Wildlife Disease Association
62\textsuperscript{nd} Annual International Conference

White-Nose Syndrome Workshop

World Fair Park
Knoxville, Tennessee
July 27, 2013
## AGENDA

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(Bat Identification; WNS Sampling Techniques and Protocols)
Economic Importance of Bats in Agriculture

Justin G. Boyles,1* Paul M. Cryan,2 Gary F. McCracken,3 Thomas H. Kunz4

White-nose syndrome (WNS) and the increased development of wind-power facilities are threatening populations of insectivorous bats in North America. Bats are voracious predators of nocturnal insects, including many crop and forest pests. We present here analyses suggesting that loss of bats in North America could lead to agricultural losses estimated at more than $3.7 billion/year. Urgent efforts are needed to educate the public and policy-makers about the ecological and economic importance of insectivorous bats and to provide practical conservation solutions.

Infectious Disease and Wind Turbines

Insectivorous bats suppress populations of nocturnal insects (1, 2), but bats in North America are under severe pressure from two major new threats. WNS is an emerging infectious disease affecting populations of hibernating cave-dwelling bats throughout eastern North America (3). WNS is likely caused by a newly discovered fungus (Geomyces destructans). This fungus infects the skin of bats while they hibernate and is thought to trigger fatal alterations in behavior and/or physiology (e.g., premature depletion of energy reserves) (3, 4). Since February 2006, when WNS was first observed on bats in upstate New York, G. destructans has spread west of the Appalachian Mountains and into Canada. To date, over one million bats have probably died, and winter colony declines in the most affected region exceed 70% (5). Populations of at least one species (little brown bat, Myotis lucifugus) have declined so precipitously that regional extirpation and extinction are expected (5).

At the same time, bats of several migratory tree-dwelling species are being killed in unprecedented numbers at wind turbines across the continent (6, 7). Why these species are particularly susceptible to wind turbines remains a mystery, and several types of attraction have been hypothesized (6). There are no continental-scale monitoring programs for assessing wildlife fatalities at wind turbines, so the number of bats killed annually by wind turbines in the Mid-Atlantic Highlands alone (7). Obviously, mortality from these two factors is substantial and will likely have long-term cumulative impacts on both aquatic and terrestrial ecosystems (5, 7). Because of these combined threats, sudden and simultaneous population declines are being witnessed in assemblages of temperate-zone insectivorous bats on a scale rivaled by few recorded events affecting mammals.

Economic Impact

Although much of the public and some policy-makers may view the precipitous decline of bats in North America as only of academic interest, the economic consequences of losing so many bats could be substantial. For example, a single colony of 150 big brown bats (Eptesicus fuscus) in Indiana has been estimated to eat nearly 1.3 million pest insects each year, possibly contributing to the disruption of population cycles of agricultural pests (8). Other estimates suggest that a single little brown bat can consume 4 to 8 g of insects each night when active (9, 10), and when extrapolated to the one million bats estimated to have died from WNS, between 660 and 1320 metric tons of insects are no longer being consumed each year in WNS-affected areas (11).

Estimating the economic importance of bats in agricultural systems is challenging, but published estimates of the value of pest suppression services provided by bats ranges...
from about $12 to $173/acre (with a most likely scenario of $74/acre) in a cotton-dominated agricultural landscape in south-central Texas (12). Here, we extrapolate these estimates to the entire United States as a first assessment of how much the disappearance of bats could cost the agricultural industry [see supporting online material (SOM)]. Assuming values obtained from the cotton-dominated agroecosystem in Texas, and the number of acres of harvested cropland across the continental United States in 2007 (13), we estimate the value of bats to the agricultural industry is roughly $22.9 billion/year. If we assume values at the extremes of the probable range (12), the value of bats may be as low as $3.7 billion/year and as high as $53 billion/year. These estimates include the reduced costs of pesticide applications that are not needed to suppress the insects consumed by bats (12). However, they do not include the “downstream” impacts of pesticides on ecosystems, which can be substantial (14), or other secondary effects of predation, such as reducing the potential for evolved resistance of insects to pesticides and genetically modified crops (15). Moreover, bats can exert top-down suppression of forest insects (1, 2), but our estimated values do not include the benefit of bats that suppress insects in forest ecosystems because economic data on pest-control services provided by bats in forests are lacking. Even if our estimates are halved or quartered, they clearly show how bats have enormous potential to influence the economics of agriculture and forestry.

Although adverse impacts of WNS on bat populations have occurred relatively rapidly, impacts of wind energy development appear to pose a more chronic, long-term concern. WNS has caused rapid and massive declines of hibernating bats in the northeastern United States, where this disease has persisted for at least 4 years (5). Thus, the coming growing season may be the first in which the adverse effects of this disease will become noticeable. Because of regional differences in crop production, the agricultural value of bats in the U.S. Northeast may be comparatively small relative to much of the United States (see the figure) (SOM). However, evidence of the fungus associated with WNS was recently detected in the Midwest and Great Plains, where the estimates of the value of bats to agriculture are substantial (see the figure). Additionally, because this region has the highest onshore wind capacity in North America, increased development of wind energy facilities and associated bat fatalities in this region can be expected (16). Thus, if mortality of bats associated with WNS and wind turbines continues unabated, we can expect noticeable economic losses to North American agriculture in the next 4 to 5 years.

Policy
A recently stated goal of the United Nations Environment Programme is to demonstrate the value of biodiversity to policy-makers and the public (17). In keeping with this goal, we hope that the scale of our estimates and the importance of addressing this issue will resonate both with the general public and policy-makers. Bats provide substantial ecosystem services worldwide, and their benefits to human economies are not limited to North America. For example, pioneering research in tropical ecosystems shows the importance of plant-visiting bats in the pollination of valuable fruit crops (18, 19). Although the economic impacts of mass mortality of bats associated with WNS appear to be confined, at present, to North America, wind turbines are also causing bat fatalities in Europe (20), and the potential for WNS to spread to other parts of the world is unknown.

We suggest that a wait-and-see approach to the issue of widespread declines of bat populations is not an option because the life histories of these flying, nocturnal mammals—characterized by long generation times and low reproductive rates—mean that population recovery is unlikely for decades or even centuries, if at all. Currently, there are no adequately validated or generally applicable methods for substantially reducing the impacts of WNS or wind turbines on bat populations. To date, management actions to restrict the spread of WNS have been directed primarily toward limiting anthropogenic spread (e.g., cave and mine closures and fungal decontamination protocols) (21). Other proactive solutions for understanding and ameliorating the effects of WNS include developing improved diagnostic tests to detect early-stage infections and fungal distribution in the environment; defining disease mechanisms; investigating the potential for biological or chemical control of the fungus; and increasing disease resistance through habitat modification, such as creation of artificial or modified hibernacula that are less conducive to disease development and transmission (11, 22). Other approaches, such as culling of infected bats have been widely discussed and dismissed as viable options for control (23). New research also shows that altering wind turbine operations during high-risk periods for bats significantly reduces fatalities (24, 25). Specific action on these issues will benefit from scientific research carefully aimed at providing practical conservation solutions for bats in the face of new threats and at assessing their economic and ecological importance. We as scientists should also make concerted efforts to develop and use more effective methods for educating the public and policy-makers about the ecosystem services provided by bats.

Bats are among the most overlooked, yet economically important, nondomesticated animals in North America, and their conservation is important for the integrity of ecosystems and in the best interest of both national and international economies. In our opinion, solutions that will reduce the population impacts of WNS and reduce the mortality from wind-energy facilities are possible in the next few years, but identifying, substantiating, and applying solutions will only be fueled in a substantive manner by increased and widespread awareness of the benefits of insectivorous bats among the public, policy-makers, and scientists.

References
17. The Economics of Ecosystems and Biodiversity, www.teebweb.org/.

Supporting Online Material
www.sciencemag.org/cgi/content/full/332/6025/41/DC1
Bat White-Nose Syndrome in North America

Since 2007, infections by a previously unrecognized, perhaps imported fungus killed an estimated 1 million bats in North America

David S. Blehert, Jeffrey M. Lorch, Anne E. Ballmann, Paul M. Cryan, and Carol U. Meteyer

In 2007 bats in eastern North America began dying in unprecedented numbers from a previously undocumented disease, now called white-nose syndrome (WNS). Although the ecological and economic impacts of this disease are not fully elucidated, this severe loss of insectivorous bats threatens decreased crop yields, forest defoliation, and a rise in insect-borne diseases. The recent emergence of WNS in bats of eastern North America, its rapid spread, and the severity of the outbreak highlight the importance of wildlife disease as an integral component of ecosystem health.

Biologists with the New York State Department of Environmental Conservation first recognized WNS as a problem in late winter 2007 at five hibernation sites near Albany, N.Y. Subsequently, a recreational caver furnished a photograph from February 2006 in nearby Howes Cave depicting bats with clinical signs of WNS, implicating this location as the likely index site and suggesting disease emergence the winter before New York state biologists drew public attention to the disease. By 2011 WNS had spread south along the Appalachian Mountains into eastern Tennessee, as far west as southern Indiana and western Kentucky, and north into the Canadian provinces of Quebec, Ontario, and New Brunswick (Fig. 1). Experts estimate that more than 1 million bats have died from WNS thus far. Modeling studies show that, if such mortality trends continue, one of the most abundant bat species in eastern North America, the little brown bat (Myotis lucifugus), could disappear from this region within 16 years. Sustained killing of this magnitude from an infectious disease is unprecedented among the approximately 1,100 species of bats known worldwide.

Summary

• The newly described fungus, Geomyces destructans, causes an invasive skin infection in bats and is the likely agent of white-nose syndrome (WNS).
• With immune system functions and body temperatures reduced during hibernation, bats may be unusually susceptible to a pathogenic fungus such as G. destructans.
• WNS was first observed in a popular show cave near Albany, New York, leading some investigators to suspect that a visitor inadvertently introduced G. destructans at this site, triggering a wider WNS outbreak in North America.
• Biologists trying to manage WNS within North American bat populations face major challenges, including the variety of susceptible host species, incredible dispersal capabilities of bats, difficulties in treating such populations, and persistence of the pathogen in their vulnerable underground habitats.

The Host, Pathogen, and Environment

The likely agent of WNS is a newly described fungus, Geomyces destructans, which causes an invasive skin infection that is the hallmark of this disease (Fig. 2). G. destructans belongs to the order Helotiales within the phylum Ascomycota. Characteristics that distinguish it from
other Geomyces spp. include curved conidia (Fig. 2), slow growth on laboratory medium, cold adaptation, and pathogenicity to bats. Species of Geomyces exist in soils worldwide, especially in colder regions.

Any infectious disease involves interactions among a susceptible host, pathogen, and the environment. To comprehend the ecology of WNS, we must consider the physiological and behavioral aspects of bats that make them susceptible to the disease, the characteristics of the fungus that allow it to act as a pathogen, and the role of underground sites (hibernacula) such as caves and mines in providing conditions conducive to maintaining this pathogen and enabling it to infect these hosts.

WNS appears to occur only in bats, suggesting they possess unique traits that make them a suitable host. Bats are nocturnal and the only mammals capable of powered flight. Their forelimbs are highly modified, consisting of elongated phalanges connected by a thin layer of skin to form wings. This body plan provides bats with selective advantages that allow them to dominate the night skies, making them the second most diverse group of mammals, accounting for approximately 1,100 of 5,400 mammalian species. Of 45 bat species in the United States, at least 6 of the approximately 25 that hibernate have been documented with WNS, including the little brown bat, the northern long-eared bat (M. septentrionalis), the eastern small-footed bat (M. leibii), the endangered Indiana bat (M. sodalis), the tricolored bat (Perimyotis subflavus), and the big brown bat (Eptesicusfuscus).

All six of those species are insectivorous and cope with winter food shortages by hibernating in cold and humid, thermally stable caves and mines. When hibernating, the animals typically congregate in large numbers, dramatically reduce metabolic functions, and assume a body temperature...
Blehert: White-Nose Syndrome Mechanisms in Bats, Mechanics of Vintage Vehicles

If not microbiology, David Blehert might well have studied mechanical engineering instead. While a child, he liked nothing better than to help his father repair cars. When he was in high school, the two of them rebuilt a 1968 sports car and then restored another car from the 1950s. Blehert, 40, continues to collect and repair vintage vehicles, saying this process helps him when “diagnosing and solving problems, understanding how things work, and seeing projects to completion. [It is] a sound basis for conducting laboratory research.”

Blehert heads the diagnostic microbiology laboratory within the disease investigation branch of the U.S. Geological Survey (USGS)-National Wildlife Health Center, in Madison, Wis. His lab works with a team that includes wildlife pathologists and other specialists whose expertise ranges from diagnostic virology to parasitology and chemistry. Together, they investigate and find the causes of wildlife illnesses and unexplained deaths. “Essentially, we function as the nation’s [Centers for Disease Control and Prevention (CDC)] for wildlife,” he says. “Our work provides scientific support for the management of wildlife disease and the promotion of ecosystem health.”

Blehert and his collaborators are investigating what led to emergence of bat white-nose syndrome (WNS) in North America, an outbreak that experts attribute to the fungus *Geomyces destructans*. “With bat population declines exceeding 70% in the eastern United States, WNS presents a significant threat to hibernating bat species of North America,” he says. “Bats are primary predators of insects, including crop and forest pests. Thus, reduced bat populations could adversely impact agriculture and the health of forests with consequent economic and ecologic repercussions.”

Blehert suspects that humans accidentally transported *G. destructans* from Europe to or near a popular tourist cave in New York, enabling the fungus to establish itself among North American bats. “Global travel and trade have effectively eliminated natural barriers, such as mountain ranges and oceans, that once prevented the spread of disease agents around the world, and are today recognized as one of the most significant drivers in the emergence of infectious diseases worldwide,” he says. Interest in bat WNS also “serves to highlight the importance of this wildlife disease as part of the ‘One Health’ concept, that is, the recognition that wildlife health, domestic animal health, human health, and ecosystem health are inextricably linked.”

In addition to his work on WNS, Blehert’s laboratory is conducting collaborative research with a local company, BioSenti nel, to develop rapid in vitro methods for detecting botulinum neurotoxins types C and E, the cause of avian botulism, one of the most significant causes of waterfowl mortality in North America. “The current gold-standard test for detecting botulinum neurotoxins uses live mice, and an in vitro assay would significantly decrease costs, increase throughput, and eliminate the need to use animals for botulinum detection,” he says.

Blehert grew up in Minneapolis. His mother is a teacher, and his father is a mechanical engineer. “My parents strongly support education and encouraged me and my brothers—I have younger identical twin brothers—to take school seriously,” he says. “As an engineer, my father especially encouraged us to pursue course work in math and science. I came close to heeding his advice by choosing to study biology.”

Long interested in the outdoors, Blehert received a B.S. in biology in 1993 from the University of Minnesota and a Ph.D. in bacteriology in 1999 from the University of Wisconsin, Madison. He did postdoctoral research at the National Institute for Dental and Craniofacial Research at the National Institutes of Health from 1999 until 2003, when he joined the USGS.

Blehert is married to Regina Vidaver, a cellular and molecular biologist who is executive director of the National Lung Cancer Partnership, a nonprofit research advocacy group. They have two children, a daughter, 8, and a son, 5. In his spare time, he indulges his love for the outdoors by hiking, fishing, gardening, ice-skating, skiing, and camping. And he still works on antique vehicles, a collection that now includes two vintage motorcycles and a sports car. “I maintain all of these vehicles in running condition, but given my current work and family responsibilities, finding time to drive and work on them is challenging,” he says. “For now, it makes me happy to have them in my garage, complete periodic maintenance and improvements, and collectively drive or ride them a couple of hundred miles per year.”

Marlene Cimons
Marlene Cimons lives and writes in Bethesda, Md.
close to that of their surroundings (2–7°C). These physiological adaptations and behaviors likely predispose bats to infection by *G. destructans* and consequent development of WNS. Because approximately half the bat species of the United States are obligate hibernators, another 19 species are at risk for infection by *G. destructans* if it spreads beyond its current range.

*G. destructans* colonizes the skin of bat muzzles, wings, and ears, then erodes the epidermis and invades the underlying skin and connective tissues. This pattern is distinctive and is more severe than that caused by typical transmissible dermatophytes. Although the disease was named for the characteristic white growth visible around an infected animal’s nose, the primary site of infection is the wing (Fig. 3a). Gross damage to wing membranes such as depigmentation, holes, and tears are suggestive of WNS, but these lesions are nonspecific, and histopathologic examination is necessary to diagnose the disease.

Specifically, fungal invasion of wing membranes ranges from characteristic cup-like epidermal erosions filled with fungal hyphae to ulceration and invasion of underlying connective tissue, with fungal invasion sometimes spanning the full thickness of the wing membrane (Fig. 3b). Fungal hyphae can also fill hair follicles and destroy skin glands and local connective tissue. Bat wings play an important role in the pathogenesis of WNS by providing a large surface area for the fungus to colonize. Once infected, the thin layer of skin that composes the bat wing is vulnerable to damage that may catastrophically disrupt homeostasis during hibernation.

In North America, bat hibernacula range in temperature from approximately 2–14°C, temperatures all permissive to growth of *G. destructans*. Within this temperature range, *G. destructans* exhibits increasing growth rates with increasing temperature (Fig. 4), but the fungus does not grow at temperatures of approximately 20°C or higher. This temperature sensitivity helps to explain why...
WNS is observed only among hibernating or recently emerged bats and why the disease is not diagnosed in bats during their active season when body temperatures are consistently elevated above those permissive to growth of *G. destructans*.

**Looking for Other Host and Environmental Susceptibility Factors**

Hosts with impaired immune functions tend to be susceptible to opportunistic fungi in their environments. Guided by this concept, some investigators suspected that insults such as exposure to environmental contaminants or infections by viral pathogens compromised bat immunity and made them vulnerable to *G. destructans*. However, neither contaminant exposure nor viral coinfections can be consistently identified in bats infected with that fungus.

Hibernating bats with WNS generally do not exhibit signs of an inflammatory response. However, severe inflammation typifies fungal skin infections of bats aroused from hibernation, providing evidence that such animals are not immunocompromised. Although studies of bat immune functions are in their infancy, studies of other mammalian species indicate that their immune functions are naturally suppressed during hibernation. Thus, rather than suggesting immune-function impairment, the lack of inflammatory response to fungal infection by hibernating bats may reflect an immune suppression that is part of hibernation physiology.

In addition, the body temperature of hibernating bats drops dramatically, providing another vulnerability to infection by *G. destructans*. Fatal fungal diseases are relatively rare among endothermic, or warm-blooded, animals because their tissues are too warm to support the growth of most fungal species. However, fungi are more apt to cause fatal diseases in ectothermic, or cold-blooded, organisms such as insects, fish, amphibians, and plants. Bats and other mammals that hibernate are unique in that they are warm-blooded when metabolically active, but cold-blooded during hibernation—a period when their metabolism and body temperatures are dramatically suppressed. Although lowered body temperatures may predispose torpid bats to infection by *G. destructans*, the mechanism enabling this specific fungus to be a pathogen for bats while other cave-associated fungi remain innocuous is not known.

How *G. destructans* kills bats is under active investigation. One possibility is that fungal infection disrupts how bats behave while hibernating, leading to more frequent or longer arousals from torpor and thus accelerating usage of fat reserves. However, fat depletion is not consistently observed among all bats with WNS. Infected bats also may exhibit other aberrant behaviors midway through the hibernation season, such as shifting from thermally stable roost sites deep within hibernacula to areas with more variable temperatures near entrances. Sometimes, they depart early from hibernacula. Thus, exposure to cold could account for some WNS-associated mortality.

Further, fungal damage to wing membranes, which can account for more than 85% of the total surface area of a bat, may increase fatality rates. In addition to the key role that wings play in flight, wing membrane integrity is essential for maintaining water balance, temperature, blood circulation, and cutaneous respiration. Disrupting any of these functions could increase WNS mortality rates.

As with so many other diseases, the environment affects the progress and transmission of...
WNS. Some pathogenic fungi such as *Histo-
plasma capsulatum*, *Cryptococcus* spp., and *Bat-
trachochytrium dendrobatidis* can persist in the
environment without an animal host for sur-
vival. This independence contrasts with host-
requiring viruses or other pathogens for which
transmission dynamics tend to moderate as in-
fected hosts are removed from a population. *G. de-
structans* likely does not require bat hosts to
survive and can persist in caves by exploiting
other nutrients.

The cool and humid conditions of under-
ground hibernacula provide ideal environmen-
tal conditions for *G. destructans* or other fungal
growth. While most *G. destructans* isolates were
cultured from skin or fur of bats collected in or
near underground hibernacula during winter,
DNA from the same fungus is found in soil
samples from several hibernacula that harbor
WNS-infected bats in the northeastern US. Also,
*G. destructans* has been cultured from soil sam-
ples from hibernacula in three states where
WNS occurs, supporting the hypothesis that bat
hibernacula are reservoirs for this pathogen and
that bats, humans, or fomites may transport *G.
destructans* between hibernacula. How temper-
ature and humidity differences among hiber-
nacula influence *G. destructans* and WNS is not
known.

**Uncertainties about WNS Emergence**

What caused WNS to emerge in a North Amer-
ican cave during the winter of 2005 to 2006?
Bats with clinical signs consistent with WNS
were first observed in Howes Cave, a hibernacu-
num connected to a popular North American
show cave. Because of its high human traffic, a
tourist might have inadvertently introduced *G.
destructans* at this site.

Europe might be the source for the fungus
causing WNS. Reports dating back several de-
cades describe hibernating bats in Germany
with white muzzles resembling bats with WNS
in North America. Recent culture and PCR sur-
vveys indicate that *G. destructans* is widespread
in Europe, including among hibernating bats in
hibernacula in the Czech Republic, France, Ger-
many, Hungary, Slovakia, and Switzerland. Un-
like in North America, however, mortality rates
and population declines remain normal among
European bat species. This sharp contrast be-
tween disease manifestation among bats in Eu-
rope and North America provides an opportu-

**Challenges in Managing WNS, Conserving Bat Populations**

Bat conservation efforts have historically fo-
cused mainly on reducing human causes of bat
mortality, including habitat destruction, detri-
mental intrusions into roosts, and intentional
extermination of colonies. Bat census figures
prior to the emergence of WNS in North Amer-
ica indicate many populations of cave-hibernat-
ing bats were stable or increasing. However, the
current WNS outbreak brings an even more
serious threat to bat populations of North
America, confronting biologists with a new set
of conservation and management challenges.

Mitigating diseases in free-ranging wildlife
populations requires very different approaches
from those applied in agriculture for domestic
animals. Once established, diseases in free-rang-
ing wildlife are rarely, if ever, eradicated. Biolo-
gists trying to manage WNS within bat popula-
tions face multiple challenges, including the
need to deal with numerous host species, long-
distance migrations of infected hosts, poor ac-

...
cesses that might yield behaviorally or immunologically resistant bats.

However, “first, do no harm” does not mean “do nothing.” State and federal agencies already are taking measures to combat WNS, including closing caves and mandating decontamination procedures. Such steps are intended to prevent people from disturbing hibernating bats and to reduce the chance that intruding humans will transfer *G. destructans* from one hibernaculum to another. For example, taking a proactive approach prior to the appearance of WNS, state wildlife officials in Wisconsin conferred threatened status on four cave bat species that hibernate within its borders and designated *G. destructans* a prohibited invasive species providing state resource managers with legal authorities to take disease management actions.

Since the first description of *G. destructans* in 2008, its genome has been sequenced, and WNS pathology has been more fully defined. Additionally, hibernacula are being surveyed internationally, and ongoing analyses are revealing much about the biodiversity of fungi associated with bat hibernacula. With these and other advances in understanding WNS, opportunities will arise to better manage the disease cycle. The sudden and unexpected emergence of WNS exemplifies the importance of monitoring, investigating, and responding to emerging wildlife diseases and the ecological and societal threats that they present.

**SUGGESTED READING**


Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-Nose Syndrome

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Abstract

White-nose syndrome (WNS), an emerging infectious disease that has killed over 5.5 million hibernating bats, is named for the causative agent, a white fungus (Geomyces destructans (Gd)) that invades the skin of torpid bats. During hibernation, arousals to warm (euthermic) body temperatures are normal but deplete fat stores. Temperature-sensitive dataloggers were attached to the backs of 504 free-ranging little brown bats (Myotis lucifugus) in hibernacula located throughout the northeastern USA. Dataloggers were retrieved at the end of the hibernation season and complete profiles of skin temperature data were available from 83 bats, which were categorized as: (1) unaffected, (2) WNS-affected but alive at time of datalogger removal, or (3) WNS-affected but found dead at time of datalogger removal. Histological confirmation of WNS severity (as indexed by degree of fungal infection) as well as confirmation of presence/absence of DNA from Gd by PCR was determined for 26 animals. We demonstrated that WNS-affected bats aroused to euthermic body temperatures more frequently than unaffected bats, likely contributing to subsequent mortality. Within the subset of WNS-affected bats that were found dead at the time of datalogger removal, the number of arousal bouts since datalogger attachment significantly predicted date of death. Additionally, the severity of cutaneous Gd infection correlated with the number of arousal episodes from torpor during hibernation. Thus, increased frequency of arousal from torpor likely contributes to WNS-associated mortality, but the question of how Gd infection induces increased arousals remains unanswered.

Introduction

White-nose syndrome (WNS) is estimated to be responsible for the deaths of at least 5.7 to 6.7 million hibernating bats in the eastern United States and Canada [1,2]. Clinical signs of WNS were first observed at a single cave in New York State during the winter of 2006–2007 and as of April 2012, WNS has spread to over 200 hibernacula in 19 U.S. states and four Canadian provinces (Fig. 1 [2,3]). Bats with WNS display a number of aberrant behaviors, and in many instances they have depleted fat stores. Thus far, WNS affects at least six (and possibly nine) species of hibernating insectivorous bats [2], including some classified as endangered or threatened. The little brown bat (or, little brown myotis, Myotis lucifugus), which was once the most common hibernating bat in the American Northeast (NE), has incurred an average of 91% mortality in sites that have been affected for at least two years [2] and mathematical models indicate that this species may go extinct in the NE within 16 years [4]. A white fungus identified as Geomyces destructans (Gd) grows on the muzzle, wings, and ears of bats suffering from WNS starting in late January/early February [3,5,6]. Recent laboratory experiments have demonstrated that cutaneous infection with this fungus is the cause of WNS, but it is not fully understood how such an infection produces mortality during hibernation [7]. It is hypothesized that infection by Gd disrupts normal physiological functions, such as water balance [8] or other aspects of hibernation physiology, including use of torpor [9].

For insectivorous bats that live in northern temperate zones, such as those affected by WNS, food is primarily available from late spring to early autumn and absent during winter. Bats survive...
this winter energetic bottleneck by building stores of body fat (depot fat) in late summer and early autumn and by conserving metabolic energy through hibernation. In little brown bats, body fat increases from approximately 7% of total mass (~6 g) during summer to 27% of total mass (~9 g) prior to hibernation, an increase of 3 g or more in body mass [10,11]. This depot fat is the sole energy source during the hibernating period, when body temperature (Tb) and metabolic rate are both greatly reduced. Because their energetic costs in the subsequent spring are greater than those of males, female little brown bats enter hibernation with higher body mass indexes (BMI) and manage their energy stores during hibernation more efficiently than males [12]. Minimum metabolic rates during mammalian torpor can be, 5% of basal metabolic rate with Tb close to ambient temperature (2 to 8°C for bats) [13,14]. However, hibernators do not remain torpid throughout hibernation; instead bouts of torpor last from days to weeks, interrupted by brief arousal episodes involving periods of high metabolic rate and euthermic Tb [15]. Earlier studies demonstrated that healthy, free-ranging little brown bats hibernating at ambient temperatures of 5–6°C have torpor bouts lasting between 12.4 and 19.7 days [16,17], with arousal episodes lasting 1–2 hours.

Although euthermic periods account for approximately 1% of the total time budget during winter, about 80–90% of the energy (depot fat) used during hibernation is consumed during these periodic arousals from torpor, because metabolic rate greatly increases with increased Tb [13,18]. The amount of depot fat expended during each arousal episode (not including flight) for hibernating little brown bats is about 107.9 mg [18]. While the function of arousal episodes in hibernators is poorly understood and likely multifactorial [19], the fact that every mammalian

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**Figure 1. Distribution and spread of WNS throughout North America.** Spread of WNS by hibernation season through the winter of 2010–2011 is shown along with locations of study sites, indicated by stars (see also Table 1). Confirmed sites have been officially reported by each state or province based upon histological confirmation of infection with the fungal pathogen Geomyces destructans (Gd); bats from suspect sites have clinical signs of WNS but lack laboratory confirmation. The inset shows a little brown bat infected with Gd from site #1 in Vermont. This site was WNS confirmed in 2008–2009, when bats were studied. Bats from site #2 in Pennsylvania were studied in 2008–2009 (for 8 weeks only in the spring), when no signs of WNS were present, in 2009–2010, when a single bat from this site showed infection with Gd without mass mortality and in 2010–2011, when bats in this site were heavily infected. Bats from site #3 in Pennsylvania were studied in 2008–2009 (no WNS), 2009–2010 (when Gd was noted but without mass mortality) and in 2010–2011, when bats in this site were heavily infected. Bats from site #4 in Pennsylvania were studied in 2009–2010 (for 8 weeks only in the spring), when bats were heavily infected. Bats from site #5 in West Virginia were studied in 2008–2009, when there was no evidence of Gd presence – which was also the case for bats from site #6 in Michigan, which were studied all three years. doi:10.1371/journal.pone.0038920.g001
Table 1. Temperature-sensitive datalogger deployment and retrieval (bat recapture) data, by study year, hibernacula site number (see Fig. 1), and sex for the 504 loggers deployed.

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Whether data were successfully downloaded from the logger and ultimately used in the analyses of this paper, are also described.

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**bats were occasionally excluded from analyses due to incomplete data (e.g., BMI not recorded) or problems with downloaded data.

+loggers only deployed mid-winter (January–March) as opposed to the full hibernation season (November–March).

Materials and Methods

Permits and Permissions

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Bucknell University [protocol number DMR-02]. In the states of VT and WV, research was conducted by state wildlife officials (SRD with Vermont Fish and Wildlife Department and CWS with WV Department of Natural Resources) on non-endangered bats; thus numbered permits were not required or issued. In Michigan, research was conducted each year under MI Scientific Collector’s Permit SC620 from the Michigan Department of Natural Resources to AK. In PA, research was conducted each year under PA Game Commission permits to DMR (64-2008; 70-2009; 183-2010), in collaboration with GGT, a wildlife biologist for the state of PA. In accordance with the permits and with state wildlife policies, research was either conducted on state land or on private property, with the explicit permission of private landowners.

Temperature Tracking

Temperature-sensitive dataloggers were programmed to read skin temperature (Tsk) every 30 min and were attached to 504 bats over the course of three winters at six different hibernacula using standard methods [22]. Temperature readings could not be collected more frequently due to constraints on datalogger memory and the need to record continuous data for up to five months. To maximize recapture rates, bats with loggers were recaptured in March of each year, several weeks prior to the normal time of emergence from hibernation. Loggers weighted about 1.1 g and were either purchased commercially (iBBat or iBTagLites, AlphaMach, Inc., British Columbia, Canada) or were constructed by the authors (DMR and GGT). Appendix S1 describes and illustrates the methods for making these dataloggers from Thermochron DS1922L iButtons (Maxim Integrated Products, Inc., California, USA), modified from the techniques of Lovegrove [23]. Table 1 provides a summary of loggers deployed, retrieved, and downloaded successfully, by site, year, and sex.
Study sites were widely distributed and located in Vermont, West Virginia, Pennsylvania, and the Upper Peninsula of Michigan (Fig. 1). Among loggers retrieved, success rates varied. WeeTagLites failed at a rate of up to 90% whereas loggers constructed by the authors failed about 20% of the time. Overall 111 of 190 loggers retrieved yielded usable data, an average of 58.4%. We expected to recover less than half the loggers placed in the field and expected datalogger failure as well, which is why so many loggers were deployed. Of the 190 bats from which loggers were retrieved, 17 were found dead (four of which were in suitable post-mortem condition to perform histology analysis). For the 175 live bats recaptured in the spring, loggers were removed, and the animal was either released (N = 126) or euthanized for measurement of immune function and other physiological parameters for a separate study (N = 25) or for histology analysis (N = 22), as described below.

PCR and Histology
Wing skin samples (approximately 3 mm X 3 mm each) were collected from a subset of freshly euthanized animals (N = 26). Nucleic acid was extracted from each skin sample using the Gentra Puregene genomic DNA purification kit (Qiagen Inc., Valencia, CA) per the manufacturer's instructions (solid tissues protocol), with the following modifications: protease K was added to a final concentration of 0.5 mg/ml during the cell lysis procedure and no RNAse treatment was performed. To determine presence/absence of DNA from Gd on each sample of wing skin (within the defined sensitivity limitations of the technique used), extracted nucleic acid was analyzed by PCR as previously described by Lorch et al. [24].

Wing membrane from these same animals was also analyzed by histology [5] to determine WNS infection status. The entire wing membrane was stripped from the right forearm and digits, rolled onto 2 dowels 2.5 cm in length, trimmed into three approximately 0.8 cm-wide sections, placed on trimmed edge, sectioned at 0.4 µm-thickness, and stained with Periodic Acid Schiff [5]. This preparation technique yields six whorls of wing membrane on each slide. White-nose-syndrome was diagnosed based on previously published microscopic criteria [5]. A histologic scoring system was developed to classify severity of WNS on a scale of 0 to 4 as described and illustrated in Appendix S2. Briefly, a score of 0 indicates the sample is negative for WNS, and there are no diagnostic cupping erosions in the tissues. A score of 1 indicates the tissues are positive for WNS with cupping erosions diagnostic for WNS but erosions are mild, occasional, and are limited in both depth and extent of wing membrane involved. The presence of even one characteristic WNS erosion is sufficient for a diagnosis of WNS. A severity score of 2 indicates moderate WNS with more frequent and deeper fungal cupping erosions diagnostic of WNS, but distribution over wing membrane is still limited. A WNS severity score of 3 indicates moderately severe fungal infection with deeper and coalescing cupping erosions that are deep enough to be considered ulcers, and the extent of the wing membrane with fungal invasion is greater. A severity score of 4 indicates a severe fungal infection with deep tissue invasion and coalescing of cupping erosions; as many as 100 or more erosions/ulcers can be present in one roll of wing membrane. Scores ranging from 1 to 4 were identified as WNS.

Analyses
Calculations and initial statistics. Usable data for our analyses were recovered from 99 of the 504 loggers deployed (see Table 1). Although data downloaded from 111 loggers, data from 12 of these bats were removed from final analyses for a variety of reasons, including having temperature data recorded for too short of a time period to be comparable to other groups and missing body mass data. Prior to datalogger attachment, each bat was weighed using a portable battery-operated scale (accuracy to 0.1 g), and the length of their right forearm was measured (in triplicate) to the nearest mm using calipers; from these data BMI (weight in g/length of right forearm in mm) [10] was calculated. As most analyses included BMI as a covariate, only bats for which we were able to calculate BMI at the beginning of hibernation (November) were included in the final analysis (N = 83). Data from an additional 16 bats for which we had recordings from only January through March (see Table 1) are also described in the results.

Torpor was defined as when a bat's T_s was 10°C or more below its highest temperature (T_max). Duration of an arousal episode (when T_s was within 10°C of T_max) was calculated to be 30 min. Although recording T_s every 30 min was sufficient to detect arousal episodes, it did not provide sufficient resolution to describe precisely the true length of an arousal bout, as arousal episodes averaged less than 90 min in length (see results). Thus, we did not attempt to determine if there were significant differences in arousal episode length by WNS status. Torpor bout length (TBL, in days) was defined as the period between two arousal episodes. For both arousal bout length and TBL, values were first averaged for each bat and then averaged across all bats. Data on TBL were log_{10} transformed to achieve normality and homogeneity of variance, as determined by Shapiro-Wilk's test for normality and examination of skew and kurtosis and by Levene's test for equality of variances. BMI data were normally distributed. TBL data from multiple years are combined in our analysis, which is supported by the lack of a year-to-year difference in TBL in bats from a given hibernaculum when the WNS status did not change between years (e.g., from site 6 (Table 1; Figure 1): 10.52±1.62 days (2008–2009) vs. 12.47±3.09 days (2009–2010); F_{1,16}=3.091, p = 0.098; partial eta squared = 0.162, power = 0.380). For all analyses, power and effect size are reported for non-significant results. All data are presented as the mean ± standard deviation (SD).

WNS status and TBL. For the initial analysis, bats for which we had data on TBL, BMI, and sex were grouped into three ‘WNS status’ categories: (1) unaffected [N = 57], (2) WNS-affected (as determined by histology and/or visible fungus) and alive at time of datalogger removal [N = 14], and (3) WNS-affected and found dead at time of datalogger removal [N = 12]. Bats were assigned to the ‘unaffected’ category either when the presence of fungal infection with Gd was not detected with PCR or histology [N = 10] or when they were from a hibernaculum presumed to be unaffected and not located in the WNS zone at the time of study [N = 47] (Fig. 1). Combining the two groups of ‘unaffected’ bats for further analyses is supported by the lack of a difference in TBL between them (17.55±4.56 days (PCR/histology) vs. 16.06±2.03 days (presumed unaffected); F_{1,25}=1.111, p = 0.297; partial eta squared = 0.020, power = 0.179). Effects of WNS status on TBL were tested with ANCOVA, with BMI (random), site identity (fixed), and sex (fixed) as covariates. Post-hoc examination of sex differences in BMI was conducted using a Student’s t-test (with df and p values adjusted for unequal variance).

TBL and date of death. Within the WNS-affected bats that were found dead at the time of datalogger removal, the relationships between TBL and BMI and date of death were analyzed using Pearson Product Moment Correlations (PPMC) (after confirming normality and homoscedasticity for each variable). Date of death was measured as the date on which T_s < 0°C for the first time, since the T_s of little brown bats always remains
above 0°C during torpor [17,18]. P values were adjusted for multiple comparisons using sequential Bonferroni correction [25], and the coefficient of determination ($r^2$) was calculated by squaring significant correlations.

**TBL and WNS severity score.** Using a subset of animals for which a ‘WNS severity score’ could be calculated and for which BMI at the start of hibernation was available (N = 26), the effects of severity score, BMI, and site on TBL were examined with ANCOVA. A significant relationship between severity score and TBL was examined using the Gamma Correlation Statistic, which allows for multiple ‘tied rankings’ [26]. Of these 26 bats, 10 were classified in the first analysis as ‘unaffected’ 13 were classified in the first analysis as ‘WNS-affected and alive at time of datalogger removal’ (of these three bats received a severity score of 1, four bats a severity score of 2, two bats a severity score of 3, and four bats a severity score of 4), and three were classified in the first analysis as ‘WNS-affected and found dead at time of datalogger removal’ (of these two bats received a severity score of 2 and one bat a severity score of 3).

**Results**

**Arousing to Euthermic Temperatures**

During the course of this study, when bats aroused from torpor, they remained at euthemeric temperatures for a short period, averaging 78.3 ± 27.3 min. The range of average arousal bout length per bat was from 38.18 to 180 min (N = 83 bats), while the shortest recorded arousal bout lasted 30 min (the shortest period that could be discerned by our methods) and the longest 330 min. We were unable to test for differences in arousal bout length in relation to WNS status (or severity score) due to the limited data storage capacity of our dataloggers (and thus insufficient resolution for precisely quantifying arousal bout length).

**WNS Status and TBL**

Although female bats were in significantly greater body condition than males at the start of hibernation (BMI: 0.2284 ± 0.0293 g/mm (N = 32) vs. 0.2073 ± 0.0210 g/mm (N = 51); t = −3.635, adjusted df = 52.2, p = 0.001), there were no detectable influences of sex on TBL (F(1,76) = 0.031, p = 0.861; partial eta squared = 0.000, power = 0.053). Likewise, we did not detect a relationship between BMI at the start of hibernation and TBL (F(1,76) = 0.140, p = 0.710; partial eta squared = 0.000, power = 0.066). Our BMI analyses were not biased by recapture dynamics as there was no significant difference in BMI at the time of datalogger attachment between bats for which loggers were retrieved and bats that were not recovered (Mann-Whitney U = 3.339, Z = 1.259, p = 0.208). However, both WNS-status and site identity significantly influenced TBL. Site identity heavily influenced the model (F(1,76) = 25.027, p<0.001) as two of the sites contained only one category of bat (site 1 had only ‘WNS dead at time of datalogger removal’ and site 6 only ‘unaffected’ bats). Despite the strong influence of site identity, a significant WNS status main effect was still apparent (F(1,76) = 7.569, p = 0.007).

Unaffected bats had a mean TBL of 16.32 ± 6.65 days (Fig. 2). Limited data collected from an additional 12 unaffected bats from field sites where dataloggers were deployed for only eight weeks late in the hibernation season in 2009 are similar with a mean TBL of 15.62 ± 8.07 days (sites 2 and 3, Fig. 1). As predicted, having WNS was associated with decreased TBL (Fig. 2). Bats that were affected by WNS but still alive at the collection of dataloggers (March) had shorter TBLs than unaffected bats, although the difference was small and not statistically significant (13.96 ± 3.30 days vs. 16.32 ± 6.65 days; F(1,69) = 1.491, p = 0.226, partial eta squared = 0.021, power = 0.226). However, these affected but alive bats had significantly longer TBLs than WNS-affected bats that were found dead at the time of datalogger collection (7.93 ± 2.49 days; F(1,24) = 17.191, p<0.0001). Limited data collected from an additional four WNS-affected bats found dead from a field site where dataloggers were deployed for only eight weeks late in the hibernation season in 2010 are similar with a mean TBL of 6.17 ± 1.79 days (site 4, Fig. 1).

**TBL and Date of Death**

Within the 12 WNS-affected bats found dead at the time of datalogger collection, there was a very strong positive relationship between TBL and the number of days that a bat lived (Fig. 3; PPMP, r = 0.763, corrected p = 0.012). Based upon the calculated coefficient of determination ($r^2 = 0.582$, TBL significantly predicted the date of death, explaining 58% of the variance. Similar to the findings of our full ANCOVA, we did not detect a relationship between BMI at the start of hibernation and TBL (PPMP, r = 0.157, power = 0.090) or between BMI at the start of hibernation and date of death (PPMP, r = −0.026, p = 0.936). While the power to detect significant differences at these low effect sizes (correlation coefficients of 0.178 and 0.084) is extremely low (<0.05), even if they were statistically significant, they are not biologically significant. In each bat, mortality was observed immediately after the last arousal to euthermic temperatures. While several bats (Fig. 2C) displayed frequent arousals just before death, most did not, and arousals were spread throughout their hibernation period.

**TBL and WNS Severity Score**

In the subset of animals for which the WNS severity score could be calculated (N = 26), TBL was not related to BMI (F(1,23) = 0.111, p = 0.743, partial eta squared = 0.005, power = 0.062) or site identity (F(2,22) = 2.515, p = 0.104, partial eta squared = 0.186, power = 0.045), but was related to severity score (F(1,24) = 6.509, p = 0.018). Bats with more severe fungal infections had significantly shorter torpor bouts (gamma correlation statistic = −0.383, p = 0.022; Fig. 4).

**Discussion**

Our results support the hypothesis that WNS causes alterations in bat torpor patterns that likely contribute to death. Our prediction that increased mortality/disease state is associated with abnormally short torpor bouts due to frequent arousal episodes was supported by our larger dataset, in which bats were placed into the WNS status categories of ‘unaffected,’ ‘WNS-affected and alive at time of datalogger collection at the end of hibernation,’ and ‘WNS-affected and dead at the time of datalogger collection.’ While our ‘unaffected’ bats had an average TBL that falls within the previously documented range for this species (16.32 days [16,17]), TBL was shortened (at the low end of previously described TBLs) in WNS-affected bats (13.96 days), and significantly reduced in WNS-affected bats that died between mid-December and late-February (7.93 days). An average torpor bout length of 7.93 days is presumably not sustainable. In fact, within those WNS-affected bats found dead at the time of datalogger removal, TBL was a very strong predictor of the date of death, explaining 58% of the variance in timing of mortality. The distribution of death dates for these bats (Fig. 3) is earlier than that reported in the USA [7] and earlier than seasonal changes in Gd prevalence reported for Europe [27,28]. However, this was at least the second year of infection at this site, which might shift the
Figure 2. Torpor bout length (TBL) in days by WNS status. WNS was associated with decreased TBL: bats that were affected by WNS but still alive at the collection of dataloggers (March) had shorter TBLs than unaffected bats (but this difference was not significant). Significantly shorter TBLs were seen in WNS-affected bats that were found dead at the time of datalogger collection compared to affected but alive bats (2A). Bats were categorized as: unaffected, WNS-affected and alive at time of datalogger removal ('WNS-alive'), and WNS-affected and dead when loggers were removed in the spring ('WNS-dead'). Numbers in brackets indicate sample size and boxes sharing the same letter are not significantly different from each other. Boxes depict the 25th and 75th percentiles, lines within boxes mark the median, and whiskers represent 95th and the 5th percentiles. Outliers are indicated with open circles. Additional panels illustrate sample temperature profile of an unaffected (B) and an affected (C) bat, during the winter of 2009. The bat illustrated in C displayed daily arousals at the end of its life, which was seen in several of these animals. Each of the ‘WNS-dead’ bats died at the end of their last arousal.

doi:10.1371/journal.pone.0038920.g002
Figure 3. Torpor bout length (TBL) as a function of date of death and BMI. For the 12 bats that died from WNS, BMI at the beginning of hibernation was not related to TBL (3A), nor was BMI predictive of the date of death (3B). However, TBL significantly predicted date of death in WNS-affected bats that were found dead at the time of datalogger retrieval (3C) ($r^2 = 0.58$). Bats that died sooner were arousing to euthermic temperatures much more frequently than those that lived longer.

doi:10.1371/journal.pone.0038920.g003
roosting sites, which allows for decreased metabolic rates and thus lower body masses at the beginning of hibernation selected colder significantly influenced hibernation energetics such that bats with patterns would be influenced by the amount of energy stores arousal bout length do not occur in WNS-affected animals.

utes for all bats tested indicate that biologically important shifts in other mortality events within our study animals. May have prevented us from detecting in the field [7], may have prevented us from detecting other mortality events within our study animals.

Our analysis of WNS severity based upon histological confirmation of the degree of fungal invasion and infection further supported and strengthened our conclusion – as the severity of infection increased, so did the frequency of arousals from torpor. Our data mirror the independently derived mathematical model of Boyles and Willis [9], for which an estimated shift in TBL to every 8.33 days resulted in a prediction of 81.9% mortality. Relative to this model, our finding of a TBL of 7.93 days for WNS-affected bats found dead, and field observations of 91% mortality support the linkage between TBL and death, as significant body fat is lost with each arousal [13,18]. Boyles and Willis [9] also proposed that significant changes in arousal bout duration in WNS-affected bats could lead to mortality. Bats are unlike other hibernators [13,18] in that their arousal bouts are typically measured in minutes rather than hours (or even days). Thus, an increase in the duration of euthermy would incur significant energetic costs. Although we were unable to statistically validate differences in arousal bout length in bats of variable WNS status, our finding of an average arousal bout of 78.3 ± 27.3 minutes for all bats tested indicate that biologically important shifts in arousal bout length do not occur in WNS-affected animals.

We also predicted that relationships between WNS and torpor patterns would be influenced by the amount of energy stores available to the bat. In a previous study of little brown bats, BMI significantly influenced hibernation energetics such that bats with lower body masses at the beginning of hibernation selected colder roosting sites, which allows for decreased metabolic rates and thus lower energy expenditure [29]. Other studies have demonstrated that bats roosting at colder temperatures arouse from torpor less often, allowing them to conserve even more energy [19,30,31]. Thus, it is reasonable to expect that bats with lower BMIs would display greater TBL and expend less energy.

These energetic arguments underlay the model of Boyles and Willis [9] that our data so closely match. However, contrary to our predictions, we did not find a relationship between BMI and TBL or BMI and ‘WNS status’, death date, or ‘severity score’. As the power for BMI effects in our models was low (driven by the strong site effects), BMI may still play a role in hibernation patterns and in a bat’s ability to withstand Gd infection. However, even within a site (WNS-affected bats that were found dead at the time of datalogger attachment from site 1 in Vermont), we failed to find a relationship between BMI and WNS. If a higher BMI could ‘buffer’ a bat from the effects of WNS by allowing it to withstand more arousals to euthermy, then we should have detected a relationship between BMI and the number of arousals prior to death – but we did not.

Although statistical analyses confirmed the significance of our findings, studies of behavior and physiology in free-ranging animals are often fraught with unknowns and potential biases, which likely underlie the significant site effects in our statistical models. One potential source of bias in our dataset is BMI at the start of the hibernation season. While one could predict that bats in poorer body condition would find datalogger attachment more physiologically stressful than bats in greater body condition (and thus be less likely to be recaptured), there was no difference in starting BMI between bats that were recaptured and those that were not. Another source of bias in our WNS-affected bats could have been ambient temperature of hibernacula, because TBL generally decreases with increased ambient temperature [30]. Although the exact ambient temperature at the exact roosting site of each individual studied during hibernation was unknown, our WNS-affected field sites were generally colder than our unaffected sites (e.g., 7.29°C vs. 9.77°C). This would presumably bias bats with WNS toward longer TBLs, but we observed the opposite pattern. Within our unaffected bats, TBLs varied greatly (Fig. 2A), likely due to a number of site-, individual-, and population-specific factors. However, these factors appear to be overridden in the WNS affected bats, especially those found dead at the time of datalogger removal – as variability decreased and all bats exhibited shortened TBLs.

Collectively, our data indicate that one proximate mechanism of the mortality associated with WNS is decreased TBL. Warnecke et al. [21], in a study of captive bats experimentally infected with Gd during the third year of our field study, found a similar TBL shift. The challenge that lies before us is to determine how infection by Gd induces altered torpor patterns and why significant variation in TBL between affected bats occurs. While too-frequent arousal is clearly associated with WNS, not all bats that died displayed the severely shortened TBL characteristic of some that died, and some bats that displayed very short TBL did not die.

In other mammalian hibernators, mechanisms associated with immunity are reduced during hibernation, when the conservation of energy is critical [32,33], and the periodic arousals from hibernation may activate the dormant immune system. Thus, immunological responses to fungal infection may be triggering arousals more frequently than normal [34]. Additionally, physical damage to wing skin caused by fungal infection may disrupt other physiological functions, such water balance, resulting in dehydration, another trigger for arousal from torpor in hibernating animals [8]. Equally important to understanding how Gd infection leads to altered torpor patterns is the need to understand how
these too-frequent arousals to euthermia may be contributing to death – in ways that are not clearly related to energy balance, but are potentially related to the disruption of other homeostatic mechanisms [9].

A detailed understanding of the mechanism(s) by which infection with Gd causes mortality in hibernating bats may provide insights to develop interventional strategies to mitigate this unprecedented wildlife disease. Insectivorous bats perform significant ecosystem services because they are primary predators of nocturnal insects [33–37]. As such, we believe that the loss of cave-dwelling hibernating bats in North America will be ecologically significant.

Supporting Information

Appendix S1 Instructions for producing temperature sensitive dataloggers for attachment to bats, including figures. (PDF)

Appendix S2 Description of WNS histopathology and assignment of wing damage severity scores (SS), including figures. (PDF)

References

Please contact the diagnostic laboratory prior to submitting samples from mortality events. Please consult with the diagnostic laboratory prior to collecting samples for live-bat surveillance.

**Photo documentation:**
Surveyor moves through cave observes and photographs bats. Digital images are later examined by surveyors for visible evidence of fungal growth missed by visual observation alone. **Diagnostic value** – poor; this is a good method for detection of suspicious bats but is not definitive for WNS and therefore should be followed by other sampling methods (see below). Other methods will require handling bats, and therefore the consequences of disturbing the bats must be evaluated before further action is taken.

**Fungal tape lift:**
Surveyor handles individual bats to collect samples from muzzles of animals with visible evidence of fungus. This is a nonlethal sampling method designed to identify the type of fungus present rather than determine if fungus exists on an animal; personnel with basic knowledge of light microscopy and fungal morphology can analyze samples. **Diagnostic value** - limited; **this method is only suitable for testing animals with visible fungus.** Sample quality affects analysis, sample method does not detect mild/early WNS infection.

**Wing punch biopsy:**
Surveyor handles individual bats to collect suspicious area on wing membrane using sterile technique. This is a nonlethal sampling method which provides only a small area of tissue to evaluate. **Diagnostic value** - moderate; good quality samples allow for PCR testing. There is good probability of detecting *G. destructans* DNA if sample from wing contains the fungus.

**Whole carcass:**
Fresh carcasses can be collected outside of hibernaculum without disturbing hibernating bats or sick bats within hibernacula showing suspicious clinical signs (visible fungus, abnormal behaviors, etc.) may be euthanized for thorough examination. **Diagnostic value** - high; all available test methods (PCR, fungal culture, histopathology) can be employed on fresh carcasses allowing the highest probability of detecting *G. destructans* and diagnosing WNS based on histopathology. Other tissues can be evaluated and carcasses can be saved for follow-up investigations.

Whole carcass submission is the only method suitable for the early detection (bats with no visible fungus) of WNS. In order to provide the greatest opportunity to detect WNS, visible fungus on bats should be observed in the field. Surveyors will need to evaluate the necessity of handling or collecting bats for the detection of WNS.
### Within the WNS Endemic Area
(Appendix A Map – Pg. 9)

**Unusual bat mortality/behavior not associated with WNS (NOV-APR) Pg. 6**

**Priority Samples**
- Any species
- Any county
- ≥ 5 dead/sick bats at one location
- For other situations- consult with NWHC

**Samples to submit**
- (5-8 bats)
- Photos AND
- Fresh, intact carcasses
- Maximum of 3 euthanized non-T/E bats per site

**Bats with signs suggestive of WNS* (NOV-APR) Pg. 7-8**

**Priority Samples**
- Species not previously confirmed with WNS at/near a contaminated hibernaculum in a confirmed county
- Any species at a hibernaculum of suspect or unknown status in an unconfirmed county

**Samples to submit**
- (1-5 bats)
- Photos AND
- Fresh, intact carcass of any species
- Wing biopsies or fungal tape from live T/E species, banded bats, or species were WNS confirmation is NOT required
- Euthanasia of sick, live bats is not advised except for species not previously confirmed with WNS (maximum of 3 euthanized bats per site)

Species confirmed with WNS - *Myotis lucifugus, M. septentrionalis, M. sodalis, M. leibii, M. grisescens, Perimyotis subflavus, Eptesicus fuscus*

* WNS signs include visible fungus, UV fluorescence, WDI ≥2, suspicious behaviors (day flight activity, entrance roosting, delayed arousal)

pg. i
**Outside of the WNS Endemic Area**  
(Appendix A Map – Pg.9)

<table>
<thead>
<tr>
<th>Unusual bat mortality/behavior not associated with WNS (NOV-APR)</th>
<th>Bats with signs suggestive of WNS* (NOV-APR)</th>
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<tr>
<td>Priority Samples</td>
<td>Priority Samples</td>
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<tr>
<td>• Any species</td>
<td>• Species with confirmed susceptibility to WNS at a suspect positive hibernaculum</td>
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<tr>
<td>• Any county</td>
<td>• Any cave species at/near a hibernaculum of unknown status in any county of unconfirmed status</td>
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<tr>
<td>• ≥ 5 dead/sick bats at one location</td>
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<td>• For other situations- consult with NWHC</td>
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<tr>
<td>Samples to submit</td>
<td>Samples to submit</td>
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<td>(5-8 bats)</td>
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<td>• fresh, intact carcasses</td>
<td>• fresh, intact carcass of any species</td>
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<tr>
<td>• MAXIMUM of 3 euthanized non-T/E bats per site</td>
<td>• wing biopsies or fungal tape from T/E species or banded bats</td>
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<td>• MAXIMUM of 3 euthanized non-T/E bats per site</td>
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</tbody>
</table>

Species confirmed with WNS- *Myotis lucifugus, M. septentrionalis, M. sodalis, M. leibii, M. grisescens, Perimyotis subflavus, Eptesicus fuscus*

* WNS signs include visible fungus, UV fluorescence, WDI ≥2, suspicious behaviors (day flight activity, entrance roosting, delayed arousal)
Bat “White-Nose Syndrome” (WNS) Submission Guidelines
Winter 2012/2013 (November – April)

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The following sample submission guidelines are for use when surveying bat hibernacula or evaluating unusual bat morbidity or mortality during the winter 2012-2013. They are meant to assist with prioritizing appropriate field samples for laboratory submission based on geographic location and prior knowledge of WNS status at survey sites. The primary objectives of this targeted surveillance are to identify new geographic locations and bat species affected with WNS. This document replaces the 2012 Summer Submission Guidelines for Bats and all previous winter submission guidelines from the USGS- National Wildlife Health Center (NWHC). The level of diagnostic evaluation depends on 1) the presence of unusual numbers of sick or dead bats, and 2) the distance from confirmed contaminated sites with greater emphasis on suspect WNS bats found outside the current disease boundaries. These guidelines will be periodically reviewed to ensure that they meet the needs of the field and the laboratory. Please contact your regional FIT member with any questions, suggestions, or concerns (Eastern US: Anne Ballmann, 608-270-2445, aballmann@usgs.gov; Central US: LeAnn White, 608-270-2491, clwhite@usgs.gov; Western US: Barb Bodenstein, 608-270-2447, bbodenstein@usgs.gov).

Winter field signs associated with WNS in bats:

- White or gray powdery fungus seen around the muzzle, ears, wing/limbs, and/or tail;
- Excessive/unexplained bat mortality at the winter hibernaculum;
- Delayed arousal from torpor following disturbance;
- Aberrant bat behaviors (found on ground inside or outside the hibernaculum, roosting near hibernaculum entrance, increased bat activity outside the hibernaculum during cold weather);
- Thin body condition and/or dehydrated (wrinkled and flaky appearance of furless areas);
- Wing damage (membrane thinning, depigmented areas, holes, tears, flaky appearance) in cave bat species found outside the hibernaculum through May

WNS has been confirmed in the following 7 species:

- Little brown bats (*Myotis lucifugus*)
- Tri-colored bats (*Perimyotis subflavus*)
- Northern long-eared bats (*Myotis septentrionalis*)
- Indiana bats (*Myotis sodalis*)
- Small-footed bats (*Myotis leibii*)
- Big brown bats (*Eptesicus fuscus*)
- Gray bats (*Myotis griseascens*)

Potentially susceptible species (only *Geomyces destructans* DNA detected):

- Cave bats (*Myotis velifer*)
- Southeastern myotis (*Myotis austroriparius*)
Key components of the diagnostic effort:

1. **Hibernaculum data collection.**

Fill out the hibernaculum data collection sheet ([Appendix B.2](#)) whenever hibernacula are surveyed, regardless of what state or county you are in and whether or not you see fungus on bats. These data will increase our understanding of the epidemiology of WNS and records of negative data (no fungus or abnormal behaviors observed) are important in this effort. Also complete the 2nd page (Individual Bat Specimen Collection Datasheet Winter 2012/2013) whenever samples are collected for laboratory analysis for WNS. If observed bat mortality does not appear to be related to WNS, please submit specimens with the NWHC Specimen History Form (Appendix B.1). E-mail the appropriate completed datasheets to the appropriate FIT contact (608-270-2415 fax) when submitting samples to NWHC.

If there is no unexplained bat mortality and there is no evidence of fungal growth or unusual behaviors in live bats at the hibernaculum, no photos or diagnostic samples are requested for submission. The NWHC does not accept samples from normal populations of hibernating bats without prior knowledge of purpose and agreement to participate in healthy bat surveillance. Disturbance of hibernation sites can compromise survival of bats.

2. **Field photographs.**

Handling bats may cause much of the visible fungus to disappear before specimens arrive at the lab. Please take good quality field photographs of representative affected bats, particularly in regions where WNS has yet to be identified, to be included with all bat submissions. Digital photos can be e-mailed to the appropriate FIT contact for further submission consultation.

When non-lethal samples (tape-lift or biopsies) are collected, we request close-up images of individual live bats to be sampled. E-mail photos to the appropriate FIT contact (608-270-2415 fax) along with the Hibernaculum/Bat data sheets ([Appendix B.2](#)) including the date photos were taken, site name, and the photographer’s name.

3. **Carcass collection.**

Advised application- whenever laboratory confirmation of WNS is required (suspicious field signs of WNS in a species not previously confirmed with the disease or in a new geographic area).

Once WNS has been confirmed in a federal or state-listed endangered species, only specimens of that species that are found dead will be accepted for diagnostic testing except in extenuating circumstances where necessary permits allow.

Collect the freshest carcasses (intact body, no evidence of scavenging, fur does not pull out easily) of all affected species. If fresh carcasses are unavailable, mummified carcasses are preferable to wet, slimy carcasses and may be accepted upon consultation with a FIT member. Follow carcass collection instructions described in Appendix F. If carcasses are being submitted for diagnostic evaluation, keep individual carcasses chilled in separate bags with ID labels containing the following information:

- date died & date collected (if different)
- location (hibernaculum or nearest town, county, state)
- collector name & phone
- species
- unique animal ID number (standard format: state, MMDDYY, collector, ###; ex: WI061610AE8001)
- found dead or method of euthanasia

Group all individually bagged carcasses destined for laboratory shipment in a 2nd clean bag upon exiting the hibernaculum but prior to traveling to another site. If you plan to visit additional sites on the same day, follow the current recommendations described in the USFWS WNS Decon Supplement for Researchers (http://www.whitenosesyndrome.org/sites/default/files/resource/national_wns_revise_final_6.25.12.pdf).

Contact the appropriate FIT member to arrange shipping. If additional intact carcasses are being saved for future evaluation, triple-bag the labeled specimens, freeze carcasses and store locally. Keep record of frozen bat carcass inventory on datasheets (Appendix B.2).

Please contact the NWHC prior to submitting bat samples. See Appendix F for NWHC shipping instructions.

4. Non-lethal sampling techniques:

NOTE: Bats from WNS confirmed counties with visual evidence of WNS (white material on muzzle and wing membranes) are presumptively positive. Disturbance of these bats that are likely infected with WNS compromises survival and further testing is not advised unless there are special circumstances. Most current non-lethal sampling techniques cannot confirm WNS and may have a reduced reliability of detection as compared to whole carcass evaluation.

☐ Fungal tape-lift sample collection (see Appendix C for detailed instructions)

Advised application- known susceptible bat species in an unconfirmed county within the WNS confirmed region with visible fungus; any threatened/endangered bat species with visible fungus on muzzle; histological confirmation of the disease is not necessary.
Wear clean gloves to handle each bat to reduce the risk of cross-contamination of diagnostic samples. Collect tape-lifts only from visibly affected muzzles of bats (alive or dead) with fungal growth when carcasses cannot be submitted. E-mail hibernaculum data collection sheet (Appendix B.2) and specimen history form (Appendix B.1) to the appropriate FIT contact and send fungal tape slides with a hard copy of the datasheet to the NWHC.

- **Ultraviolet light (UVA) screening of wing membranes (see Appendix E for detailed instructions)**

  Advised application - any dead bat or live bat with physical or behavioral signs suggestive of WNS but lacking visible fungal growth examined mid-winter through spring. **This is an INVESTIGATIONAL screening technique with unknown specificity outside the WNS endemic area.**

  This technique requires handling individual bats to examine extended wings and thus results in hibernation disturbance as well as unknown safety risks to bats. Detection of pale yellow-orange fluorescence spots on wings IS NOT definitive for diagnosing WNS and therefore should be used in conjunction with other techniques for targeted sample collection. **It is not recommended for use in apparently healthy bat populations outside of the WNS endemic area.**

- **Wing punch biopsy (see Appendix D for detailed instructions)**

  Advised application - any threatened/endangered bat species with visible fungus or characteristic fluorescence on wing membranes under UVA light; known susceptible species in an unconfirmed county within the WNS confirmed area with physical evidence (visible fungus, wing damage). This non-lethal sampling is the preferred, more sensitive method to fungal tape lifts for diagnostic evaluation when fungus is present on both flight membranes and muzzle as PCR and/or histopathology may be performed.

  To reduce the risk of cross-contamination among bats, all equipment (i.e.: gloves, tissue punches, biopsy boards, and forceps) should be cleaned or changed between each sampled bat. Collect wing biopsies only on live bats with visible fungal growth or characteristic UV fluorescence when whole carcasses cannot be submitted. Biopsy punches should be collected from portions of the wing membrane that exhibit fungal growth or other types of visible lesions (Appendix D & E). E-mail hibernaculum data collection sheet (Appendix B.2) and specimen history form (Appendix B.1) to the appropriate FIT contact and overnight ship samples to the NWHC.

  **Note:** Non-lethal sampling techniques are meant to serve as adjunct or alternative diagnostic methods to evaluate for the presence of G. destructans among suspect bats at a particular location. The maximum number of individuals (in any sample combination of carcasses, tape lifts, or wing biopsies) per site that will be accepted for WNS/Gd diagnostic evaluation is 10 per season unless prior arrangements have been made with the lab. Not all of the submitted samples may be tested; this will be at the discretion of the lab.
5. **Biosecurity concerns:**

A site contaminated with *Geomyces destructans* retains this designation indefinitely regardless of the presence of affected bats. Follow the most current [protocols for containment and decontamination of field gear and personnel](http://www.whitenosesyndrome.org/sites/default/files/resource/national_wns_revise_final_6.25.12.pdf) described in “National White-Nose Syndrome Decontamination Protocol Version 06.25. 2012)” prior to leaving each survey site. If you plan to visit a potentially uncontaminated hibernaculum after conducting survey work at a contaminated hibernaculum, use clothing, footwear, gear, and vehicles dedicated for use at clean sites.

---

**UNUSUAL BAT MORTALITY/BEHAVIOR NOT ASSOCIATED WITH WNS**

Before entering hibernacula of endangered Indiana bats or any other listed bat species, appropriate Federal and State permits (or authorizations) must be obtained. For listed species, authorization is needed to collect and possess dead specimens, to handle live bats, or to euthanize sick bats.

Priority samples to submit for laboratory diagnostics:

1. Any species in any county nationwide where 5 or more dead or sick bats are observed at one location within 1-2 weeks.

- **If no fungal growth on live bats is observed at the site where unexplained bat mortalities are detected**, collect 5 - 8 freshly dead bats, chill and ship to NWHC as soon as possible for evaluation according to packaging and shipping instructions in Appendix F. A maximum of 3 affected non-T/E species may be euthanized per site for submission if the quality of available carcasses is questionable. Complete a specimen history form (Appendix B.1).
BATS WITH CLINICAL SIGNS SUGGESTIVE OF WNS

Before entering hibernacula of endangered Indiana bats or any other listed bat species, appropriate Federal and State permits (or authorizations) must be obtained. For listed species, authorization is needed to collect and possess dead specimens, to handle live bats, or to euthanize sick bats.

☐ Sites within the WNS confirmed/endemic area (see Appendix A)-
Priority samples to submit for laboratory diagnostics:
1. Bat species not previously confirmed with WNS with suspicious fungal lesions or aberrant behavior in a confirmed county
2. Any bat species with suspicious signs at/near a hibernaculum of suspect or unknown status in an unconfirmed county

Site prioritization recommendations:
Only hibernacula of critical biological or management significance that require conclusive laboratory confirmation of WNS should be surveyed for clinically affected bats within the WNS confirmed area. Notification of need for diagnostic confirmation at sites within this region should be communicated to the laboratory prior to collection of bats. Take field photos and submit 3-5 bats (fresh dead or euthanized) with physical or concurrent behavioral evidence suggestive of WNS along with a completed Hibernaculum/bat datasheets (Appendix B.2). Once WNS is confirmed in the county, only bat species of unknown susceptibility will typically be accepted for WNS diagnostic evaluation from that county.

☐ Sites outside the WNS confirmed/endemic area (see Appendix A)-
Note: It is recommended that all previously identified G. destructans contaminated hibernacula outside the WNS endemic area be surveyed between late February-March for the development of WNS. Do not submit samples if no signs of WNS are observed in the bat population without prior consultation with NWHC.

Priority samples to submit for laboratory diagnostics:
1. Species with confirmed susceptibility to WNS at a suspect positive hibernaculum
2. Any cave bat species with suspicious fungal lesions or aberrant behavior at/near a hibernaculum of unconfirmed status

Site prioritization recommendations:
To be determined by the land resource management agency. Please consult the National Surveillance Implementation Plan (Dec 2011) for prioritization recommendations.
The following sample collection descriptions apply to bats with clinical signs suggestive of WNS regardless of the area they are detected. Consult the NWHC Bat Submission Quick Reference Charts (pg. i-ii) for a summary of sample prioritization recommendations.

- **If fungus, wing damage or characteristic UV fluorescence on wing membranes is observed on dead bats**, fill out hibernaculum/bat datasheets (Appendix B.2) and e-mail to the appropriate FIT contact (608-270-2415 fax). Submit 3-5 fresh carcasses of new bat species of unknown susceptibility only that appear affected from a confirmed county. If county is of suspect or unknown WNS status, submit 3-5 carcasses of any affected species. (See pg. 2 for list of confirmed susceptible species).

- **If live bats have behavioral or physical evidence of suggestive of WNS but no mortality is observed AND**

  - **Histological confirmation IS required**, euthanize up to 3 bats (representative of affected non-T/E species) with evidence of fungus for submission to NWHC. Please see AVMA Guidelines on Euthanasia 2007 at [http://www.avma.org/issues/animal_welfare/euthanasia.pdf](http://www.avma.org/issues/animal_welfare/euthanasia.pdf) and [www.michigan.gov/documents/emergingdiseases/Humane_Euthanasia_of_Bats-Final_244979_7.pdf](http://www.michigan.gov/documents/emergingdiseases/Humane_Euthanasia_of_Bats-Final_244979_7.pdf). Submit photos (clusters and live individuals) and bat carcasses to NWHC (Appendix F). Include completed hibernaculum/bat datasheets (Appendix B.2). **NOTE:** UV-guided wing punch biopsies stored in 10% formalin may be used in lieu of euthanasia (Appendices D & E) although diagnostic reliability for WNS detection may be reduced as compared to whole carcass evaluation.

  - **Histological confirmation is NOT required**, follow one of the methods below:

    1. Perform punch biopsies on 3-5 individuals (2 biopsies per each individual – See Appendix D) per field site from an affected portion of the flight membranes only and store samples chilled or frozen. Photograph the bat prior to biopsy and record associated geographic, demographic, and physical data (Appendix B.2). **NOTE:** Wing punch biopsies continue to be evaluated as a definitive diagnostic & surveillance method for detecting *Geomyces destructans*, the fungus causative of WNS. Thus, negative results do not rule out the possibility of an animal being infected.

    2. Collect fungal tape-lifts of grossly visible white fungal growth on the muzzles of 3 - 5 affected live bats (See Appendix C for detailed instructions). A new tape strip and gloves should be used for each individual bat. Tape-lift slides can be stored and shipped at room/ambient temperature. Follow packaging and shipping instructions for slides only in Appendix C. Include the completed datasheets from Appendix B.2. **NOTE:** The sensitivity of tape-lift samples to detect *Geomyces destructans*, the fungus causative of WNS, is highly dependent on the slide quality; thus, negative results do not rule out the possibility of an animal being infected.
MAP A: Counties and districts with confirmed or suspect (likely) WNS since disease emergence. WNS-confirmed (endemic) region is denoted by shaded area.

Most current map updates are posted at [http://www.fws.gov/WhiteNoseSyndrome/maps.html](http://www.fws.gov/WhiteNoseSyndrome/maps.html)

Modified from map by Cal Butchkoski, PA Game Commission.
SPECIMEN HISTORY FORM
For mortality events please e-mail a USGS Field Investigation Team member before shipping
Western States: Barb Bodenstein, bbodenstein@usgs.gov, 608-270-2447
Central States: LeAnn White, clwhite@usgs.gov, 608-270-2491
Eastern States: Anne Ballmann, aballmann@usgs.gov, 608-270-2445
For single animal cases, Nationwide: Jennifer Buckner, jbuckner@usgs.gov, 608-270-2443

Submitter’s name: 
Address:

Collector’s Name: 
Affiliation: 
Telephone: 
E-mail:

Date collected:

Method of animal collection: ☐ Found Dead, ☐ Died in Hand, ☐ Euthanized
Method of euthanization:

Species:
Number Submitted: Condition: ☐ Chilled, ☐ Frozen, ☐ Preserved Tissues

Specific die-off location (refuge unit, pond, address, intersection, park, etc):

State: County: Nearest City:
Latitude/longitude (Decimal degree in WGS 84): Zone:

Disease onset date: (Best estimate) Disease end date: (best estimate)

Species affected: (The diversity of species affected may provide clues to the disease involved.)

Age/sex: (Any pattern noticed that is related to age and sex?)

Known dead: (Actual number counted) Known sick:

Estimated dead: Estimated sick:
(Consider removal by scavengers or other means, density of vegetation, etc.)

Clinical signs: (Any unusual behavior and physical appearance.)

Population at risk: (Number of animals in the area that could be exposed to the disease.)

Population movement: (Recent changes in number of animals on area and their source or destination, if known.)

Problem area description: (Land use, habitat types, and other distinctive features.)

Environmental factors: (Record conditions such as storms, precipitation, temperature changes, or other changes that may contribute to stress.)

Comments: (Additional information/observations of value such as past occurrences of disease in area, photographs or videos)
APPENDIX B.2 Winter 2012/2013 Hibernaculum Survey Datasheet

Investigator Name(s): ___________________________ Date: ________________
Phone /e-mail: ____________________________________________

<table>
<thead>
<tr>
<th>State:</th>
<th>County:</th>
<th>Site Name:</th>
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**Observations at the hibernaculum entrance (within area impacted by daylight)**

# of bats observed flying at entrance in 5 minutes ________

<table>
<thead>
<tr>
<th>Bat species</th>
<th>Bands observed</th>
<th># live(^1)</th>
<th># dead(^1)</th>
<th># moribund(^1)</th>
<th># with fungus visible(^1)</th>
<th>Distribution of affected bats (Solitary, Clustered(^2))</th>
<th>Photo #(#s) of affected bats</th>
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\(^1\)Indicate if number is an estimate count; \(^2\)Cluster defined as \(\geq 2\) bats in direct contact

**Bat observations inside the hibernaculum**

<table>
<thead>
<tr>
<th>Bat species</th>
<th>Bands observed</th>
<th># live(^1)</th>
<th># dead(^1)</th>
<th># moribund(^1)</th>
<th># with fungus visible(^1)</th>
<th>Distribution of affected bats (Solitary, Clustered(^2))</th>
<th>Photo #(#s) of affected bats</th>
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Comments:

PLEASE ATTACH A MAP OF THE HIBERNACULUM WITH LOCATIONS OF BATS WITHIN THE SITE MARKED & COMPLETE THE INDIVIDUAL BAT DATASHEET (ON BACK) FOR ALL SPECIMEN COLLECTIONS
### APPENDIX B.2 - USGS NWHC Individual Bat Specimen Collection Datasheet Winter 2012/2013

<table>
<thead>
<tr>
<th>ID or Band# (state, MMDDYY, collector, ###)</th>
<th>Species (4 letter code)</th>
<th>Onsite location (Outside, Entrance, Inside)</th>
<th>Sex (Male, Female)</th>
<th>Status (Live, Dead, Euth)</th>
<th>Age Class (Juv, Adult, Unk)</th>
<th>Weight (g)</th>
<th>Forearm length (mm)</th>
<th>Visible fungus (Muzzle, Ear, Wing, Tail)</th>
<th>Wing Damage Score (circle one)</th>
<th>Photo file ID</th>
<th>Sample Type (Fungal Tape, Wing Biopsy, Whole Carcass, Archived)</th>
<th>Comments/Notes Key</th>
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Additional Notes/Diagrams: [use key code in last column to link this information to specific animal(s)]
APPENDIX C - Fungal tape-lift protocol for bats

Protocol: Tape-Strip Sampling of Bats for Identification of Geomyces destructans Fungal Infection

Authors: David S. Blehert and Anne Ballmann, USGS – National Wildlife Health Center

Date: 6 December 2010 (modified)

Purpose: The following procedure is designed to collect visible fungi from the muzzles of bats for later microscopic analyses while minimizing harm to the sampled bat. This technique will NOT confirm White-nose Syndrome (WNS) on bats and should not be used as the sole sampling methodology in areas where WNS has not been previously confirmed in the bat population.

Required materials:

NOTE- Neither the USGS nor the NWHC endorse these vendors as the only sources of these products. This information is provided only as a guideline.

1) Glass microscope slides with white label (25 mm (W) X 75 mm (L); 1 mm thick). Fisher Scientific Catalog #12-552. Fisher list price $58.34 pack (144/pack).

2) Fungi-Tape (25 yards X 1 inch; approximately 1 mm thick). Fisher Scientific Catalog #23-769-321 (Scientific Device Laboratory No. 745). Fisher list price $35.59 per box.

3) Plastic 5-slide transport mailers. (Maximum capacity is 10 slides per mailer – see instruction #9 below). Fisher Scientific Catalog #12-569-35 ($31.00 for pack of 25) or #12-587-17B ($185.35 for pack of 200).

4) Pencil and permanent marker

Procedure:

1. Wear new disposable gloves when handling each individual bat to reduce the risk of cross-contamination.

2. Label the end of a microscope slide in pencil with an animal ID number, date, and anatomical sample location. Muzzle samples yield the clearest results and are the preferred sample location.

3. Remove a precut piece of Fungi-Tape from the box being careful not to contaminate the adhesive surface.

4. Bend the tape-strip (without creasing), adhesive-side out, between your thumb and index finger so that the tape forms the shape of a “U” (Fig. 1).

5. Sample grossly visible areas of fungal growth on the muzzles of bats. When possible, avoid collecting samples from wing membranes as analyses of unfurred skin have not been reliable in detection of Geomyces destructans.

6. Lightly touch the adhesive surface of the tape-strip, at the bottom of the “U”, to an area of suspect fungal growth on bat surface (Fig. 2). DO NOT use your finger to press the tape down onto the bat’s muzzle. Attempt to maximize adherence of fungus to the tape adhesive while minimizing adherence of hair (Fig. 3).
APPENDIX C - Fungal tape-lift protocol for bats – con’t

7. If only a small area is transferred to the tape, use a different portion of the same tape “U” to touch another area of visible fungal growth on the bat. DO NOT attempt to obtain more than 3 lifts per tape strip. Collect only 1 tape-strip per live bat.

8. Align the tape-strip containing the fungal sample, adhesive-side down, over the microscope slide. Ensure that the edges of the tape-strip do not protrude beyond the edges of the microscope slide when laid flat, and do not remove any portion of the tape-strip from the glass slide once it has adhered (Fig. 4).

9. Lightly wipe over the top surface of the tape-strip using a clean paper or cloth towel to consistently adhere the strip to the slide. Circle the area(s) on the tape with a permanent marker containing the material sampled from the bat.

10. Place each slide into a slide mailer for safe transport. If 2 slides are placed per slot, ensure that the tape surfaces of each slide are facing outwards (only the non-tape sides should be in contact so as not to crush the tape). Seal the slide mailer shut with standard tape or rubber bands prior to shipment.

11. Place slide mailer(s) into a clean Ziploc bag and seal closed to transport from the hibernaculum. Place in a second clean Ziploc bag to store or mail to the lab.

12. The slide mailers can now be held at ambient temperature and shipped to the NWHC for microscopic examination. Ship mailers in a padded envelop with a completed specimen history form. If including slide mailers in a cooler shipment with bat carcasses, ensure that the slide mailers are not in contact with the blue ice. Send an electronic copy of the completed specimen history form to the appropriate FIT contact. Contact your regional FIT if you have any additional questions (Eastern US: Anne Ballmann, 608-270-2445; Central US: LeAnn White, 608-270-2491; Western US: Barb Bodenstein, 608-270-2447).
APPENDIX C. Illustrations – Fungal tape-lift protocol for bats
Photographs by D. Berndt and D. Johnson, USGS – NWHC

Fig. 1

Fig. 2

Fig. 3

Fig. 4
APPENDIX D - Instructions for Taking a Wing Tissue Biopsy
Updated by Pat Ormsbee (NFS) and Jan Zinck 5/14/09 (original: Shonene Scott, Portland State University 5/2003)
Modified by Anne Ballmann (USGS-NWHC) 12/21/12

NOTE: If punch biopsies are the only sample type to be submitted to the lab for PCR testing of \textit{G. destructans} in a particular case, it is highly recommended that 2 biopsies per bat be collected (from different wings). Additional population genetic sampling should not be attempted in these individuals to reduce the number of holes in the wings. \textbf{This technique may NOT confirm White-nose Syndrome (WNS) on bats and should not be used as the sole sampling methodology in areas where WNS has not been previously confirmed in the bat population.}

1. When taking biopsies it is important to reduce the potential for cross-contamination between bats. In order to do this, use a small clean piece of sturdy cardboard that can be discarded after each animal, a new tissue punch for each bat, sterilized forceps, and disposable gloves.

2. Label a sterile vial: Use a black ultra-fine Sharpie permanent marker and a sticky paper label. Be careful that once the label is adhered to the tube the entire identifier is visible. Use the following naming convention to uniquely identify the bat:

   State, Date (MMDDYY), Collector initials, bat number (ex: WI061609AE8001)

3. Have a fresh cardboard square, a labeled tube, a new tissue punch, and a sterilized forceps ready for each bat. Do not touch (contaminate) the end of the punch, the forceps, or the inside of the tube lid with fingers or environmental debris.

4. Identify 2 representative lesions to biopsy on the affected wings/tail of the bat. Place the bat on the cardboard on its back and extend one wing membrane (Avoid sampling from bats with large wing tears). For people inexperienced in this technique, it works best when one person holds the bat and another person collects the biopsy.

5. When collecting wing tissue biopsies, avoid bones and major blood vessels. (Figure 1). \textit{Long-wave UV light can optimize biopsy placement and allows for additional histopathological evaluation (target areas exhibit faint yellow-orange fluorescent spotting-See APPENDIX E).} If possible, locate an affected area near the body wall within the lower half of the wing membrane or uropatagium. These locations have been demonstrated to have faster healing rates and are less disruptive to flight aerodynamics (Faure PA et al. 2009. J Mammalogy 90(5): 1148-56.) Press the punch firmly through the membrane and twist the punch slightly to ensure a complete punch. Apply direct pressure to biopsy site for several minutes if bleeding occurs.

Figure 1: “X” marks ideal sample locations for collecting tissue biopsies from bat flight membranes.
APPENDIX D - Instructions for Taking a Wing Tissue Biopsy - con’t

6. Carefully lift the bat off the biopsy board and look for the tissue sample. It should either be on the board or inside the tip of the punch. Be careful on windy days since the wind can blow the tissue off of the board. A new 25 ga needle or sterile forceps can be used to pick up the tissue and transfer each biopsy to separate storage vials. If fungal PCR is desired for diagnostic analysis, place tissue into an empty vial (no storage media) for storage. If histopathological evaluation is desired, place tissue into a storage vial containing 10% buffered neutral formalin (1 part tissue to 10 parts formalin).

7. Release the bat only after tissue samples have been placed into the tubes, the tubes have been closed, and any bleeding has stopped. The number of biopsies has been limited to 2 per bat to prevent compromising flight.

8. While in the field, sample tubes should be stored on ice. Subsequently, unfixed samples should be frozen until submitted for fungal PCR analysis. Formalin-fixed samples should be held at room temperature (not frozen).

9. Dispose of the used biopsy punch after each animal. DO NOT reuse the same biopsy punch on multiple bats. The punches are very sharp. Be careful to not cut yourself. Change into new gloves before handling each bat.

10. Before reusing forceps while in the field, rinse in alcohol and flame sterilize. Allow forceps to cool before contacting bat tissue. Upon returning to the office, perform a more thorough cleaning and disinfection of nondisposable biopsy equipment with detergent washing followed by soaking in a 10% bleach solution for 10 min with a thorough clean water rinse. Once dry, forceps can be placed into a clean hard surface container (not plastic bags), free of contaminants, marked for cleaned forceps, and with handles all pointing in the same direction.

11. Ship wing tissues to NWHC. Ensure that all cryovials are labeled and lids are secured in place to prevent cross-contamination of samples. Wrap lid of cryovials in parafilm and place in a Ziploc bag. If parafilm is not available double-bag specimens before placing in cooler. Specimens should be chilled and shipped overnight in a cooler with blue ice. If unfixed samples cannot be shipped overnight freeze them and ship as soon as possible.

Send an electronic copy of the completed hibernaculum/bat datasheets (Appendix B.2) to the appropriate FIT contact. Shipping address and examples of appropriate shipping materials are in Appendix F. Contact Anne Ballmann (aballmann@usgs.gov, 608-270-2445) if you have any additional questions.

SUPPLIES: NOTE- Neither the USGS nor the NWHC endorse these vendors as the only sources of these products. This information is provided only as a guideline.

- 3-5 mm biopsy punches Fisher Scientific Catalog # NC9515874 ($106.73/pack of 50)
- Forceps OR 25 gauge needles and sharps collection container
- 10% bleach solution (can be made fresh each time, or can be stored in opaque containers for 24 hours, it begins to break down after this)
- 10% buffered neutral formalin (if histopathological analysis is desired)
- Sterile rinse water
- 2 ml sterile plastic vials with caps
- 95% ethanol and flame source such as cigarette lighter (for sterilizing metal sampling equipment)
- Fine point permanent marker
- Vial labels
- Disposable gloves
- Paper towels/gauze
- Nonporous cutting board
- Ziploc bags and cooler with blue ice
APPENDIX E – Longwave ultraviolet (UVA) fluorescence screening of bat wings

Authors: Anne Ballmann, Carol Meteyer (modified from G. Turner & J. Gumbs 2011)
Date: 5/7/2012, revised 12/21/12

Purpose: To examine bat wings with little to no visible fungal growth for evidence of yellow-orange fluorescence areas suggestive of an infection by Geomyces destructans. This is an INVESTIGATIONAL screening technique with unknown specificity outside the WNS endemic area. It will NOT confirm White-nose Syndrome (WNS) on bats and should not be used as the sole sampling methodology in areas where WNS has not been previously confirmed.

Equipment:
NOTE- Neither the USGS nor the NWHC endorse these vendors as the only sources of these products. This information is provided only as a guideline.
- 380-385 nm wavelength UV 51 bulb LED flashlight (LED Wholesaler #7202UV385-$35) or 368 nm wavelength 9 V UV box (Contact Greg Turner [grturner@pa.gov] for more details on UV box system)
- Disposable exam gloves
- Digital camera
- Permanent marker
- PPE: UVA blocking safety glasses, SPF 15+ sunblock on exposed human skin

Additional equipment for non-lethal sample collection
- 2 ml sterile vials with screw cap lids
- 10% buffered neutral formalin
- 3-5 mm sterile punch biopsies

Procedure: (To reduce potential cross-contamination, use clean exam gloves when handling each bat.)

1. In complete darkness, shine the UV flashlight facing down approximately 3-5 inches (7.5-12.5 cm) above the extended ventral surface of the flight membranes (Fig. 1A). If using a UV box, place the bat on its back and extend the wing and corresponding foot over the UV light source to transilluminate the wing surface. Disinfect surface of UV box between bats. Avoid shining the light into the unprotected eyes of the bat or people or exposing bat skin to UV light for more than 3 minutes.

2. Examine wing membrane for circular areas of yellow-orange fluorescence (Fig. 1B). Fluorescence will be faint when viewed with the naked eye using a hand-held UV flashlight. Visualization is greatly enhanced by examining a digital photograph of the UV-illuminated wing surface when using the UV box. Photography does not improve visualization with the UV flashlight.

3. If the bat is to be euthanized, use a permanent marker to circle representative areas of fluorescence on the wing membrane to target sampling in the laboratory. Place marks outside of the fluorescent border.

4. If live-sampling techniques are used, collect paired wing punch biopsies (3-5 mm diameter, See Appendix D) that incorporate areas of UV fluorescence. Place one wing biopsy into a 2ml vial containing 1.5 ml of 10% buffered neutral formalin for histology. Place the second wing biopsy into an empty vial for PCR and keep chilled in the field. Label vials with the unique bat ID number.

5. Submit samples along with any digital photos of fluoresced wings to the appropriate FIT contact at NWHC.

Figure 1. A) UV flashlight examination of ventral bat wing to be conducted in total darkness. B) Digital photo of backlit extended wing held over 368 nm UV light box. Arrows identify yellow-orange fluorescent areas of various diameters associated with suspect G. destructans infection.
APPENDIX F

USGS – National Wildlife Health Center

INSTRUCTIONS FOR COLLECTION AND SHIPMENT OF AVIAN AND MAMMALIAN CARCASSES

Contact your USGS Field Investigation Team (FIT) member first!
Eastern states – Dr. Anne Ballmann aballmann@usgs.gov, 608-270-2445
Central states - Dr. LeAnn White, clwhite@usgs.gov, 608-270-2491
Western states – Barb Bodenstein, bbodenstein@usgs.gov, 608-270-2447
Single animal cases, Nationwide: Jennifer Buckner jbuckner@usgs.gov, 608-270-2443
Emergency Contact Number 608-270-2400

The following instructions should be used for collecting and shipping wildlife carcasses, carcass parts, and samples extracted from animals to the National Wildlife Health Center (NWHC) to insure adequate and well preserved specimens.

Freezing/thawing impedes isolation of some pathogens and damages tissues. NWHC prefers unfrozen specimens if they can be sent within 24-36 hours of collection or death. We will provide guidance on freezing samples on a case-by-case basis. As a general guideline: if you cannot call or ship within 24-36 hours, freeze the animal(s).

☐ Contact FIT to get shipping approval and discuss shipping arrangements. Typically, ship specimens by 1-day (overnight) service, Monday through Wednesday, to guarantee arrival at NWHC before the weekend. If specimens are fresh and need to be shipped on Thursday or Friday, special arrangements can be made.

☐ Email/fax history and tracking number to FIT. Packages will not be opened if history does not arrive first!

☐ Use rubber, vinyl, or nitrile gloves when picking up sick or dead animals. If you do not have gloves, insert your hand into a plastic bag.

☐ More than one disease may be affecting the population simultaneously. When possible, collect both sick and dead animals. Note behavior of sick animals before euthanizing.

☐ Collect specimens that are representative of all species affected and geographic areas.

☐ Collect the freshest dead specimens. Decomposed or scavenged carcasses are usually of limited diagnostic value. If you plan to collect animals in the field, take along a cooler containing ice to immediately chill carcasses.

☐ Collect animals under the assumption that an infectious disease or toxin is involved and other animals may be at risk. Protect yourself as some diseases and toxins are hazardous to humans.

☐ Place each animal in a plastic bag, close, and seal the bag. Twist non-zipper bags closed, fold over on itself, and secure with package strapping or duct tape. Label the outside of this bag with the following information in waterproof ink:
  - Date collected
  - Location (specific site, town, county, state)
  - Collector (name/address/phone)
  - Species
  - Found dead or euthanized
  - Your reference #

☐ Place 1st bag inside a 2nd bag, close and seal. More than one individually bagged animal can be placed in the 2nd bag. This prevents cross-contamination of individual specimens and leaking shipping containers.

☐ Tag the outside of 2nd bag and number of animals and type, date collected, location, and name of collector. Reminder order: TAG, BAG, BAG, TAG.

☐ Use a hard-sided cooler in good condition for shipment. Close the drain plug of cooler and tape over inside. Line cooler with a thick bag (1 mil thickness, 3rd layer of bags).
Place absorbent material in the 3rd plastic bag to absorb any liquids that might leak during shipping.

Place the individually bagged animal(s) that are contained within the 2nd sealed bag into the 3rd bag with enough FROZEN BLUE ICE PACKS or similar coolant to keep carcasses cold. Use enough coolant to keep samples chilled if there is a delay in delivery.

- Blue ice (unfrozen) can be obtained at hardware, sporting goods, or grocery stores.
- Wet ice can be used if frozen in a sealed plastic container (i.e., soda or water bottle).
- DO NOT USE DRY ICE.

Seal the 3rd bag with methods described for 1st bag.

Place the completed specimen history and return shipping label in a ziplock bag and tape to the inside lid of the cooler (if you want the cooler returned). NWHC CANNOT PAY FOR SHIPPING.

Using packing or duct tape, tape the cooler shut around the lid and at each end using a continuous wrap around the cooler.

Attach the shipping document (airbill) with the DOT information below to the outside of each cooler in a resealable pouch:

Address:
National Wildlife Health Center
Necropsy Loading Dock
6006 Schroeder Road
Madison, WI 53711

Emergency Contact:
NWHC FIT emergency
608-270-2400

Supplementary Labels:
Keep Cold

Mark the cooler with the appropriate information:
(Carriers that died of unknown causes:
BIOLOGICAL SUBSTANCE, CATEGORY B and UN 3373.

- Blood and tissue samples from apparently healthy animals (hunter-killed, live captured):
EXEMPT ANIMAL SPECIMENS.

- Blood and tissue samples from dead or sick animals:
BIOLOGICAL SUBSTANCE, CATEGORY B and UN 3373.

Note the tracking number in case packages are delayed.

These instructions cover federal shipping regulations for commercial carriers.

Appendix:
Example of bags available at large supermarkets (list not all inclusive):

Inner and second layer bags:
- Hefty Big Bag – 22 gal
- Hefty Freezer – 1 gal
- Hefty Jumbo – 2.5 gal

Third layer for cooler liner:
- Hefty Cinch Sak (1.1 mil) – 33 and 39 gal
- Hefty Lawn and Leaf (1.1 mil) – 33 and 39 gal
- House brand large trash (1.1 mil) – 30 gal

Absorbent material:
- Super absorbent packet or pads for water
- Paper towels
- Do not use packing peanuts or shredded paper.

- Ziplock Freezer – 1 gallon
- Ziploc Big Bag – 20 gallon
- Glad Freezer – 1 qt, 2 qt, 1 gal

- Glad Force Flex (1.05 mil) – 25 gal
- Hefty Ultra Flex (1.3 mil) – 30 gal
- House Lawn - Leaf (1.2 mil) – 39 gal

Cellulose wadding
Cotton batting or cotton balls
UN3373

BIOLOGICAL SUBSTANCES, CATEGORY B

EXEMPT ANIMAL SPECIMENS
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<td>Ontario/Nanavut Atlantic Quebec Western/Northern</td>
<td><a href="mailto:dgcampbe@uoguelph.ca">dgcampbe@uoguelph.ca</a> <a href="mailto:smcburney@upei.ca">smcburney@upei.ca</a> <a href="mailto:andre.dallaire@umontreal.ca">andre.dallaire@umontreal.ca</a> <a href="mailto:trent.bollinger@usask.ca">trent.bollinger@usask.ca</a></td>
<td>Doug Campbell 519-824-4120 x54556 Scott McBurney 902-566-0959 Andre Dallaire 450-773-8521 x8474 Trent Bollinger 306-966-5153</td>
<td>N/A at time of publication</td>
<td>N/A at time of publication</td>
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<tr>
<td>Colorado State University Diagnostic Medical Center</td>
<td>CSU Veterinary Diagnostic Lab 200 West Lake St 1644 Campus Delivery Fort Collins, CO 80523</td>
<td><a href="mailto:Terry.spraker@colostate.edu">Terry.spraker@colostate.edu</a> <a href="mailto:Colleen.duncan@colostate.edu">Colleen.duncan@colostate.edu</a></td>
<td>Terry Spraker or Colleen Duncan 970-297-4155 970-297-5422</td>
<td>8-5 M-F Weekend cases done in mornings, pathologist on duty 7d/wk</td>
<td>- Private citizen/org - State Agency - Federal Agency - Tribal Agency - In state - Out of state</td>
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<tr>
<td>New York State Animal Health Diagnostic Lab, Cornell University</td>
<td>AHDC, CVM Cornell University Upper Tower Rd Ithaca, NY 14853</td>
<td><a href="mailto:elb36@cornell.edu">elb36@cornell.edu</a></td>
<td>Elizabeth Buckles 607-253-3319</td>
<td>Receive for necropsy 24hr/day, 7 days/wk Mail/courier delivery Mon-Sat. Call ahead for Saturday delivery.</td>
<td>- Private citizen/org if from agency or vet. - State agency - Federal agency - Tribal agency - In state - Out of state</td>
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<tr>
<td>Southeastern Cooperative Wildlife Disease Study (SCWDS)</td>
<td>589 DW Brooks Dr SCWDS College of Veterinary Medicine Athens, GA 30602</td>
<td><a href="mailto:lalast@uga.edu">lalast@uga.edu</a></td>
<td>Lisa Last 706-542-1741</td>
<td>8-5 M-F. Offices cannot receive packages on weekends.</td>
<td>- State agency (Members of SEAFWA, Ohio, Kansas) - Federal agency (USFWS) - In state - Out of state</td>
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<tr>
<td>USGS National Wildlife Health Center (USGS-NWHC)</td>
<td>Necropsy Loading Dock 6006 Schroeder Rd Madison, WI 53711</td>
<td><a href="mailto:aballmann@usgs.gov">aballmann@usgs.gov</a></td>
<td>Anne Ballmann 608-270-2445 Main number: 608-270-2400</td>
<td>8-5 M-F, excluding federal holidays. Saturday delivery only by special prior arrangement.</td>
<td>- Private citizen only if coordinated through appropriate state agency - State agency - Federal agency - Tribal agency - In state - Out of state</td>
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by desquamation of palms and soles (Figure). Laboratory tests detected leukopenia and thrombocytopenia. Test results for *Plasmodium* spp. and dengue virus were negative, and blood culture results were negative as well. By using ELISA, anti-CHIKV IgM antibodies were detected 10 days after onset of symptoms, and anti-CHIKV IgG antibodies (titer 25,600) were detected 8 months later.

Both patients were diagnosed after the viremic period; no virus could be isolated or genotyped. Nevertheless, health authorities were alerted and appropriate control measures were taken.

Travelers can serve as sentinels for the introduction of viruses into previously non–disease-endemic areas. Several reports have been made of travelers carrying CHIKV to and from many regions of the world (2,4–6). Recent identification of the expansion of infested areas by *Ae. aegypti* and *Ae. albopictus* mosquitoes, population susceptibility for the virus, and the constant journeying of travelers from affected areas are relevant indications of the risk for introduction and sustained transmission of CHIKV in Brazil.

Health care professionals and public health authorities should be aware of the epidemiologic and clinical aspects of CHIKV infection and diagnoses to adopt prompt control measures to avoid CHIKV transmission in Brazil. Healthcare facilities and epidemiologic surveillance teams have jointly implemented CHIKV prevention and control measures. To date, no autochthonous transmission of CHIKV has been reported in Brazil.

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Enhanced Surveillance for White-Nose Syndrome in Bats

To the Editor: White-nose syndrome (WNS) is an emerging fungal disease in bats that was first described near Albany, New York, USA, in February 2006 (1). The causative agent, *Geomyces destructans*, is a psychrophilic (cold-loving) fungus that infects the skin of bats and leads to depletion of their fat stores during hibernation (2). WNS has caused dramatic cumulative mortality rates (up to 99%) in some winter hibernacula and has killed millions of
bats among 6 cave-roosting species in 19 central and eastern US states and 4 Canadian provinces (3). In addition, the fungus has been identified in 2 additional US states, although bat deaths have not been associated with it. No evidence has been found that WNS is transmitted from bats to humans, although humans may play a role in translocation of the fungus between caves (4,5).

Current surveillance for WNS is time- and labor-intensive. Wildlife personnel typically enter caves, inspect hibernacula, and collect bats with clinically compatible signs for testing (4). In July 2010, the National Park Service (NPS) Office of Public Health proposed an expanded WNS surveillance strategy that involved using opportunistic sampling of bats already submitted to state public health laboratories for rabies testing; the bats submitted include species known to be susceptible to WNS. The pilot study focused on the region around Mammoth Cave National Park, the world’s longest known cave system and home to 13 bat species (2 endangered), in south-central Kentucky (6). At the time of initial discussions, Kentucky was WNS-free, but the bordering state of Tennessee had recently reported its first WNS cases in spring 2010 in a cave system located <130 km from Mammoth Cave. WNS was first detected in Kentucky in April 2011 in Trigg County (180 km from Mammoth Cave) (7).

The goals of this pilot study were to 1) enhance WNS surveillance in counties in and near Mammoth Cave and 2) demonstrate a feasible, cost-effective surveillance system. NPS Office of Public Health staff coordinated meetings in Kentucky and Tennessee with representatives from the state departments of wildlife and health and other partnering organizations. Key representatives at one or both of these meetings included the state epidemiologist, the state public health veterinarian, the public health laboratory director, state wildlife biologists, and NPS and state wildlife veterinarians. Also attending both meetings was a veterinary pathologist from the Southeastern Cooperative Wildlife Disease Study (SCWDS) in Athens, Georgia, USA, one of 3 laboratories that test most samples for WNS in the United States. The surveillance concept was well received in both states, and state-specific protocols were developed for submitting rabies-negative bats to SCWDS only during hibernation months (November–April) when WNS is more likely to be detected (8). In Kentucky, a memorandum of understanding was drafted that outlined roles and responsibilities of collaborating agencies. The memorandum was reviewed by legal advisors and signed by public health and wildlife officials.

Both protocols outlined key elements of the submission process, including how laboratory personnel were to submit rabies-negative bats to SCWDS for WNS testing (fungal culture, histopathologic examination, and PCR), how bats were to be stored or destroyed after testing, and the chain of communication for reporting test results. Whenever possible, bats were frozen at −20°C within 48 hours following rabies testing, and their muzzles and forearms were left intact to maximize the yield for G. destructans and to facilitate species identification. Protocols included additional criteria to improve testing efficiency (e.g., prioritizing submissions on the basis of known WNS-susceptible species or counties where cave-roosting colonies are located). A project-specific version of the standard SCWDS submission form was completed for all samples. All funding and resources were provided in kind by respective agencies.

In October 2010, the Tennessee State Public Health Laboratory submitted 34 rabies-negative bats (archived during January–April 2010, before pilot study discussions) from 18 counties to SCWDS; all were WNS-negative. Twenty-one additional rabies-negative bats from 9 Tennessee counties collected during November 2010–April 2011 also tested negative for WNS. In Kentucky, 64 rabies-negative bats (from 22 counties) were submitted during November 2011–January 2012; all were WNS-negative except 1 bat tested on January 13, 2012, which was the first known WNS-positive bat from Fayette County, a primarily urban area in northern central Kentucky where little cave-based WNS surveillance is conducted. Overall, although the sample of bats tested to date is modest and likely insufficient as a stand-alone surveillance system, these results supplement other data and can inform the development of interventions, prevention messages, and transmission models.

This pilot study highlights several observations and implications. First, it demonstrates that opportunistic testing of rabies-negative bats for WNS can be facilitated between state departments of wildlife and health through interagency collaboration. Second, the surveillance system is low cost and could potentially be expanded to other states where WNS is likely to emerge and where statewide cave-based surveillance is cost-prohibitive. Last, this project showcases a unique interdisciplinary collaboration in wildlife and human health, disease ecology, and environmental stewardship. Such partnerships are championed by the One Health approach (9) and are central to the mission of NPS to protect the health of all species and our environment (10).

Acknowledgments

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NDM-1-producing Klebsiella pneumoniae, Croatia

To the Editor: The novel metallo-β-lactamase named New Delhi metallo-β-lactamase (NDM-1) was identified from Klebsiella pneumoniae and Escherichia coli isolates in Sweden from a patient previously hospitalized in India (/). NDM-1 is spreading rapidly worldwide to nonclonally related isolates, many of which are directly or indirectly tracked to the Indian subcontinent (2). A carbapenem-resistant K. pneumoniae strain, KLAZ, was isolated in May 2009 from the culture of a blood sample from a 40-year-old man on the day after his admission to a surgical intensive care unit of the Clinical Hospital Center in Zagreb, Croatia. The patient had been transferred after 5 days of hospitalization in Bosnia and Herzegovina following a car accident. The clinical history mentioned antimicrobial drug treatment that did not include carbapenems (gentamicin, meropenem, and ceftriaxone) and no link to the Indian subcontinent. Antimicrobial drug susceptibility testing was performed by Vitek2 (bioMérieux, Marcy-l’Etoile, France) and broth microdilution and interpreted according to the latest documents from the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org/clinical_breakpoints/, version 1.1).

The strain proved resistant to imipenem and meropenem, to all broad-spectrum cephalosporins, and to aminoglycosides and susceptible to ciprofloxacin and ticarcycline (Table). We checked for bla<sub>NDM</sub>, bla<sub>TEM</sub>, bla<sub>SME</sub>, bla<sub>GIM</sub>, bla<sub>IMP</sub>, and bla<sub>NDM</sub> resistance genes by using PCR. A PCR product was obtained only with the NDM primers, after being purified (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany), its sequence showed 100% identity with bla<sub>NDM</sub>.

Strain genotyping was performed by multilocus sequence typing to determine the sequence type (ST) of the isolate and to establish a comparison with previously reported NDM-1-producing isolates. Allelic numbers were obtained on the basis of sequences of 7 housekeeping genes at www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.
The fungus *Geomyces destructans* (*G.d.*) is the cause of white-nose syndrome (WNS), a disease that has devastated populations of hibernating bats in eastern North America. Since its discovery in New York in 2007, WNS has spread rapidly through northeastern, mid-Atlantic, and Midwest states and eastern Canada. It continues to threaten bat populations across the continent. For the protection of bats and their habitats, comply with all current cave and mine closures, advisories, and regulations on the federal, state, tribal, and private lands you plan to visit. In the absence of cave and mine closure policy, or when planned activities involve close/direct contact with bats, their environments, and/or associated materials, the following decontamination procedures should be implemented to reduce the risk of transmission of the fungus to other bats and/or habitats. For the purposes of clarification, the use of the word “decontamination,” or any similar root, in this document entails both the 1) cleaning and 2) treatment to disinfect exposed materials.

Under no circumstances should clothing, footwear, or equipment that was used in a confirmed or suspect WNS-affected state or region be used in a WNS-unaffected state or region. Some state/federal regulatory or land management agencies have supplemental documents\(^1\) that provide additional requirements or exemptions on lands under their jurisdiction.

**I. TREATMENTS TO REDUCE RISK OF TRANSFERRING *GEOMYCES DESTRUCTANS*\(^2\):**

**Applications/Products:**

The most universally available option for treatment of submersible gear is:

**Submersion in Hot Water:** Effective at sustained temperatures \(\geq 50^\circ C\) (122°F) for 20 minutes

Secondary or non-submersible treatment options (for a minimum of 10 min.) include:

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

\(^1\) To find applicable addenda and/or supplemental information, visit [http://www.whitenosesyndrome.org/topics/decontamination](http://www.whitenosesyndrome.org/topics/decontamination)

\(^2\) The use of trade, firm, or corporation names in this protocol is for the information and convenience of the reader. Use of some treatments which utilize such method need to be applied carefully, especially in confined spaces, due to inhalation or contact risks of the product. All users should be aware of these risks.
Other effective disinfectant(s) with similar chemical formulas (e.g., a minimum of 0.3% quaternary ammonium compound) or water based applications may exist but are unknown and not recommended at this time.

**REMEMBER, the product label is the law!**

*It is the responsibility of the users of this protocol to read and follow the product label and MSDS.*

**Products must be used in accordance with the label:**

Ensuring the safety of those who use any of the above products for treatment is of utmost importance. Material safety data sheets (MSDS) developed by product manufacturers provide critical information on the physical properties, reactivity, potential health hazards, storage, disposal, and appropriate first aid procedures for handling or working with substances in a safe manner. Familiarization with MSDS for chemical products prior to use will help to ensure appropriate use of these materials and assist in emergency response.

It is a violation of federal law to use, store, or dispose of a regulated product in any manner not prescribed on the approved product label and associated MSDS.

- Disinfectant products, or their contaminated rinse water, should be managed and disposed of as per product label directions to avoid contamination of groundwater, drinking water, or non-municipal water feature such as streams, rivers, lakes, or other bodies of water. Follow all local, state and federal laws. State-by-state requirements for product disposal may vary. Note: Quaternary ammonium wastewaters should not be drained through septic systems because of the potential for system upset and subsequent leakage into groundwater.

**II. PLAN AHEAD AND CAVE CLEAN:**

**Dedicate your Gear:** Many types of rope and webbing have not been thoroughly tested for integrity after decontamination. Dedicate your gear to a single cave/mine or don’t enter caves/mines that require this gear.

**Bag it Up:** Bring bags on all of your trips. All gear not decontaminated on site should be isolated (quarantined) in a sealed plastic bag/s or container/s to be cleaned and disinfected off-site.

**Before Each Cave/Mine or Site Visit:**

1.) Determine G.d./WNS status\(^5\) of the state/county(s) where your gear was previously used.

2.) Determine G.d./WNS status\(^5\) of state/county(s) to be visited.

3.) Determine whether your gear is permitted for your cave/mine visit or bat related activity, as defined by the current WNS case definitions\(^6\) and the flowchart below.

4.) Choose gear that can be most effectively decontaminated [i.e., rubber wellington type (which can be treated with hot water and/or secondary treatment options in section I.) vs. leather boots] or dedicated to a specific location. **Remember, under no circumstances should any gear that was used in a WNS-affected state or region be used in a WNS-unaffected state or region.** Brand new gear can be used at any location where access is otherwise permitted.

5.) Determine if any state/federal regulatory or land management agency addendum or supplemental document\(^1\) provides additional requirements or exemptions on lands under its jurisdiction that supplement the final instruction identified in the flowchart below.

6.) Prepare a “Clean Caving” strategy (i.e., how and where all gear and waste materials will be stored, treated and/or disposed after returning to your vehicle and base area) for your particular circumstances that provides for cleaning and treatment of gear on a daily basis **unless** instructed above to do so more frequently throughout the day.

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\(^1\) Visit [http://www.whitenosesyndrome.org/resources/map](http://www.whitenosesyndrome.org/resources/map) to determine the WNS status of a county or state.

\(^5\) Visit [http://www.whitenosesyndrome.org/resources/map](http://www.whitenosesyndrome.org/resources/map) to determine the WNS status of a county or state.


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7.) When visiting multiple caves/mines or bat research sites on the same day, clean and treat all gear between each cave/mine/site, unless otherwise directed in an agency/landowner addendum. It is recommended that known confirmed or suspect caves/mines be visited only after those sites of unknown G.d. status have been visited, to further reduce the risk of inadvertent transmission.

**Flowchart to Determine Gear Use or Decontamination**

- **Start**: Will your visit or activity take place in a suspect or confirmed state?
  - Yes
    - Will your visit or activity take place in a suspect or confirmed county?
      - Yes
        - Has your gear previously been used in a suspect or confirmed state?
          - Yes
            - Has your gear previously been used in the same state you are planning to visit?
              - Yes
                - Has your gear previously been used in a confirmed county?
                  - Yes
                    - Check with the applicable state/federal regulatory agency to determine whether properly decontaminated gear may be used.
                  - No
                    - No
                      - No
                        - No
                          - No
                            - No
                              - No
                                - No
                                  - No
                                    - No
                                      - Yes
                                        - DO NOT use gear, even if it has been properly decontaminated.
                                      - No
                                        - Yes
                                          - DO NOT use gear, even if it has been properly decontaminated.
                                          - No
                                            - Yes
                                              - DO NOT use gear, even if it has been properly decontaminated.
                                              - No
                                                - Yes
                                                  - DO NOT use gear, even if it has been properly decontaminated.
                                                  - No
                                                    - Yes
                                                      - DO NOT use gear, even if it has been properly decontaminated.
                                                      - No
                                                        - Yes
                                                          - DO NOT use gear, even if it has been properly decontaminated.
                                                          - No
                                                            - Yes
                                                              - DO NOT use gear, even if it has been properly decontaminated.
                                                              - No
                                                                - Yes
                                                                  - DO NOT use gear, even if it has been properly decontaminated.
                                                                  - No
                                                                    - Yes
                                                                      - DO NOT use gear, even if it has been properly decontaminated.
                                                                      - No
                                                                        - Yes
                                                                          - DO NOT use gear, even if it has been properly decontaminated.
                                                                          - No
                                                                            - Yes
                                                                              - DO NOT use gear, even if it has been properly decontaminated.
                                                                              - No
                                                                                - Yes
                                                                                  - DO NOT use gear, even if it has been properly decontaminated.
                                                                                  - No
                                                                                      - Yes
                                                                                         - DO NOT use gear, even if it has been properly decontaminated.
                                                                                         - No
After Each Cave/Mine or Site Visit:

1.) Thoroughly scrub and remove sediment/dirt from clothing, footwear, and other gear immediately upon emerging from the cave/mine or bat research site. Avoid contamination of vehicles; store exposed gear separately from unexposed gear.

2.) Once fully scrubbed and rinsed of all soil and organic material, clothing, footwear, and any appropriate gear should be sealed, bagged in a plastic container and once at home, machine or hand-washed/cleaned using a conventional cleanser like Woolite® detergent or Dawn® antibacterial dish soap in water (the use of Dawn® antibacterial dish soap is not intended for use in conventional washing machines.) Once cleaned, rinse gear thoroughly in water. Clean/treat gear used in a suspect or confirmed state prior to transport when traveling back to or through a state without known cases of G.d./WNS. Use the treatments listed under Applications/Products on page 1 for a minimum of 10 (products) or 20 (hot water) minutes.

Remember: Many types of rope and webbing have not been thoroughly tested for integrity after decontamination. Dedicate your gear to a single cave/mine or don’t enter caves/mines that require this gear.

A.) Submersible Gear (i.e. clothing, footwear, and/or equipment that can be submerged in liquid):

Clothing, footwear, and other submersible gear:

Following steps 1 and 2 above, the primary treatment for all submersible gear should always be submersion in water of at least 50ºC (122ºF) for a minimum of 20 minutes, where possible. Some submersible gear (depending on material) could be soaked for a minimum of 10 minutes in the appropriate products listed in the Applications/Products chart on page 1, rinsed thoroughly in water again, and air dried. Note: Although commercially available washing machines with sanitation cycles often sustain desirable water temperatures, their efficacy for killing the conidia of G.d. is unknown.

B.) Non-submersible Gear:

Gear that may be damaged by liquid submersion should be cleaned according to the manufacturer’s recommendation between cave/mine visits and when appropriate, follow steps 1 and 2 above in addition to following:

Cameras and Electronic Equipment:

Until effective techniques are developed to comprehensively disinfect cameras and electronics, it is recommended that these items only be used in caves when absolutely necessary. Regardless of the cave/mine visited, clean/treat cameras and electronics after each visit using an appropriate product listed in the Applications/Products chart on page 1. Equipment that must be used in the cave/mine may be placed in a sealed plastic casing (i.e., underwater camera housing), plastic freezer bag, or plastic wrap that permits operation of the equipment (i.e., glass lens is exposed) and reduces the risk of exposure to the cave environment. Prior to opening or removing any plastic protections, wipe the outside surfaces with an appropriate product described in the Applications/Products chart on page 1. Plastic freezer bag or wrap should be removed and discarded after each visit. A sealed plastic casing may be reusable if properly submersed in appropriate product as described in the Applications/Products chart and the functionality and protective features of the casing are not sacrificed (check with manufacturer). After removal of any outside plastic protection, all non-submersible equipment surfaces (i.e., camera body, lens, etc.) should be wiped using an appropriate product described in the Applications/Products chart.

3.) Reduce the risk of vehicle contamination and transport of G.d. to new areas by making sure to

A) transport gear in clean containers,
B) remove outer clothing/footwear and isolate in a sealed plastic bag or container prior to entering a vehicle. Storage container options vary considerably depending on the type of vehicle; but always clean and disinfect the outside surfaces of storage containers prior to putting them in the vehicle.
C) remain outside of the vehicle after exiting a cave/mine or completing field work,
D) change into clean clothing and footwear prior to entering the vehicle, and
E) clean dirt and debris from the outside of vehicles (especially wheels/undercarriage).
OBSERVATION OF LIVE OR DEAD BATS
If you observe live or dead bats (multiple individuals in a single location) that appear to exhibit signs of WNS, contact a wildlife professional in your nearest state (http://www.fws.gov/offices/statelinks.html) or federal wildlife agency (http://www.fws.gov/offices/, http://www.fs.fed.us/, http://www.blm.gov/wo/st/en.html, or http://www.nps.gov/index.htm). Do not handle bats unless authorized in writing to do so by the appropriate government agency.

Note on the use of Pesticides/Products listed above:

http://www.epa.gov/oecaagct/lhra.html

defines a pesticide as follows:

(u) Pesticide
The term “pesticide” means (in part)
(1) any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest.

FIFRA defines a pest at §136:
(t) Pest
The term “pest” means (in part)
(1) any insect, rodent, nematode, fungus, weed, or (2) any other form of terrestrial or aquatic plant or animal life or virus, bacteria, or other micro-organism (except viruses, bacteria, or other micro-organisms on or in living man or other living animals) which the Administrator declares to be a pest under section 25(c)(1).

This document is the product of the multi-agency WNS Decontamination Team, a sub-group of the Disease Management Working Group established by the National WNS Plan (A National Plan for Assisting States, Federal Agencies, and Tribes in Managing White-Nose Syndrome in Bats, finalized May 2011). On 15 March 2012 a national decontamination protocol was adopted by the WNS Executive Committee, a body consisting of representatives from Federal, State, and Tribal agencies which oversees the implementation of the National WNS Plan. This version of the protocol contains some modifications to the 15 March version, intended to clarify the recommendations for the appropriate use of treatment options. This decontamination protocol will continue to be updated as necessary to include the most current information and guidance available.
I. GENERAL INFORMATION

The US Fish and Wildlife Service (USFWS) strongly recommends, first and foremost, compliance with all cave closures, advisories, and regulations in all Federal, State, Tribal, and private lands. However, where such closures are not required or recommended, the following protocol outlines the best known procedures to help reduce the transmission of the fungus *Geomyces destructans* (*G.d.*), believed to be the cause of white-nose syndrome (WNS), to important bat habitat and populations. WNS is responsible for significant bat mortality in eastern North America, and threatens bat populations across the continent.

While adoption of the following decontamination procedures found in this supplement are likely to add extra time and money and decrease the overall life expectancy of equipment; the gravity of the situation necessitates that anyone who visits caves must be willing to do everything possible to avoid potentially contributing to the further spread of the disease.

**If not permitted or trained by the appropriate government agency; then please do not handle bats.** However, if you should observe live or dead bats (multiple individuals in a single location) that potentially are exhibiting characteristic signs of WNS (see Section 1), contact a wildlife professional in your state wildlife agency ([http://www.fws.gov/offices/statelinks.html](http://www.fws.gov/offices/statelinks.html)) or contact your nearest USFWS Ecological Services Field Office ([http://www.fws.gov/offices/](http://www.fws.gov/offices/)).

II. RATIONALE FOR DECONTAMINATION

The USFWS asks that anyone entering caves for recreational purpose read and understand the premise for taking further precautions to prevent the possible spread of the fungus, *G.d.* (use of "cave", here forth, includes all caves, fissures, mines, portals, etc.):

- The USFWS recommends that all cave visitors observe cave closures and advisories on all Federal, State, Tribal, and private lands. Some agencies have instituted management policies stipulating annual or seasonal cave closures. Other states have instituted, or are considering instituting, selective cave closures based on bat population density and/or usage. **Please visit** [http://www.fws.gov/WhiteNoseSyndrome/cavers.html](http://www.fws.gov/WhiteNoseSyndrome/cavers.html) **for a list of current cave closures.** If closure information from a state in which you plan to go caving is not listed, contact that state's wildlife agency to obtain the latest information on cave access.

- Currently, WNS and/or *G.d.* is found in Connecticut, Delaware, Indiana, Massachusetts, Maryland, Missouri, New Hampshire, New Jersey, New York, Oklahoma, Pennsylvania, Tennessee, Vermont, Virginia, and West Virginia, and the provinces of Ontario and Quebec, Canada.

- Bat-to-bat transmission is believed to be the primary vector for the spread of WNS. However, large distance jumps of WNS and/or *G.d.* have occurred to caves in West Virginia, Virginia, Missouri and Oklahoma that are not easily explained by the natural movement of bats themselves, and are potentially the result of human transmission.

- Research is underway to improve our understanding of WNS transmission, including the human component, and results of pilot studies have become available:

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1 The use of the word “cave” in this document includes natural caves, man-made mines, or any other site that may harbor *G.d.* spores.
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- Work conducted by the U.S. Geological Survey, National Wildlife Health Center has found viable fungal spores in cave sediment.
- Research conducted by the New York State Department of Environmental Conservation, Wildlife Pathology Unit has isolated fungal spores off of a backpack, coveralls and a fabric instrument bag upon exiting a cave.
- Other research has demonstrated that bats can develop WNS through infection directly from an affected cave environment, and in the absence of infected bats.

### III. PREVENTING SPREAD OF WNS

To help prevent spread of WNS to unaffected caves, it is important that you NOT transport or use any exposed clothing or gear outside of a WNS-affected state or region for use in a WNS-unaffected state or region. Clothing or gear that has been or is suspected of being exposed to *G. d.* may be reused in other affected caves; however, the WNS decontamination procedures provided in this document should always be followed for items used in affected caves prior to entering another affected cave or leaving the affected state or region. Used gear that must be transported out of affected states or regions should be decontaminated, contained, and sealed prior to leaving the affected area and should not be stored or transported in close proximity with unexposed equipment. If gear cannot be decontaminated, either for safety reasons or fear that equipment may be damaged, it should not enter subsequent caves but rather be designated for use in that one specific cave.

As stated in the WNS Decontamination Protocol v. 01.25.2011 (Available at: http://www.fws.gov/WhiteNoseSyndrome/pdf/WNSDecontaminationProtocol v01252011.pdf), the most effective course of action to guard against the transportation of *G. d.*, or any similar microbe, is to fully decontaminate clothing and gear after exiting each and every cave visited. In areas of high cave density, however, circumstances may allow for multiple caves to be visited on the same day. Assuming that bat-to-bat transmission will likely account for a greater level rapid spread of the fungus between caves in close proximity, and that only aspects of decontamination are going to be feasible within cave complexes visited on the same day in remote locations. Since limited hibernacula data show that bats easily move upwards of 10 miles in search of resources (i.e., food, caves, mates) during portions of the fall, winter, and early spring, cave visitors should, at the very minimum, use full decontamination procedures on a daily basis and between any two caves more than 10 miles apart when visiting multiple caves on the same day. Whenever there is a question on the distance or status of caves (affected vs. unaffected), visitors should always choose the most conservative approach of decontaminating gear, clothing, and equipment between each individual cave visited, especially in already affected areas. Care should be exercised at all times to prevent contamination of clean clothing, equipment, and/or vehicles.

### IV. RECOMMENDED DECONTAMINATION PRODUCTS:

All necessary and appropriate precautionary, use, storage, and disposal information should be apparent on each of the product labels. It is critical that all users read and follow all label instructions provided on the products mentioned in this protocol. It would be a violation of federal law to use, store, or dispose of a regulated product in any manner not prescribed on the approved label/MSDS.

The following chemical (with a minimum of 0.3% quaternary ammonium compound, unless otherwise denoted) and natural products were tested in the laboratory and found to be effective for killing the conidia of *Geomyces* spp.:

1. Lysol® IC Quaternary Disinfectant Cleaner - (A product effective at 1:128 dilution, or 1 ounce of concentrate per gallon of water.)

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2 Use of some products which contain quaternary ammonia, isopropanol, and other potentially harmful chemicals or boiling water in confined spaces needs to be approached carefully due to inhalation or

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*Version 01.25.2011*
2. Professional Lysol® Antibacterial All-purpose Cleaner (A product effective at 1:128 dilution, or 1 ounce of concentrate per gallon of water.) 2
3. Formula 409® Antibacterial All-Purpose Cleaner (Off-the-shelf concentrations as specified by label.) 2
4. A 10% solution of household bleach (A product effective at 1 part bleach to 9 parts water) 2
5. Lysol® Disinfecting Wipes (0.28 % di-methyl benzyl ammonium chloride) 2 & 3
6. Boiling in water for 15 minutes 2

V. DECONTAMINATION PROCEDURES:

Given the increasing evidence presented in Section I on the possible role of human transmission, the USFWS asks that cave visitors please follow these procedures for containment and decontamination to reduce the transfer of the fungus from cave to cave. Please periodically check http://www.fws.gov/WhiteNoseSyndrome/cavers.html for updates to these procedures.

Any clothing, footwear and/or equipment, including outer clothing, should never be used in subsequent caves unless a thorough cleaning and decontamination recommended below can be performed between each cave or exceptions (e.g. within 10 miles, affected vs unaffected) discussed in Section III are met. A cave should only be entered with clothing, footwear, and equipment that have been fully cleaned using the protocol below and rinsed prior to entry to remove residue of chemical product used. Upon entering and exiting any cave, scrub off all dirt and mud from your clothes, boots, and gear. Prior to leaving the cave, ensure that clothing, boots, and equipment that were used in the cave are placed in a sealed plastic bag or plastic container with lid to be cleaned and decontaminated off site, if all decontamination is not feasible at cave entrance. In all cases, outer clothing should be removed prior to entering a vehicle and after/between cave visits. A clean change of clothing is recommended. Companion animals should be kept out of caves as fungal spores could adhere to fur and be transferred to a subsequent cave.

As mentioned, the first step of decontamination is to remove all soil and organic material from equipment, clothing, and boots using a brush and preferably water (best done at entrance of cave upon exiting). This is especially important since organic material (i.e. clay soils) can prevent the chemical products from penetrating clothing, boots, and equipment.

A. Submersible Gear (i.e. clothing and equipment that can be submerged without damage):

Wash all clothing and any appropriate equipment in washing machine or by hand using conventional detergents. Washing can be done in cold, warm or hot water. Laboratory testing has found Woolite® fabric wash to be an effective detergent for this procedure. Rinse thoroughly, and then follow by soaking for a minimum of 10 minutes in one of the recommended decontaminating products listed under Section III, then rinse and air dry. Please notice when boiling water is selected as the decontamination method, all gear must be submerged for 15 minutes, then followed by air drying.

contact risks of the product. Since products/procedures may also cause damage to clothing, gear, and sensitive electronic equipment, all users should be aware of these risks prior to entering cave environments. Use of personal protective equipment to reduce contact with the product is strongly encouraged, particularly if extended contact is anticipated or as recommended by the manufacturer. Always read and follow the MSDS information and all safety/use criteria for every product used. 3

The active ingredient is considered to be at the effective concentrations known to kill the conidia of G. pannorum; however, the efficacy of field application remains to be demonstrated. So, equipment decontaminated with Lysol® Disinfecting Wipes should be used with extra precaution until laboratory results are finalized.
If multiple entries into a single cave are planned, the trip necessitates extended efforts in a remote location, with NO vehicular travel to new or additional caves, and full decontamination is not possible, then visitors should, at a very minimum, swap out and/or use disposable equipment between cave visits. Similarly, other sensitive equipment (i.e., camera, headgear, lights) should be swapped out or wiped using an appropriate decontamination product (i.e., Lysol® disinfectant wipes) prior to entering each new successive cave. All footwear should be intensively scrubbed to remove all dirt and debris, and then wiped using Lysol disinfectant wipes (if procedures listed below are not feasible).

1. **Footwear:**

   When safety permits, rubber (wellington-type) caving boots (which withstand harsh decontaminating products and are easily cleaned) are recommended. Boots need to be fully scrubbed and rinsed so that all soil and organic material is removed. The entire boot, including soles, leather uppers and other portions, should then be decontaminated with an appropriate product listed under Section III for a minimum of 10 minutes, then rinse and air dry.

2. **Ropes and Harnesses:**

   It is the responsibility of each person using vertical gear, including caving or life-support equipment (e.g., harnesses, webbing, and ropes), to ensure that the decontamination protocols in use are chemically compatible with their equipment. **To date, only Sterling rope and webbing have been shown not to be damaged by the following decontamination protocol:** Wash rope/webbing in a front loading washing machine on the gentle cycle using Woolite® Extra Delicates detergent. Treat by immersion in a 1:128 dilution of Lysol IC Quaternary Disinfectant Cleaner for 10 minutes. Rinse in fresh, clean water for a minimum of two rinses and allow to air dry.

   If you are using other brands of rope and webbing not mentioned above, these products have yet to be tested for integrity after decontamination. In cases where safety following decontamination has not yet been evaluated, then ropes and webbing should be dedicated to one cave or not used at all to prevent the spread of WNS.

B. **Non-submersible Gear** (i.e. equipment that will be damaged by submersion):

   Clean thoroughly with soap and water (or use Lysol® Disinfecting Wipes). Decontaminate by applying one of the recommended chemical products listed under Section III to the outside surface for a minimum of 10 minutes, then rinse and air dry.

   1. **Cameras, Computers, and Other Electronic Equipment:**

      If possible, do not bring electronic equipment into a cave. If practical, cameras and other similar equipment that must be brought in a cave should be placed in plastic casing (i.e., underwater camera housing) or wrapped in plastic wrap where only the lens is left unwrapped to allow for photos to be taken. The plastic casing should be decontaminated using one of the appropriate products found above. The plastic wrap should be discarded after use and followed up by decontaminating the camera surface with Lysol® Disinfecting Wipes, realizing this product could damage the body of the camera.

   2. **Vehicles:**

      In addition to gear, vehicles used to transport equipment can also harbor spores. Keep vehicles as clean as possible by taking extra precautions (e.g. storing gear in clean containers, bringing a change of clothes, conducting all work outside of the vehicle once in the cave) and decontaminating storage containers along with all other clothing, gear, and misc. equipment using the appropriate decontamination products found in Section IV.

      It should be noted that product guidelines should be consulted for compatibility before using any decontamination product listed under Section IV. **In order to insure the user’s safety and most effective measures are taken to prevent unintentional spread, it is very important that all users**
understand these products were tested independent of each other. For example, detergents and quaternary ammonium compounds (i.e. Lysol® IC Quaternary Disinfectant Cleaner) should not be mixed directly with bleach as this will inactivate the bleach and in some cases produce a toxic chlorine gas.

VI. SIGNS OF WNS

First and foremost, G.d. may be present with NO apparent signs at all, so always take the necessary and appropriate precautions in decontaminating equipment, clothing, and associated items. If signs are present, realize that the white fungus is only one of the many signs. Other possible WNS signs commonly observed in winter and spring include:

- Abnormal behaviors including:
  - daytime activity on the open landscape, including bats on buildings and structures
  - population shift to entrance of the hibernaculum (cave or mine)
  - decreased arousal with disturbance inside hibernaculum
- Excessive or unexplained numbers of dead or dying bats at/near cave entrances
- Visible fungus on flight membranes, muzzle, and/or ears of live or fresh dead bats

Such conditions and/or observations should always be put in context of the season. Many variables (e.g., warmer temperatures during winter, different species specific habits, and seasonal life cycles) can cause bats to fly about on the landscape during daylight hours. For example, during summer months, bats are normally viewed near dusk and dawn, but during winter healthy bats may be observed out during the day. Furthermore, different bat species naturally go into varying degrees of torpor (a sleep state) during hibernation, and therefore, can arouse and exit hibernacula more frequently especially under warm conditions. This may not be unusual behavior for bats.

VII. CLOSING REMARKS:

Please, understand the effectiveness of this protocol depends solely on the adherence and successful implementation by each cave visitor. Everyone must FIRST consider the risk of transmission and decontaminate, to the extent necessary, their clothing, boots, and equipment prior to entering and/or upon exiting the cave environment, both within and between States. Furthermore, no decontamination procedure can ever be 100% effective, so it is essential that cave visitors take the initiative to understand and practice the intricacies of this decontamination protocol prior to entering cave environments.

Important Note: The most updated information on known WNS affected states (mapped version), protocols, and cave closures are posted on the U.S. Fish and Wildlife Service White Nose Syndrome website at: http://www.fws.gov/whitenosesyndrome/. We recommend that you visit the website periodically to ensure that you are using the most recent protocol.

Note: Protocol updated as of 1-25-11

Please visit http://www.fws.gov/WhiteNoseSyndrome/ for the most updated information.
Histopathologic criteria to confirm white-nose syndrome in bats

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Abstract. White-nose syndrome (WNS) is a cutaneous fungal disease of hibernating bats associated with a novel Geomyces sp. fungus. Currently, confirmation of WNS requires histopathologic examination. Invasion of living tissue distinguishes this fungal infection from those caused by conventional transmissible dermatophytes. Although fungal hyphae penetrate the connective tissue of glabrous skin and muzzle, there is typically no cellular inflammatory response in hibernating bats. Preferred tissue samples to diagnose this fungal infection are rostral muzzle with nose and wing membrane fixed in 10% neutral buffered formalin. To optimize detection, the muzzle is trimmed longitudinally, the wing membrane is rolled, and multiple cross-sections are embedded to increase the surface area examined. Periodic acid–Schiff stain is essential to discriminate the nonpigmented fungal hyphae and conidia. Fungal hyphae form cup-like epidermal erosions and ulcers in the wing membrane and pinna with involvement of underlying connective tissue. In addition, fungal hyphae are present in hair follicles and in sebaceous and apocrine glands of the muzzle with invasion of tissue surrounding adnexa. Fungal hyphae in tissues are branching and septate, but the diameter and shape of the hyphae may vary from parallel walls measuring 2 μm in diameter to irregular walls measuring 3–5 μm in diameter. When present on short aerial hyphae, curved conidia are approximately 2.5 μm wide and 7.5 μm in curved length. Conidia have a more deeply basophilic center, and one or both ends are usually blunt. Although WNS is a disease of hibernating bats, severe wing damage due to fungal hyphae may be seen in bats that have recently emerged from hibernation. These recently emerged bats also have a robust suppurative inflammatory response.

Key words: Bats; emerging disease; fungus; Geomyces sp.; hibernation; Myotis; skin erosion.

White-nose syndrome (WNS) has caused mortality in hundreds of thousands of little brown bats (Myotis lucifugus) since first reported by biologists in 2007, yet the disease is still poorly understood.1 Initially detected near Albany, New York, this fungal disease of cave- and mine-hibernating bats has spread rapidly to hibernacula in Vermont, Massachusetts, Connecticut, Pennsylvania, New Jersey, Virginia, West Virginia, and New Hampshire. In addition to little brown bats, other species in which WNS has since been diagnosed include tricolored bats (Pipistrellus subflavus), northern long-eared bats (Myotis septentrionalis), and endangered Indiana (Myotis sodalis) and big brown (Eptesicus fuscus) bats.

The classic presentation of WNS in affected bats living in caves and mines includes the delicate, exuberant, white filaments that obscure the muzzle.1 Fungus on the wings of these bats can appear as an opaque white, tacky film of varying density (Fig. 1A). Grossly visible fungus is not always seen on bats with WNS, and the white facial plume and white fungus on the surface of wings is usually lost when bats are removed from hibernacula and prepared for shipping. Once received in the laboratory, gross signs of fungal infection in bats are subtle and difficult to detect. The small size of little brown bats (5–7 g) make lighted magnifying loops an asset for examining skin. Changes seen in the skin of affected bats are inconsistent and nonspecific, including patches of rough skin on the face, ears, forearms, wing membranes, and feet as well as pinpoint white foci that resemble comedones on the muzzle. Less obvious changes are loss of sheen on glabrous skin and irregular pigmentation with areas of contraction or small tears in wing membranes. Back-lighting of extended wings using a light box improves detection of these changes (Fig. 1B).

Although gross lesions can be suggestive of WNS, histopathologic examination is necessary to confirm this disease. To optimize microscopic detection of fungal hyphae, surface area of examined skin was maximized. Muzzle and nose, once dissected from underlying bone and fixed in 10% neutral buffered formalin, were trimmed into multiple longitudinal sections perpendicular to the surface of skin. The skin
was embedded with trimmed surfaces presented for sectioning to maintain the orientation of the fungus to dermal structures.

Wing membrane was sampled in multiple rectangular pieces. Each piece of membrane was dipped in formalin, carefully unfolded over a gloved finger, and rolled along the short end onto wooden dowels approximately 0.2 cm in diameter and 3 cm long. After fixation, the dowels were removed from the rolls of wing membrane, and skin rolls were trimmed into multiple cross-sections with the cut surfaces embedded for sectioning and staining. Various fungal stains were initially applied to the tissue sections, but periodic acid–Schiff stain (PAS) proved superior for the microscopic detection of nonpigmented fungal hyphae, appreciation of the pattern of fungal skin invasion (Fig. 2A–2C), and recognition of conidia (Fig. 2D).

The wing membrane is composed of 2 single-cell layers of epidermis separated by a thin layer of connective tissue with elastin fibers. Adnexa are only present in wing membrane near the arms and legs. The mildest microscopic changes seen in the wing membranes were cup-like epidermal erosions that were filled with fungal hyphae. Ulceration and fungal invasion of underlying connective tissue was common (Fig. 2B–2D) and could span the full thickness of the wing membrane. When the muzzle was involved, fungal hyphae filled hair follicles, invaded sebaceous and apocrine glands, and extended into the regional connective tissue obscuring epithelial boundaries of the adnexa (Fig. 3A, 3B). Typically, there was an absence of inflammation in the skin of hibernating bats even with extensive fungal invasion (Figs. 2, 3), which was random and nonangiotrophic. Samples of wing membranes from bats euthanized in caves and immediately fixed in formalin had more abundant aerial fungal growth and conidia on the surface of the skin, as well as the characteristic epidermal ulceration and connective tissue invasion (Fig. 2C, 2D).

Lack of inflammation in response to fungal hyphae was surprising. However, tissue invasion noted in samples fixed immediately after euthanasia of bats within hibernacula provided evidence that invasion of living tissue was an antemortem event. When inflammation was present in the skin of hibernating bats, edema and neutrophils were observed microscopically in the regional connective tissue, occasionally with intradermal abscesses. The presence of bacteria was inconsistent but common in bats with inflammation. Typical transmissible dermatophytes of mammals only invade the nonliving structures of skin (keratin, hair, and nails), in contrast to the extensive invasion of skin and underlying connective tissue in bats with WNS. Winter bats with heavy fungal burdens were emaciated, but there were no consistent microscopic lesions in tissues other than skin.

Unlike bats in hibernation, bats with visibly damaged wings that were collected outside hibernacula in May had severe inflammation associated with fungal infection. Histologic changes included suppurrative dermatitis with folliculitis, edema, and scattered infiltrates of macrophages. Frequent serocellular inflammatory crusts containing fungal hyphae were present over the intact epidermis (Fig. 3C). Bats collected shortly after hibernation also had small quiescent packets of fungal hyphae within the dermis that were surrounded by a thin layer of acellular material (Fig. 3D).

Fungal hyphae in tissue sections were branching and septate (Fig. 3B) with variable morphology ranging from parallel walls measuring 2 μm in diameter to irregular, bulging, or globose walls measuring 3–5 μm in diameter (Fig. 3B). When present on short aerial hyphae, curved conidia were
approximately 2.5 μm in diameter and 7.5 μm long measured along the curve of the conidia. The conidia also had a central region of basophilia, and one or both ends were blunt (Fig. 2D). A recently identified *Geomyces* sp. fungus was cultured from the skin of bats with WNS and formed conidia in culture that were identical to those seen in histologic sections (Fig. 2D). The conidia measure approximately 2.5 μm in diameter and 7.5 μm in curved length, have one or two blunt ends, and have a deeply basophilic central region (arrowheads). These conidia are identical to those of *Geomyces* sp. fungus isolated from bats with WNS. A focal cluster of fungal hyphae is present within the epithelium on opposite wing margin. PAS stain. Bar = 15 μm.

Figure 2. Wing membranes of *Myotis lucifugus* bats infected with white-nose syndrome (WNS). A, histologic section of wing membrane from the same bat as in Figure 1B. Invasive fungus (arrow) stains poorly with hematoxylin and eosin stain, and inflammatory infiltrates are not present. Bar = 15 μm. B, periodic acid-Schiff (PAS) stain of serial section from same tissue as in panel A. Fungal hyphae stain bright magenta. Hyphae are associated with cup-shaped epidermal erosions (arrowhead) and ulcers (arrow) with invasion of the underlying connective tissue. Bar = 15 μm. C, section of wing membrane, collected while inside the cave, from a little brown bat immediately after euthanasia. Exuberant fungal growth is present on the surface of the skin (arrow) and penetrates the wing membrane (arrowheads) without associated inflammation. PAS stain. Bar = 15 μm. D, conidia on the surface of the wing membrane of a cavedwelling little brown bat fixed immediately after euthanasia in the cave. The characteristic curved conidia measure approximately 2.5 μm in diameter and 7.5 μm in curved length, have one or two blunt ends, and have a deeply basophilic central region (arrowheads). The body temperature of bats in torpor drops to within a few degrees of the ambient temperature in their hibernaculum (usually 2–10°C), with a concomitant 96–98% reduction of metabolic rate. Research has shown that mammals in torpor also down-regulate their immune response, which does not return to normal responsiveness until basal metabolic rates and core temperatures return to euthermic levels. The drop in body temperature and depression of the immune response would provide ideal conditions for a psychrophilic fungus, such as *Geomyces* sp., to use hibernating bats as an opportunistic host. Experimental replication of WNS in hibernating bats is having early success in artificial hibernacula, and investigations into a more complete picture of the life
cycle of the fungus and its associated host response through multiple seasons are planned.

Histopathologic confirmation of WNS is efficient, cost effective, and reliable. Awareness, surveillance, and early diagnosis of this emerging infectious disease of hibernating bats will be essential for subsequent management efforts to contain the spread of WNS and its impact on populations of cave-hibernating bats.

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References

Bat white-nose syndrome: a real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*

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**Abstract:** The fungus *Geomyces destructans* is the causative agent of white-nose syndrome (WNS), a disease that has killed millions of North American hibernating bats. We describe a real-time TaqMan PCR test that detects DNA from *G. destructans* by targeting a portion of the multicopy intergenic spacer region of the rRNA gene complex. The test is highly sensitive, consistently detecting as little as 3.3 fg genomic DNA from *G. destructans*. The real-time PCR test specifically amplified genomic DNA from *G. destructans* but did not amplify target sequence from 54 closely related fungal isolates (including 43 *Geomyces* spp. isolates) associated with bats. The test was qualified further by analyzing DNA extracted from 91 bat wing skin samples, and PCR results matched histopathology findings. These data indicate the real-time TaqMan PCR method described herein is a sensitive, specific and rapid test to detect DNA from *G. destructans* and provides a valuable tool for WNS diagnostics and research.

**Key words:** diagnostic test, IGS, pathogen, PCR, wildlife disease

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**INTRODUCTION**

White-nose syndrome (WNS) is an emergent disease causing unprecedented mortality in several species of North American hibernating bats (Turner et al. 2011). First photo-documented in 2006 at a cave in east central New York (Blehert et al. 2009), the disease has spread to 19 US States and four Canadian provinces (see http://www.fws.gov/whitenosesyndrome/maps/WNSMAP04-27-12_300dpi.jpg). Bat mortality has approached 100% at some affected hibernacula (Turner and Reeder 2009). Although insectivorous bats are often inconspicuous to humans, they provide natural pest-control services (Kunz et al. 2011) valued at approximately 22.9 billion dollars per year to the United States agricultural industry (Boyles et al. 2011).

The causative agent of WNS is the recently described psychrophilic fungus *Geomyces destructans* (Gargas et al. 2009, Lorch et al. 2011, Warnecke et al. 2012). The fungus grows on bats while they hibernate, initially colonizing the skin, then penetrating the epidermis of the wings, ears and muzzle (Meteyer et al. 2009). At present, the gold-standard for diagnosing WNS is identification of characteristic skin lesions by histopathology. However, this method is time-consuming, requires specialized training, and for a thorough analysis a large amount of wing tissue (approximately 1.5 cm × 3.0 cm; Lorch et al. 2010) must be collected from each bat, either restricting analyses to dead animals or necessitating euthanasia.

PCR provides an alternative diagnostic tool to rapidly detect DNA from *G. destructans* in association with small amounts of bat skin (approximately 3 mm × 3 mm). Two PCR methods have been described (Lorch et al. 2010, Chaturvedi et al. 2011). The method developed by Lorch et al. (2010) targets the internal transcribed spacer (ITS) region of the rRNA gene complex using conventional PCR technology. However, because of the recently discovered diversity of other fungi closely related to *G. destructans* in bat hibernacula (Lindner et al. 2011, Lorch et al. 2013), this method is best conducted in conjunction with sequencing of PCR product when analyzing diagnostic samples from bats, and it cannot be used to specifically detect DNA from *G. destructans* in environmental samples without cloning and sequencing. A real-time TaqMan PCR that targets the alpha- L-rhamnosidase gene (hereafter referred to as the ALR test) was developed by Chaturvedi et al. (2011). Although the ALR test has been screened against 16 isolates of
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*Geomyces* and related teleomorphs, the relationships of these fungal isolates to *G. destructans* has not been phylogenetically defined. Thus, specificity of the ALR test for use with environmental samples containing *Geomyces* spp. closely related to *G. destructans* (Lindner et al. 2011, Lorch et al. 2013) remains unknown. In addition, the ALR test targets a gene that likely exists only as a single copy, which may reduce sensitivity compared to a PCR test targeting a multicopy gene region.

The goal of this study was to develop a highly specific real-time TaqMan PCR test with greater sensitivity than existing tests to detect DNA from *G. destructans*. The intergenic spacer (IGS) region of the rRNA gene complex was chosen as the target for this test due to its high copy number and its interspecific sequence variability compared to other portions of this gene region (Jackson et al. 1999).

### MATERIALS AND METHODS

**Fungal culture and DNA extraction.**—Fungal isolates were grown as pure cultures on Sabouraud dextrose medium (BD Diagnostic Systems, Sparks, Maryland) incubated at 7 C. Genomic DNA (gDNA) was extracted from fungal isolates with three methods. To produce high purity gDNA from the type isolate of *G. destructans* (American Type Culture Collection number ATCC MYA-4855) for generation of standard curves, fungal biomass (approximately 800 mg) grown in liquid Sabouraud dextrose medium (BD Diagnostic Systems) was washed twice with 1 mL sterile deionized water, then ground approximately 10 min in liquid nitrogen to yield a fine powder. Genomic DNA extraction was initiated with a commercial kit (Genta® Puregene® Genomic DNA Purification Kit, QIAGEN Inc., Valencia, California) according to the manufacturer's instructions for cell lysis, proteinase K digestion, and RNase treatment steps except the amount of proteinase K was increased to 20 mg/mL (final concentration). Genomic DNA from the resulting lysate was further purified with a second kit (OmniPrep™ for Fungi, G-Biosciences, Maryland Height, Missouri) following the manufacturer's instructions beginning at the chloroform extraction step. Concentration of type isolate gDNA was measured with the Quant-IT™ High-Sensitivity DNA Assay Kit (Invitrogen, Carlsbad, California). Genomic DNA was extracted from nine additional isolates of *G. destructans* (for PCR and DNA sequencing applications) using the OmniPrep™ for Fungi kit (G-Biosciences) according to the manufacturer's instructions. Genomic DNA also was extracted from a group of near-neighbor fungi consisting of 54 isolates (Table I), including 40 identified as congeneric with *G. destructans* based on analysis of ITS sequences by Lorch et al. (2015). An additional three isolates, cultured according to the methods of Lorch et al. (2010) and identified as *Geomyces* spp. based on ITS sequence analysis, also were included. These three isolates were cultured from animals collected in 2010, including a tricolored bat (*Perimyotis subflavus*) from Tennessee, a silver-haired bat (*Lasionycteris noctivagans*) from Tennessee and a tricolored bat from Wisconsin. Genomic DNA extractions for these 54 isolates were conducted with the protocol of Lindner and Banik (2009) with reagent volumes modified proportionally for use with eight-well 0.2 mL PCR strip tubes as described by Lorch et al. (2013).

**IGS analysis, primers and probe.**—Complete IGS region was amplified by PCR of gDNA extracted from the type isolate of *G. destructans*. Amplification primers (synthesized by the University of Wisconsin Biotechnology Center, Madison, Wisconsin) were complementary to the 3'– end of the large subunit (LSU) rRNA gene (CNL12: 5'– CGT AAC GCC TCT AAG TCA G–3'; Anderson and Stasovski 1992) and the 5'– end of the small subunit (SSU) rRNA gene (CNS1: 5'– GAG ACA AGC ATA TGA CTG –3'; White et al. 1990). Polymerase chain reaction was conducted per the manufacturer’s instructions with TaKaRa ExTaq proofreading DNA polymerase (Clontech Laboratories Inc., Madison, Wisconsin). Reactions included 1 μL gDNA in a volume of 50 μL. Cycling conditions consisted of an initial denaturation at 98 C for 2 min, followed by 35 cycles of 98 C for 10 s, 50 C for 30 s, and 72 C for 4 min, with a final extension at 72 C for 7 min. Full-length amplification product (2793 nt) was directly sequenced in both directions with a primer-walking strategy. Sequencing reactions were run by the University of Wisconsin Biotechnology Center with the BigDye Termina¬tor® 3.1 system (Applied Biosystems, Foster City, California), and reaction products were analyzed with a 3730xl DNA Analyzer (Applied Biosystems). Complementary strand sequencing reaction results were assembled and edited with SeqMan Pro 9.0.5 (DNASTAR, Madison, Wisconsin). The IGS region sequence with flanking nucleotides from the type isolate of *G. destructans* is deposited in GenBank (accession No. JX415267).

The IGS region of the type isolate of *G. destructans* was aligned with IGS region sequences from 34 fungal isolates previously identified as close relatives of *G. destructans* based on analysis of their ITS sequences (Table I; Lorch et al. 2013). All IGS nucleotide positions were numbered starting with position 1 following the 3'– terminal nucleotide of the LSU. A variable region spanning nucleotides 114–310 was identified within the IGS, and specific primers and probe were developed with the sequence generated from the type isolate of *G. destructans* (Fig. 1). This region was analyzed with commercial software (Primer Express 3.0, Applied Biosystems), and the following primers and probe were synthesized by commercial laboratories (University of Wisconsin Biotechnology Center and Biosearch Technologies Inc., Novato, California, respectively): forward primer nu-IGS-0169-5'–Gd: 5'– TGC CTC TCC GCC ATT AGT G –3'; reverse primer nu-IGS-0235-3'–Gd: 5'– ACC ACC GCC TCG CTA GGT A –3'; and probe nu-IGS-0182/0204-Gd: 5'– (FAM) CGT TAC AGC TTG CTC GGG CTG CC (BHQ-1) –3'. To ensure the nucleotide region targeted for primer and probe development did not exhibit intraspecies variability, sequence of the 5'– end of the IGS region (622 nt) was determined for nine additional isolates of *G. destructans* cultured from the skin of bats collected in Connecticut,
its specificity sequenced, were screened with the IGS test to assess isolates, including those for which the IGS region was not real-time TaqMan polymerase chain reaction assay. All were used for development and specificity testing of the bats (fungal isolates 22984-1-I1, 23014-1-I2, 23342-1-I1) that isolated from caves and mines (Lorch et al. 2013) or from TABLE I. GenBank accession numbers of the rRNA gene internal transcribed spacer (ITS) region and if applicable

<table>
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<tr>
<th>Fungal isolate</th>
<th>Genus</th>
<th>ITS accessions nos.</th>
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<td>Geomyces</td>
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Massachusetts, New York, New York, Pennsylvania, Vermont, Virginia and West Virginia with primers nu-IGS-0033-’Gd: 5’–TCT CCG ATT AAC TTG CAG GCT AG–3’; and nu-IGS-0613-’Gd: 5’–AGT GCC TCT CGC CCT AGA AC–3’. The portion of the IGS regions for all isolates of G. destructans were identical and did not match any other sequences in GenBank, including those of near-neighbor isolates described by Lorch et al. (2013).

Development of the real-time IGS PCR test.—Real-time IGS PCR (hereafter referred to as the IGS test) was conducted using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) and commercial master mix (QuantiFast™ Probe PCR + ROX Vial Kit, QIAGEN Inc.) according to the manufacturer’s instructions. Standard laboratory practices to avoid cross contamination of samples (e.g. unidirectional workflow) were followed. Each 25 μL PCR reaction contained 12.5 μL 2× master mix, 0.5 μL 50× ROX dye solution, 0.5 μL of each 20 μM PCR primer solution, 0.25 μL 20 μM dual-labeled BHQ probe and 5 μL template DNA. A positive control of gDNA from G. destructans and a no-template control were included in each 96-well assay plate. PCR cycling conditions included an initial Taq polymerase activation step of 95°C for 3 min followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. For the purpose of standardization between assays (i.e. IGS vs. ALR), plate runs and sample types (as background fluorescence levels occasionally differed), the threshold baseline was set to 10% of the maximum fluorescence as determined by positive control samples (King and Guidry 2004). Any reaction that crossed the threshold baseline within 40 cycles was considered positive.

Tenfold serial dilutions 3.3 ng–3.3 μg gDNA from the type isolate of G. destructans were used as template to generate a standard curve. Each dilution of gDNA was loaded in triplicate into a 96-well assay plate and amplified by the IGS test. The lowest concentration of gDNA template that was detected in three out of three replicates (for which the replicates did not vary by greater than one Cₜ value) was identified as the lower detection limit.

To determine specificity of the real-time IGS PCR test, a near-neighbor panel consisting of purified gDNA template from fungal isolates cultured from bats or from soil collected within bat hibernacula (TABLE I; Lorch et al. 2013) was diluted tenfold and assembled in a 96-well assay.
plate. Triplicate positive and negatives controls containing gDNA from *G. destructans* and no template respectively also were included. To rule out the possibility of PCR inhibition, duplicate samples of three representative gDNA extracts prepared according to Lindner and Banik (2009) were spiked with 33 pg gDNA from the type isolate of *G. destructans* and amplified by the IGS test. Spiked samples within one C_t value of the average C_t of identical samples lacking added gDNA were identified as non-inhibitory.

Real-time IGS PCR analysis of bat skin samples were compared to histological analyses conducted previously with the same skin samples from bats submitted to the U.S. Geological Survey National Wildlife Health Center for diagnostic testing 2009–2011. Ninety-one bats of 12 species from 57 collection events in 19 states (Alabama, California, Connecticut, Florida, Indiana, Maryland, Maine, Missouri, New Jersey, New Mexico, New York, Ohio, Pennsylvania, Tennessee, Texas, Virginia, Vermont, Wisconsin, and Virginia), both within and outside the known range of WNS at the time of collection, were analyzed. Of these 91 samples, 42 were previously identified as WNS negative and 49 were WNS positive by histology. All tissues were stored frozen at either –20 C or –80 C until extracted with the Gentra® Puregene® Genomic DNA Purification Kit (QIAGEN Inc.) as described by Lorch et al. (2010), and the extracted gDNA was diluted 100-fold. To rule out the possibility of PCR inhibition, duplicate aliquots of gDNA from WNS negative skin samples each were spiked with 33 pg gDNA from the type isolate of *G. destructans* and amplified by the IGS test. Spiked histology-negative skin samples within one C_t value of the average C_t of identical samples lacking added gDNA were identified as non-inhibitory. Triplicate positive control reactions each containing the same amount of gDNA from *G. destructans* also were amplified.

Comparison of IGS and ALR PCR tests.—A direct comparison of the sensitivity and specificity of the ALR (Chaturvedi et al. 2011) and IGS PCR tests was conducted. Primers for the ALR test were as described by Chaturvedi et al. (2011), and the probe was synthesized by a commercial laboratory (Biosearch Technologies Inc.) as follows: 5’–(FAM) TTC GGC GGC CAG CCG CG (BHQ-1) –3’. Reagents, cycling conditions and analyses for the ALR PCR were otherwise as described above. The ALR test sensitivity was compared to that of the IGS test by simultaneously generating two concentration curves in the same 96-well assay plate. The two concentration curves each were prepared in triplicate by serially diluting gDNA from the type isolate, as described above, and each set of serially diluted gDNA templates was amplified with the appropriate primer/probe combination (IGS or ALR) together in the same 96-well assay plate. Because the maximum fluorescence of the ALR assay was lower than that of the IGS assay, the threshold baseline was manually set at 10% of the maximum fluorescence for each respective assay.

The specificity of the ALR test was assessed with DNA templates from the near-neighbor panel as described above. A subset of the diagnostic samples described above also was used to further assess the sensitivity and specificity of the ALR test. These samples included wing skin from 22 bats diagnosed as WNS negative by histopathology and 35 bats diagnosed WNS positive by histopathology. As with all PCR runs, both positive and negative controls were included.

Results

Standard curve.—A standard curve was prepared by PCR amplifying triplicate serial dilutions (3.3 ng–3.3 fg) of gDNA from *G. destructans* (Fig. 2). The assay was consistently linear over seven logs with an R^2^ value greater than 0.99. Amplification efficiency was calculated as 104.86% (efficiency = –1 + 10^((–1/slope))). Product also was amplified at the 10–7 template dilution (330 ag) but was not included in the standard curve because it was detected only in one of three replicate samples. Because the 10–6 template dilution (3.3 fg) was the
lowest concentration yielding amplification for all three replicates, 3.3 fg gDNA (per 25 μL reaction) was identified as the lower limit of detection.

Near-neighbor screen.—Target DNA was not amplified with the IGS PCR test from any of the 54 fungal isolates designated as near neighbors to G. destructans. Positive and negative controls in this screen performed as expected. Duplicates of three of the samples containing fungal gDNA were spiked with gDNA from G. destructans to test for PCR inhibition. Spiked samples varied by < 1 Ct value from identically prepared positive control samples that lacked near-neighbor fungal gDNA, indicating that inhibition did not occur.

Diagnostic screen.—Genomic DNA extracts from 42 wing skin samples previously diagnosed as WNS negative by histopathology all were confirmed negative by the real-time IGS PCR test. Genomic DNA extracts from 49 wing skin samples previously diagnosed WNS positive by histopathology all were positive by the real-time IGS PCR test. To test for inhibition, gDNA extracts from the 42 negative samples were spiked with gDNA from G. destructans and retested by real-time IGS PCR. PCR results for spiked diagnostic samples differed by < 1 Ct value from identically prepared positive control samples that lacked wing skin DNA, indicating that inhibition did not occur.

Test comparison.—Sensitivity and specificity of the IGS and ALR (Chaturvedi et al. 2011) tests were compared with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Using purified gDNA as template, the ALR test consistently detected 330 fg gDNA in all three replicates at its lower detection limit while the IGS test consistently detected 3.3 fg gDNA in all three replicates at its lower detection limit. When amplifying replicate gDNA samples, Ct values for the ALR test were on average 7.8 cycles higher than those of the IGS test. When amplifying gDNA extracts from diagnostic samples, both tests responded equivalently with samples from bats determined to be WNS negative by histopathology (no amplification). However, compared to the IGS test, Ct values were higher in all instances when using the ALR test to amplify diagnostic samples from bats determined to be WNS positive; Ct values for the ALR assay were on average 7.1 cycles higher than those resulting from the IGS assay. In addition, the ALR test yielded false-negative results for extractions from seven of 35 (20%) diagnostic samples determined to be positive for WNS by histopathology. Despite lower sensitivity, the ALR test was equally specific as the IGS test based upon analysis of gDNA extracts from near-neighbor fungi; no amplification of gDNA from the near neighbor panel was detected.

DISCUSSION

As WNS continues to spread across North America, development of enhanced diagnostic tests to detect the causative agent, G. destructans, improves the ability to conduct disease surveillance and provides researchers with needed laboratory tools. We developed a specific and sensitive real-time TaqMan PCR test (the IGS test) to detect DNA from G. destructans by targeting the multicopy IGS region of the rRNA gene complex. The IGS test proved to be specific, agreeing with histopathologic diagnoses for 91 of 91 previously analyzed bat skin samples and appropriately discriminated target DNA from a panel of 54 closely related fungal isolates. In addition, the IGS test was approximately 100-fold more sensitive than a previously published real-time TaqMan PCR test (the ALR test; Chaturvedi et al. 2011).

The IGS region of the rRNA gene complex has been shown to be a useful target for development of PCR tests suitable for identifying fungal species/subspecies (Radford et al. 1998, Williamson et al. 2000, Suarez et al. 2005, López-Flores et al. 2008). Because the IGS region is not transcribed, there may be less selective pressure for conservation of nucleotide sequence yielding high interspecific variability (Hillis, et al. 1991, Jackson et al. 1999). In addition, as a high-copy DNA target (approximately 100–400 copies per fungal genome [Boyle et al. 2004]), IGS
region offers greater PCR sensitivity compared to single or low copy-number genes. Furthermore, should spontaneous mutation or intraspecific sequence variation occur within a gene, multicopy PCR primer/probe binding sites are less likely to lose primer/probe specificity than are single-copy DNA targets.

To verify suitability of the IGS region as a PCR target for the detection of \textit{G. destructans}, IGS regions from 10 isolates of \textit{G. destructans} were PCR amplified. The 5′– end (622 nt) of all resulting amplification products were directly sequenced and determined to be identical. Subsequently equivalent partial IGS sequences of two fungal isolates cultured from bats and 32 isolates cultured from soil collected in bat hibernacula and identified as close relatives of \textit{G. destructans} based on analysis of their ITS sequences (Table I; Lorch et al. 2013) were compared. This comparison revealed that, although these isolates exhibited low ITS region variability, there was high variability among analyzed portions of their IGS regions. Based upon this analysis, a variable region within the IGS, downstream of the LSU of the rRNA gene region, was identified for development of primers and probe (Fig. 1).

The lower limit of detection of the IGS test was 3.3 fg of gDNA per 25 μl reaction, or approximately 0.1 genome equivalents (based on an estimated size of 30.65 Mb for the genome of \textit{G. destructans} [see http://www.broadinstitute.org/annotation/genome/Geomyces_destructans/GenomeStats.html] and the assumption that an average base pair is 650 Daltons). In our laboratory the lower limit of detection of the ALR test was 330 fg gDNA per 25 μl reaction, or approximately 10 genome equivalents. This was higher than the detection limit of two conidia (presumably two genome equivalents) per real-time PCR reaction reported for the ALR test by Chaturvedi et al. (2011). This disparity could result from differences in the platforms used to conduct the real-time PCR or from discrepancies between using viable conidia as compared to purified gDNA to determine the lower limit of detection. Nonetheless, when compared with standardized methods the IGS test was approximately 100 times more sensitive than the ALR test. This difference in sensitivity most likely results from differences in copy numbers of the DNA targets for each PCR test. Specifically, the IGS test targets the multicopy IGS region, while the ALR test targets the alpha1-Rhamnosidase gene, which presumably exists only as a single copy per genome.

The ALR test (Chaturvedi et al. 2011) was specific when assessed against gDNA from the near-neighbor panel. However, the ALR test failed to detect DNA from \textit{G. destructans} in 20 percent of diagnostic samples previously determined to be positive for WNS by histopathology. In contrast, results of the IGS test for the same samples agreed with the previous histopathology findings. In addition, ALR test samples consistently crossed the threshold baseline approximately seven cycles (approximately two orders of magnitude) behind those of the IGS test. The observed reduced sensitivity of the ALR test compared to the IGS test when analyzing diagnostic samples was consistent with measured differences in lower limits of detection for both tests determined through concentration curve analyses.

Our analyses demonstrate that the real-time Taq-Man PCR method described herein (the IGS test) provides a sensitive, specific and rapid tool for detecting DNA from \textit{G. destructans}. This test thus provides an enhanced tool for both WNS diagnostic investigations and research. Furthermore, by demonstrating the IGS test did not amplify target DNA from closely related fungal isolates cultured from soil collected in bat hibernacula, we show this test likely has specificity sufficient for the analysis of environmental samples.

**ACKNOWLEDGMENTS**

We thank A. Minnis (U.S. Forest Service) for assistance with phylogenetic analyses. We acknowledge technical assistance provided by B. Berlowski-Zier (U.S. Geological Survey) and M. Banik (U.S. Forest Service). D.S.B. was financially supported by the U.S. Geological Survey and D.L.L. by the U.S. Forest Service. Use of trade, product or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

**LITERATURE CITED**


# Characteristics of Eastern Bats

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<th>Species</th>
<th>Fur</th>
<th>4arm Avg (mm)</th>
<th>Weight (gr)</th>
<th>Foot (mm)</th>
<th>Ear (mm)</th>
<th>Tragus shape</th>
<th>Calcar</th>
<th>Other ID Traits</th>
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<tr>
<td>Brazilian Free-tailed Bat (<em>Tadarida brasiliensis</em>)</td>
<td>Short, brown to dark gray</td>
<td>43</td>
<td>11-14</td>
<td>8.6</td>
<td>19-20</td>
<td></td>
<td></td>
<td>Tail free from membrane</td>
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<tr>
<td>Rafinesque’s Big-eared Bat (<em>Corynorhinus rafinesquii</em>)</td>
<td>Gray or brown above and whitish below</td>
<td>41</td>
<td>8-14</td>
<td>8-13</td>
<td>27-37</td>
<td>Broad and Long</td>
<td></td>
<td>Huge ears</td>
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<td>Townsend’s Big-eared Bat (<em>Corynorhinus townsendii</em>)</td>
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<td>9-13</td>
<td>9-13</td>
<td>27-39</td>
<td>Broad and Long</td>
<td></td>
<td>Huge ears</td>
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<tr>
<td>Big Brown Bat (<em>Eptesicus fuscus</em>)</td>
<td>Long brown - two toned black base</td>
<td>47</td>
<td>13-25</td>
<td>10-12</td>
<td>17-18</td>
<td>Broad and Rounded</td>
<td>Keeled</td>
<td>Large, prominent glands on muzzle</td>
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<td>Evening Bat (<em>Nycticeius humeralis</em>)</td>
<td>Dark chocolate or dull brown, = above buff brown below. Juv darker</td>
<td>36</td>
<td>5-14</td>
<td>7-8</td>
<td>14-15</td>
<td>short and rounded</td>
<td>Not Keeled</td>
<td>Strong odor!, Looks like small big brown bat. Look for swollen glands on muzzle. Often identified by process of elimination. Only two upper incisors</td>
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<td>Eastern Pipistrelle (<em>Pipistrellus subflavus</em>)</td>
<td>Sandy brown to orange on back - tricolored,</td>
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<td>4-7</td>
<td>8-10</td>
<td>13-15</td>
<td>Blunt and Straight</td>
<td>Not Keeled</td>
<td>Pink forearms, black wings</td>
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## Characteristics of Eastern Bats

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<td>Red Bat <em><strong>(Lasiurus borealis)</strong></em></td>
<td>Bright red or rust color, females with frosting, males without. White patches on shoulders</td>
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<td>9-10</td>
<td>8-13</td>
<td>short, blunt and curved</td>
<td>Keeled</td>
<td>Membrane between rear legs is completely furred</td>
</tr>
<tr>
<td>Seminole Bat <em><strong>(Lasiurus seminolus)</strong></em></td>
<td>Mahogany brown with frosted tips above, paler below</td>
<td>40</td>
<td>8-15</td>
<td>6-11</td>
<td>7-14</td>
<td>short, blunt and curved</td>
<td>Membrane between rear legs is completely furred</td>
<td></td>
</tr>
<tr>
<td>Hoary Bat <em><strong>(Lasiurus cinereus)</strong></em></td>
<td>Yellowish brown to dark brown with very heavy frosted tips. White patches on shoulders</td>
<td>53</td>
<td>20-35</td>
<td>6-13</td>
<td>17</td>
<td>short, blunt and curved</td>
<td>Very Large Bat!!!!, Membrane between rear legs is completely furred</td>
<td></td>
</tr>
<tr>
<td>Northern Yellow Bat <em><strong>(Lasiurus intermedius)</strong></em></td>
<td>Yellowish brown, no white shoulder or wrist patches no frosting</td>
<td>48</td>
<td>14-20</td>
<td>10</td>
<td>?</td>
<td>short, blunt and curved</td>
<td>Membrane between rear legs is furred only on basal half</td>
<td></td>
</tr>
<tr>
<td>Silver-haired Bat <em><strong>(Lasionycteris noctivagans)</strong></em></td>
<td>Dark blackish brown, with silver frosted tips</td>
<td>41</td>
<td>7-16</td>
<td>7-11</td>
<td>14</td>
<td>Blunt and rounded</td>
<td>Membrane between rear legs is furred on anterior half</td>
<td></td>
</tr>
</tbody>
</table>
## Characteristics of Eastern Bats

<table>
<thead>
<tr>
<th>Species</th>
<th>Fur</th>
<th>4arm Avg (mm)</th>
<th>Weight (gr)</th>
<th>Foot (mm)</th>
<th>Ear (mm)</th>
<th>Tragus shape</th>
<th>Calcar</th>
<th>Other ID Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray Bat (Myotis grisescens)</td>
<td>Solid gray above, paler below</td>
<td>43</td>
<td>7-12</td>
<td>9-12</td>
<td>14-16</td>
<td>Sharp</td>
<td>Not Keeled</td>
<td>Wing attached to ankle, all gray, notch in claw on rear feet.</td>
</tr>
<tr>
<td>Southeastern Bat (Myotis austroriparius)</td>
<td>Wooly dull, little contrast between base and tips, yellowish to gray above, orangeish in mid-late summer, white below</td>
<td>40</td>
<td>5-12</td>
<td>10-13</td>
<td>13-16</td>
<td>Slender Pointed</td>
<td>Not Keeled</td>
<td>Gray-black wings, long toe hairs Females orange - Males brown??</td>
</tr>
<tr>
<td>Little Brown Bat (Myotis lucifugus)</td>
<td>Long glossy tan to dark brown above, gray to buff below</td>
<td>38</td>
<td>7-8</td>
<td>8-11</td>
<td>13-16</td>
<td>Slender Pointed</td>
<td>Not Keeled</td>
<td>Long toe hairs (beyond claws), contrasting wings with body,</td>
</tr>
<tr>
<td>Indiana Bat (Myotis sodalis)</td>
<td>Fine fluffy, not glossy dark gray to brown above, grayish below</td>
<td>38</td>
<td>5-11</td>
<td>7-10</td>
<td>11-16</td>
<td>Slender Pointed</td>
<td>Keeled</td>
<td>short toe hairs, pink nose, uniform in color, wings do not contrast with body. Never has condensation in winter.</td>
</tr>
<tr>
<td>Northern Long-eared Bat (Myotis septentrionalis)</td>
<td>Not glossy, brown above, grayish below</td>
<td>35</td>
<td>5-10</td>
<td>7-10</td>
<td>16-19</td>
<td>Very Long and Slender</td>
<td>Keeled</td>
<td>Large propatagium, ears extend beyond nose (3mm)</td>
</tr>
<tr>
<td>Eastern Small-footed Bat (Myotis leibii)</td>
<td>Blackish brown above, paler below</td>
<td>32</td>
<td>4-6</td>
<td>6-8</td>
<td>13-15</td>
<td>Sharp</td>
<td>Sharply Keeled</td>
<td>Black mask, longish tail (33 mm)</td>
</tr>
</tbody>
</table>
KEY TO THE BATS OF COLORADO

R. Schorr and K. Navo (revision April 2012)
Figures 3, 4, and 28 from Barbour and Davis. 1969. Bats of America. Prior to beginning please familiarize yourself with Fig 30-32 of the Appendix. ACF = average forearm length of Colorado specimens; AWT = average weight of Colorado specimens; HF = hindfoot from heel to tip of claw/nail).

1. a. Tail extends "free" past tail membrane (free-tailed bats) [Fig 1a, Fig 2] .......................................................... 2
   b. Tail enclosed within tail membrane (uropatagium) [Fig 1b] ........................................................................... 3

Figure 1

2. a. Large bat, ears connect at mid-forehead, forearm > 55mm; (ACF = 60.3mm; AWT = 21.7g) [Fig 3]
   Nyctinomops macrotis (big free-tailed bat)
   b. Smaller than Nyctinomops; ears do not connect, ammonia smell, forearm 36 - 46mm; (ACF = 42.5mm; AWT = 11.6g) [Fig 4]
   Tadarida brasiliensis (Mexican free-tailed bat/Brazilian free-tailed bat)

3. a. Bat has very long ears (> 30mm), extend past nose ......................... 4
   b. Bat ears < 30mm (warning: Myotis evotis and M. thysanodes have longer ears but < 30 mm) .......................................................... 7

4. a. Bat with large pinkish ears, 3 large white spots on back (ACF = 50.7 mm; AWT = 16.2 g) [Fig 5, 6]
   Euderma maculatum (spotted bat)
   b. Bat without large white spots on back ...................... 5
5. a. Eyes large and conspicuous; bumps or warts on face; rounded nostrils on long snout; pale color; forearm 48-60mm; ears do not connect at bases (ACF = 55.2 mm; AWT = 18.9 g) [Fig 7]  
   *Antrozous pallidus* (pallid bat)  

   b. Bat without large eyes; ears connect at the base…………………………..6  

6. a. Lack of leaf-like structures extending from forehead; calcar not keeled; weight 9-11g; forearm 41-46mm; (ACF = 43.6mm; AWT = 9.9 g; Avg Wt ♂ = 9.2g; Avg Wt breeding ♀ = 10.6g) [Fig 8]  
   *Corynorhinus townsendii* (Townsend’s big-eared bat)  

   b. Leaf-like structures that extend from forehead over face; white patch of fur at the base of each ear; dark shoulder patch usu. Present; calcar keeled; (ACF = unk; AWT = unk). *Only known from southwestern region of Colorado.* Documented based on vocalizations; no physical specimens have been collected [Fig 9]  
   *Idionycteris phyllotis* (Allen’s big-eared bat/Allen’s lappet-browed bat)  

7. a. Tail membrane heavily furred ..........................................................8  

   b. Tail membrane not heavily furred ..................................................10  

8. Bat has black hair with silver tips; hair on tail membrane usually only extends a third of the length of the membrane (ACF = 40.85mm; AWT = 9.8g) [Figs 9b, 10]  
   *Lasionycteris noctivagans* (silver-haired bat)  

   b. Bat without black hair and silver tips and hair to end of tail membrane [Fig 9a].................................................................................................9  

9. a. Large bat; salt & pepper color with light orange around face; forearm 50 - 58mm; rounded ears edged with black; (ACF = 53.0mm; AWT = 25.3g) [Fig 11]  
   *Lasiurus cinereus* (hoary bat)
b. Medium size bat; reddish color; long pointed wings; rounded ears, not edged with black. *Only known from eastern half of the state* (ACF = 39.4; AWT = 10.0g) [Fig 12]  
*Lasiurus borealis* (eastern red bat)

**MOUSE-EARED BATS**  
(usually short ears, without “tail”, without well-furred uropatagium)

**NOTE:** For this group it is helpful to ensure you are identifying an adult bat because there may be overlap in size between large young bats and small adult bats. Prior to calcification, the finger bones of juvenile bats will be translucent when backlit and will not show the rounded, opaque calcified joints seen in adults [Figs 13a, b and 14a, b, see Fig 30 also].

Figure 13. From Nagorsen and Brigham 1993  
*Mammals of British Columbia*

10. a. Forearm > 32 mm (big/medium mouse-eared bats)........... 11
b. Forearm < 32 mm (small mouse-eared bats).................... 16

11. a. 1st tooth behind canine ≥ ½ of canine [Fig 15a]............ 12
b. 1st tooth behind canine small; 2 small premolars [Fig 15b]. 13

12. a. Large bat; forearm > 40mm; 2 upper incisors [Fig 16a]; 32 teeth, keeled calcar (ACF = 47.2mm; AWT = 16.7g)  
*Eptesicus fuscus* (big brown bat)

b. Medium-sized bat; forearm 34-38 mm; 1 upper incisor [Fig 16b]; 34 teeth; calcar variable; tragus short and blunt; hairs with black bases *Not yet documented in Colorado* (likely to be found in southeast or northeast corner of the state).  
*Nycticeius humeralis* (evening bat)

![Figure 16a](Eptesicus fuscus)

![Figure 16b](Nycticeius humeralis)
13. a. Bat has well-developed keeled calcar [Fig 17b]; ears short and roundish; heavy bodied bat; fur on back extends onto tail membrane; forearm 37-41mm, (ACF = 39.0mm, AWT = 8.3g); heavily furred under wing from knee to the elbow (“hairy armpits”) [Fig 18] **Myotis volans** (long-legged myotis/hairy-winged myotis)

b. Bat without noticeable keeled calcar [Fig 17a].

14. a. Fringe of stiff hairs on trailing edge of uropatagium (Fig 19); ears long 17-20mm; forearm > 40mm, (ACF=42.8, AWT=7.6g) **Myotis thysanodes** (fringed myotis)

b. No fringe of stiff hairs, or if hairs are present they are fine and non-distinct.................................................................15

15. a. Ears long and black (21-24mm) that extend well past nose when laid flat [Fig 20]; dark black membranes; hair lead grey at base; forearm approx. 37 -41mm; sometimes a very slight fringe of hair visible on trailing edge of tail membrane, but is very fine and not easily noticeable (ACF = 39.2mm, AWT = 6.2g ) [Fig 20] **Myotis evotis** (long-eared myotis)

b. Ears not long; foot > 10mm; large myotis; forearmusu. > 41mm (not yet documented in CO; likely to be found in the southeastern corner of the state); fur short and coarse **Myotis velifer** (cave myotis)

16. a. Bat has well-developed keeled calcar [Fig 17b]..................................................................................................17

b. Bat without noticeable keeled calcar [Fig 17a].................................................................................................19

17. a. Very small bat [Fig 21]; black mask and ears [Fig 22]; 1st premolar miniscule; forearm ≤32mm; tragus <5mm, blunt and curved; 34 teeth; keeled calcar; weight usu. < 5g ; HF < 7mm; grayish to light brown/yellow color (ACF = 31.0mm; (AWT = 4.5g; avg wt non-breed = 4.0g; avg wt breeding ♀ = 5.5g) **Parastrellus hesperus** (canyon bat, formerly western pipistrelle)
18. a. Ears dark; foot very small (< 8mm); keeled calcar; skull does not rise abruptly above the rostrum; sometimes black mask noticeable; forearm 28-39mm, (ACF = 33.4mm, AWT = 4.6g); the naked part of snout wide, about 1.5x as long as the width of the nostrils when viewed from above [Figs 24, 25, 26]  
   *Myotis ciliolabrum* (western small-footed myotis)  

b. Ears lighter; small foot but can be > 8mm; keeled calcar; skull rises abruptly above the rostrum; naked part of snout narrower, about as long as the width of nostrils [Figs 24, 25, 26]; dorsal fur usually tri-colored (dark/light/medium – dark closest to skin) [Fig 27]; (ACF = 32.4mm, AWT = 3.7g)  
   *Myotis californicus* (California myotis)

19. a. Very small; tri-colored dorsal hairs (dark at tips and bases with light band between) [Fig 28]; 32 teeth; forearm 30-35mm; weight 5-8g; found in eastern half of Colorado.  
   *Perimyotis subflavus* (tricolored bat, formerly eastern pipistrelle)  

b. Not very small, or if small then features not as above…………………………………….. 20

20. a. Small to medium sized bat; hair on toes often extends past the tips of claws [Fig 29]; forearm about 35 - 40mm; weight > 6g; fur long and glossy; (ACF = 38.6 mm, AWT = 7.6 g)  
   *Myotis lucifugus* (little brown bat)

b. Smaller than above; hair short and pale (Front Range specimens darker); forearm 34-38mm; weight < 7.0g; foot large for its size approx.10 mm; (ACF = 36.2mm; AWT = 6.1g; AWT non-breeding = 5.8g; Avg wt breeding ♀ =7.3g)  
   *Myotis yumanensis* (Yuma myotis)
APPENDIX

COLORADO SPECIES LIST

- **Antrozous pallidus**: pallid bat
- **Corynorhinus townsendii**: Townsend’s big-eared bat
- **Eptesicus fuscus**: big brown bat
- **Euderma maculatum**: spotted bat
- **Idionycteris phyllotis**: Allen’s big-eared bat/Allen’s lappet-browed bat
- **Lasionycteris noctivagans**: silver-haired bat
- **Lasiurus borealis**: eastern red bat
- **Lasiurus cinereus**: hoary bat
- **Myotis californicus**: California myotis
- **Myotis ciliolabrum**: western small-footed myotis
- **Myotis evotis**: long-eared myotis
- **Myotis lucifugus**: little brown bat
- **Myotis thysanodes**: fringed myotis
- **Myotis velifer***: cave myotis
- **Myotis volans**: long-legged myotis/hairy-winged myotis
- **Myotis yumanensis**: Yuma myotis
- **Nycticeius humeralis***: evening bat
- **Nyctinomops macrotis**: big free-tailed bat
- **Parastrellus hesperus**: canyon bat
- **Perimyotis subflavus**: tricolored bat
- **Tadarida brasiliensis**: Mexican free-tailed bat/Brazilian free-tailed bat

*not documented in CO

Figure 30 (left) from Kunz and Parsons 2009 *Ecological and Behavioral Methods for the Study of Bats*. Column A – transilluminated finger joints of *Myotis lucifugus*; Column B – Xrays of same joints; First Row (I) – neonate; Second Row (II) – juvenile; Third Row (III) - adult

Figure 31. Proper ear measurements. Tragus (tr) and ear starting from internal notch at base of ear

Photo by Kirk Navo

Figure 32. Proper measurement of bat hindfoot. From ankle to end of toenails (*Mystacops tuberculatus*). Image from Dobson 1876 *Proc. Zool. Soc.*