Isolation and analysis of proteases secreted by *Geomyces destructans* for assessment of function in White-nose Syndrome in bats

PROJECT SUMMARY

Arkansas is home to three federally endangered cave dwelling bats. *Geomyces destructans* is a dermatophytic fungus and the putative agent for White-nose Syndrome (WNS) in bats. WNS may infect Arkansas bat populations as early as winter 2011. This project addresses the need to understand the basis for virulence of WNS. We propose to isolate putative protease activity(-ies) that may mediate this disease and subsequently identify by mass spectrometry analysis. These studies will aid in the identification of possible virulence factors released by *G. destructans* and future WNS control measures.

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Total Project Cost = $79,025
Total Arkansas SWG request = $48,400 (61.2%)
Total Matching Funds* provided = $32,600 (38.8%)
*these are non-federal dollars supplied by Arkansas State University and Arkansas Biosciences Institute

Isolation and analysis of proteases secreted by *Geomyces destructans* for assessment of function in White-nose Syndrome in Bats

Introduction: *Geomyces destructans* is a fungus isolated from cave-dwelling bats and is the putative pathogen responsible for the disease White-nose Syndrome (WNS) and the associated mass mortality of North American cave bats. WNS was first discovered in Howe Cave, NY, in 2006 and has since spread to 13 states and 3 Canadian provinces. The disease is projected to reach caves in AR as early as next year (Figure 1). AR is home to large groups of three federally endangered cave dwelling bats: the Ozark Big-eared Bat (*Corynorhinus townsendii*), the Gray Bat (*Myotis grisescens*), and Indiana Bats (*Myotis sodalis*). The mortality rates (up to 90-98%) observed from bats infected with *G. destructans* have been devastating to local populations of once common bat species. The little brown bat (*Myotis lucifugus*) was once the most widespread bat species in North America, but now it may be at risk of local extinctions and has been suggested to be placed on the endangered species list. WNS has the potential to decimate populations of AR bats.

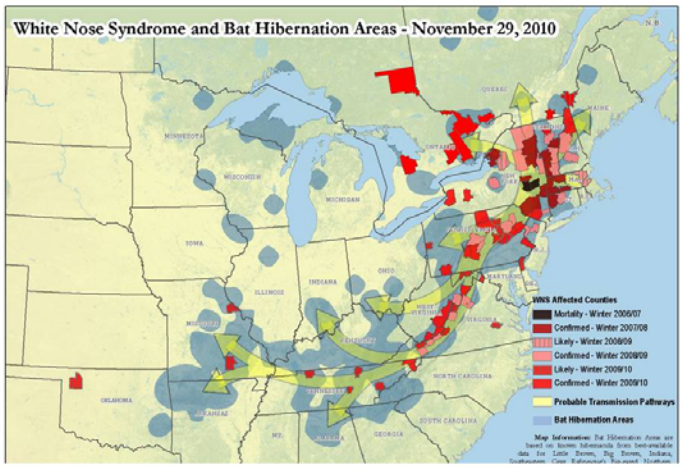

Figure 1. Counties where WNS has been documented and projected spread of WNS (Formatted from Bat Conservation International.)

G. destructans is a dermatophyte that causes skin infections and has been observed to be keratinophilic (has an affinity for hair). Dermatophytosis normally requires the organism adhere to the extracellular matrix of a host organism (which normally consists of hard cornified insoluble structural proteins), allowing the dermatophyte to activate a suite of genes expressing proteolytic enzymes. A common saprophytic fungus closely related to *G. destructans*, *G. pannorum*, has been documented to be keratinolytic (capable of hair digestion) and is a rare superficial infection of humans and animals. One of the main clinical signs observed from *G. destructans* infection is the ability to invade sebaceous and apocrine glands of the muzzle into underlying connective tissue. Fungal hyphae also grow into the wing interior, which is primarily connective tissue (composed of collagen, elastin, and keratin) between two epidermal cell layers and leads to visible lesions. *Geomyces* species are reported to possess proteolytic enzymes that are capable of damaging integumentary tissue.

Dermatophytes can exhibit pathogenicity and virulence through the secretion of extracellular enzymes and may become pathogenic if the host is immunocompromised. It is thought that *G. destructans* attaches to bat integument and grows during torpor bouts when the hosts immune system is suppressed. Pathogenic microorganisms living on host integument utilize structural proteins for carbon and nitrogen sources and must secrete a mixture of proteases capable of degrading the host extracellular matrix. To date, no study has attempted to characterize if *G. destructans* produces proteases capable of metabolizing and degrading the extracellular matrix of bat integument.

What priorities does this proposal address and Need for Research? This proposal directly addresses the need to protect AR bats from WNS, which is listed as Emerging Issues (priority 2) in: 2011 AR State Wildlife Grants request for pre-proposals to implement priorities identified in the AR Wildlife Action Plan, Appendix A. The USFWS draft framework for managing WNS includes a greater understanding of WNS pathogenesis and host/disease ecology. While pathogenesis can involve studies on either host or pathogen, the interface between the two organisms is almost unavoidably linked. Currently, there is a lack of research on the virulence and pathogenicity of WNS. This proposal will offer insight into how the

pathogen detects host tissue and establishes infection. Thus, these studies may provide insight into possible virulence factors associated with WNS and how this disease may affect AR bats.

What are your objectives? The overall goal of this study is to determine if the necrosis of bat wing tissue is partially the result of *G. destructans* proteases, and if fungal proteases play a role in WNS pathogenicity by allowing nutrition to be derived from the host. *G. pannorum* (ATCC #14556) is a close relative of *G. destructans* and will be investigated in parallel to allow for detailed comparisons. To achieve our goal we will first establish an *in vitro* system to isolate extracellular enzymes secreted by *Geomyces* species and then subsequently detect and identify extracellular proteins that may be markers for fungal colonization of host tissue. We hypothesize that *G. destructans* secretes extracellular proteases in response to integumentary substrates.

Objectives:

1- Establish an axenic *in vitro* system for growing *Geomyces* in defined culture media.
2- Perform electrophoresis analyses to detect proteolytic activities by *Geomyces* secreted enzymes and associate to specific proteins.
3- To identify targeted putative proteases of *G. destructans* by structural basis with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

What are the expected results of this study and benefits to species of concern? The expected outcomes of objective 1 are development of a well-established methodology to generate detectable amounts of secreted enzymes from *Geomyces* in an *in vitro* system. This system will allow for the collection and storage of aliquots of fungal proteases that will be available for current and future research.

The expected outcomes of objective 2 are the confirmation if *Geomyces* secretions contain proteases capable of integumentary degradation that may play a role in the virulence of *G. destructans*. New information will be made available on molecular weight of fungal enzymes capable of digesting integumentary structural proteins.

The expected outcomes of objective 3 are the identification of *Geomyces* proteolytic enzymes, providing new targets for alleviating the devastation of WNS. We will provide information on homology of *Geomyces* proteases among the Kingdom Fungi. By comparing putative virulence factors with other disease models, we may obtain further insight into possible WNS therapeutic strategies and properly strategized governmental action plans.

The combined results of this study will provide preliminary information on the presence and activities of enzymes secreted by *G. destructans* allowing for investigation on the possibility of these enzymes playing a role in virulence. Ultimately this project is expected to aid in WNS containment by supporting treatment programs focused on alleviating the effects of the disease on North American and AR wildlife.

What is your approach? This project consists of integrating classical microbiological and biochemical methods combined with advanced proteomic techniques. Each step and a time frame are listed below. Additional details on methodology have been previously described by the Savary Laboratory.

Approach Objective 1: This objective aims to establish an *in vitro* system capable of sustaining *G. destructans* filamentous growth and recovering secreted enzymes into an aqueous media supplemented with major structural proteins (collagen, keratin, and elastin). Media will be inoculated with *Geomyces* and incubated. Hyphal tissue will be separated from the supernatant and extracellular enzymes in the media will be concentrated for determining protein content and enzyme activity (-ies). Protease activity of the supernatant will be screened for the four major classes of proteases at three pH levels.

Approach Objective 2: This objective aims to evaluate extracellular secreted proteases of *Geomyces* in aqueous media and visualize proteolytic activity. Native (zymographic) and SDS-PAGE will be used to quantitatively and qualitatively analyze the extracellular proteins recovered from culture media. Protein bands of interest will be trypsin digested for MALDI-TOF MS analysis.

Approach Objective 3: The aim of objective 3 is to use mass spectrometry to identify putative fungal proteases by structure with the generation of peptide mass fingerprints (PMFs). Protein targets will be separated by electrophoresis and will be used to generate a PMF from a MALDI-TOF mass spectrum. PMF will be used for putative fungal enzyme identification. Samples will be processed in the ABI Protein Chemistry lab and their quality will be first evaluated with the Waters MicroMX MALDI-TOF MS. Likely samples will be submitted to UAMS to generate details sequence information by MS/MS. Database searches using peptide sequence information obtained will be performed using the BLAST program at NCBI (www.ncbi.nlm.nih.gov/blast/) to determine sequence identity and correlate identity to known fungal proteases.

What is the location of your work? The proposed study is laboratory based but the results will affect all species of bats affected by WNS.

Time frame: *Geomyces* will be continually cultured fall 2011-spring 2012 (Objective 1). Secreted proteins will be quantified and electrophoretically resolved from spring 2012-summer 2012 (Objective 2). Protein bands will be analyzed and identified by structure from summer 2012-fall 2012 (Objective 3). Results will be achieved, analyzed, and published by spring 2013. *References are available at your request

What is your proposed budget?

Budget Items	SWG Request	Match Funds	Total
Staff (salary & benefits)	**$15,000**	**$4,030∞**	**$19,030**
Graduate Student Summer Salary (6 months @ $1667/month = $10,000)	**$9,900** **1% Fringe ($100)**	**$4,030∞**	
ABI Laboratory Personnel (5% Personnel Time = $5,000)	**$3,923** **27.45% Fringe ($1,077)**		
Analytical Supplies & Materials	**$18,000**	**$0**	**$18,000**
Standards and Chemicals	**$4,000**		
ABI Savary Laboratory Supplies	**$5,000**		
Electrophoresis Consumables	**$4,000**		
Mass Spectrometry Laboratory Fees	**$5,000**		
Fungal Supplies & Materials	**$9,000**	**$21,670¥**	**$30,670**
Microbiological Consumables and Cultures	**$4,000**		
Refrigerated Incubator	**$5,000**		
Type A2 Biosafety Hood		**$8,572¥**	
Incubator Shaker		**$13,098¥**	
Presentation of Data	**$2,000**	**$0**	**$2,000**
Publication cost of 2 peered reviewed papers	**$1,000**		
Travel to meetings	**$1,000**		
TOTAL DIRECT COST	**$44,000**	**$25,700**	**$69,700**
ASU INDIRECT RATES		**($1,975*)**	**$9,325**
WAIVED INDIRECT (MATCH)		**$2,950**	
INDIRECT CHARES TO AGFC (10% TOTAL DIRECT)	**$4,400**		
TOTAL PROJECT COST	**$48,400 (61.2%)**	**$30,625 (38.8%)**	**$79,025**

¥Equipment Supplied by the Arkansas Biosciences Institute (State Funds)
∞30.8% of One Year of Nine Month Fellowship Supplied by EVS Graduate Program to EP
*ASU Indirect Rate is 49% of Salaries and Fringes

Qualifications of the individual(s) and organizations(s) involved

Arkansas State University is supplying lab space, equipment, and assistance to this project. Risch's lab has studied Arkansas bats for over six years and has students that will assist in field work. Pannkuk is a Ph.D. student at ASU and has coordinated efforts from four labs ranging in expertise from field ecology to organic chemistry in an interdisciplinary attempt to gain a greater understanding of WNS pathogenesis and host/disease ecology through analyzing biochemical metabolism of bats by fungus. Through the integration of ecology, physiology, pathology, and biochemistry the team at ASU offers unique insights into the spread and control of WNS. Permits have been received from the Center for Disease Control (#2009-09-136), U.S. Fish and Wildlife (#LE227131-0), and Institutional Animal Care and Use Committee. Standard Operating Procedures (SOP) for the handling of bats and fungus have been peer reviewed by the Institutional Biosafety Committee and are available at your request.

Dr. Thomas Risch has a Ph.D. in Zoology from Auburn University. He is an associate professor of environmental biology, curator of mammals, the Director of the Graduate Program in Environmental Sciences, and Chair of the Department of Biological Sciences at ASU. Risch's strength is in field ecology with over six years' experience studying bats in the Eastern United States. He currently serves on the Board of Directors of the Southeastern Bat Diversity and is this groups WNS committee chair. Additionally, Tom is the Arkansas representative to the Midwestern Bat Working Group. He will provide materials and assistance from the field ecology lab at ASU.

Dr. Brett Savary has a Ph.D. in Plant Physiology from Pennsylvania State University. He previously worked as a USDA-ARS research scientists and has been Research Associate Professor of Protein Chemistry in the Arkansas Biosciences Institute since 2006. His laboratory specializes in bioanalytical chemistry, including enzymology, protein purification and structure analysis, and he has published over 35 papers in the subject area. He will provide supervision of electrophoresis techniques, microchemical analytical methods, and MALDI-TOF mass spectrometry. Dr. Savary is supplying lab space, equipment, and personnel in the Arkansas Biosciences Institute.

Mr. Evan Pannkuk has procured funding, isolated, and performed chemical analysis of carotenoids and porphyrins in vertebrate tissue. This project is his central hypothesis to a M.S. thesis at ASU. His recent Ph.D. studies involve protein fragments released from WNS infected bat tissue and the mechanics of infected tissue. He has a B.S. and a M.S. in Biology from Appalachian State University studying comparative anatomy and biomechanical properties of owl feathers and he contractually served on the Chemistry faculty at Appalachian State University for one year teaching General and Organic Chemistry labs. He is currently working on a Ph.D. in Environmental Science and a M.S. in Chemistry at ASU.

Dr. David Gilmore has a Ph.D. in microbiology from the University of Connecticut. He specializes in microbial physiology and detergent microbial breakdown into plastics. He has sixteen publications in the microbiological and biochemical sciences. He will provide the microbiology lab at Arkansas State University (Laboratory Science West), some culturing materials, and assist with culturing techniques.