NORTHEAST REGIONAL CONSERVATION NEED GRANT FINAL REPORT

Grant Number: **2010-01**
Grant Title: **"*Laboratory and Field Testing of Treatments for White Nose Syndrome: Immediate Funding Need for the Northeast Region*"**
Grant Receipt**: Bucknell University**
Grant Contact Name: **DeeAnn M. Reeder, Department of Biology, Bucknell University**

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ABSTRACT

White-nose Syndrome (WNS), an emerging infectious disease caused by the novel fungus *Pseudogymnoascus destructans* (*Pd*), has devastated North American bat populations since its discovery in 2006. The little brown myotis, *Myotis lucifugus*, once the most common bat in North America, has been especially affected. The goal of this study was to address bat declines by developing and optimizing treatments for WNS. We report the results of two *in vitro* studies of pharmaceutical/organic compounds and the results of two studies of treatments in control and *Pd infected* little brown myotis *in vivo,* performed at the bat research facilities in the Reeder lab at Bucknell University. As has been found by other laboratories, a number of chemical agents, including, from this study 5,7-hexadecadiynoic acid and heptadecanoic acid, are effective at killing or inhibiting the growth of *Pseudogymnoascus* (but *Pichia* spent medium (PSM) was not effective). Unfortunately, both systemic and dermatophyte (superficial) fungal infections in humans and animals are notoriously difficult to treat, and often require prolonged chemical application to achieve a cure. Traditional antifungal agents are also known to have relatively high side effect profiles and the use of these drugs in hibernating animals is novel. While treatment with the strong antifungal agent voriconazole was very clearly harmful to bats, trials with subcutaneously terbinafine implants were more promising. We can make no specific treatment recommendations at this time, but rather urge the research community to continue to pursue mitigation options for WNS. Clearly the WNS problem is complex, especially when one considers any sort of treatment option – which will affect the entire cave ecosystem.

INTRODUCTION/BACKGROUND

Hibernating bats in the NE USA have experienced sudden and dramatic declines over the past seven winters due to an emerging infectious disease dubbed "White Nose Syndrome" (WNS). At the time this grant was funded, an estimated one million bats had died; a 2012 estimate put this number at 5.7 million (USFWS 2012; Coleman and Reichard 2014) and likely a million or more bats have died since then. In 2011, colleagues and I (headed by Jeff Lorch) published the seminal study that definitively determined that the newly described cold-loving fungus *Geomyces destructans* (*Gd*) was the infective agent; this was further supported by Warnecke et al. (2012). This fungus was reclassified to the genus *Pseudogymnoascus* in 2013 (Minnis) and is thus now know as *Pseudogymnoascus destructans* (*Pd*). Bats are vulnerable to *Pd* because their immune system is suppressed along with nearly all physiological processes during hibernation (Bouma et al. 2010; Carey et al. 2003; Storey et al. 2010) and because the cold temperature and high humidity typical in many bat hibernacula represent ideal conditions for *Pd* growth (Webb et al. 1996; Verant et al. 2012; Reeder and Moore 2013). *Pd* is not native to North America, and is believed to have been introduced from Europe (Puechmaille et al. 2010; Warnecke et al. 2012). Although large-scale mortality of bats has never been documented in Europe, >90% mortality of bats occurs in North American hibernacula after *Pd* is introduced (Puechmaille et al. 2011; Turner et al. 2011). Such high mortality rates have led to predictions of regional extinctions, although not all bat species appear equally affected by the disease (Frank et al. 2014; Frick et al. 2010, 2015; Turner et al. 2011).

At least seven species of bats are affected, but little brown myotis (*Myotis lucifugus*) are especially susceptible, with an average 91% decline in northeastern North America (Turner et al. 2011). Little brown myotis infected with *Pd* arouse more frequently from hibernation than unaffected bats, resulting in exhaustion of fat reserves needed to survive the winter (Reeder et al. 2012; Warnecke et al. 2012) Although the trigger for this increase in arousals has yet to be confirmed, there is evidence that hypotonic dehydration of infected bats may influence arousal behaviors (Cryan et al. 2010, 2013).

The goal of this proposal was to address these declines by developing and optimizing treatments for WNS. Tasks included: (1) testing potential treatments for efficacy against cultured *Pd* under laboratory conditions; (2) testing potential treatments for safety in healthy bats, and (3) testing potential treatments for efficacy against *Pd* in hibernating bats. Tasks 2 and 3 were performed simultaneously, in *Pd* infected and uninfected (presumably healthy) bats. This report will first summarize the laboratory testing for *Pd* treatments (2 studies under "I" below) and then will summarize our findings of treatments on uninfected *Pd* infected bats (2 studies under "II" below).

I. Studies of the effects of treatment agents on *Pd* growth.

Both systemic and dermatophyte (superficial) fungal infections in humans and animals are notoriously difficult to treat, and often require prolonged medication application to achieve a cure. Bats are susceptible to WNS only while at hibernation temperatures, and thus may require a lower dose of antifungal agent since metabolism of the drug will likely be slow. Alternatively, application of a topical antifungal agent could prevent a new infection from establishing on bats during hibernation, and could prevent hyphal spread once infection is established. The ideal anti-fungal drug is highly effective at low doses over a short period of time, has minimal adverse

affects, is easy to apply and is low cost. Both pharmaceutical (Ghannoum Lab) and non-pharmaceutical (Barton Lab) compounds were tested.

IA. **Evaluation of the Antifungal Activity of Pichia Spent Medium (PSM) Against *Pseudogymnoascus destructans* in an *In vitro* Agar Diffusion Assay.**

Performed by Co-PI: Mahmoud A. Ghannoum, PhD

The objective of this study was to determine the *in vitro* antifungal activity of *Pichia* spent medium (PSM) against *Pseudogymnoascus destructans* using an agar diffusion assay. *Pichia Spent Medium:* In a recent study, we demonstrated that secretory products present the spent medium (media supernatant) of Pichia (a non-pathogenic colonizer of the oral cavity) inhibits a wide range of pathogenic fungi including *Candida*, *Aspergillus*, and *Fusarium* (Mukherjee et al. 2014**)**. Since *Pichia* is a known biocontrol agent with activity against plant fungal pathogens, we decided to determine whether *Pichia* spent medium (PSM) has utility as an antifungal agent against *G. destructans*. In the first phase of the study, we optimized the assay using activity of PSM against *C. albicans*; next we evaluated its activity against *G. destructans* using used an agar diffusion based method.

METHODS:

Test Isolate: *Candida albicans* and *P. destructans* isolates were from the culture collection of the Center for Medical Mycology.

***Pichia* Spent Medium (PSM):** *Pichia farinosa* was grown in Sabouraud dextrose broth (SDB) 48 h, and the culture supernatant was obtained by centrifugation (3000 rpm for 15 min). The resulting supernatant was filter sterilized through a 0.22 micron filter, and used for antifungal activity testing.

Activity Assay: To test the activity of PSM against *Candida albicans* (SC5314), cells were standardized to a suspension of 5 x 10^5 cells /mL in yeast nitrogen medium (YNB, supplemented with glucose) and exposed to diluted (25%, 50%) and undiluted PSM for 24 h. At the end of the incubation period, *Candida* growth was determined by metabolic activity (XTT dye) assay and counting the colony forming units (CFUs) (Chandra et al. 2008). For testing activity against *Pd*, we used an agar diffusion-based assay, as described previously by our group and others (Nweze et al. 2010; Espinel-Ingroff et al. 2011**)**. Briefly, potato dextrose agar (PDA) plates were seeded with *C. albicans* or *Pd*, (1×10^7 conidia), and 8-mm diameter well was cut into the center of the plate. Different dilutions of PSM (100, 75, 50 and 25%) were prepared and added (150 πL) into the well. Plates were then placed in an enclosed container over a saturated salt solution to promote hydration. The plates were incubated at 30°C for 4 days and zones of inhibition were measured.

RESULTS: As shown in Figure 1, the number of CFUs was significantly reduced for *C. albicans* grown in the presence of PSM (undiluted, 50% or 25%), compared to untreated control (Fig. 1). Our results showed that exposure to undiluted PSM reduced *Candida* growth by as much as 94.5% when exposed for 22 h (Table 1).

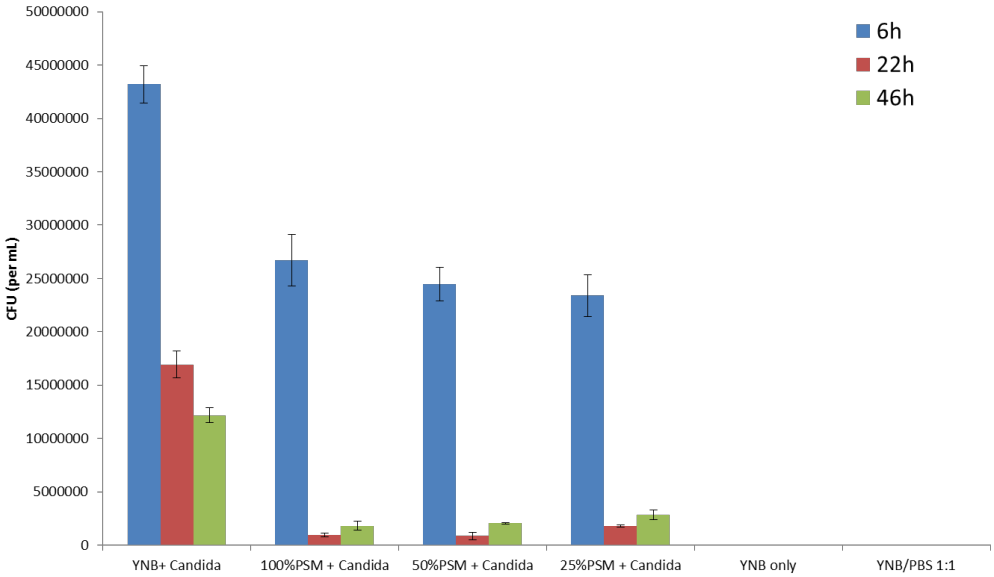

Figure 1. Effect of PSM on growth of *C. albicans*. Growth was measured as colony forming units (CFUs) per mL for *Candida* cells exposed to different dilutions of PSM.

Table 1. Effect of PSM on *Candida* growth (CFUs)			
Exposure Time	Reduction (%) in growth of *Candida* exposed to:		
	Undiluted PSM	50% PSM	25% PSM
6 h	38.1%	43.3%	45.8%
22 h	94.5%	94.9%	89.6%

The assay for metabolic activity using XTT dye revealed a similar trend, with exposure to increasing concentration of PSM resulting in increasing reduction in metabolic activity of *C. albicans* (Fig. 2). The effect of PSM on *Candida* growth was also evaluated using the agar diffusion assay, which confirmed the findings of the metabolic activity assay.

Activity against *P. destructans*

Testing for activity of PSM against *Pd* using the agar diffusion assay revealed no zone of clearance on plates treated with PSM. These results showed that while PSM exhibits potent activity against *Candida*, this agent showed no activity against *Pd* (Figure 3, Table 2).

Table 2. Zone of inhibition in *Pd* culture exposed to PSM	
PSM Concentration (%)	Zone of inhibition (mm)
100	0
75	0
50	0
25	0

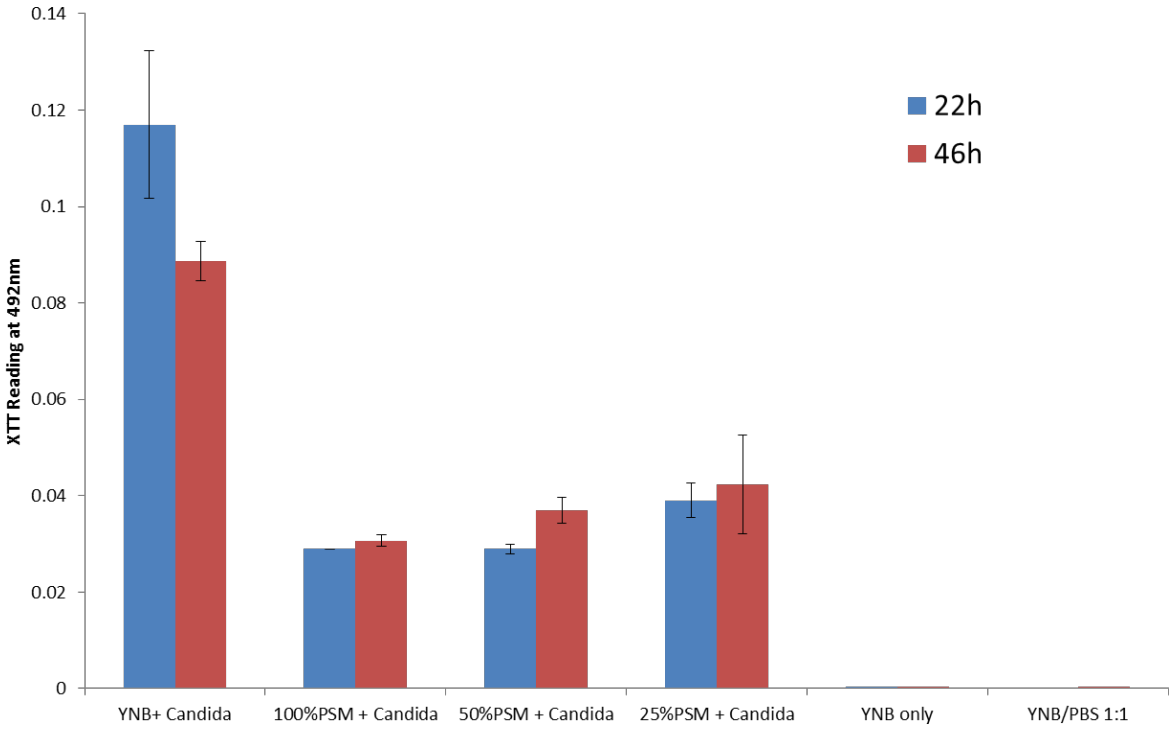

Figure 2. Effect of PSM on metabolic activity of *C. albicans*. *Candida* cells were exposed to different concentrations of PSM and their metabolic activity was measured using an XTT-based assay.

CONCLUSION**:** PSM exhibits potent activity against *Candida*, but no activity against *Pd*. This lack of activity against the latter could be due to the psychrophilic nature of *Pd*, which likely renders the PSM active component inactive. More studies need to be performed to identify potent agents that can target *Pd*.

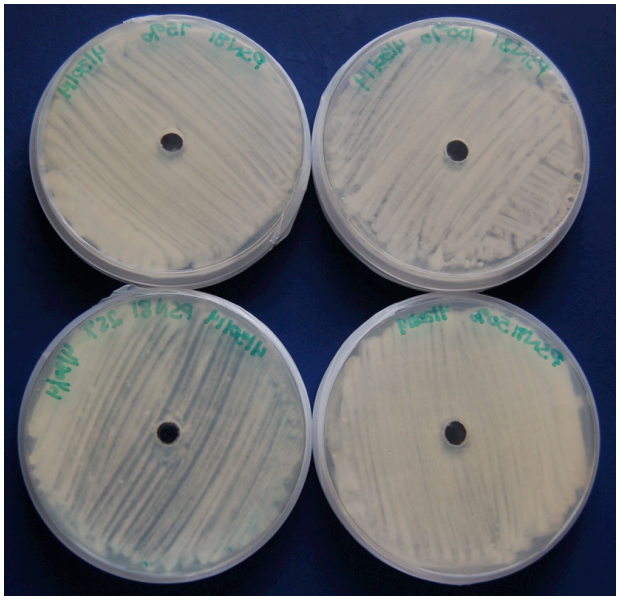

Figure 3. Effect of PSM against *P. destructans* (agar diffusion assay).

IB. **Evaluation of the Antifungal Activity of Organic Compounds Against *Pseudogymnoascus destructans*.**

Performed by Co-PI: Hazel Barton, PhD

METHODS:
To determine the effectiveness of various antifungal compounds at preventing the growth of the *Pd,* we tested the anti-fungal agents on the sister-taxa *P. pannorum* in our lab at NKU. Effective compounds were then tested against *Pd* in the lab of Dr. Kevin Keel (at the time at SCWDS, University of Georgia):

1) The spore suspensions used were derived from isolated *P. pannorum, P. destructans,* and the control species *Aspergillus brasiliensis* and *Penicillium pinophiolium* as described in Shelley et al. (2011). The spores' concentration was determined using a hemocytometer to 1 x 10^5 spores/mL. These spore suspensions were subsequently used in all of the tests.
2) The organic chemical compounds were tested through 2 types of competition assays:

    A) A disk-diffusion assay in which the chemical was added to sterile paper disks and then placed onto an agar plate, streaked with the fungal spore suspension (similar to that used by Ghannoum in study IA).
    B) A direct application of the chemical onto the *Geomyces* agar, allowing the chemical to soak directly into the media.

Organic Antifungal Agents

Thirteen organic antifungal agents were tested to determine the susceptibility of *P. pannorum* and *P. destructans* (Table 3). Twelve of these compounds were obtained from Dr. Xing-Cong Li at the University of Mississippi due to their anti-fungal activity, similarity to organic fatty acids found in bat pelage, and low toxicity. Each compound was diluted with Dimethyl Sulfoxide (DMSO) to a final concentration of 10 mg/mL. Additional compounds, including Heptadecadiynoic acid (Acros Organics, Fairlawn, New Jersey), Carvone, Carvomenthenol**,** Hexana, and Trans-2-Hexanal (Sigma Chemical Company, St. Louis, Missouri), were also tested.

Table 3. Antifungal Fatty Acids Tested

• 5,7-Dodecaydiynoic acid (A)	• 10,12-Pentadecadiynoic acid (H)
• 10,12-Tricosadiynoic acid (B)	• 6-Octadecynoic acid (I)
• 5,7-Eicosadiynoic acid (C)	• 5,7-Hexadecadiynoic acid (J)
• 6,8-Heneicosadiynoic acid (D)	• 4,6-Heptadecadiynoic acid (K)
• 9-Octadecynoic acid (E)	• 6,8-Nonadecadiynoic acid (L)
• 5,7-Octadecadiynoic acid (F)	• Heptadecanoic 98% (M)
• 6-Nonadecynoic acid (G)	

RESULTS:

The disk diffusion assays using fatty acids demonstrated that the organic compounds had no effect on preventing the growth of *Pseudogymnoascus* species. Every compound produced a similar result, all displaying no zone of inhibition. It was hypothesized that the disks may not be allowing the solution to be properly dispensed onto the media. To determine if this was the case the fatty acids were also added directly to the *Geomyces* media and then streaked with the fungi. The results demonstrated two compounds that prevented of growth of the fungi,: 5,7-hexadecadiynoic acid and heptadecanoic acid. To determine the minimum concentration of fatty acids needed, they were diluted with dimethyl sulfoxide (DMSO). Both compounds displayed similar results in that, although the 1:10 dilutions allowed for the growth of *P. pannorum*, the number of colonies that grew was significantly less than 1:100 and 1:1,000 dilution areas.

The disk diffusion assay was also performed using the volatile organic compounds (VOCs) carvone, carvomenthenol, hexanal and trans-2-Hexanal. All of these VOCs displayed inhibition of *Pseudogymnoascus* growth (Table 4).

Table 4. Disk Diffusion

Chemical	Inhibition (mm)
4-Carvomenthenol	10
Hexanal (98%)	12
Trans-2-Hexanal	No Growth
Carvone (98%)	No Growth

While these compounds displayed good killing of the fungi, we need to determine any effect they had on the native cave species *Aspergillus* and *Pencillium*. The results suggested that there was some specificity in the activity of carvone for the *Pseudogymnoascus* species, making it a potential for *in vivo* hibernacula studies; however, carvone is a major ingredient in mint and has been used extensively as a deterrent compound to exclude bats for houses, arguing as to whether this compound may be an effective agent to use in hibernacula.

CONCLUSION:

In situ treatment of bats against WNS represents a challenge for finding chemicals that won't harm the bats or the native ecology of the cave. Our results suggest that the fatty acid heptadecanoic acid, which is naturally excreted by bats during grooming, can inhibit the growth of *P. pannorum* while not affecting other fungal species; however, the concentration that this compound would need to be used at to be effective is prohibitively expensive for field applications. The cheaper volatile organic compound trans-2-Hexanal does appear to work, but also effectively kills other fungal species, which could be potentially damaging to the native cave fauna. Carvone, which is normally secreted as antifungal agent by the mint plant completely inhibits *Pseudogymnoascus* growth; however, carvone in particular is used as an anti-bat scent, bringing into question its effectiveness within the environment.

II. Studies of the effects of treatment agents on *Pd* affected and unaffected bats.

The ultimate goal of studies such as those described in section I, which test the fungicidal efficacy of various compounds on *Pd,* is to develop candidates for *in vivo* testing. Building upon previous pilot studies in my lab (and upon pilot studies of test compounds), two experimental trials were conducted, as described below. Trials of two different products (voriconazole; with Dr. Alison Robbins and terbinafine, with Dr. Marcy Souza) were tested in live bats

IIA. **Voriconazole treatment trial for *Myotis lucifugus* infected with *Psuedogymnoascus destructans*.**

Performed with Co-PI: Alison Robbins, MS, DVM (research conducted at Bucknell University)

The purpose of this project was to determine whether the antifungal drug Voriconazole (VFend©, Pfizer) administered to *Psuedogymnoascus destructans* (Pd) infected Little Brown Bats (*Myotis lucifugus*) can reduce fungal infection load and increase survival rates during hibernation. Additionally we compared survival of voriconazole treated bats to those treated with the topical antifungal drug terbinafine.

For this project we begin studies using a new generation antifungal agent, voriconazole (VFend©, Pfizer), which has been employed successfully to treat the most difficult disseminated fungal infections in humans. These disseminated fungal infections, such as invasive aspergillosis in immunocompromised patients, are completely unresponsive to other antifungal agents and are usually fatal. Voriconazole is in the triazole class of antifungal compounds, which inhibits ergosterol synthesis, a necessary component to fungal cell walls. *Psuedogymnoascus destructans* (Pd) is susceptible to Voriconazole in i*n vitro* testing at very low concentrations with a Mean Inhibitory Concentration (MIC) of 0.003 to 0.008 micrograms/ml (Chatuverdi et al., 2011). *Candida albicans fungi* isolates are susceptible to voriconazole with mean inhibitory concentrations (MIC) ranging from 0.007 to 0.25 micro g/ml in *in vitro* testing (Andes et al., 2003). A mouse model of disseminated *Candida albicans* was used to test the efficacy *in vivo* of voriconazole, and treatments were successful as long as the MIC values were reached in the affected tissues for a specified period of time. Side effects of this drug may include visual disturbances, rash, vomiting and abdominal pain, and death (http://www.centerwatch.com/drug-information/fda-approvals/drug-details.aspx?DrugID=784), and this drug is known to have a narrow tolerance range for use in humans. Voriconazole has been administered to humans and animals in a nebulized form to treat pulmonary fungal infections. Voriconazole is available as a lyophilized power for IV injection which is convenient for administration for subcutaneous injection into bats. The reported LD 50 for IV voriconazole is >100 mg/kg for rats http://www.pfizer.com/files/products/material_safety_data/605.pdf).

Terbinafine (Lamisil Once©, Novartis) is a safe and well tolerated drug that initially had been shown to be effective against Pd in non-published *in vitro* trials. Lamisil Once is a single dose treatment approved for athletes foot cure in humans, and thus was selected as a good antifungal choice for application to bats with Pd. Previous treatment trials in the Reeder lab with topical terbinafine showed mixed results, and suggested that a repeat trial was warranted.

METHODS:

Little brown myotis were collected from 2 separate hibernacula in Tennessee (East Fork Slip cave (42), and New Mammoth Cave (50)) on December 15 and 16, 2011, placed in individual bags and transported to the bat vivarium at Bucknell University, in Lewisburg, PA. One hibernacula was a known WNS infected site, and the second was not yet known to be infected with WNS. Bats were transported in coolers held at temperature ranging from 7 to 20 C. Bats from each hibernacula were mixed in equal numbers in the groups. All bats were inoculated with an infectious dose of *Pd* of cultured *Pd* by applying a solution to the wings on December 17, 2011. Each bat had a wing band placed and a temperature data loggers glued to the skin of the dorsal thoracic region. Bats were assigned to one of 4 treatment groups, and were placed in a single cage of 8 to 10 individuals per cage per treatment group. Bats were then placed in screen mess cages 14in (H) X16in (W) X16in (D), then put into environmental chambers held at 8°C and 95% humidity until January 17, 2012.

On 31 days after inoculation (January 17, 2012), bats were removed from the hibernation chambers, weighed, had wings photographed under both incandescent and UV light conditions to record extent of fungal infection and wing condition. Bats were then transferred to a different building with an animal care facility modified for working with bats, and held in the screen cages at room temperature (68-72 °F). Bats were provided with fresh water and gut loaded meal worms fed on a diet nutritionally appropriate for feeding to bats.

Bats were treated with doses of voriconazole given by subcutaneous (SQ) injection and some with topical treatment on wings and ears in addition to SQ injections. Injections were given on the back with a 0.3 ml tuberculin syringe, with volumes injected not greater than 150 ul (0.15 ml, target volumes for drugs range from 0.05 ml to 0.15). Drug concentrations were adjusted to keep injected volumes the same for each dosage group. Dosages ranged from 4 mg/kg body weight of bat per day to 40 mg/kg body weight of bat per day, equivalent to 1x, 5x, and 10x, the therapeutic intravenous dose used in humans. Lamisil Once ® 1% cutaneous topical solution (Novartis) was administered to bats by placing approximately 1 ml on wings and gently massaging into tissue on ventral and dorsal surfaces and on ears.

Treatment groups, 6 groups of 8 to 9 bats each:

1) True control group (n=9): Bats were inoculated with *Pd* following the methods outlined in Johnson et al. (2014), then placed in environmental chambers, and checked and handled on same schedule as other groups (Feb 16, Feb 28, Mar 23). This group remained in chambers and were not handled or fed the week of Jan 17.
2) Sham injection control group (n=8): bats were handled, fed, and injected with 0.9% saline (drug vehicle with no voriconazole) daily. On the first treatment day bats bodies were wiped with normal saline on a gauze pad.
3) Terbinafine topical treatment (n=9): approximately 1 ml solution was placed on wings and gently massaged into tissue on ventral and dorsal surfaces, uropatagium, and on ears.

4) 4 mg/kg injection (n=8): 4 mg voriconazole/kg body weight of bat per day by SQ injection. On the first day of treatment bats were wiped down with 2% chlorhexidine on a gauze pad. (N=10)
5) 20 mg/kg injection (n=9): 20 mg voriconazole/kg body weight of bat per day by SQ injection. On the first day of treatment bats were wiped down with 2% chlorhexidine on a gauze pad. (N=10)
6) 20 mg/kg injection Plus 20 mg/kg topical (n=9): 20 mg/kg voriconazole by SQ injection plus topical application of 0.5 ml of 0.4 mg/ml voriconazole solution equal to a dose of 20 mg/kg for a 10 g bat (total of 40 mg/kg combined). On the first day of treatment bats were wiped down with 2% chlorhexidine on a gauze pad.

Bats were treated and feed daily for 5 days, then returned to hibernation on January 21, 2012. On February 16, 2012 the cages were checked and dead animals were remove. On February 28 2012 all bats were removed from cages, photographed under UV light, then returned to hibernation. On March 23 all remaining live bats were euthanized, and remaining dead collected.

Data loggers malfunctioned and recorded temperature profiles until February 10. Exact date of death was not possible to determine after Feb 10, so survival was calculated by directly checking cages for dead animals on the specified dates.

RESULTS:

The 'true control' group had the most surviving to 84 days post inoculation with *Pd*, with 3 out of 9 bats in the group surviving to the experiment end date (Table 5). Two of the 8 sham injected bats, which were handled with the same protocol as the voriconazole treated bats, survived to the experiment end point (84 days post inoculation). Two of the group of 9 bats that were treated with terbinafine and handled and feed for the period of 5 days survived to the experiment end point. There is no significant difference in the survival between these three groups.

Table 5: Bat survival by treatment group

Group/treatment	% survival to 16 Feb (59 days post inoculation)	% survival to 28 Feb (71 days post inoculation)	% survival to 23 March (84 days post inoculation)
True Control (n=9)	100% (9/9)	88% (8/9)	38% (3/9)
Lamisil (n=9)	67% (6/9)	67% (6/9)	22% (2/9)
Sham injections (n=8)	88% (7/8)	38% (3/8)	25% (2/8)
4mg/kg injections (n=8)	75% (6/8)	13% (1/8)	0% (0/8)
20 mg/kg injections (n=9)	11% (1/9)	0% (0/9)	0% (0/9)
20 mg/kg Inject plus 20mg/kg topical (n=9)	67% (6/9)	0% (0/9)	0% (0/9)

The voriconazole treated bats had a lower survival rate than either control or the terbinafine treated group (Figure 4). Out of the 3 voriconazole treatment groups combined (26 bats), only 1 bat survived to day 71, and none to day 84.

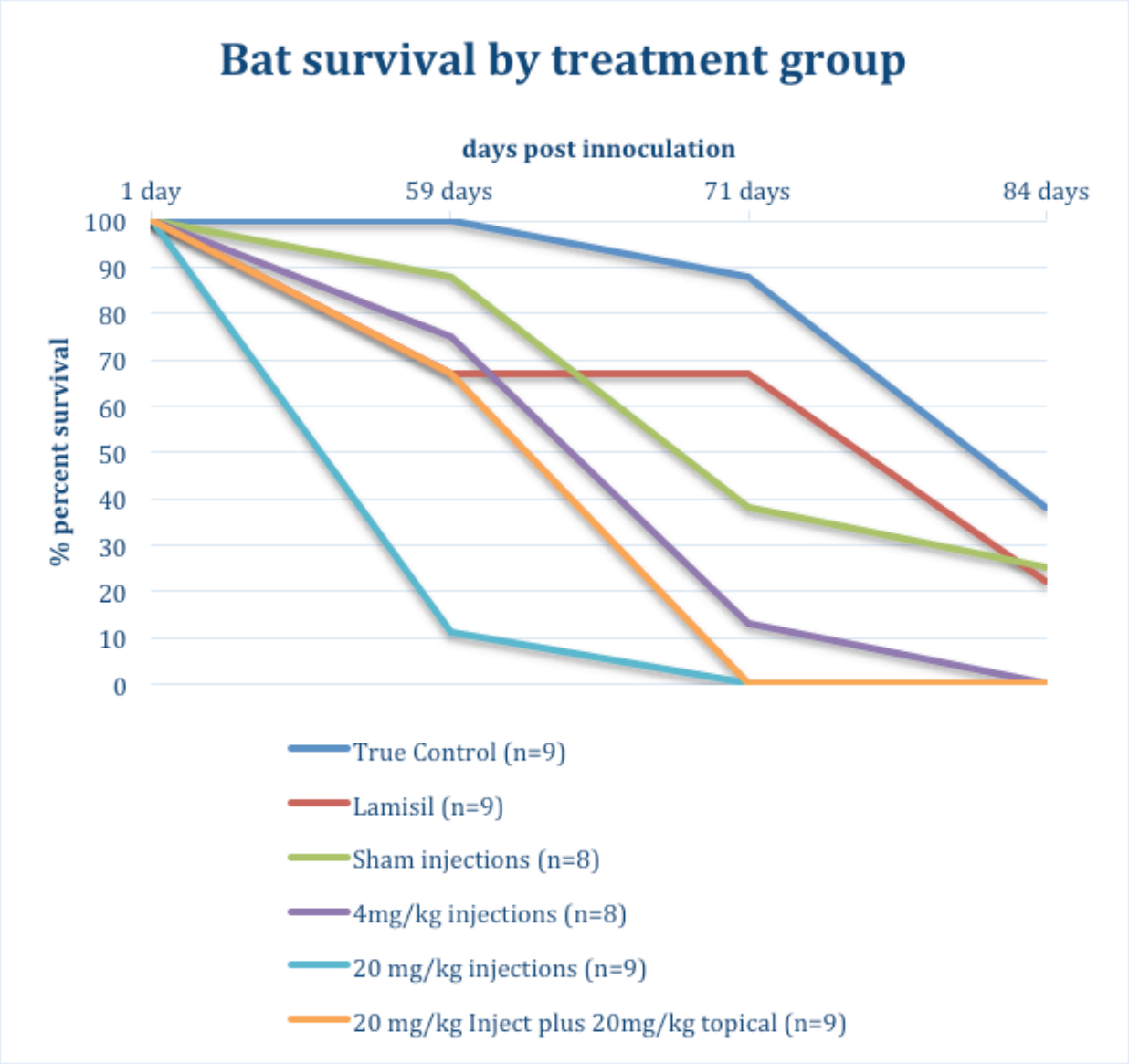

Figure 4: Bat survival by treatment group.

We also observed behavior in the bats that suggested voriconazole immediately made the bats feel ill, within 24 hours of the first dose even in the lowest dose group (4 mg/kg voriconazole by body weight). Voriconazole treated bats did not cluster together in their cages as the control bats did, and did not groom themselves as control bats did. Treated bats held their wings slightly away from their bodies, roosted away from other bats in their cage, and did not eat meal worms readily or ate very few (Figure 5); these are classic "sickness" behaviors in bats. This observed behavior suggests that little brown myotis cannot tolerate voriconazole even at the lowest doses needed to inhibit fungal growth. Additionally, voriconazole-treated bats did not survive as long

Figure 5: Voriconazole treated bats after 2 days of treatment even at the lowest dosage of 4 mg/kg displayed signs of illness by holding wings slightly away from body, roosting in separate locations within the cage, not grooming fur, and very poor appetite. In a previous experiment (conducted by A. Robbins) bats treated with varying dosages of terbinafine displayed normal behavior in captivity by roosting in groups, grooming fur, and good appetites. (Photos A. Robbins).

as the sham-injected bats, suggesting that voriconazole could be toxic to bats at the lowest dosages and increase mortality. This observed reaction may be specific to voriconazole, as in this researchers experience, terbinafine is tolerated at very high dosages, with no observable behavior changes, normal clustering, grooming, and feeding behaviors (A. Robbins personal observation in terbinafine treatment trials).

CONCLUSION:

True controls survived longer than voriconazole treated bats. The true control, sham injected and terbinafine (Lamisil) treated bats survived for a similar length of time, suggesting that the handling and feeding regimen in January did not cause increase in mortality compared to the true control group. All three groups of voriconazole treated had shorter survival times than the control and terbinafine treated groups. Mortality among the sham injected bats was lower than the dosages for the voriconazole treated bats. This indicates that the handling and feeding during January did not increase mortality, and is tolerated by bats. Bats treated with voriconazole at all dosages displayed behavior of ill bats within 24 hours of the first treatment. Treated bats roosted separately, held wings away from their bodies, did not grooming fur, and had a decreased to no appetite. Survival was lower for all dosages of voriconazole treated bats compared to the true control, sham injected and the terbinafine (Lamisil) treated bats, suggesting that voriconazole may be toxic to Little Brown MYOTIS. **Voriconazole at a minimum is not tolerated by bats at expected therapeutic dosages, and may be toxic causing increased mortality. Voriconazole is not a safe or effective drug in *Myotis lucifugus* and should not be used in further *in vivo* experiments in this species.** One time topical treatment with terbinafine did not improve survival compared to controls, and is not an effective treatment regimen in this setting. Fungal UV pattern and QPCR analysis has not yet been evaluated in treated bats, and may provide further insight into infection pattern and spread.

One factor that has not been evaluated at all is the timing of antifungal treatment in the development of the disease. As new information becomes available about the cascade of physiological events set off by *Pd* infection, there may be windows of opportunity to apply therapeutic agents that could interrupt this cascade. Further research into the timing and application of antifungal agents is needed to identify effective strategies for preventing the continuing mass mortalities of bats across North America. The next logical research approach is to develop *in vitro* assays for testing antifungal compounds so bats are not used at early treatment trial stages. Further research is needed to discover antifungal and other therapies for captive bats and for wild bats.

IIB. **Evaluation of a Terbinafine Impregnated Subcutaneous Implant In *Pseudogymnoascus destructans* Infected Bats**

Performed with Co-PI: Marcy Souza, MPH, DVM (research conducted at Bucknell University)

The objective of this study was to evaluate the treatment potential of terbinafine-impregnated subcutaneous (SQ) implants to treat infection with *Pd* in little brown myotis. Terbinafine disrupts fungal cell wall growth and inhibits the ergosterol synthesis pathway. We evaluated the extent of lesions associated with infection, the clinical response to treatment, and toxic effects of implant use. The terbinafine implants were developed by the Souza lab to provide a slow-release of this drug under the skin of the bat; in vitro experiments demonstrated that they release the drug over a six-month period (Souza et al. 2012). Topical fungal infections are difficult to treat and often require repeat treatments. By developing a slow-release implant, we hoped to provide continued treatment of bats throughout the hibernation season in order to slow or even halt the growth of the fungus.

METHODS:

Forty little brown myotis without gross *Pd* infection were captured from two hibernacula in Tennessee in December 2011. Bats were placed in individual bags and transported to the bat vivarium at Bucknell University, in Lewisburg, PA. Bats were transported in coolers held at temperature ranging from 7 to 20 C. Each bat received a SQ implant containing 1 of 4 terbinafine concentrations (0, 2, 4, 8 mg) on the day of capture. These implants are approximately the size of a grain of rice and were injected under the skin between the shoulder blades using a specialized needle delivery system (similar to that used with putting "pit" RFIC tags under the skin of bats. Bats were inoculated with 20µL (125,000 conidia) of *Pd* the next day and housed in artificial hibernacula for 3 months at Bucknell University, PA.

While histologic confirmation or PCR are considered the gold standard, gross visualization of fungal growth under ultra-violet (UV) light is also possible in live bats (Turner et al. 2014). UV and white light photos of wings were taken once per month; at the end of the study, bats were euthanized and fixed in formalin. Histologic samples (wing, muzzle) were stained with Periodic acid Schiff (PAS) stain to highlight *Gd* conidia. Liver, kidney, and gonads were stained with hematoxylin and eosin to assess possible drug toxicity. Wing and muzzle lesions were analyzed

by (1) visual photo exam, (2) CellSens Digital Imaging, and (3) Image Pro Plus 7.0 analysis software (IPP). Histologic lesions were defined as a cluster of >5 conidia, data are represented as the number of lesions per area of tissue as measured by IPP. The lesion number/mm^2 for each wing and muzzle was compared across treatment groups with a Kruskal-Walis test (P of < 0.05 significance). A limited field trial was conducted the following winter, with preliminary results below.

Table 6. Mean lesion number/mm^2 for wings & muzzles. No significant differences between groups were found.

	Wing Tissue		Muzzle Tissue	
	Mean # of lesions per mm^2 (SD)	Range	Mean # of lesions per mm^2 (SD)	Range
0 mg	5.7 (3.9)	0-24.6	0.2 (0.3)	0-0.9
2 mg	13.1 (8.4)	0-55.9	0.3 (0.4)	0-0.7
4 mg	14 (8.5)	0-79.3	0.2 (0.3)	0-0.7
8 mg	8.9 (2.8)	0-63.6	0.1 (0.3)	0-0.8

RESULTS:

Captive study: UV and white light photos showed moderate wing scarring of unknown cause and minimal to no fluorescence (Figure 6). Conidia were seen histologically in all treatment groups (Figure 7). Lesion number/mm^2 was typically lower in the 8mg treatment group for both wing and muzzle tissue compared to other treatment groups, but differences were not statistically significant (Table 6). No histologic lesions consistent with toxic effects were present on any tissues.

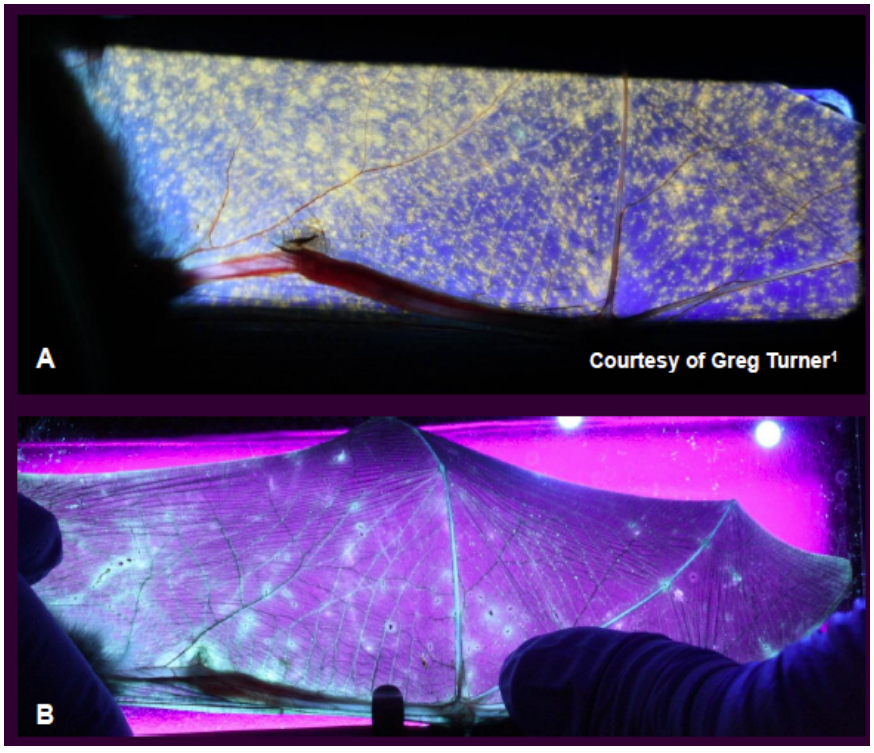

Figure 6. Comparison of fungal UV fluorescence. A. Severe WNS infection (from a field positive bat). B. Scarring without gross infection.

Of note for general studies of bat disease, kidney samples in the control group (0 mg implant) contained *Klossiella* sp. parasites.

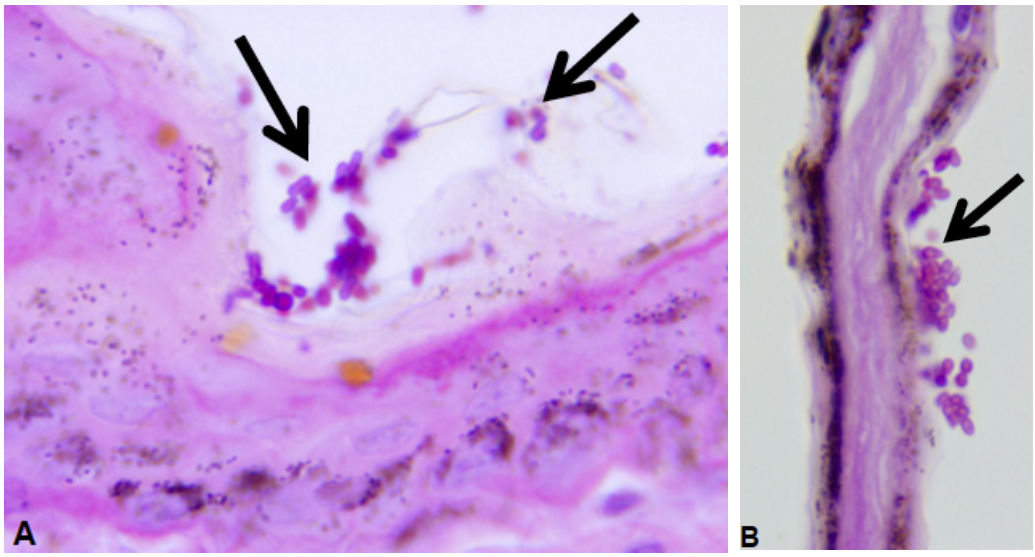

Figure 7. Fungal Infection of Muzzle (A) and Wing Fold (B), Arrows indication fungal conidia, PAS Stain 100x.

Field trial: The results of the field trial (with comparable groups housed at Bucknell) were nothing short of abysmal (Table 7); high mortality was seen across all groups. This may be due to the fact that these bats were naturally infected and already too sick to respond to treatment, or, more likely, due to the fact that bats were caged within the cave.

Table 7. Summary data for field trials with terbinafine implants.

	KY 8mg	KY 0mg	KY Control	Bucknell 8mg	Bucknell 0 mg
Mean Weight at capture (g)	7.91 (0.9)	8.41 (0.5)	ND	8.48 (0.54)	8.3 (0.58)
Mean Survival Time of those that died (days)	20.12	23.63	23.14	22.8	22.3
# Euthanized	3	12	0	10	13
# Died	17/20	8/20	7/20	10/20	6/19
Mortality	85%	40%	35%	50%	32%

CONCLUSION:

Our study evaluated a potential treatment for WNS with digital (IPP) and histologic analyses to quantify WNS infection. There is inherent difficulty in quantifying pathologic lesions with current diagnostic methods (PCR, culture, histology, wing damage index). UV light can be used to see *Pd* lesions in field studies and determine relative fungal growth (Turner et al. 2014) however, in this study, infection severity was not sufficient to produce UV fluorescence. Our analysis methods quantified infection load by the number of lesions/mm^2 on histology. Concurrent use of digital analysis (IPP) in gross & histologic lesion evaluation could lead to a standard quantification method of WNS infection. In order to buffer the subjectivity of quantifying histologic lesions, 2 separate observers counted wing and muzzle lesions. Low sample size (10 bats/treatment group) & inherent subjectivity in manually counting lesions most likely contributed to no statistically significant differences in lesion number/mm^2 between treatment groups. Future studies should have larger number of animals to increase the power of detecting differences between treatment groups. PAS staining highlighted the fungal lesions in the tissues (Figure 7). Futures studies using another method to better contrast fungal lesions from the background tissue would allow IPP to automatically quantify lesions and would simultaneously remove the subjectivity associated with manually identifying and counting lesions. Under UV light, the wings showed minimal to no fluorescence and only areas of moderate scarring (Figure 1). Future studies should use higher inoculation doses or use naturally infected animals to increase probability of finding fluorescent fungal lesions with UV light. Although the cause of the wing scarring seen in this study is unknown, previously healed wild-type *Pd* infections could potentially produce similar lesions. Further studies examining terbinafine implants should use larger groups of bats, naturally infected bats, and a natural cave setting. **No toxicity was associated with terbinafine treatment**. Gross photos showed moderate wing scarring of unknown cause and minimal to no fluorescence. With further research, we hope to validate the efficacy and safety of a lasting, single-administration treatment to minimize handling stress and prevent further mortality from WNS.

OVERALL DISCUSSION

In this study we hoped to find an intervention of white nose syndrome in bats. Treatment trials so far have shown little progress in slowing the course of this highly fatal disease. Developing a successful treatment strategy is at best a slow and discouraging trial and error process.

We can make no specific treatment recommendations at this time, but rather urge the research community to continue to pursue mitigation options for WNS. Our study focused on pharmaceutical or organic compounds as treatments; other laboratories are now pursuing biological control agents as possible treatment (and are having only limited success). Clearly the WNS problem is complex, especially when one considers any sort of treatment option – which will affect the entire cave ecosystem.

Yet we must continue to dedicate effort to the development of a treatment or intervention for this devastating disease. If we do not, then we face the possible extinction of some bat species.

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Acknowledgements: Additional assistance for various aspects of the project was provided by: Missy Meierhofer, Sarah Brownlee-Bouboulis, Laura Grieneisen, and Chelsey Musante (Bucknell University), Rachel Dutkosky, Kim Newkirk, and Edward Ramsay (University of TN College of Veterinary Medicine), Kevin Keel (SCWDS, University of Georgia), Brooke Hines (Bat ecologist, KDFWR)