Biology Summer

PREVIOUS RESEARCH:

Bats play important ecological roles in insect control, plant pollination, seed germination, and forest regeneration (1, 2). Bat populations have declined drastically since 2006 in Europe and in the northeastern US due to the emergence of white-nose syndrome (WNS) (1, 2, 3, 5, 6, 9, 11, 12, 13, 14). Over 1,000,000 bats have died in the affected areas (3, 12). No mass mortalities in bats have been reported before (1, 3, 5,). WNS has become a global problem (9, 13). Fading of the North American bat populations is likely to have far-reaching ecological consequences (1, 5, 13). Thus, immediate conservation measures need to be taken to minimize spread of the disease (2, 9, 13).

WNS has been characterized as a condition in hibernating bats (1, 2, 3, 9, 11, 13, 14). WNS disrupts the hibernating pattern of bats, eroding the epidermal layer of skin, depleting energy reserves, and promoting dehydration (2, 3, 9, 13). WNS is also characterized by the presence of the fungal hyphae and abundant conidia on ears, muzzles, forearms, and wing membranes (1, 6, 9, 11, 13). Small tears in wing membranes, rough patches of skin on face, ears, and wing membranes, irregular pigmentation in the affected areas, are all manifestations of the disease (3, 5, 11, 13). Damaged wings significantly reduce thermoregulatory, foraging, and reproductive capacities of bats after hibernation (3, 5, 13). Fungus also affects hair follicles, connective tissues, sebaceous and apocrine glands (1, 3, 9, 11). Drastically decreased fat reserves and suppressed immune system significantly decrease bats' survival during and after hibernation and provide ideal conditions for the spread of infection by *Geomyces* fungus (3, 6, 9, 11, 12). The mortality rates are much higher among Northern American bats than in European bats (3, 9, 12, 14). Although the physiological differences between European and American bats are not known, some researchers suggest that death rate is lower in European hibernating bats because they coevolved with G. Destructans, and therefore, are more immunologically resistant to the fungus (3, 9, 14).

White Nose syndrome (WNS) in US was documented for the first time in New York on 16 February 2006 (1, 2, 3, 5, 6, 9, 13). The sudden decline in bat populations was alarming; therefore, bats were examined in laboratories for the presence of pathogenic microorganisms or toxic chemicals (2). Although, WNS is claimed to affect about seven species of hibernating bats, the WNS fungus has been most frequently isolated from the three species of bats: *Myotis lucifugus* (little brown bat)*, Myotis septentrionales* (northern long-eared), *and Perimyotes subflavus* (1, 3, 5, 6). The histological studies revealed that fungal hyphae eroded the epidermis of ears and wings (1, 2, 3, 5, 6, 9). According to some studies, fungus was isolated from hibernating bats was cultured at an average temperature of 3 °C; it grew optimally between 4 ° and 10 ° - 15 °C, with upper limit for growth at about 20 or 24°C (1, 2, 6, 14). Phylogenetic studies and ribosomal RNA analysis from gene sequences of psychrophilic fungi placed the isolated fungus near the representatives of genus *Geomyces*, and named it G. destructans (1, 2, 6, 11, 12).

G. Destructans form conidia and grow slowly in artificial media (3, 6, 11). A few reports claim *Geomyces* spp., to be causative agents of many skin infections in animals in cold climates (1, 2, 14). *G. pannorum,* a member of *Geomyces* genus, is suspected to be involved in some humans mycoses, although, no human diseases have been recorded in the WNS areas. (2,14).

The mechanism of WNS transmission and persistence are still poorly understood (2, 5, 9, 11, 13). Chaturvedi et al. suggested that proteolytic enzymes identified in G. Destructans could promote fungal growth and distribution (2). We still do not know if the fungus is primarily responsible for deaths of bats or is a secondary infection. We do not know how the fungus appeared: whether it has recently emerged in Europe or was there for a long time but not casing massive die-offs of bats (9, 12). We are not certain if delayed recovery rates are caused by bacterial co-infection in WNS bats (13).

Researches claim that growth of the fungus in pure culture and characterization of it is necessary for understanding WNS and bat mortality, and for the development of control measures (3, 5, 6, 9, 14). Therefore, most research was concerned with the fungus itself and not with microbiota in general in the affected bats. Some researchers declare that neither pathology, nor virology studies conducted revealed

any known pathogenic microbes associated with WNS; thus, most studies were primarily concerned with *G. Destructans* (2, 3, 14). Symbiotic relationships have been mostly ignored in WNS bats. Symbiotic relationships help to support health and survival of many species (4, 8, 10). For example, *Staphylococcus epidermitis,* living on human skin, guards it from various pathogens. *Pseudomonas* spp. engaged in mutualistic relationship with its host, provides bacterial protection from pathogens (4). Human symbiont *Bacteroides fragilis* protects from colitis caused by *Helicobacter hepaticus* (10). Environmentally induced shifts in microbiota (due to changing temperature, pH, etc.) may promote emergence of new diseases (4, 8). Coral bleaching, for example, associated with rising temperature, is interconnected with loss of mutualistic algae and decreased antibiotic activity of mutualistic bacteria *Acropora.* The antibiotic activity of *Pseudomonas fluorescence* that benefits its host plant at 35 °C is greatly reduced at 30 °C degrees (4). Therefore, more research needs to be conducted on microbiota of WNS bats.

CURRENT AND PROPOSED RESEARCH:

We are focusing not as much on the previously described fungus as on microbes, especially fluorescing microbes, obtained from hibernating bats. Most of the samples we received were taken from bats fluorescing under the UV light. Therefore, we decided to pay especial attention to surveying fluorescing bacteria. Most of the samples were taken from bats in deep torpor, with the exception of a few specimens, taken from a euthermic bat. We received dry and wet tissue samples, which included: ears, wing, forewings, and forearms. We made S1 medium with intent to grow the fluorescent bacteria, according to the procedure described by Gould et al., except that we did not add any antibiotics to it (7). We did not use a selective isolation medium of any kind because we wanted to isolate and survey as many microbes as we can culture. We added sterile saline in the amount of 1 ml to dry samples and 4 ml to wet samples. Then, we plated 100 microliters of undiluted samples on the prepared agar and on blood agar (tryptic soy agar with 5 % defibrinated sheep blood). Then, we preserved the original samples in glycerol and placed them in a freezer. We incubated four plates of each of sample: one on blood agar, one on S1 agar at room temperature; another one on blood agar and one more on S1 agar at 4°C. We incubated samples for two weeks and then examined all 61 plates under UV light. We discarded 24 plates that had no visible growth on them. Five plates had visible fluorescence under UV light. We took pictures of all plates and isolated pure cultures. The best growth was present on forewings and ears. So, we decided to compare fluorescing and non-fluorescing samples from forewings and ears in addition to the surveying of microorganisms. The next logical step would be to check all pure cultures under UV light and take note of fluorescing plates. We would then grow fluorescing cultures in pure culture, isolate DNA, amplify small subunit rRNA genes by polymerase chain reaction, and perform BLAST analysis to identify fluorescing cultures based on comparisons of DNA sequence. Thus far, no survey of microbes has been performed in WNS bats and no comparison studies on changes to the microbiota in diseased animals have been performed. One of the goals for our future research is to compare microorganisms found in healthy bats with the ones in WNS bats. Surveying of microbes and comparison studies will help to shed some light on the origin of WNS and the potential role of other microorganisms in addition to *G. Destructans* in the development of WNS.

TIMELINE:

February – April 2012. Initial culture of samples from WNS – infected bats to detect fluorescent microorganisms; growth of selected microorganisms in pure culture.
May 1 to July 31 2012 Isolation of DNA from pure cultures, amplification of small subunit rRNA genes by polymerase chain reaction, and identification of pure cultures using BLAST analysis of small subunit rRNA gene sequences.

RESULTS:

Results will be presented at UNCA-Fall 2012 Symposium of Undergraduate Research and in the UNCA Journal of Undergraduate Research.

BUDGET:

Stipend for 8 weeks (Yelena Litvinchuk)	$1,500.00
Blood agar, Fisher Scientific	$100.00
Bacteriological agar powder, 500 g, Fisher Scientific	$200.00
Plasmid DNA miniprep kit, Qiagen	$200.00
TA cloning vector and competent cells, Invitrogen	$500.00

REFERENCES

1. Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Ziert, B. M., Buckles, E. L., LastColeman, T. H., Darling, S. R. et al. (2009). Bat white-nose syndrome: An emerging fungal pathogen? *Science*, *323*, 227.
2. Chaturvedi, V., Springer, D. J., Behr, M. J., Ramani, R., Xiaojiang, L., Peck, M. K., Ren, P., Bopp, D. J., et al. (2010). Morphological and molecular characterizations of psychrophilic fungus *Geomyces destructans* from New York bats with white - nose syndrome (WNS). *Plosone*, *5*(5), 1-12.
3. Cryan, P. M., Meteyer, C. U., Boyles, J. G., & Blehert, D. S. (2010). Wing pathology of white – nose syndrome in bats suggests life – threatening disruption of physiology. *BMC Biology*, *8*, 123-131.
4. Danskin, J. H., & Alford, R. A. (2011). Context - dependent symbioses and their potential roles in wildlife diseases. *Proc.R.Soc.B*, *10*, 2276-81.
5. Frick, W. F., Pollock, J. F., Hicks, A. C., Landwig, K. E., Reynolds, D. S., Turner, G. G., Butchoski, C. M., & Kunz, T. H. (2010). An emerging disease causes regional population collapse of a common north american bat species. *Science*, *329*, 679-682.
6. Gargas, A., Trest, M. T., Christensen, M. J., Volk, T. J., & Blehert, D. S. (2009). Geomyces destructans sp. nov. associated with bat white - nose syndrome. *Mycotaxon*, *108*, 147 - 154.
7. Gould, W. D., Hagedorn, C., Bardinelli, T. R., & Zablotowicz, R. M. (1984). New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Applied and Environmental Microbiology*, *49*(1), 28 - 32.
8. Marcot, B. G., & Heyden, M. V. (2011). Key ecologicalfunctions of wildlife species. *Ecology*, *6*, 168- 182.
9. Martinkova, N., Backor, P., Bartonicka, T. R., Blazkova, P., Cerveny, J., Falteisek, L., Gaisler, J., & Hanzal, V. (2010). Increasing incidence of geomyces destructans fungus in bats from the czech republic and slovakia. *Plos One*, *5*(11), 1-7.
10. Mazmanian, S. K., Round, J. L., & Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*, *453*(29), 620-625.
11. Meteyer, C. U., Buckles, E. L., Blehert, D. S., Hicks, A. C., Green, D. E., Shearn - Bochsler, V., Thomas, N. J., & Gargas, A. (2009). Histopathologic criteria to confirm white - nose syndrome in bats. *J Vet Diagn Invest*, *21*, 411-414.
12. Puechmaille, S. J., Verdeyroux, P., Fuller, H., Gouilh, M. A., Bekaert, M., & Teeling, E. C. (2006). White - nose syndrome fungus (geomyces destructans) in bat, France. *EID*, *16*(2), 290-293.
13. Reichard, J. D., & Kunz, T. H. (2009). White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (myotis lucifugus). *Acta Chiropterologica*, *11*(2), 457–464.
14. Wibbelt, G., Kurth, A. C., Hellman, D., Weishaar, M. A., Barlow, A., Veith, M. K., Pruger, J., & Gorfol, T. H. (2010). White - nose syndrome fungus (*Geomyces destructans*) in bats, europe.

EID, *16*(8), 1237-1242.