DERMATOLOGICAL ANALYSIS OF WINGS FROM BATS WITH WHITE NOSE SYNDROME

HONORS THESIS

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ABSTRACT

White Nose Syndrome (WNS) is an emerging disease that is killing hibernating bat populations in the Northeast U.S. It is characterized by an early loss of subcutaneous fat in hibernation, abnormal hibernatory behaviors, and white fungal growths on the nose, ears, and wing membranes. A collaborative effort is underway to find the cause in order to form a management plan to save the bats. To evaluate the pathogenic role of the WNS fungus, a new species of *Geomyces*, wing membranes were collected from 166 bats in New York, Vermont, and Missouri over a period from September to March. Each was given an independent objective score from 0 (absent) to 4 (severe) for amount of fungus, degree of fungal invasion, amount of bacteria, inflammation, and necrosis, and total score was calculated by summing these. Data showed that amount of fungus, degree of invasion, and consequently total score increased between October and December and remained high through winter to March. Amount of bacteria, inflammation, and necrosis had no obvious patterns over time. Only 14.3% of the time that necrosis and inflammation were present was fungus clearly associated with it. However, of the cases with necrotic and inflammatory lesions, bacteria were clearly associated 38.1% of the time. Furthermore, there was an apparent direct relationship between amount of bacteria and necrosis. This data suggested that the fungus is not the source of WNS, and that bacteria may be of interest as a causative agent.

ACKNOWLEDGEMENTS

I would first like to thank my research mentor, Dr. Beth Buckles; without her help this project would have been nowhere near possible for me to accomplish. She introduced me to the field of veterinary pathology and held my hand only to the point when I could begin to direct myself. Her guidance, patience, understanding, and encouragement have helped to shape me as a researcher and as a person. I would also like to thank Dr. Shankar Mandel for helping me in the lab in my first year conducting research and Dr. Michael Piepenbrink for being there for me in my second. The staff of the Cornell School of Veterinary Medicine Necropsy Lab has been nothing but friendly and helpful as I have used their facilities, and those at the Histology Lab have been incredibly patient and hard working in providing my slides in a timely manner. I am greatly appreciative of their services. I would also like to thank the United States Fish and Wildlife Service and Bat Conservation International for their funding and commitment to the cause, and all of the many people around the United States, especially the New York State Department of Environmental Conservation, who are collecting the samples and conducting experiments of their own to help save our precious bats.

Finally, I have to thank all of those who have supported me throughout this process. My advisor, Dr. Ron Butler, has always had only positive words for me, and I have looked forward to our meetings as a chance to smile and relax a little. His encouragement has been a vital force in putting me on my current path, and I will always be grateful for it. Last, but certainly not least, are all of my friends and family who have been there for me every step of the way. I cannot thank them enough for helping me through my undergraduate career and life in general.

TABLE OF CONTENTS

INTRODUCTION

White Nose Syndrome (WNS) is a poorly understood syndrome that has spread through populations of hibernating bats across the eastern United States. The syndrome was first detected in one location in New York and has since spread throughout New York and neighboring states.[2] The syndrome is characterized by abnormal hibernation patterns in the bats, bats flying onto the winter landscape and obvious fungal growth on the muzzles and wings of the bats.[2] Once a hibernacula becomes affected large numbers of the bats will die either in the hibernacula or on the surrounding landscape.[2]

Since its first appearance three years ago, there has been a serious decline in bat populations of affected areas, making it a major threat for the species involved. Post mortem examinations of affected bats have revealed that the animals have premature depletion of winter fat stores as well as skin colonization by a distinctive previously undescribed fungus of the genus *Geomyces*. It s thought that early loss of subcutaneous fat reserves during hibernation results in bats leaving the hibernacula early and starving to death in the cold winter months. The role of this fungus has yet to be determined. It is possible that the fungus grows secondary to the depletion of fat reserves or that the fungal infection results in abnormal metabolism in hibernating animals. A management plan may be necessary to contain or control the disease if possible, but in order to form such a plan, the source and mechanism of disease must be known. Currently, a multidisciplinary disease investigation involving veterinarians, veterinary pathologists, field biologists, physiologists and microbiologists is underway. The aim of this effort is to determine the cause of WNS and determine how it can be managed in order to save at risk bat populations.

The rapid spread of the syndrome across the Eastern United States certainly suggests a contagious disease; however, this has yet to be proven in the laboratory setting. In order to establish an infectious etiology for the disease, Koch's postulates must be proven. According to this, a sample of the organism in question, in this case possibly the fungus, must be taken from the host, isolated, and cultured in a laboratory setting. Then, a healthy host organism is inoculated with the newly cultured disease-causing agent, and the host must exhibit the same symptoms as the original hosts. After this, the organism must be isolated and cultured from the new host and identified as the same as the originally isolated organism. If this is true, the agent satisfies Koch's postulates and is proven to be the source of the disease. Additionally, in the case of WNS, a statistical association must be established between affected bats in the natural setting and the presence of fungus. Since the major potential pathogen in this case is a fungus, we must establish that the fungus is associated with bats with WNS and has a statistically significant association with lesions seen on the skin of the bats.

The fungus of interest in this study is a newly isolated species. Collaborators at the National Wildlife Health Center have isolated it and sequenced its DNA, finding it to be a new species of the genus *Geomyces*, a pyschrophilic fungus that grows best at 5-10^{o}C, similar to the body temperatures of hibernating bats.[2] If the fungus is the causative agent of WNS, it must be present on a statistically significant number of bats, cause lesions that can lead to the death of the bat, and be absent on bats not affected with WNS. Additionally, a mechanism consistent with the physiology and biology of bats must be present by which the fungus can spread between hibernacula. Thus, the fungus must not only be present on bats that die during the winter, but also on bats that survive and can

transmit it to unaffected bats during the summer breeding season when bats from different hibernacula interact in the maternity colonies.

The purpose of this study was to histologically evaluate the wing skin of bats during different times of the year for the *Geomyces* fungus. The wing membranes were scored for amount of fungus, degree of fungal invasion, amount of bacteria, inflammation, and necrosis, allowing quantification of severity of skin lesions. By using statistical comparison of the lesion scores for each of these criteria, we would be able to determine if the *Geomyces* is associated with WNS, if it causes lesions that could result in the death of bats and if it is present on the skin during times of year when it could be spread to naive bats. Thus, the hypothesis of this study is that if the newly isolated species of *Geomyces* is the sole cause of WNS, it should be present on bats during all times of year, and be associated with lesions that could result in the death of the affected bats.

REVIEW OF THE LITERATURE

Bat Ecology

The little brown bat (*Myotis lucifugus*, MYLU), is the most prevalent bat in eastern hibernacula and has been the population of bats most affected by WNS.[23] It is also the species examined in this study. However, a number of other species hibernate with the MYLU and may also be at risk for development of WNS. These species include the northern long-eared bat (*Myotis septentrionalis*), the eastern pipistrelle (*Perimyotis subflavis*), the small-footed bat (*Myotis leibii*), the big brown bat (*Eptesicus fuscus*), and the Indiana bat (*Myotis sodalis*).[23] The latter is a federally listed endangered species,[20]

making its involvement in the disease of particular interest for those concerned with conservation. Additionally, New York is home to some of the largest hibernating colonies of *Myotis leibii* and loss of these bats in New York could have significant implications for the health of the eastern population.

Adult MYLU weigh approximately 5 to 14 grams, females being heavier than males in winter,[6] and have an average lifespan of 6 to 7 years in the wild, although individuals over 10 are common[3] and the oldest wild bat recorded was 31 years old.[12] They undergo a period of pre-hibernatory fattening in the late summer and early fall, when they eat enough to gather the majority of the fat reserves that will be used to sustain them throughout the winter.[14] The majority of this energy is used for spontaneous arousal from periodic bouts of winter torpor.[6] Sometime around late October MYLU go into hibernation, and hibernation continue until the following March.[8] Exact dates are subject to variation across different areas due to climate and latitude. For example, in Ontario, hibernation lasts from early September to mid-May.[8] While hibernating, they commonly decrease their body temperature to between 0 and 10 ^{0}C,[11] but raise it periodically throughout the winter. These periods of torpor last from an average two weeks to as much as 90 days,[16] and during the brief episodes of awakening bats may fly to warmer parts of the hibernacula to preserve energy and males may copulate with torpid females.[21] Hibernation occurs in large hibernacula, roosting areas that may contain numbers of bats in the hundreds of thousands over the winter months. The bats always return to the same hibernacula every winter, but in the summer they travel to maternity colonies to breed.[7] They are highly gregarious, and mate many times, resulting in a sperm pool that is stored in the female and used to fertilize the egg the following spring.[21]

Females produce one pup per year in the early summer.[24] This close contact with many animals in different areas, along with their unique ability among mammals to fly, makes bats prime hosts for an infectious contagious disease, unequaled only by the human race in their ability to spread pathogens among the mammalian world.[17]

While once a common animal in the northeast, the numbers of MYLU have severely declined since the outbreak of WNS.[2] Conservational efforts aside, preservation of these animals is essential not only for the sake of saving wildlife, but also for the essential ecological niche they fill. Bats play important roles in pollination and seed dissemination, thereby helping plant propagation. In addition to this, one little brown bat is capable of eating 50% of its body weight in insects every night; a lactating female is capable of eating as much as 110% of her body weight.[1] This equates to one bat eating over 3000 mosquitoes in one night,[3] and considering that there are roosts with hundreds of thousands of bats, one can imagine their importance in controlling insect populations in those areas. Without these animals, there could be huge increases in populations of many insects including crop pests and mosquitoes, which are well known for their disease-transmitting capabilities.

White Nose Syndrome

The disease was first documented via a photograph taken February of 2006 at Howes Cave, 52 kilometers west of Albany, New York.[2] In the following winter of 2006/2007 it became more prevalent in central New York, presenting in a few colonies. The following winter, it appeared in over 25 colonies throughout New York, Vermont, Massachusetts, and Connecticut.[2] In the winter of 2008/2009 we have seen an even greater spread involving most of the Northeast region. The disease is devastating to

affected colonies, killing 70-100% of the bats in each colony it infects.[3] A two year population survey shows that this has led to an estimated decline of over 75% of bat populations in areas in which the disease is present.[2] The severity of this disease to bat populations and the speed with which it is spreading has prompted a rapid response by researchers from many fields of study to attempt to determine the cause and what can be done.

The first clinical indications that WNS is present in a hibernacula is exhibition of abnormal hibernation behavior. Affected bats hibernate in parts of the caves and mines that have not previously been used, they congregate later in the season than usual and at the entrance to the hibernacula as opposed to well inside. Unlikely healthy bats, affected animals do not arouse when disturbed. Later in the hibernation season, affected bats leave the hibernacula unusually early and fly out into severe winter weather where they starve or freeze to death. At post mortem examination there are two major lesions associated with affected bats. First, there is a distinct lack of subcutaneous fat early in the hibernation season.[2] While bats normally accumulate enough fat stores to last them the entire winter, affected bats either do not get enough food in the summer or burn through it too quickly, leaving them without energy supplies as early as February.[2] Having nothing to sustain them, it is thought that these bats awaken from hibernation early and leave the hibernacula in search of food. Because insects are not readily found in the dead of winter, they then starve or freeze to death. This would explain the abnormal behavior mentioned above. The most visible sign of the syndrome to people entering the hibernacula is the plume of white fungus covering the nose of the animals. It is this latter attribute from which the syndrome derives its name.[2] On further inspection

the bats have fungal growth on other areas of hairless skin including the wings and ears.[2] However, during the winter months, lesions associated with the fungal growth are mild or non-existent. Thus it is not clear if the fungus is causing any damage that could result in the death of the bat.

Fungal Infections

The fungus in question is of the genus *Geomyces,* a psychrophilic fungus that grows best at 5-10^{o} Celsius.[2] It is perhaps not coincidental that the body temperature of a hibernating bat fits in this range, making an ideal environment for infection. The fungus does not tolerate higher temperatures, however, making its role as an active agent to spread infection of WNS during the summer questionable. Furthermore, it is not clear if this fungus is a normal commensile on bats that has simply overgrown because the host has become sick. In other species, dermatophytes, a particular group of skin-infecting fungi, may colonize skin and not result in skin lesions or even infection in otherwise healthy individuals.[18]

For example, *Geomyces pannorum*, the closest known relative to the species of *Geomyces* found on the bats. Commonly found in soils around the world, including waste-contaminated areas of Poland[22] and India,[13] it is most suited to areas of temperate or cold climates,[5,9] and is especially prevalent in soil from areas of Antarctica frequented by birds.[4,15,19] However, this strain is rarely reported to infect skin, and even in those cases in which infection occurred, its pathogenicity often remained unknown.[10] The fungus may just as easily be a secondary infection taking advantage of ideal preexisting conditions. There is no reason as of yet to believe that the WNS fungus is anything other than a secondary opportunistic infection.

When dermatophytes first infect the epidermis, they invade growing hair follicles.[18] The fungal hyphae degrade the hair and replace it, spreading into associated sweat and sebaceous glands.[18] The WNS fungus, based on preliminary studies conducted to determine its nature, was found to operate the same way, breaking through the basement membrane and invading the regional tissue.[2] The skin's response to a dermatophyte is normally hyperplasia of the epidermis and folliculitis.[18] This inflammation is characterized by edema and invasion of lymphocytes associated with the innate immune system.[18] The immune response of bats has not yet been extensively studied, and there has been no attempt to characterize the inflammatory response to WNS until the current study. Dermatophytes are spread via contact of a non-infected surface with an infected one,[18] which makes spread across a bat colony simple considering how tightly packed they are within their roosts. The nature of the White Nose Fungus and the ecology of the bats it is associated with create ideal conditions for spread of a fungal pathogen, but do not indicate whether this fungus is capable of being the primary cause of this disease.

MATERIALS AND METHODS

Tissue Collection and Preparation

Little brown bats (*Myotis lucifugus*) were collected via mist netting as they left their hibernacula by field biologists with permits to collect wild bats. They were anesthetized and then humanely euthanized using procedures approved by the individual agencies collecting the bats. Submissions to our laboratory consisted mainly of wings. The bodies of these bats were sent to Dr. Tom Kunz at Boston University for body fat

analysis. In a few cases, the whole bat was submitted to our laboratory. Full necropsies were performed on these bats. The wings were retained for the current study and the remaining tissues saved for additional analyses.

Once wing tissues arrived in our laboratory they were handled aseptically and divided into three sections. One section was placed in 10% neutral buffered formalin (NBF) for histology and two sections were frozen for future culture and molecular analysis. After fixation, tissues were trimmed into multiple thin strips and processed routinely for histology by the Cornell College of Veterinary Medicine Histology Lab. Tissues were embedded on end to ensure that full thickness sections of skin were visible on the slides. Sections were stained with hematoxylin and eosin (H+E) stain for routine analysis and a periodic acid-Schiff (PAS) stain in order to highlight fungal elements. Hematoxylin stains basophilic molecules, such as nucleotides, blue, while eosin stains eosinophilic molecules, such as proteins, red, creating a contrast between cell nuclei and cytoplasm. The PAS stain oxidizes polysaccharides and causes them to show as bright purple. Thus, the highly-glycosylated cell walls of fungi show purple, while other cells remain a dark blue.

Scoring System

H+E and PAS stained wing sections were examined under a light microscope to grade amount of fungus, degree of invasion, amount of bacteria, inflammation, and necrosis of the tissue. All slides were objectively scored a value of 0 (normal), 1 (minimal), 2 (mild), 3 (marked), or 4 (severe) for each histological parameter using the following guidelines. These are also detailed in Table I for clarity. The entire section of wing was examined in 20X and 60X in order to ensure that no abnormalities were

missed. The amount of fungus present was determined using the PAS stain: A score of 1 indicates the occurrence of a few cells or scattered colonies; a score of 2 indicates a relatively mild infection with at least a small colony present in most 20x microscope fields; a score of 3 indicates multiple colonies present in most 20x fields; a score of 4 indicates that fungus has spread out to cover the majority of the wing and there are multiple very large colonies invading the tissue. Degree of invasion indicates how deeply embedded in the tissue the fungus is, also assessed using the PAS stain. A score of 1 indicates that all the fungus is superficial, merely spread out on top of the epidermis (**Fig. 1A**). When the fungus begins to form pockets extending through the epidermis into the dermis, the score given is 2 (**Fig. 1B**). A score of 3 is assigned when there is fungus isolated within the dermis, whether it is just a few cells or an isolated colony (**Fig. 1C**), and a score of 4 is assigned when the fungus cuts through the entire wing from one side of epidermis to the other (**Fig. 1D**). Amount of bacteria measures the occurrence of bacteria in the wing tissue using the H+E stain, which causes bacterial cells to appear blue. A score of 1 indicates a visible concentration of bacteria

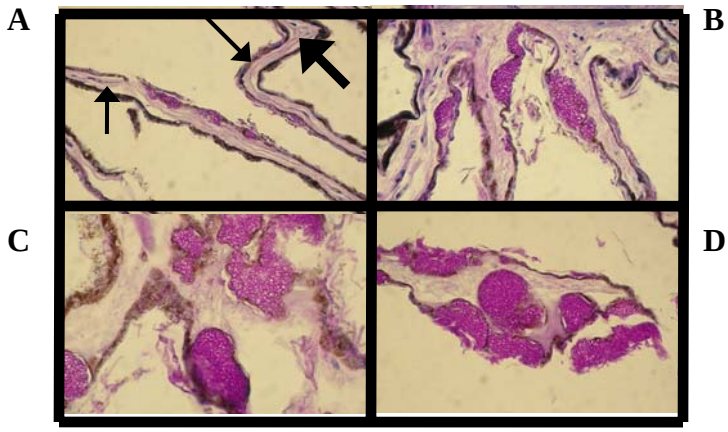


Figure 1. Degree of Invasion scoring of fungus on bat wing tissue. Areas of purple PAS-positive staining are fungal colonies. Thin arrows indicate epidermis; thick arrows indicate dermis. (A) Score of 1 has fungus only superficially atop the epidermis. (B) Score of 2 has fungus penetrating the epidermis and cupping into the dermis. (C) Score of 3 has fungus isolated within the dermis. (D) Score of 4 has fungus nearly or completely penetrating through the entire width of the wing.

either scattered in the dermis as a weak infection or present in the wing as a colony. A score of 2 means that the wing has multiple small colonies or one large colony or a mild infection scattered through a small portion of the dermis, while a score of 3 means several large colonies are present or there is a heavy infection in the dermis. A score of 4 indicates that a large portion of the wing tissue is heavily infected by bacteria or many large colonies are present. To score the degree of inflammation, the H+E stain was used to evaluate the size and type of cellular inflammatory response present in the wing tissue, inflammatory cells being recognizable from other cells by their unique nuclei. A score of 1 constitutes a minor response with slight edema and more than basal levels of inflammatory cells within, while a score of 2 indicates a mild edema with inflammatory cells or a mild focal dermatitis. A score of 3 is given when either a large area of edema with many inflammatory cells, multifocal dermatitis, or one heavy focal dermatitis are found. A score of four indicates a severe inflammatory reaction involving multiple areas of strong dermatitis alongside edema with inflammatory cells throughout. The necrosis score was designed to evaluate the amount of damage done to the wing tissue and was assessed using the H+E stain. A score of 1 indicates a small area of necrosis that covers less than half the width of the wing, while a score of 2 indicates that half the width of the wing or more degraded via necrosis. When necrosis has gone through the entire width of the wing, it is scored a 3. A score of 4 is given when a particularly large area of wing is subject to necrosis covering the full width of the wing.

A total score was determined for each wing by calculating the sum of these five individual scores. Mean scores were determined for each collection group, consisting of wings collected in the same geographical area and time. All comparisons for statistical

difference are made using t-tests. Data points given in figures are the means and bars show the standard error of the mean (SEM). To compare scores of bat wings collected in Vermont and New York, fall data sets used were based on bats collected 10/19/2008 in Vermont and 10/27/2008 in New York, and winter data sets used were bats collected 11/18/2008 in Vermont and 12/17/2008 in New York. Proportions are used to compare lesion association with fungus and lesion association with bacteria.

Table I. Description of scoring method for grading White Nose Syndrome infection.

Variable	Score	Definition
Amount of Fungus	0	Fungus is not present.
	1	Fungus is present as only a few cells or 1-5 scattered colonies throughout the wing.
	2	Fungus is present as multiple colonies throughout the wing, at least one in most 20X focal fields.
	3	Fungus is present as multiple colonies in most 20X fields.
	4	Fungus covers the majority of the wing tissue.
Degree of Invasion	0	Fungus is not present.
	1	Fungus is found superficially on the epidermis.
	2	Fungus penetrates the epidermis pocketing into the dermis.
	3	Fungus has cells or colonies suspended within the dermis.
	4	Fungus has completely penetrated through the wing tissue.
Amount of Bacteria	0	Bacteria are not present.
	1	Bacteria are present in small scattered colonies or as a weak infection scattered throughout an area of the dermis.
	2	Bacteria are found in 1 large colony or as a mild infection scattered throughout the dermis.
	3	Bacteria are found as multiple large colonies or as a heavy infection throughout an area of the dermis.
	4	Bacteria is found as many large colonies or as a heavy infection throughout most of the wing tissue.
Inflammation	0	Basal levels of inflammatory cells may be found throughout the tissue without any clear inflammatory response present.
	1	Wing tissue has slight edema accompanied by few scattered inflammatory cells.
	2	Wing tissue has moderate edema accompanied by inflammatory cells throughout or a mild focal dermatitis.
	3	Wing tissue has strong edema accompanied by many inflammatory cells or multifocal mild dermatitis or strong focal dermatitis.
	4	Strong inflammatory response as described in 3 is found throughout most of the wing tissue.
Necrosis	0	No necrosis is present.
	1	Wing tissue has a small area of necrosis covering less than half of the wing width.
	2	Wing tissue has a larger area of necrosis covering over half but not all of the wing width.
	3	Wing tissue has a large area of necrosis covering the full width of the wing.
	4	A large portion of the wing tissue has necrosis covering the full wing width.

RESULTS

A total of 166 bats were received from three areas at several different collection dates from fall through winter: Aeolus Cave in Vermont (Sets 1 to 4), Williams Hotel Cave in New York (Sets 5 to 9), and an unspecified source in Missouri (Sets 10 and 11) (**Table II**). Missouri bats had the lowest total wing scores of all study areas. New York and Vermont study sample populations were significantly different compared to Missouri, with the exception of Set 6 from New York, which also had a value of 0 for all variables. Of the 149 bats from New York and Vermont examined, on 41.6% fungus was present (score greater than 0), on 21.5% bacteria was present, on 38.3% inflammation was present, and on 20.1% necrosis was present.

Table II. Collection data of bat samples.

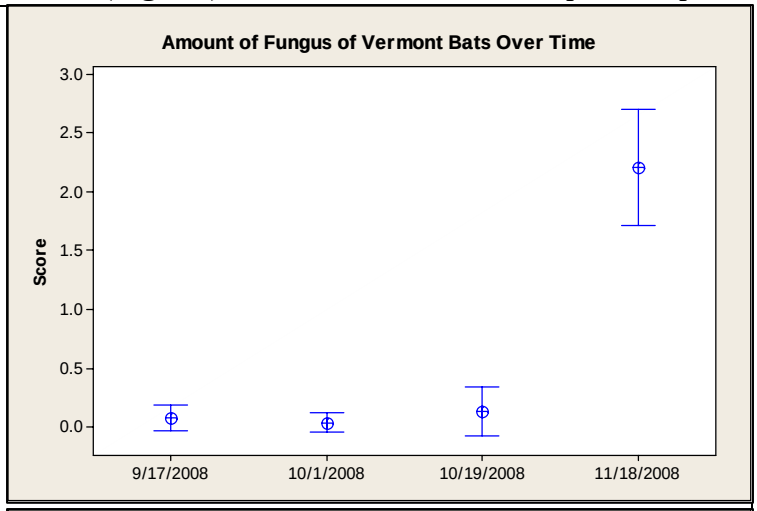
Set Number	Location	Date Collected	Sample Size (n)	Total Wing Score
1	Vermont	9/17/2008	26	1.4 ± 0.6
2	Vermont	10/1/2008	24	0.4 ± 0.3
3	Vermont	10/19/2008	22	0.5 ± 0.4
4	Vermont	11/18/2008	19	6.2 ± 0.7
5	New York	10/27/2008	24	2.3 ± 0.5
6	New York	11/3/2008	7	0
7	New York	12/17/2008	7	5.9 ± 1.7
8	New York	2/24/2009	6	9.2 ± 1.7
9	New York	3/10/2009	14	9.4 ± 0.6
10	Missouri	12/15/2008	10	0.6 ± 0.6
11	Missouri	3/2/2009	7	0.6 ± 0.3

Total wing score values are given as mean ± SEM.

Amount of Fungus

The mean scores for amount of fungus in Vermont and New York at different times of collection are shown in Figure 2. As time progresses into winter there is an increase in amount of fungus present on the wings of bats in both Vermont (**Fig. 2A**) and New York (**Fig. 2B**). New York bats exhibit a possible plateau as the winter progresses.

A.



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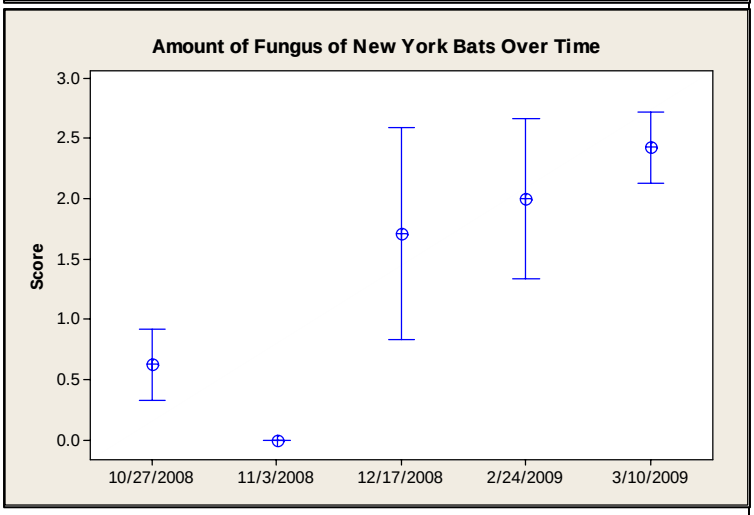


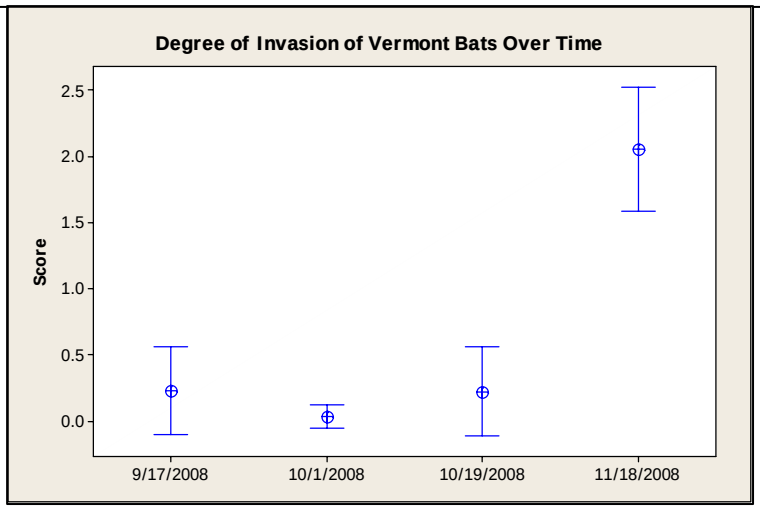
Figure 2. Interval plots of the Amount of Fungus score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed on Table II.)

In Vermont amount of fungus is low until November when there is a sharp increase from 0.1 ± 0.1 to 2.2 ± 0.2 (**Fig. 2A**). In New York amount of fungus starts low in October at 0.6 ± 0.2 and gradually rises and plateaus to 2.4 ± 0.1 in March. New York showed significantly more fungus than Vermont in fall ($P < 0.05$), but not in the winter.

Degree of Invasion

The mean scores for degree of invasion in Vermont bats and New York bats at different times of collection are shown in Figure 3. Degree of invasion follows similar trends to amount of fungus. In Vermont degree of invasion is low until November when there is a sharp increase from 0.2 $\pm$ 0.2 to 2.1 $\pm$ 0.2 (**Fig. 3A**). In New York amount of fungus starts low in October at 0.9 $\pm$ 0.2 and gradually rises and plateaus to 2.6 $\pm$ 0.2 in March (**Fig. 3B**). The value of bats from New York is significantly higher than that of bats from Vermont in fall ($P < 0.05$), but not in winter. There is a direct relationship between the amount of fungus and the degree of invasion (**Fig. 3C**). Degree of invasion score is always 0 when there is no fungus, and it increases with an increase in amount of fungus.

A.



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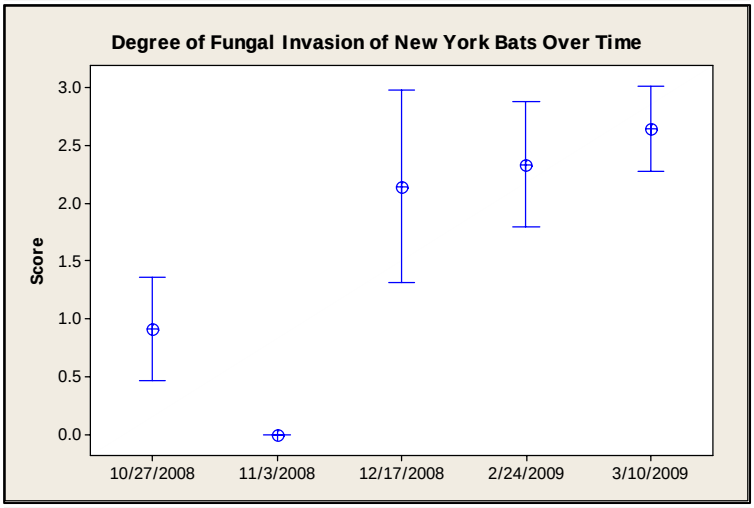

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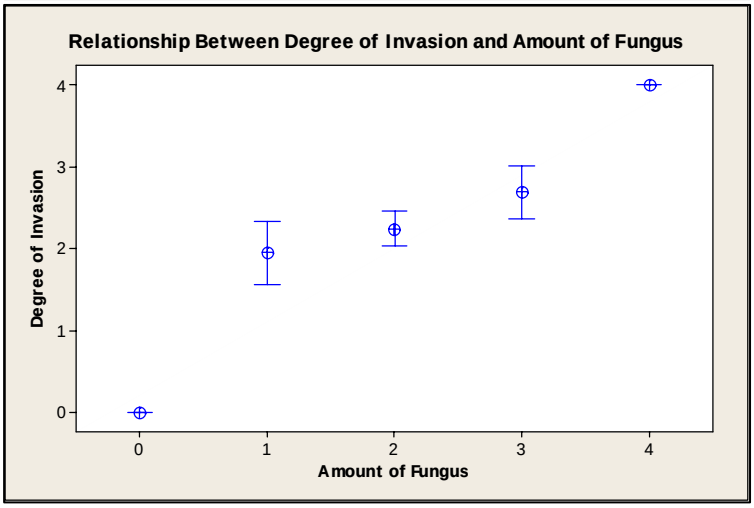


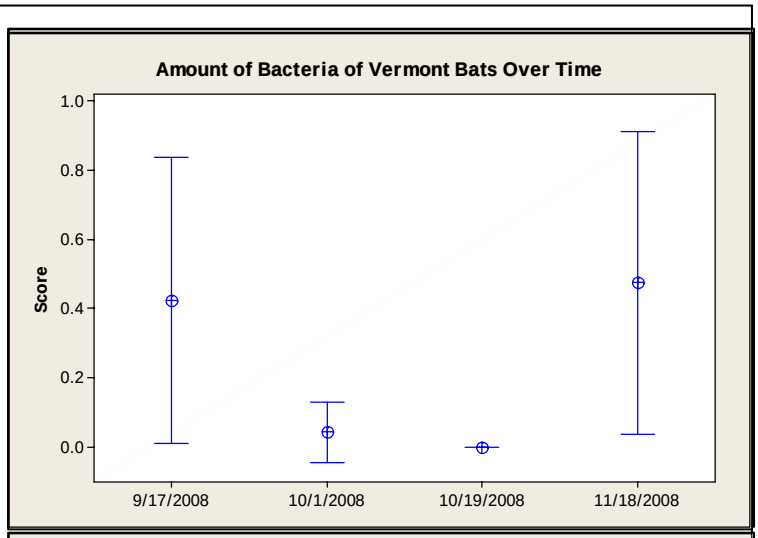
Figure 3. Interval plots of the Degree of Invasion score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed on Table II.) (C) Degree of invasion scores plotted against amount of fungus score in bats from New York and Vermont. There is an apparent direct relationship between the two such that the more fungus that is present, the deeper it penetrates into the wing tissue. (n = 104, 20, 25, 16, 1 in order of Amount of Fungus score)

Amount of Bacteria

The mean scores for amount of bacteria in Vermont bats and New York bats at different times of collection are shown on Figure 4. In Vermont bats there is no apparent trend, with high points being in September and November (0.4 ± 0.2 and 0.5 ± 0.2, respectively) (**Fig. 4A**). In New York bats a small trend to increase through winter is evident, with a low in October of 0.04 ± 0.04 rising to 1.4 ± 0.2 by March (**Fig. 4B**).

Figure 4. Interval plots of the Amount of Bacteria score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed in Table II.)

A.



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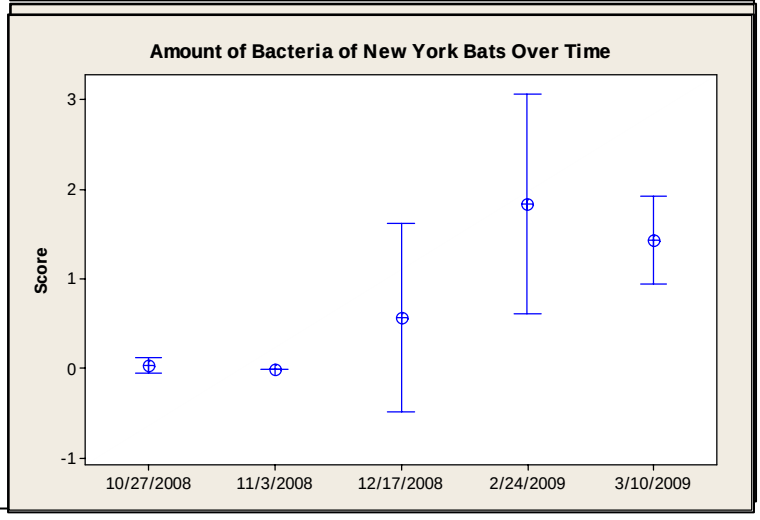
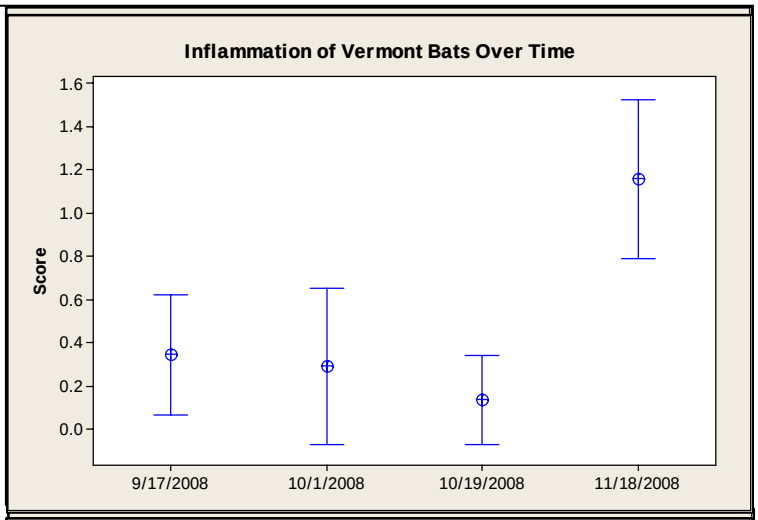


Inflammation

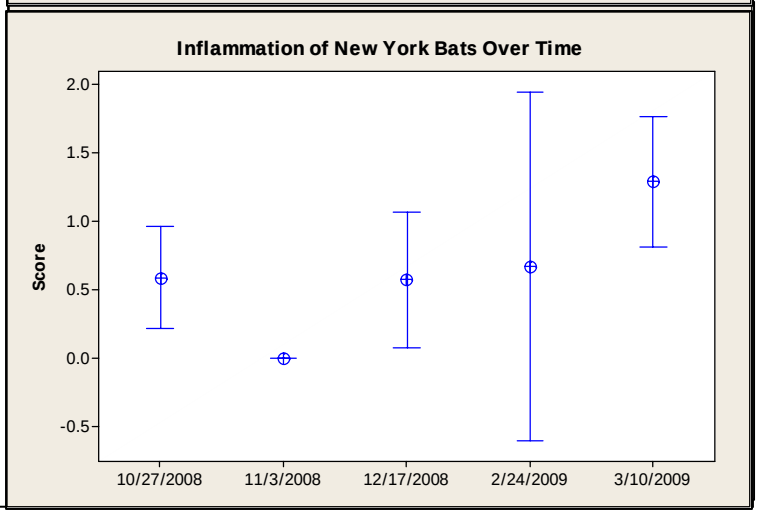
The mean inflammation scores for Vermont bats and New York bats at different times of collection are shown in Figure 5. In Vermont bats there is an increase from in October 0.1 ± 0.1 to 1.2 ± 0.2 in November (**Fig. 5A**). In New York it appears that inflammation stays at low levels through most of the year, with a possible slight upward increase in inflammation in March (0.6 ± 0.2 in October up to 1.3 ± 0.2 in March.) (**Fig. 5B**). New York has a statistically higher score for inflammation in the fall, but Vermont has a statistically higher score in the winter ($P < 0.05$).

Figure 5. Interval plots of the Inflammation score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed on Table II.)

A.

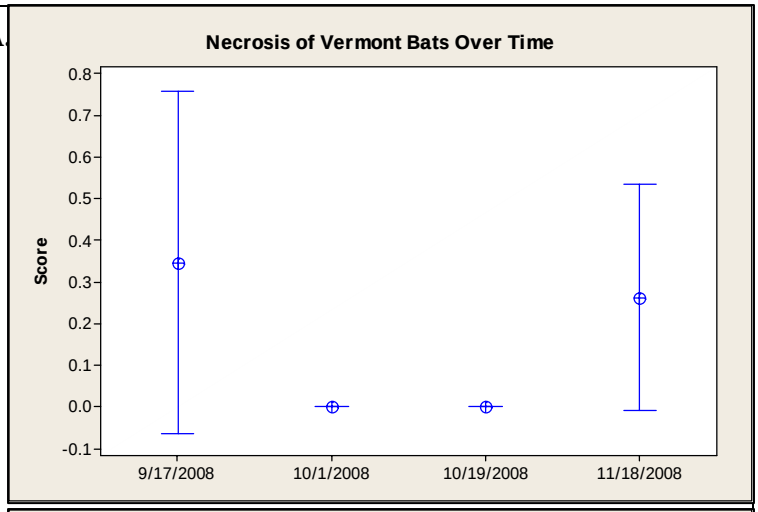


B.



Necrosis

The mean necrosis scores for Vermont bats and New York bats at different times of collection are shown in Figure 6. Vermont bats had no necrosis on collection dates 10/1/2008 and 10/19/2008, and exhibited no apparent trend (**Fig. 6A**). In New York bats a small trend to increase through winter is evident, with a low in October of 0.08 ± 0.06 rising to 1.6 ± 0.3by March (**Fig. 6B**). Lack of necrosis in the fall in Vermont made statistical comparison impossible, and there was no statistical difference between states in the winter.



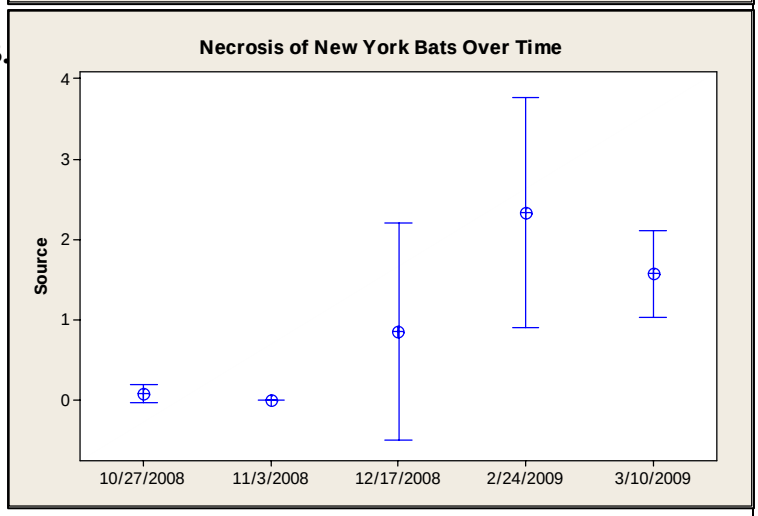


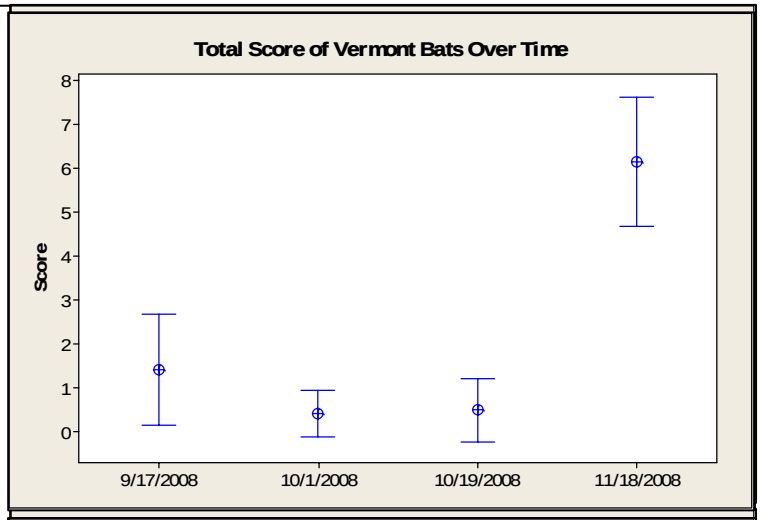
Figure 6. Interval plots of the Necrosis score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed on Table II.)

Total Score

The mean total scores for Vermont Bats and New York bats at different collection times, also found on Table II, are shown graphically on Figure 7. Vermont bats exhibit a sharp increase in Total score in November from 0.5 ± 0.4 to 62 ± 0.7, similar to the trends of amount of fungus (**Fig. 7A**). In New York bats amount of fungus starts low in October at 2.3 ± 0.5 and gradually rises and plateaus to 9.4 ± 0.6 in March (**Fig. 7B**). Bats from New York have a significantly higher score than bats from Vermont in fall ($P < 0.05$), but scores of the bats from the two states are not different in the winter.

Figure 7. Interval plots of the Total score over time for bats in (A) Vermont and (B) New York. Values are given as mean ± SEM. (n is detailed on Table II.)

A.



B.

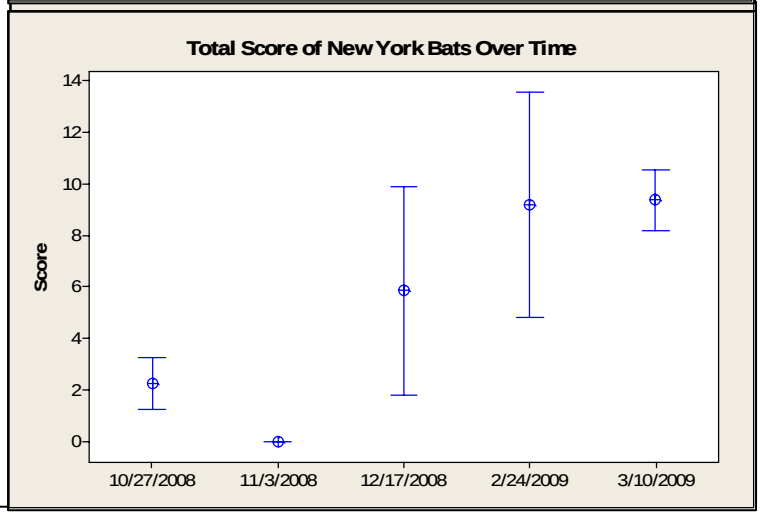


Table III compares the incidence of bacteria and fungus on wings exhibiting necrosis, wings exhibiting inflammation, and wings exhibiting both necrosis and inflammation. Wings with inflammation were more likely to have fungus on them than to have bacteria, and wings with necrosis and inflammation were more likely to have a lesion associated with bacteria than with fungus ($P < 0.10$). Although a similar trend may be evident in wings with necrosis, data is inconclusive ($P = 0.110$).

Table III. Comparison of incidence of bacteria and fungus in wings with necrosis and inflammation.

	Exhibiting bacteria	Exhibiting fungus	Pathology associated with bacteria	Pathology associated with fungus
Wings with necrosis	.87	.93	.30	.13
Wings with inflammation	.51[a]	.74[b]	.053	.053
Wings with necrosis and inflammation	.86	.95	.38[c]	.14[d]

To exhibit necrosis, inflammation, bacteria, or fungus, a wing must have a score greater than 0 for that variable. Wings with necrosis, n = 30. Wings with inflammation, n = 57. Wings with necrosis and inflammation, n = 21. Values are expressed as proportions.

[a] and [b] are statistically different groupings across rows at $P < 0.05$.

[c] and [d] are statistically different groupings across rows at $P < 0.10$.

DISCUSSION

The data clearly indicates that the overall score for Vermont bats increases as winter begins (11/18/2008 sample; **Fig. 7A**). This is mostly due to the increases in amount of fungus (**Fig. 2A**) and depth of fungal invasion into the wing (**Fig. 3A**). This correlates with the onset of hibernation, which typically starts in late October for MYLU in temperate climates like New York.[8] Thus, it would appear that when temperature drops and the bats begin relying on their fat stores for nutrients rather than hunting, fungal infections become worse, implying a possible lack of resistance of the bats or increased ability of the pathogen to infect during the colder winter months. Considering the change in ambient temperature of the time period and the psychrophilic nature of the fungus,[2] the latter seems more likely. Inflammation shows similar trends of increasing in November (**Fig. 4A**) but is a little more variable. Amount of bacteria and necrosis, on the other hand, show no apparent trend as time progresses (**Fig. 4A** and **5A**).

A similar trend is seen in the bats from New York. While the levels of infection are low in October, by December they increase to a plateau state that continues through to March. This trend can be seen in amount of fungus (**Fig. 2B**), degree of invasion (**Fig. 3B**), and total score (**Fig. 7B**). After this initial rise in severity between October and December, these scores reach a stable high point that persists until March. Although the fungus may show signs of getting steadily worse as time passes, as evidenced by the increasing means and decreasing SEM shown in Figure 2A, there is only a small statistical difference between December and March collection samples ($P = 0.106$). After the sharp increase in fungus and total disease score seen in early hibernation, any increase after that is gradual at best. There does appear to be a rise in necrosis as the winter

progresses (**Fig. 6B**), which makes sense considering that any pathogens present are given time to act on and degrade the wing. Inflammation, however, does not appear to show any great trend as winter progresses (**Fig. 5B**). Several reasons could cause this: The bats might not have the capability for significant immune responses during hibernation, the observed fungus or bacteria may somehow block an immune response to prevent reaction, the observed fungi or bacteria are common antigens present in more than normal amounts so the bat's body does not recognize them as serious invaders, or perhaps the bats are immunosuppressed, thereby allowing other infections to rampage through their systems.

Few significant trends could be found when comparing New York and Vermont bats over time. New York bats receive a consistently higher score in the fall than Vermont bats, but while this may be indicative of a worse disease in New York than in Vermont, it may also be due to the difference in collection dates of the samples. The bats from late October in New York were collected eight days after those collected in Vermont, which may be when the disease progressed the most. This would be consistent with the data charting the course of disease over time, in which there is a sharp increase in most variables shortly after October collection dates. Thus, bats have eight more days to develop worse symptoms in New York, accounting for the difference. However, these differences are unimportant when the November bats of Vermont are compared with the December bats of New York. Throughout all categories there is no significant difference between the two sets from different states, excepting for the inflammation score, which reverses this trend, Vermont bats having a higher score than those from New York. Even

if the disease is worse for New York bats in the fall at the start of hibernation, Vermont bats catch up such that the disease presents itself to the same degree in either state.

The 11/3/2008 data set for New York bats (Set 6) presents an anomaly. All of the bat wings observed from this period were perfectly healthy when one would expect a significant amount of infection. Little brown bats go through periods of arousal in their torpor, at which point they may fly to a warmer area in order to conserve energy.[8] The bats for this study were collected via mist netting outside the cave entrances. It is possible that these seven bats, perfectly healthy, arose from torpor on a particularly warm November day and left the cave to be caught by the nets. Alternatively, the disease was mild enough that a cross-section of wing did not cut through an area containing pathology, so no infection was observed. Either way, considering that there is disease in October and December, it is safe to assume that the disease was present in November, but by some chance was undetected by our histological methods. Another possibility is that these bats were undetectably affected by WNS, but did not have any fungal infection, supporting the theory that the disease is not caused by the fungus.

In order to further explore this new hypothesis, that the fungus is not an active agent in killing the bats, the tissues containing necrosis and inflammation were analyzed to see if fungus or bacteria were clearly associated with the lesions. Necrosis and inflammation were associated with bacterial infection in 38.1% of the wings that had both necrosis and inflammation, while fungal infection was clearly associated with lesions in only 14.3% of those wing samples (**Table III**). Compared to fungal infections, bacterial infections are associated with necrosis and inflammation in a statistically larger proportion of wings. Furthermore, although lesions were just as likely to be found on

wings with fungus (28 out of 30 wings with necrosis exhibit fungus) as those with bacteria (26 out of 30 wings with necrosis exhibit bacteria), by observing the shapes of the interval plots in Figures 4A, 4B, 6A, and 6B, amount of bacteria appears to increase when necrosis increases. While these two factors do not follow any apparent trend over time like fungus so obviously does, they do compare to each other in a strikingly similar manner. In addition, observations show that the fungus, which is indeed overly abundant, does not appear to cause much necrotic damage to the wing as would be suspected from a serious infection. These relationships suggest that the bacteria are linked to the cause of the damage on the wing. The fungus in this case is more likely a commensal organism presented with conditions in which it can thrive. This information pushes us to reject our former hypothesis of fungus as a cause of WNS and to adopt the alternative hypothesis that fungus represents a secondary infection while some other agent causes the disease.

One problem that became evident while evaluating this data was that the amount of bacteria is difficult to score using H+E and PAS stains. While bacteria are detectable in appreciable amounts, quantification in the tissue is difficult. Use of a Gram stain to clearly highlight the bacteria apart from the tissue would be useful in assessing this variable and exploring their possibility as causes for necrosis in wing tissue. However, lacking the time to prepare Gram stains for this study, analysis of H+E-stained was used, making results of the amount of bacteria category questionable. Despite this, the score is still valuable as a detector of the presence of appreciable amounts of bacteria, and thus the comparisons made between scores for bacteria and necrosis still hold firm.

This data suggests a far more complex underlying story in the case of WNS than a single primary pathogen. It appears to support the theory that the fungus is merely a

secondary infection, opportunistically attacking while the bats are somehow weakened to its infiltration. Perhaps, like *Geomyces pannorum*,[4,5,9,13] this new species is actually widespread around the world, but some aspect of the WNS disease has created an ideal environment for it to become pathogenic. In order to test this it is necessary to test soil samples around the United States in sites with and without White Nose Syndrome to detect this new *Geomyces*. One bat from Missouri was found to have a few fungal cells embedded in its wing tissue; it would be beneficial to develop a PCR assay to see if the WNS *Geomyces* could be identified as the one of interest or whether some other dermatophyte was present. In addition to this, PCR can be used to make sure that all of the fungus found in minimal amounts in tissues obtained during warmer periods is actually the same fungus as the *Geomyces* under scrutiny.

Several other steps are also necessary for a more thorough examination of the roles of fungus and bacteria in the case of WNS. As previously stated, Gram stains of all slides containing bacteria are necessary for evaluation of amount and types of bacteria present in the wing. Another way to improve this examination would be to increase its breadth. Our lab has collected wing tissue from over 400 additional bats from various times between September and April over various states. Each of these will eventually undergo the same analysis as the 166 wings described here, but to use all of them was beyond the scope of this preliminary study. Once all are evaluated, a more sophisticated statistical method may be used to determine regression models and draw answers for further inquiries. Also, it is necessary to obtain samples during the rest of the year, starting in later April and continuing through May, June, July, August, and September to see how the disease regresses in the spring and summer to the point where it is no longer

visually observable until the following fall. Although efforts are underway, it is an increasingly difficult task. The bats are harder to catch during these time periods because they are not contained within their hibernacula. The greatest concentrations of bats for harvesting can be found in maternity colonies during the early summer, but setting up mist nests in this area would be risky because we do not want to upset the breeding cycles of species already at such great risk of devastation by intruding in their mating areas. Collecting samples from a population already under stress may have negative consequences for bats that have survived hibernation in a colony plagued by WNS. It would effectively be culling some of the survivors of the disease, and although they may also harbor it and be spreading it, the more effective management plan in the long run would be to help boost the populations that are strong enough to resist WNS.

In addition to expanding the research presented here, much more must be done in order to determine the source of WNS. Thankfully, many other researchers are working on projects to do just that. One group is testing Koch's Postulates on the fungus in vivo to see what pathogenesis form and to compare that with wild specimens. Other factors must be considered as a potential source of the disease, though, constituting a broad-range effort to find the cause. Some investigators are examining the parasite load associated with bats as possible energy drains (personal communication with Dr. Buckles), and this study suggests that someone it will be worthwhile to examine the types of bacteria found in necrosis of the wing tissue. Another group is performing phytohaemagglutinin (PHA) tests on healthy bats to determine the characteristics of a normal inflammatory response in bats (personal communication with Dr. Buckles). There are no existing reports in which the immune response of bats has been studied in

great detail.[17] This information could prove valuable as a positive control for determining whether or not immunosuppression has any role in WNS. All in all, much is being done to determine the origin of this destructive disease, but much more work needs to be done before a management plan is fathomable. Some scientists plan on placing heaters in caves to help prevent disruption of periods of torpor, another possible mechanism of disease (personal communication with Dr. Buckles), but plans like this are more likely to allow weaker bats to survive. This would merely help spread WNS faster, if it is in fact a contagious disease. Management plans like this are ineffective; we need to find the cause of WNS before we can begin to treat it or control its spread, and the information from the current study is one of many first steps towards doing so.

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