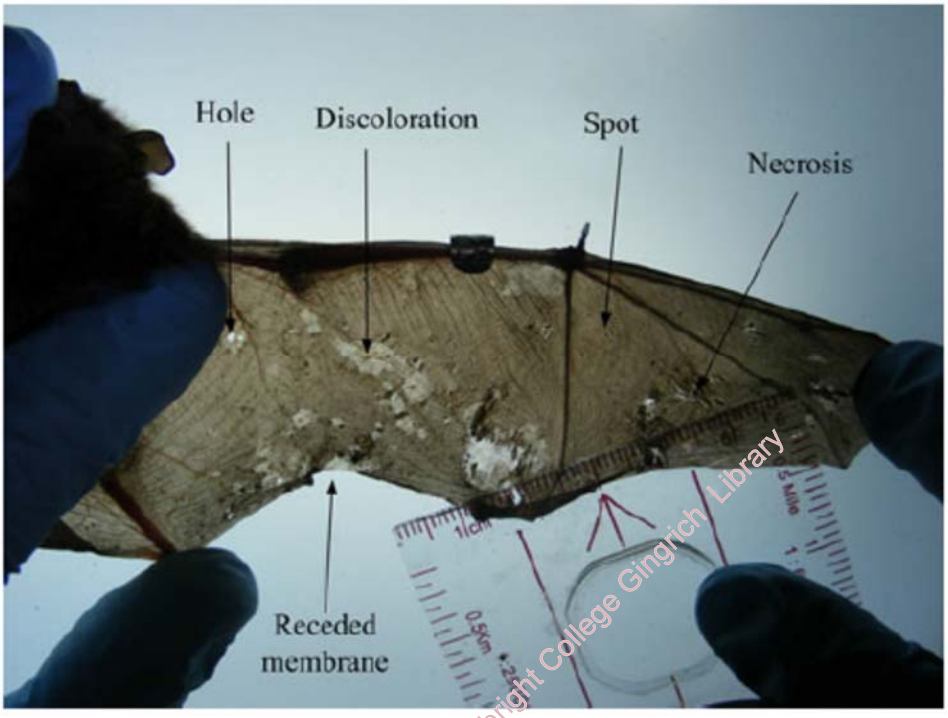

Figure 5. Wing damage defined by discoloration, tears, holes, flaking, necrosis, receded areas, and missing tissue. (Fuller *et al.* 2011).

The later stages of WNS are identified by presence of delicate, white filaments of fungus that obscure the muzzle, and on the wing membranes, the fungus appears as a dense, white film with varying degrees of thickness. Changes in the epithelium of infected bats are inconsistent and nonspecific, but patches of rough skin on face, ears, forearms, and wings have been observed. Less noticeable signs are dullness of glabrous skin and irregular pigmentation, areas of contraction, or small tears in wing membranes (Meteyer *et al.* 2009).

Major inflammation is usually not observed in infected tissues, most likely due to immune system repression during hibernation that prevents a response to the invading pathogen (Warnecke *et al.* 2012); although in some rare cases, bats have exhibited edema, neutrophilic granulocytes, and intradermal abscesses in connective tissue (Wibbelt *et al.* 2013). Infectious fungal agents can be primary pathogens or invade secondarily due to predisposing factors, such as co-infections by other pathogens. However, WNS bats display no significant changes to internal organs, and no bacterial or viral pathogen has been detected that would indicate a cofactor of infection. Experimental infection of bats with *Pd* produces similar lesions that are found on infected bats in the wild so WNS is a certainly a result of *Pd* infection, but it is still possible that the disease is a result of other pathogenic agents, as well (Wibbelt *et al.* 2013).

Physical examination of infected bats shows very little remaining body fat. Typically, mortality is observed in the latter half of the hibernation season, and the bats that survive the winter with infection emerge from hibernation emaciated and dehydrated (Warnecke *et al.* 2012). Additional anecdotal observations of infected bats include altered sensory thresholds, tremors of the forearms when crawling, flight during daytime and collisions with large stationary objects (building walls), and extreme thirst (evidenced by licking of snow or prolonged flying over areas of open water). These symptoms may be results of starvation and/or electrolyte imbalance (Turner *et al.* 2011).

Torpor patterns are possibly altered with WNS infection, and irregular torpor characteristics may result in increased activity during hibernation. More frequent arousals, longer arousals, elevated body temperatures, and aberrant behaviors like irregular flight patterns have been observed likely as consequences of behavioral or immune responses to the disease as well as physical wing damage that disrupts water balance (Britzke *et al.* 2010; Reeder *et al.* 2012).

Mortality associated with WNS has caused 30-100% declines in bat populations within infected hibernacula, causing collapse of regional populations (Figure 6) (Puechmaille *et al.* 2011). Although *Pd* has been established with certainty as the causal agent of WNS in North America, the exact pathomechanisms of the fungus in causing mortality are relatively unknown. Death associated with WNS might be related to close relationships between physical damage caused by *Pd* and the time and stored energy available to hibernating bats for coping with infection (Cryan *et al.* 2013). Different hypotheses exist for the mechanism of mortality regarding behavioral aberrations and physiological disruption during hibernation (Zukal *et al.* 2014).

Figure 6. Carcasses of infected bats piled on the floor of a hibernaculum in Vermont, United States, demonstrating the mass mortality caused by WNS (Puechmaille *et al.* 2011).

Arousal and fat depletion

Infection of bat wings may contribute to WNS mortality through physical or behavioral disruptions of homeostasis during hibernation that result in arousal (Flory *et al.* 2012). Increase in arousal frequency during hibernation due to *Pd* infection may lead to premature fat depletion and subsequent death of infected bats by starvation or freezing. Observations of wild bats and laboratory testing has confirmed that bats indeed arouse from torpor more often when infected with WNS, especially in the later stages of infection; however, fungal infection of the skin may not coincide with death (Flory *et al.* 2012). Depletion of energy caused by increased arousal certainly contributes to mortality in WNS bats, but the exact mechanism of triggering arousal is still unknown (Cryan *et al.* 2013).

Evaporative water loss and dehydration

There is increasing evidence that evaporative water loss (EWL) during hibernation causes dehydration and electrolyte depletion that leads to death (Cryan *et al.* 2013). The dehydration hypothesis asserts that cutaneous infection of wing membranes causes dehydration that increases arousal frequency during hibernation, suggesting that high EWL increases susceptibility to WNS (Willis *et al.* 2011). Loss of water by evaporation occurs across the cutaneous and pulmonary membranes of hibernating bats, such as on the wing membranes. EWL and dehydration caused by WNS could stimulate arousal of hibernating bats to drink and maintain water balance, thus increasing energy expenditure (Ehlman *et al.* 2013). Some studies have shown that there is a close relationship between EWL and periodic arousals during hibernation, making it likely that EWL is associated with mechanisms of mortality of the disease.

Diagnosis

Although useful, visual inspection of bats cannot distinguish between healthy and infected bats. Laboratory tests for detection of *Pd* include PCR, culture methods, and histology. Histopathology is commonly used to diagnose WNS by analysis and identification of aggregates of *Pd* fungal hyphae that form cutaneous erosions and ulcerations in the wing membrane (Turner *et al.* 2014). In order to conduct a thorough histopathologic analysis, euthanasia is usually needed to collect adequate samples of wing membrane because large amounts of tissue are required. Though this method produces definitive results for diagnosis of WNS, the detrimental effects of the disease on bat populations necessitates the development of detection protocols that do not require euthanasia.

Previously, there were no non-lethal, on-site, preliminary screening methods available for diagnosis of WNS in bats; however, Turner *et al.* (2014) have suggested the use of ultraviolet (UV) fluorescence to detect lesions characteristic of WNS (Figure 7). The technique includes illumination/ transillumniation of wing membranes of bats with WNS using long-wavelength UV light (360-385 nm) to produce an orange-yellow fluorescence that identifies with the presence of fungal cupping erosions in the epidermis. Severe cases of WNS produce numerous and large aggregate regions of fluorescence that correlate with presence of the fungus and are easily identified upon UV examination. Mild WNS produces random, thin, and isolated areas of fluorescence, making diagnosis more difficult but still possible (Turner *et al.* 2014).

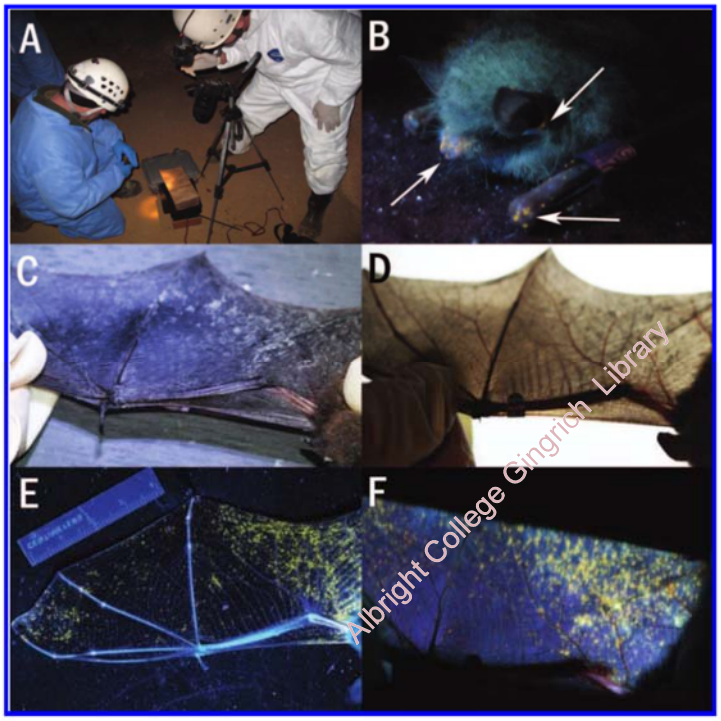

Figure 7. Long-wave ultraviolet (UV) and white-light illumination of WNS lesions. (A) Camera mounted in a cave to transilluminate bat wings with UV light. (B) Orange-yellow fluorescent spots indicated by arrows on a roosting *M. sodalis*. (C) *M. lucifugus* wing membrane lit with white lights shows areas of fungal growth. (D) Transillumination of *M. lucifugus* wing membrane shows absence of fungal infection or wing damage. (E) Wing of dead *P. subflavus* lit with UV light to show orange-yellow fluorescent points. (F) Transillumination of *M. lucifugus* with fluorescent UV light shows lesions (Turner *et al.* 2014).

UV fluorescence provides a rapid, non-lethal, field-applicable method of detecting possible WNS infection and reduces the need to euthanize bats to obtain a diagnosis (Turner *et al.* 2014). Adoption of UV fluorescence detection would not only allow rapid screening of bats for preliminary diagnosis, it could be used to assist nonlethal collection of small (4mm) biopsy samples for histopathologic tests, PCR reactions, and cultures. Furthermore, this technique would allow identification of bats with lesions while limiting the disturbance of healthy bats within hibernacula (Turner *et al.* 2014).

Environmental Factors and WNS

Transmission and pathogenicity of fungus

Prior to infection, bats must come into contact with viable infectious propagules of the fungus, believed to be conidia (fungal spores). Some pathogenic fungi produce spores that are transported to the outer surface of the host where they germinate and penetrate the host epidermal tissue. Such fungi are typically sensitive to microenvironmental conditions. *Pd* probably fits this model because spores on infected bats can be a means of transmission to healthy bats (Knudsen *et al.* 2013). Furthermore, the presence of fungal spores on cave walls (and possibly in the air or water sources) suggests that hibernacula may act as passive vectors and/or reservoirs for the fungus and might be important in transmission of WNS (Puechmaille *et al.* 2011; Knudsen *et al.* 2013). Mechanisms of within-hibernaculum dispersal of pathogen (contact with cave walls, wind, and water splash) and transmission mechanisms between hibernacula and roosting sites need to be determined (Knudsen *et al.* 2013).

Mortality of bats

WNS has been detected in new areas of North America far from the location where the disease first emerged as well as in eight European bat species (as of 2012) from different countries. However, mortality was not observed in many of these North American locations or in any part of Europe (Flory *et al.* 2012). Evidence has strongly suggested that winter environmental conditions play a role in the deaths of diseased bats and may explain differences in mortality among North American locations and between North American and European bats (Flory *et al.* 2012).

It is possible that winter conditions inside and outside hibernacula that differ between continents (such as sustained subfreezing temperatures) may influence the survival of infected bats through the winter and function as a cofactor in the virulence of the disease (Flory *et al.* 2012). In regions where there are shorter winters or mid-winter feeding opportunities that allow supplementation of energy stores, bats emerging from hibernation may not be as overwhelmed by infection when returning to euthermic levels (Cryan *et al.* 2013). Microclimate conditions vary among sites, and selection of these conditions is different among species, which may correlate with the susceptibility differences of species (Knudsen *et al.* 2013). In general, bats tend to choose locations with high humidity and temperatures ranging from 3-15°C; however, details on underground hibernacula conditions are scarce (Flory *et al.* 2012).

Regional differences in North America exist for characteristics of *Pd*, rates of disease progression, and/or physiological traits of affected species, and it is possible that certain environmental conditions must co-occur with fungal infection to cause mortality. Possible requisite environmental conditions have been modeled for mortality in addition to presence of the fungus (Flory *et al.* 2012). Models suggested that WNS is most likely to occur in landscapes that are higher in elevations, topographically diverse, dry and cold during winter, and seasonally

variable. This information is useful for selecting top-priority sites to be monitored for WNS and to predict potential spread of the disease (Flory *et al.* 2012).

Environmental limits of WNS may be a reason for the restricted distribution of mortality relative to overall distribution of infection (Flory *et al.* 2012). A likely connection exists between humidity within hibernacula and susceptibility to WNS (Cryan *et al.* 2013). Humidity and temperature conditions on the skin of hibernating bats may govern fungal virulence as conidial fungi germination typically depends on surface moisture of the growth substrate. Similarly, temperature and humidity are factors in choice of hibernacula by bats and such environmental variation of hibernacula microclimate could influence the extent and severity of *Pd* infection between bat species and hibernacula (Cryan *et al.* 2013).

WNS in Europe

Little is known about *Pd* infection in European bats, and knowledge of pathological effects of WNS in these species is even less well understood. Additionally, the different effects of WNS on bat mortality between North America and Europe are unknown (Zukal *et al.* 2014). European bats have been observed with *Pd* colonization but without the symptoms and mortality of infected North American bats. Several hypotheses exist to explain the differences in *Pd* distributions and WNS manifestations between Europe and North America which include factors such as intercontinental differences in bat physiology and behavior, environmental conditions, and growth properties of *Pd* strains (Puechmaille *et al.* 2011).

Pd has previously existed in Europe and has only recently invaded North America

The first observed case of WNS in North America occurred in Howes Cave in Albany, New York. This location is the largest commercial cave in the northeastern United States, so it is possible that high human visitation rates resulted in anthropogenic introduction of the disease. There have been reports ranging from the 1970s to 1990s of white fungal growth on bats in Europe, although there is no evidence that the fungus was *Pd.* The presence of WNS in Europe been only recently been confirmed by histopathological analyses that demonstrate fungal infection in hibernating European bats (Pikula *et al.* 2012).

In contrast, there have been no reports or photographic documentation of such fungal growth on bat species in North America prior to 2006. If *Pd* were native, closely related species of the fungus should be present in hibernacula in eastern North America. Studies suggest, however, that there are no closely related sister taxa of *Pd* in this region (Minnis and Lindner 2013). The widespread occurrence of the fungus in Europe without the mass mortality of hibernating bats supports the accumulating evidence that *Pd* is an exotic and invasive species (Minnis and Lindner 2013).

Experimental inoculation with North American or European isolates of the fungus supports that *Pd* causes WNS and mortality in North American *M. lucifugus* (Warnecke *et al.* 2012). Bats inoculated with European *Pd* developed cutaneous infections characteristic of WNS, exhibited progressive increases in hibernation arousal frequency, and were emaciated after 3-4 months of infection. Indeed, analyses by Verant *et al.* (2012) indicate that fungal isolates from the two continents do not have naturally different growth properties. Both North American and European isolate are lethal to North American bat species, so differences in WNS may be a result of environmental conditions that influence the growth performance of *Pd* rather than specific variations of the pathogen itself. Because hibernacula temperature and humidity conditions differ by bat species, there is the likelihood that microclimate within hibernacula influences the

physiology and growth of *Pd* in such a way that allows it to colonize its bat host (Verant *et al.* 2012). North American bat susceptibility to both North American and European strains of the fungus suggests that *Pd* was introduced from Europe.

Furthermore, the absence of observed mortality in European bats despite widespread presence of *Pd* in Europe may reflect different physiological and behavioral responses rather than differences in pathogenicity of the strains (Puechmaille *et al.* 2011). Skin lesions on European bats affected with *Pd* are similar to those found on their North American counterparts; however, there is no presence of deep penetration of fungal hyphae into dermal connective tissue causing ulceration (Wibbelt *et al.* 2013). It is possible that *Pd* has occurred in the past in Europe and native bats have coevolved resistance (immune system responses) or tolerance (behavioral adaptations) that protect them from the detrimental effects of WNS (Wibbelt *et al.* 2013). Along with the absence of fungal-associated mass mortalities in European bats, these observations support the current leading hypothesis that *Pd* was introduced to North America from Europe.

Pd acts as an opportunistic pathogen with an unknown primary mortality agent

Because mass mortality has not been observed in European bats, it is possible that *Pd* is not the primary cause of death associated with WNS. *Pd* has been identified as the causative agent of WNS; however, the exact mechanism of mortality is unknown. There has not been evidence of organ failure, toxic elements, or bacterial or viral agents in infected bats that act as primary mortality agents, but this does not mean that a different causal agent is absent. It is imperative that the causal mechanisms of WNS are discovered in order to identify effective management strategies (Puechmaille *et al.* 2011). A third hypothesis describes *Pd* as a newly emergent fungal pathogen that recently surfaced in North America. Since its emergence, *Pd* has spread rapidly from its epicenter in a wave-like pattern, a pattern that is typical of a newly emergent pathogen. Therefore, *Pd* is possibly a new, virulent strain of a previously nonpathogenic widespread fungus in North America (Puechmaille *et al.* 2011.

Management Strategies

Supportive care and recovery of individual bats

An encouraging discovery is that bats have the potential to recover from WNS (Figures 8 and 9). During the winter months, inflammatory cell response may be delayed until arousal from hibernation because such a response during hibernation could potentially overwhelm the host rather than reducing infection (Cryan *et al.* 2013). However, recovery is a long and energetically expensive process most likely requiring consistent euthermic body temperatures (approximately 38°C), and hibernating bats do not possess enough fat storage to remain euthermic for long periods during the winter. Experimental recovery of free-ranging bats required an increase in body temperature and supportive care in the form of warmth, food, and water (Meteyer *et al.* 2011). Captive and wild bats have overcome infection after emerging from hibernation, with complete healing of wing membranes after several weeks.

The lack of an immune system inflammatory response and euthermic body temperatures during hibernation is an advantage for the fungus, and it is during this period that *Pd* causes the most physiological damage (Flory *et al.* 2012). Thus, increase in body temperature and immune system response after arousal may be adequate for recovery in post-hibernation bats. Bats may recover from WNS after hibernation only if they survive the post-emergence healing process, and so recovery is not guaranteed. Success of the healing process, the maintenance of adequate

water and energy balance, and the avoidance of predators despite wing damage are essential for effective recovery (Meteyer *et al.* 2011).

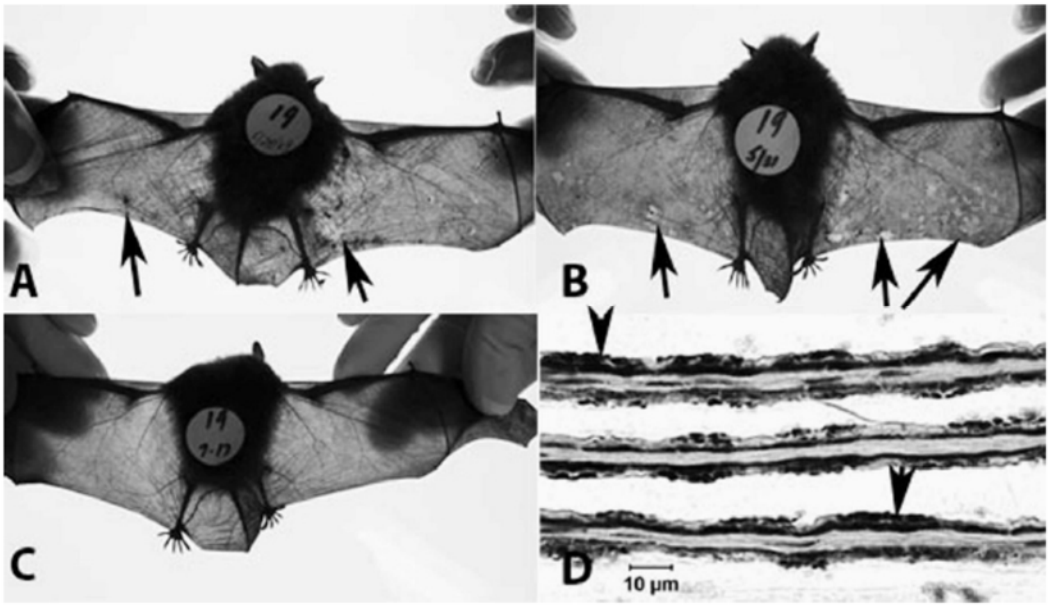

Figure 8. WNS wing damage on *M. lucifugus* from New Jersey, United States and signs of recovery. (A and B) Characteristic lesions (indicated by arrows) on infected wing membranes. (C) Signs of recovery without evidence of lesions after supportive care (Meteyer *et al.* 2011).

A large portion of recovery involves cutaneous wound healing that progresses through several stages: clotting, inflammation, re-epithelialization, wound contraction, and angiogenesis. During each stage, there is promotion of cell proliferation, microbial clearing, and tissue restructuring (Fuller *et al.* 2011). Experimentally treated bats likely have experienced accelerated healing during the rapid epithelialization and wound contraction stages. Free-ranging bats with severe wing damage healed to a condition of less severity of infection within two weeks. Thus, bats may be able to heal from wing damage caused by WNS during the active season given that they do not experience fatal complications associated with reduced wing functions or become overwhelmed by the immune system recovery response (Fuller *et al.* 2011).

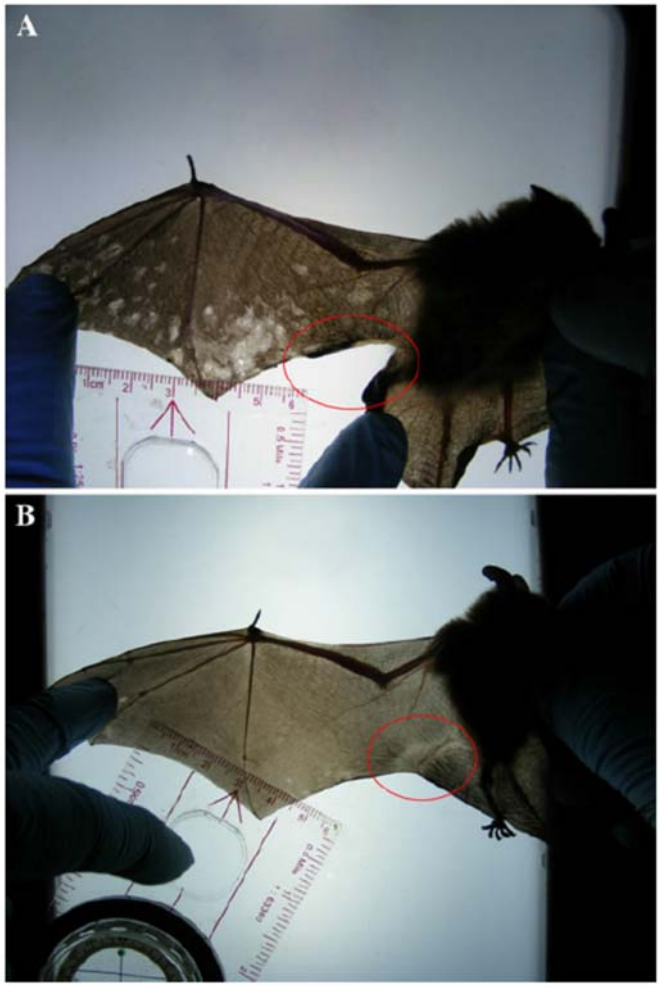

Figure 9. Transillumination of the wing membrane of an adult female *M. lucifugus*. (A) White lesions are visible and the circled region shows an area of lost tissue. Over a period of 57 days, lesions disappeared and missing tissue was recovered (B) (Fuller *et al.* 2011).

Food and water sources could be given to bats in hibernacula to provide more energy in preparation of post-hibernation recovery and to alleviate dehydration. This strategy is met with complications because hibernating species do not typically feed during the winter, and supplementing thousands of hibernating bats seems impractical. Nevertheless, reducing the amount of energy expended by infected bats may have benefits in the recovery process (Boyles and Willis 2009).

Unfortunately, viable fungal hyphae or conidia may remain associated with the epidermis and fur of infected bats even after recovery following hibernation, resulting in the chance of reinfection. The fungus may remain dormant during the active, homeothermic summer months and experience renewed growth during hibernation when conditions are cold and humid and body temperatures of hibernating bats drops below euthermic levels (Meteyer *et al.* 2011).

Environmental modification of hibernacula

In milder winter conditions, some bats with WNS infection may leave the hibernacula during arousal periods to feed and drink in order to survive the winter. However, in regions

where winters are colder and drier, insects are not active, and standing water is frozen so bats expend more energy than gained by being active. Without access to food and water in warmer conditions, WNS bats that arouse during hibernation have a greater chance of dying from starvation or dehydration. As a result, mortality of WNS infected bats is higher in these regions (Flory *et al.* 2012).

A proposed method of increasing survival of infected bats through the hibernation period is modification of microclimate within the hibernacula. Reductions in growth performance of *Pd* with changes in temperature have been documented in laboratory analyses (Verant *et al.* 2012). If such reductions were shown to decrease the pathogenicity of the fungus, a potential method of managing WNS would be to modify the ambient temperatures of hibernacula in small amounts (2-3°C) so to moderate disease progression (Verant *et al.* 2012).

Manipulation of temperature and humidity within hibernacula has its consequences. Although microclimate modification may reduce the growth of the fungus, it may also negatively affect the delicate ecosystems with caves and the bats themselves. Bats have evolved to survive hibernation in cold and humid conditions, so alterations of these conditions may actually reduce survival (Foley *et al.* 2010).

An alternative to modifying the ambient temperature of hibernacula might be to provide localized, warm-temperature refugia within affected sites, while keeping the overall hibernacula temperature cold (Boyles and Willis 2009). “Thermal refugia” could decrease heat loss during periodic arousals, reducing the amount of energy expended and thus increase survival of individual bats. The efficacy of this method would depend on the ability of bats to detect and travel to the refugia during arousals. The design of refugia would need to be specific for different environments of individual hibernacula, but the suggested general structure is a small heating unit affixed to cave walls to heat domes or crevices that are typically used by euthermic bats. Heaters would need to be temperature-regulated by thermostats to prevent detrimental increases that may harm the bats or surrounding cave microbiota that are sensitive to such changes (Boyles and Willis 2009).

Introduction of artificial heat sources to hibernacula is controversial, however, because cold temperatures seem to be essential for hibernation. It would be necessary to ensure that thermal refugia do not alter the overall temperature within hibernacula for this method of management to be successful (Boyles and Willis 2009).

Antifungal treatment of hibernacula and bats

The most effective chemical compounds against *Pd* are antifungal drugs, fungicides, and biocides. Antifungal drugs identified as active in the temperature ranges found in hibernacula and effective against certain strains of *Pd* are amphotericin B, fluconazole, intraconazole, ketoconazole, and voriconazole (Chaturvedi *et al.* 2011). However, the use of antifungal treatment in rehabilitation of affected species and decontamination of *Pd* in caves has its risks because decontamination using chemical compounds has deleterious effects on the environment. For example, some fungicides contain heavy metals such as mercury or cadmium that are toxic to organisms within cave habitats (Aley 2010). Chemical disinfectants used to decontaminate hibernacula must be chosen carefully to avoid potentially harming delicate cave microbiota (Shelley *et al.* 2013). Long-term effects of these compounds on the environment are unknown, so refinement of fungicide/biocide/antifungal drug use would be imperative to ensure that fungal organisms other than the target species (*Pd*) are not harmed (Chaturvedi *et al.* 2011). Complications may also arise in achieving complete coverage of infected areas with chemical

compounds due to the great internal volume and structural complexity of hibernacula (Foley *et al.* 2010). Furthermore, the presence of clays, sand, and silt found in mud within cave hibernacula might adsorb the reactive ions of disinfectants, counteracting the antifungal properties of the compounds and leaving surfaces vulnerable to reinfection (Shelley *et al.* 2013).

In situ treatment of bats and hibernacula using commercially-available disinfectants is also problematic (Shelley *et al.* 2013). Inhibition or killing of the pathogen on bat epidermal surface with topical fungicide would provide a break in the disease cycle at the point of infection but would likely have toxic effects on treated bats and the sensitive cave ecosystems in which they hibernate (Knudsen *et al.* 2013).

Treatment and delivery methods proven safe for bats and their habitats have not yet been developed. Possibilities include fogging of hibernacula with treatment compounds, hand delivery of treatment to bats while hibernating or during passage in and out of hibernacula, or treatment in captivity. Capture and treatment of infected bats may result in injuries to the bats or an increase in mortalities; disturbance during hibernation could cause use of infected bats' already decreased stored energy (Aley 2010). Moreover, the proportion of bat populations that must be treated to reduce disease levels and spread are not known (Foley *et al.* 2010).

Factors limiting management of hibernacula and bats

Implementation of management strategies for private caves and mines requires consent and cooperation from owners, and some sites have multiple entrances with different landowners. Management of such sites may be ineffective because gaining access is difficult due to safety and liability concerns. Some mines and caves are unstable, and bats may use the areas that are inaccessible to humans. Hundreds of hibernacula sites exist, and for effective management of WNS, a majority of the sites must be monitored – a daunting task given the number of locations. This is also true for summer roost sites, which are more numerous than winter sites. Therefore, only a small fraction of bat habitat sites could be managed (Aley 2010).

The option of preventing bats from entering infected sites is not desirable. This strategy would deprive bats of essential habitats and may enhance the spread of WNS by forcing bats to seek new hibernacula and roost sites (Aley 2010). Managing the agents of spread (bats) by controlling their movement and chosen locations of hibernation and roosting is impractical because bats are highly mysterious, dispersed for most of the year, and wide-ranging (Warnecke *et al.* 2012).

Controlling anthropogenic spread

While the primary mechanism of transfer appears to be bat-to-bat contact, it is unknown what role human activity plays in the spread of WNS. Fungal spores are durable and easily attach to clothing and equipment, and it is possible that *Pd* may have been introduced to North America from Europe by means of contaminated speleological equipment. The rapid spread of the pathogen from its epicenter in New York may be evidence of its introduction to a previously unexposed location (Turner *et al.* 2011). Anthropogenic spread remains largely anecdotal; however, there is historical precedent for the very real risk of human-assisted movement of fungal pathogens (e.g. the chytrid fungal disease) (Turner *et al.* 2011).

Currently, there is no complete understanding of *Pd* survival in the environment or the number of fungal cells needed to colonize a host or hibernacula; therefore, it is imperative to adhere to decontamination protocols to prevent spread of WNS (Shelley *et al.* 2013). Several decontamination protocols used by speleologists were shown to be effective in reducing the

chance of pathogen spread to unaffected bat populations and hibernacula (Table 1). Certain commercially available disinfectants, such as Formula 409® or bleach, are effective against *Pd*, while alcohols are ineffective. Hand washing and bathing are more practical methods of limiting the spread of *Pd* on human skin. Pre-cleaning to remove mud and sediment from equipment followed by use of disinfectants effectively removes *Pd* from caving material. In addition, immersion of material in water baths above 50°C for at least 20 minutes can destroy fungal spores (Shelley *et al.* 2013).

	PRODUCT	Clorox® (6% HOCl) Bleach	Lysol® IC Quaternary Disinfectant Cleaner	Professional Lysol® Antibacterial All-purpose Cleaner	Formula 409® Antibacterial All-Purpose Cleaner	Lysol® Disinfecting Wipes
APPROVED USES	Hard, non-porous surfaces	Yes	Yes	Yes	Yes	Yes
	Non-porous personal protective safety equipment	No	Yes (headgear, goggles, rubber boots, etc.)	No	No	No
	All surfaces, including: porous clothing, fabric, cloth footwear, rubber boots	Yes (Do not use on ropes, harnesses or fabric safety gear.)	No	No	No	No
DILUTION / TREATMENT (as per label)		Effective at 1:10 dilution (bleach : water) [3,4]	Effective at 1:128 dilution (1 ounce: 1 gallon of water) [3,4]	Effective at 1:128 dilution (1 ounce: 1 gallon of water) [3,4]	Effective at concentrations specified by label [3,4]	Effective at 0.28 % di-methyl benzyl ammonium chloride [3,4]

Table 1. Secondary or non-submersible treatment options (for a minimum of 10 minutes) for decontamination of material and equipment exposed to WNS. (National White-Nose Syndrome Decontamination Protocol Version 06.25.2012. For complete protocol: https://www.whitenosesyndrome.org/topics/decontamination).

Universal precautions include implementing decontamination procedures before leaving potentially contaminated sites, prohibiting movement of clothing and equipment between contaminated and unaffected sites, and restricting human access to sensitive habitats (Sleeman 2011). Closing of public caves and mines to human visitation has already been implemented by federal agencies, but there have been economic consequences since many caves serve as public visitation sites (Aley 2010).

Culling individuals or populations

Culling, the reduction of a population by selective extermination, is a controversial strategy for managing disease spread in wild species. In order to be successful, culling of a population must meet several criteria: 1) the pathogen should not originate from fomites, 2) a sufficiently high proportion of the infected population must be removed, and 3) the remaining population of individuals must be isolated to prevent further spread of infection (Foley *et al.* 2010). Culling is more easily conducted and with a higher rate of success as a short-term method of managing localized disease outbreaks in domestic animal populations since individuals are confined and environmental factors can be controlled. In addition to being perceived negatively by the public, wildlife culling is more difficult to conduct due to delays in diagnosis, vagility of

animals, and the inability to control environmental factors. In the past, this method of disease control has proved ineffective or exacerbated the disease (Foley *et al.* 2010).

Culling models show the ineffectiveness of the method as a management strategy (Hallam and McCracken 2010). Modeling suggests that culling may reduce the number of infected bats, but substantial numbers of infected bats could still be present in the affected hibernacula, meaning the disease would not be eradicated. For effectiveness of this method, it is suggested that response in the early stages of infection is needed (Hallam and McCracken 2010). However, culling is an unlikely control method when there is an environmental reservoir for the fungus (Hallam and McCracken 2010).

Because indications of WNS are temporary and difficult to detect, there is the chance that culling would remove individuals with resistance, reducing the likelihood that subsequent generations of would develop natural immunity to the disease (Foley *et al.* 2010). Culling might very well lead to local extinction of bat populations without ever significantly managing the spread of WNS (Foley *et al.* 2010) and so appears to be an unlikely candidate for disease control.

Educating the Public

Education of the public is necessary to avoid inadvertent spread of disease, avoid disturbance of hibernating bats and delicate hibernacula environments, and to reduce reactive and ineffective killing of bats (Foley *et al.* 2010).

Whitenosesyndrome.org provides current information to the public regarding WNS. Several resources are provided by the website to increase awareness of the disease and promote action in combating it:

- General information and news about WNS, affected bat species, and the fungus *Pd*
- Document and resources, including decontamination protocols; maps of current spread; and video, audio, and images
- "A National Plan for Assisting Sates, Federal Agencies, and Tribes in Managing White-Nose Syndrome in Bats" published by the U.S. Fish and Wildlife Service to address the spread and impact of white-nose syndrome as of May 2011
- United States federal and state response plans
- Tracking of current research, projects, and monitoring

Further information about WNS can be found on governmental websites, including:

- U.S Fish and Wildlife Service (http://www.fws.gov/whitenosesyndrome/)
- USGS National Wildlife Health Center (http://www.nwhc.usgs.gov/disease_information/white-nose_syndrome/)
- USGS Fort Collins Science Center (http://www.fort.usgs.gov/WNS/)

Importance of Management

As one-fifth of all mammal life on the planet, bats occupy a significant position in the diversity of animals. The continued loss of bat populations in North America would have enormous and unforeseen long-term impacts, but several short-term consequences have been predicted.

Insectivorous bats are top predators in their ecosystems, and as such, they play an important role in controlling insect populations in North America. Extensive mortality of bat populations by WNS poses a threat not only to bat species and their ecosystems, but to

agriculture and public health if insect populations were allowed to increase without management (Reynolds and Barton 2014).

Novel invasive pathogens like *Pd* cause significant conservation problems and WNS has direct influences on bat populations in North America that present consequences such as regional extirpation of populations or even extinction of species. Species like *M. lucifugus* and *M. sodalis* are at risk of extinction in North America because of WNS. Population viability analyses on *M. lucifugus* suggest that regional extinctions of the species will occur within the next twenty years, with complete extirpation from the northeast by 2026 (Chaturvedi *et al.* 2011; Verant *et al.* 2012). A study in 2010 documented a 78% decline in the summer activity of *M. lucifugus* that correlates with the emergence of WNS. The mortality of *M. lucifugus* during hibernation in the winter is suggested to reflect in the decreased activity of the species in the summer (Dzal *et al.* 2010). *Myotis* species are vital predators of adult nocturnal aquatic insect species and may have a significant role in population control. *Myotis* bats also transport nutrients from aquatic foraging sites to terrestrial ecosystems, and thus the loss of *Myotis* species will have unknown but enormous effects (Brooks 2011).

Likewise, the extinction of *M. sodalis* would have numerous ecological consequences. *M. sodalis* is considered endangered under the United States Endangered Species Act of 1973 and red listed according to the International Union for the Conservation of Nature (Thogmartin *et al.*2012). With WNS, population estimates of Indiana bats are not likely to reach recovery status in the near future.

Current Unknowns of WNS

Due to the recent emergence of WNS, information and understanding of the disease is severely lacking, and there are still many questions left unanswered. It is unknown how *Pd* has come to cause WNS in North American bats, but current hypotheses suggest *Pd* was introduced from Europe, is a North American fungus that recently became pathogenic, or co-infects with an unknown primary mortality agent. It is not known why the fungus targets North American bats and the primary mechanisms of *Pd* in causing infection and mortality have not yet been discerned; studies thus far suggest that mortality is correlated with fat depletion, evaporative water loss and dehydration, immunosuppression, or any combination of these factors. Furthermore, it is unknown how infection with WNS triggers increased arousal from hibernation.

The natural habitat of *Pd* is unknown, and there is little understanding of the life cycle of the fungus as there has not yet been any observed sexual stage (Minnis and Lindner 2013; Chaturvedi *et al.* 2011). It is unknown how many fungal spores are needed to colonize a new bat host or hibernaculum. Additionally, there is no knowledge of how *Pd* thrives at low temperatures or how euthermic body temperatures of bats during hibernation affect the growth of the fungus while colonizing the epithelium (Chaturvedi *et al.* 2011; Verant *et al.* 2012). Furthermore, the ecological roles of *Pd*, such as natural range, saprophytic compared to pathogenic growth potential, physiological growth constraints, reproductive potential, genetic recombination potential, and susceptibility to biological and chemical management strategies are largely unknown (Knudsen *et al.* 2013).

The impact of *Pd* on the diverse species of cave fungi in North America is not known, nor is how the fungus persists over long periods within the environment (Minnis and Lindner 2013). There is no concrete evidence how or why *Pd* moved from a soil substrate to a bat host, and the role of an infected environment in transmission of *Pd* and spread of WNS is unclear. The exact pathogenesis of the disease and the means of transmission and spread, the individual and

site factors that may contribute to the probability of disease outbreak, and the relationship between microclimate of hibernacula and progression of disease also need to be determined (Foley *et al.* 2010).

There is an equally great need of information about the affected bat species themselves. In depth information on feeding and roosting behaviors; nightly, seasonal, and annual flight distances; population carrying capacities; and age-specific survival and reproductive rates are required in order to form and implement management actions that may reduce further infection and mortality of hibernating bat populations (Foley *et al.* 2010). Little is known about roost site choice by healthy bats, much less by bats infected with the disease (Ehlman *et al.* 2013). Particularly, it is unknown how hibernacula and roost site choice is influenced by disease-induced increases in arousal frequencies (Ehlman *et al.* 2013). There is little information on hibernation ecology of bats in the wild, including hibernation body temperatures and arousal lengths and frequencies (Britzke *et al.* 2010). Transmission rates of WNS within caves are unknown and difficult to obtain while minimizing disturbance of hibernating bats (Ehlman *et al.* 2013). Information is building about physiological and behavioral response and symptoms to the disease for both North American and European bats. No mass mortality has been observed in European bats, so the relationship of these species with *Pd* needs further understanding. An investigation is ongoing regarding the roles of wing membrane microfauna in differential survival among species or sites, as well.

The best option for management of WNS has not been determined. Suggested strategies include supporting recovery of bats by providing food, water, and antifungal treatment; modification of hibernacula by adjusting internal temperature and humidity or decontaminating with antifungal compounds; and controlling anthropogenic spread. Wild bats cannot yet be treated safely and effectively for WNS. Decontamination procedures cannot be implemented on fragile cave environments upon which bats rely (Sleeman 2011). Little is known about microclimate selection of hibernacula by bats during the winter and how this selection varies geographically and among species. Microclimate selection might be a factor in the pathogenesis of WNS, and so further research is required (Verant *et al.* 2012). Studies are needed to determine if immune system suppression with WNS is normal or a cause of the disease (Meteyer *et al.* 2009). Furthermore, it is necessary to increase awareness of WNS, develop surveillance strategies, and develop a means of early diagnosis in order to successfully manage the disease since there is no rapid site screening procedure for the presence of WNS when visibly infected bats are absent (Meteyer *et al.* 2009; Sleeman 2011).

Future of WNS

Although understanding of WNS is building, the futures of the disease and affected bat populations are still unclear. Currently, WNS efforts focus on gaining understanding of the disease and monitoring bat populations. Data collected during surveillance of live bats is suggested to include individual bat sex, species, age class, clinical signs of WNS, ectoparasite loads, season, and other possible factors that may contribute to the spread and transmission of WNS (Foley *et al.* 2010).

Modeling is also a useful method for predicting the futures of bat populations. Jachowski *et al.* (2014) have shown that, in addition to direct effects on bat populations, WNS can have indirect, cascading effects on behavior and interspecific interactions of species not directly affected by the disease. These interspecific interactions are important for structuring wildlife

communities and so it is possible that WNS will weaken spatial and temporal niche partitioning in sympatric bat species.

Current hypotheses for post-WNS population dynamics support that bats acquire immunity or develop behavioral resistance after initial pathogen exposure. WNS has been predicted to continue producing periodic high-mortality outbreaks in infected populations. Management efforts to increase adult survival and reproduction may temporarily alleviate mortality effects of WNS. Short-term population growth may be achieved by increasing survival of healthy adults such as applying antifungal treatments, supplementing hibernacula and roost site food sources, and decontaminating infected sites (Maslo and Fefferman 2015).

Research that targets the understanding of the ecology of WNS and bats and their environments will aid in the development of management strategies (Blehert 2012). Hibernating and summer locations should be managed, including modification of human activity through implementation of decontamination procedures, equipment restrictions, and site closures. Because *Pd* is found in the soil of hibernacula, universal precautions dictate that the presence of the fungus must be assumed unless its absence can be proven (Puechmaille *et al.* 2011).

Increasing awareness is necessary for combating WNS. New discoveries should be made available to the public to provide current understanding and ways to become involved in the management of this lethal disease of North American bats, whose continued mortality would be a great loss to the ecosystem and the animal kingdom as a whole.

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