White-Nose Syndrome: Pathogenicity of *Geomyces destructans* and the Role of Secreted Extracellular Enzymes in Host/Pathogen Ecology

PROJECT SUMMARY

Arkansas is home to three federally endangered cave dwelling bats. *Geomyces destructans* is a fungus that causes the wildlife disease White-Nose Syndrome (WNS) in bats. This project addresses the need to understand the basis for virulence of WNS. We propose to isolate, identify, and characterize putative enzyme activity (-ies) secreted by the pathogen that may mediate this disease. These studies will aid in the identification of possible virulence factors released by *G. destructans* to support measures to control WNS.

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Total Project Cost = $109,130 (35% match): $141,949 (50% match)
Total Arkansas SWG request = $70,936
Total Matching Funds* provided = $38,194 (35%): $71,013 (50%)

*these are non-federal dollars supplied by Arkansas State University and Arkansas Biosciences Institute

White-Nose Syndrome: Pathogenicity of *Geomyces destructans* and the Role of Extracellular Enzymes in Host/Pathogen Ecology

Introduction: *Geomyces destructans* is the fungus isolated from cave-dwelling bats that is responsible for White-Nose Syndrome (WNS) [or geomycosis] and the associated mass mortality of North American cave bats. Later stages of disease include the display of fungal hyphae and conidia on bat muzzles and wings, hence the term WNS. WNS was first discovered in Howe Cave, NY, in 2006 and has since spread to 19 states and four Canadian provinces. The high mortality rates (up to 90-100% in the northeastern U.S.) from bats infected with *G. destructans* have devastated local populations of once common bat species. Extensive losses of bat populations due to WNS are implicated in major economic losses for the agriculture industry. The little brown bat was once the most widespread bat species in North America, but it is now likely to be petitioned for placement on the endangered species list. Moreover, Eastern small-footed myotis and Northern long-eared bat, both affected by WNS, have already been petitioned for the endangered species list. *G. destructans* is thought to be an invasive species from Europe. Interestingly, European bats have coexisted with *G. destructans* since at least 1995 without the observed mass mortality currently seen in NA, and NA bats experimentally infected in the lab with the European strain of *G. destructans* exhibited pathogenic effects.

G. destructans infects skin and can be keratinophilic (having affinity for hair) (Pannkuk, unpublished data). Fungal parasitism requires adhesion to the extracellular tissue matrix of a host (which normally consists of hard cornified insoluble structural proteins) and activation of suites of genes for proteolytic and lipolytic enzymes. A major clinical sign observed during *G. destructans* infection is invasion of sebaceous and apocrine glands in the muzzle and 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 and lipolytic activities that could damage integumentary tissue.

Fungal parasites can exhibit virulence through the secretion of extracellular enzymes and may become pathogenic in susceptible hosts, especially if the host is immunocompromised. It is thought that *G. destructans* attaches to bat integument and grows during torpor bouts when the host's immune system is suppressed. Pathogenic microorganisms living on host integument may utilize structural proteins for carbon and nitrogen sources by secreting proteases capable of degrading the host extracellular matrix. In addition, host integument is covered by glande-secreted lipids that can be hydrolyzed by fungal parasites for nutrition. Such lipids may also serve as signal molecules to pathogens. To date, *no one has attempted to isolate, characterize, or identify the G. destructans enzymes that damage 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 the 2012 AR SWG RFP, 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 pathology and host/disease ecology. There is lack of research on *Geomyces'* virulence factors and their role in pathogenicity in WNS. This proposal will offer insight into how this mycopathogen detects host tissue and establishes infection. Thus, these studies may provide insight into the role of virulence factors in WNS and how this disease may affect AR bats.

What is the location of your work? The proposed study is laboratory-based and located at ASU-Jonesboro.

What are your objectives? The overall goal is to determine if proteases and lipases secreted by *G. destructans* contribute to the necrosis in bat wing tissue and how enzymes function in pathogenicity by acquiring nutrition from the host. To achieve our goal we have established an *in vitro* system to isolate extracellular enzymes secreted by *Geomyces* species. Our preliminary results show enzyme activities and extracellular proteins are produced in these cultures, and these may be markers of fungal colonization of host tissue. We have also characterized the sebaceous triacylglycerides (secreted lipids) profiles from three bat species using thin-layer chromatography (TLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). (Such biochemical studies on *Geomyces* enzymes are novel within the WNS community.) We hypothesize that *G. destructans* secretes extracellular enzymes (proteases and lipases) in response to host integumentary substrates, enabling pathogen establishment in host tissue and disease progression.

Objectives:

1. Determine activity profiles of secreted proteases and associate to specific proteins.
2. Determine activity profiles of secreted lipases and associate to specific proteins.
3. Isolate enzyme targets and identify by structural basis with MALDI-TOF MS.

What is your approach? This project consists of integrating classical microbiological and biochemical methods with advanced proteomic techniques. *G. destructans* and *G. panorum* (a non-pathogenic relative) will be grown on defined substrates and also on host integumentary tissue, and the culture media will be processed to recover secreted enzymes for subsequent analysis and description of target enzymes. General methodology necessary for proposed experiments have been described in publications by the Gilmore and Savary Laboratories.

Approach Objective 1: This Objective aims to establish the yield and biochemical properties of protease activity (-ies) and associate them to electrophoretic profiles. Media concentrates from *Geomyces* cultures will be screened for the four major protease classes. Proteins will be separated and visualized by SDS-PAGE and native PAGE (for zymograms). Protein profiles will be generated for each medium treatment. Individual protein bands correlated to specific enzyme activity will be processed further for peptide mass fingerprinting by MALDI-TOF MS to correlate *Geomyces* proteins to known protease sequences (see Objective 3).

Approach Objective 2: This Objective aims to characterize extracellular lipases from culture media in a similar manner to be used for proteases. General lipolytic activity will be assayed with the pH-stat titrimetric method. More specific lipolytic activities will be assessed as necessary with fluorescence-labeled substrates. Lipase fractions will then be separated by SDS-PAGE and native PAGE (for zymograms), and protein bands associated with activity will be processed for peptide mass fingerprinting by MALDI-TOF MS analysis (see Objective 3).

Approach Objective 3: This Objective aims to use highly accurate mass spectrometry to identify putative fungal proteases and lipases based on structural (sequence) properties of proteins isolated from *Geomyces* cultures. Primary MS data (peptide ion mass lists) acquisition will be done at ASU, followed by MS/MS data (*de novo* peptide sequences) acquired through services at the University of Arkansas Medical School (Little Rock). Public sequence databases will be first queried for matching to known enzyme homologs using the Mascot search engine

with peptide ion lists and associated peptide sequences generated from putative *Geomyces* proteases and lipases. Database searches using the annotated *G. destructans* genome sequence available at the Broad Institute website will be used to match enzymes to specific genes.

Expected Results and Benefits: Experimental outcomes from these Objectives will confirm that *Geomyces* secrete proteases capable of degrading host integumentary tissues. This will provide fundamental knowledge on their identity and biochemical properties to support determining their role in the virulence and pathogenicity of *G. destructans*. Experimental outcomes will similarly determine identity and biochemical properties of *Geomyces*-secreted lipases. This new knowledge of *Geomyces* lipase will provide specific targets to determine their involvement in the growth and infection process, particularly for action on host-secreted triacylglycerides. Results from structural identification of *Geomyces* proteases and lipases will directly identify genes coding for these enzymes, opening new opportunities for determining phylogenetic relationships of the *Geomyces* enzymes among the Kingdom Fungi. This will also enable comparison to known virulence factors in other disease models; and this may enable better insight into possible WNS therapeutic strategies and properly strategized governmental action plans.

Species of Greatest Conservation Need (SGCN) in AR that will benefit include: Ozark big-eared bat (*C. townsendii ingens*), Indiana bat (*M. sodalis*), Southeastern myotis (*M. austroriparius*) Eastern small-footed bat (*M. leibii*), and Rafinesque's big-eared bat (*C. rafinesquii*).

BUDGET:

Personnel		**SWG**	**35% Match**	**50% Match**
Co-PI (D. Gilmore)	2.48 mo of Academic Salary		$16,490	$34,044
Graduate student	12 mo @ $1916/month	$23,000		
Undergraduate	625 hrs @ $8.00/hr	$5,000		
Fringes				
Co-PI (D. Gilmore)	25.48% of Academic salary		$4,202	$8,674
Graduate student	0.67%	$154		
Undergraduate	0.67%	$34		
Supplies & Services				
Protein standards & chemical reagents		$4,000		
Analytical Protein lab consumables		$5,000		
Miscellaneous lab supplies & non-capital equipment		$6,000		
Microbiological lab consumables & cultures		$5,000		
MALDI-TOF MS instrument service contract (10% per year)		$3,000		
Commercial facility fees (Edman and MS/MS sequencing)		$5,000		
Publication cost		$1,500		
Tuition remission		$4,800		
Travel				
Travel to meetings and collaborator networking		$2,000		
Total Direct Cost		**$64,488**	**$20,692**	**$42,718.40**
Indirect Cost	**10 % of TDC**	**$6,449**	**$ -------**	**$ -------**
Match Indirect Cost	**49% of SWF**	**$ ------**	**$10,139**	**$20,932**
Waived Indirect Cost	**49% of SWF**	**$ ------**	**$7,363**	**$7,363**
Total Cost		**$70,936**	**$38,194**	**$71,013**
Overall Project Cost			**$109,130**	**$141,949**

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

Arkansas State University is providing lab space, equipment, and assistance to this project within the College of Science and Mathematics and the Arkansas Biosciences Institute. Dr. Risch's lab has studied Arkansas bats for over eight years. Pannkuk is a Ph.D. student at ASU and has coordinated interdisciplinary efforts between four labs ranging in expertise from field ecology to organic chemistry in an attempt to gain a greater understanding of the biochemical basis of WNS pathogenesis and host/disease ecology. Through the integration of ecology, physiology, pathology, and biochemistry, the team at ASU offers unique multidisciplinary approaches to study 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 approved by the Institutional Biosafety Committee.

Dr. David Gilmore is a microbiologist with a Ph.D. from the University of Connecticut. He is Assistant Professor of Environmental Biology and specializes in microbial physiology and ecology with an emphasis on *Staphylococcus.* He has sixteen publications in the microbiological and biochemical sciences. He will provide the microbiology lab at Arkansas State University (Laboratory Science East), some culturing materials, and assist with culturing techniques.

Dr. Brett Savary is a biochemist with a Ph.D. from Pennsylvania State University. He is Research Associate Professor of Protein Chemistry in the Arkansas Biosciences Institute and specializes in bioanalytical chemistry, including enzymology, protein purification and structure analysis, and he has published 36 papers in the subject area. He will provide supervision of electrophoresis techniques, bioanalytical methods, and MALDI-TOF mass spectrometry. Dr. Savary is supplying lab space, equipment, and personnel in the Arkansas Biosciences Institute.

Dr. Thomas Risch is a zoologist with a Ph.D. from Auburn University. He is a Professor of Animal Ecology, Curator of Mammals, the Director of the Graduate Program in Environmental Science, and Chair of the Department of Biological Sciences at ASU. Risch's strength is in field ecology with over eight years' experience studying bats in the Eastern United States. He currently serves on the Board of Directors of the Southeastern Bat Diversity and is on this group's WNS committee. Additionally, Dr. Risch is the Arkansas representative to the Midwestern Bat Working Group. He will provide materials and assistance from the field ecology lab at ASU.

Mr. Evan Pannkuk has a B.S. and a M.S. in Biology from Appalachian State University studying comparative anatomy and biomechanical properties of owl feathers and also isolation of vertebrate tissue pigments. He contractually served on the Chemistry faculty at Appalachian State University for one year teaching General and Organic Chemistry labs. He has defended proposals for both a Ph.D. in Environmental Science and a M.S. in Chemistry at ASU and completed the required coursework for these degrees. His final two years at ASU will be spent solely on continued biochemical investigations in host/pathogen ecology of WNS.