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**Award Number:** P14AC00588

**Project Number**: UNM-99

**CFDA #:** 15.945

**Park/NPS Unit: El Malpais National Monument**

**Title of Project: Searching for Close Relatives of *Pseudogymnoascus destructans* and Clues to Natural Defenses in Bat Microbiota**

**Administered through the:**  Colorado Plateau Cooperative Ecosystem Studies Unit Cooperative Agreement Number H1200-09-0005

**CESU Partner: University of New Mexico**

**PROJECT CONTACTS:**

**Principal Investigator:** *Diana E. Northup, Ph.D., Biology Department, 1 University of New Mexico, MSC03 2020, 167 Castetter, Albuquerque, NM 87131, TEL: 505-277-5232, FAX: 505-277-6318, dnorthup@unm.edu*

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**FUNDING INFORMATION:**

**Amount Funded: $38,634**

**NPS Account Numbers (amounts in parentheses): WBS PX.XELMAWN14.00.1 ($38,634)**

**Fund Source (e.g., ONPS, FLREA, CRPP, CESU, etc.): ONPS**

[x] NPS Funding

[ ]  Is this funded using a reimbursable account number? If yes, IMR contracting needs a copy of the Interagency Agreement.

**PROJECT DATES:**

**Start Date: May 20, 2014**

**End Date: January 1, 2015**

**NPS Administrative Contacts**

**CESU Coordinator: Todd Chaudhry***, CPCESU Interim Research Coordinator, NAU P.O. Box 5765, Flagstaff, AZ 86011, 928-523-6638, Fax: 928-523-2014, todd\_chaudhry@nps.gov*

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**FEDERAL FINANCIAL REPORTS AND DRAWDOWN SCHEDULE:**

***Federal Financial Reports*** (Check as required for project based on spending plan, period of performance, risk, cooperator history, etc.)

{ } Quarterly { } Semi-annually { } Annually {X} Final

**Project SCHEDULE AND TECHNICAL REPORT DEADLINES:**

List all technical reports and products in sequential order as required in the scope (more lines and milestones can be added as needed):

*Project Start Date* – **May 20, 2014**

*Technical progress reports –* { } Quarterly { } Semi-annually {X} Annually

(Check as needed from PI to monitor progress of specific project. Content should be addressed in the scope.)

*Investigator’s Annual Report (IAR)* – **April 1, 2015**

*Database, Collections/Specimens, Archives, and Maps provided to the NPS ATR or Technical Expert* – **January 1, 2015**

*Draft Final Report* – **January 1, 2015**

*Final Report* – **January 1, 2015**

*Project End Date* – **January 1, 2015**

*Final SF425 FFR* must be submitted within 90 days of project end date

**PAYMENTS**

**CFR PART 215.22*:*** Cash advance (drawdown) to recipient organization shall be limited to the minimum amounts needed and be timed to be in accordance with the actual immediate cash requirements of the recipient organization in carrying out the purpose of the approved program or project. The timing and amount of cash advances shall be as close as is administratively feasible to the actual disbursements by the recipient organization for direct program or project costs and the proportionate share of any allowable indirect costs.

**CESU REQUIRED PRODUCTS (may be different from those products required by the ATR – See Statement of Work for Products required by the NPS unit):**

The Principal Investigator will prepare a brief report abstract suitable for public distribution and two hard copies and an electronic version (in PDF file format) of the final report and mail all toJudy Bischoff, National Park Service, CPCESU, NAU P.O. Box 5765, Flagstaff, AZ 86011. Please be sure to include the project number (e.g.; NAU-###, UMT-###, UAZDS-###) and the P number on the cover page of the final report.

**PROJECT ABSTRACT:**

Since the winter of 2006-2007, white-nose syndrome (WNS) has spread north, south and west from Albany, New York, killing hibernating bats as it continues to move westward. At present *Pseudogymnoascus destructans*, a fungus likely from Europe and causal agent of WNS, and the disease itself has been documented as far west as western Missouri and Arkansas, near the eastern Oklahoma border. The westernmost record of the fungus alone has been reported from the panhandle of Oklahoma at a roost belong to the cave myotis (*Myotis velifer*). This location is approximately 200 miles from the New Mexico-Oklahoma state line and will likely serve as a corridor into the Southwest. In light of this and based on the speed and distance the fungus and WNS has spread, we predict that movement of either the fungus or disease into New Mexico will occur within the next 10 years. Once in New Mexico, we believe that the disease will have an impact on 16 of the 27 bat species known to occur in the state.

Current research by Ms. Buecher has established the several caves in El Malpais National Monument, New Mexico possess appropriate microclimate conditions for the growth of *P. destructans*. Previous work by the Northup lab has shown that there are fungi present in the soil and guano samples from some of our 10 study caves at ELMA that test positive for *P. destructans* using Lorch primers (Lorch et al. 2010), but are negative using the more accurate real time PCR (Minnis et al. 2013). We hypothesize that these represent close relatives of *P. destructans*. Using these findings as a guide, Dr. Northup and Ms. Buecher selected sites for the investigation of bat microbiota (bacteria and fungi) on roosting bats to investigate differences in bat species with different vulnerabilities to WNS. Dr. Northup has found that naturally occurring bacteria and fungi on the surfaces of bats’ wings and fur vary among bat species. In addition, some bat species have a preponderance of *Actinobacteria*, the bacterial phylum from which two-thirds of naturally occurring antibiotics come from (Berday 1985; Netta et al. 2009). Drs. Northup and Porras-Alfaro are currently testing *Actinobacteria* cultured from twelve different bat species against *P. destrucans* to determine if these bat *Actinobacteria* may serve as a natural defense against *P. destructans* (unpub. results). We believe that the western bats’ microbiota composition may be different or greater in diversity and abundance when compared to eastern bat species that have been affected by WNS. Therefore, we propose to conduct novel research that investigates the natural occurring microbiota of bats from ELMA cave roosting bats. This study will address four main objectives:

Objective 1: Capture bats in key study caves with roosting bats and net bats at key surface locations.

Objective 2: Identify whether close relatives of *P. destructans* are present in New Mexico caves that are determined to have appropriate microclimate conditions, and if it is found on particular bats species (i.e., bat species belonging to Myotis) that hibernate and share roosts with other species;

Objective 3: Provide a baseline of fungal and bacterial microbiota that reside on bats in ELMA caves/surface prior to *P. destructans*/WNS exposure, that may provide insight to differences found on affected eastern species;

Objective 4: Determine if *Actinobacteria* found on ELMA bat species will serve as biological agents that can be used as a natural defense to infection to WNS.

Addressing Objectives 1, 2, and 3 will provide current information on occurrence, habitat use, and movement, as well as a baseline of naturally occurring microbiota of ELMA bats that can be compared to eastern species and insight on the dynamics of *P. destructans*. Our efforts to address Objective 4 will provide new information on the dynamics of antifungal treatments to existing or preventing infections from *P. destructans*)

**Scope of Work:**

**Objective 1**: Capture bats in key study caves with roosting bats and net bats at key surface locations.

*Methods for Objective 1:*

This project will involve capturing bats as individuals hanging in a torpid state in ELMA caves or netting at ELMA water sources. Northup and students will participate in all fieldwork in ELMA caves and with surface mist netting. All bats handled for this project will be covered by a 2014 New Mexico Game and Fish Department Scientific Collecting Permit (SCI#3423), a National Park Service Scientific Collecting Permit (ELMA-2014-SCI-0001) and an Institutional Animal Care and Use Committee (IACUC) Permit from The University of New Mexico (Protocol #12-100835-MCC), all of which have approved our animal handling procedures and study design. All bat work followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2011). All personnel handling bats had pre-exposure immunizations for rabies and maintained an appropriate antibody titer. In order to limit the possibility of human introduction of *Pseudogymnoascus destructans* (the causative fungal agent for White-nose Syndrome, resulting in the death of over 5.5 million bats in the eastern U.S.) to area bats, we will strictly follow USFWS WNS decontamination protocol for bat researchers (http://www.fws.gov/WhiteNoseSyndrome/pdf/WNSDecon Researchers\_v012511.pdf) including:

1. Biologists will wear new disposable gloves for each individual bat captured.
2. Each individual bat will be held in a clean holding bag.
3. Any equipment that touched a bat during measurements will be cleaned with Clorox® or Lysol® wipes/spray, rinsed and dried between each bat.
4. Nets and bat bags will be submerged in water with sustained temperatures of ≥50oC for 20 minutes between each netting session to decontaminate the equipment.
5. We will check wings and skin condition and record a ranking for Wing Damage Index (WDI – Reichard and Kunz 2009) on each bat to monitor for evidence of WNS.

Bats will be captured in caves using a hand net or on the surface by using mist nets at water sources at ELMA (Kunz and Kurta 1988). The mist nets are standard 2-ply 50 denier, 38 mm mesh nylon nets, size as required, (Avinet Inc. Dryden, NY) stretched across naturally occurring or impounded water. We will monitor the nets and remove bats from the nets upon capture. We will place animals in individual cloth bags and record time of capture. If we capture obviously pregnant females we will identify to species and release immediately to reduce stress to the animals. We will determine species, sex and reproductive condition (Racey 1988) and evaluate tooth wear (Twente 1955). Tooth wear can be used to determine general age, however care must be taken because the hardness of food items chosen by different bat species (hard beetles vs. soft moths) can also impact tooth wear. When bats were in the hand, wings, muzzle, ears and uropatagium will be evaluated for any tissue damage (necrosis), lesions, scarring or skin mottling currently attributed to *G. destructans* (Reichard and Kunz 2009, Cryan et al. 2010). Most bats will be easily identified using a key of standard morphological features (Adams 2003, Hoffmeister 1986). However, there are a few little brown bats (genus *Myotis*) that can be difficult to identify. In these cases, forearm length, ear length, foot length and other species-specific characteristics will be used. To distinguish between the cryptic California myotis and the western small-footed bat we will use the morphological characteristics specific to these two species. No bats will be taken as voucher specimens but we will use photographs to confirm species when necessary. For a short time after young bats became volant, adults can be distinguished from sub-adults by determining the amount of closure of cartilaginous epiphyseal plates in the finger bones (Anthony, 1988). We will weigh each bat using a Pesola spring scale (± 0.25 g) and measure the right forearm length with a caliper (± 0.1 mm).

*Methods for Objectives 2, 3, 4:*

Microbiota will be sampled by swabbing captured bats. The skin (wing and tail membranes) and fur surfaces of the bats will be swabbed following methods modified from Johnson et al. (2013), and using sterile nylon tipped swabs moistened with sterile Ringer’s solution. Three different swabs will be used on each bat to determine: (1) the microbiota on the membranes and fur; (2) *Actinobacteria* present on the bat and, (3) if *P. destructans* is present on either membranes or fur. After inoculation each microbiota swab will be placed in a sterile 1.7ml snap-cap microfuge tube and then frozen in a liquid nitrogen dry shipper for transport to the lab, where they will be stored in a -80°C freezer for shipment to MR DNA for DNA extraction and sequencing. Areas of caves in which bats are observed to roost or hibernate will also be swabbed for culture inoculation. Preliminary data from Fort Stanton Cave demonstrated that such areas are more successful for culturing efforts than soil and guano deposits, which produce a wealth of weedy fungi.

**Objective 2**: Identify whether close relatives of *P. destructans* are present in New Mexico caves that are determined to have appropriate microclimate conditions, and if it is found on particular bats species (i.e., bat species belonging to *Myotis*) that hibernate and share roosts with other species.

*Methods for Objective 2:*

*Culture inoculation*: Swabs will be used to inoculate potato dextrose agar (PDA) plates following the methods of Johnson et al. (2013) and using newly developed media targeting keratin degraders (horse hair agar developed by Porras-Alfaro, and peacock feather medium developed by Dr. Northup). All inoculations will be done at the capture site and will be stored in a cooler to maintain an appropriate temperature until stored in a 10°C incubator. Cultures will be monitored and subcultured as colonies develop.

*Culture DNA extraction and sequencing*: Because Dr. Porras-Alfaro has found a range of culture morphotypes that match *P. destructans*, we will select cultures for sequencing based on different morphotypes. Culture DNA will be extracted using the MoBio Ultraclean DNA Extraction Kit. To test for the presence of *P. destructans*, DNA will be initially amplified using the polymerase chain reaction (PCR) with the primers developed by Lorch et al. (2010). Samples that amplify using the Lorch et al. (2010) primers will be tested with the real-time PCR protocol developed by Muller et al. (2013), which is much more diagnostic for the presence of *P. destructans*. Any cultures that test positive on this diagnostic test will be sequenced using the methods of Minnis and Lindner (2013), to generate DNA sequence data for the internal transcribed spacer (ITS) region, nuclear large subunit (LSU) rDNA, MCM7, RPB2, and TEF1 genes to accurately place them phylogenetically.

**Objective 3**: Provide a baseline of fungal and bacterial microbiota that reside on bats in ELMA caves/surface prior to *P. destructans*/WNS exposure, that may provide insight to differences found on affected eastern species.

*Methods for Objective 3:*

*Microbiota DNA extraction and sequencing*: DNA will be extracted by MR DNA in Shallowater, TX and sequenced using next gen 454 sequencing to generate approximately 3000X reads, using 27F bacterial primers and ITS1/4 fungal primers.

*Microbiota 454 sequence analysis*: Sequence libraries generated with next generation sequencing will be analyzed using the QIIME software (Caporarso et al. 2010), followed by statistical testing and diversity analysis in the R statistical package. Analyses will be guided by testing of different parameters that are hypothesized to influence diversity patterns observed in the preliminary data: (1) location bat was caught, especially surface versus subsurface, (2) bat species and sex, (3) time of year, (4) presence of putative pathogenic bacteria and fungi on the bats, (5) percentage of *Actinobacteria* present.

**Objective 4**: Determine if *Actinobacteria* produce antifungal secondary metabolites effective against *P. destructans.*

Addressing Objectives 1, 2, and 3 will provide current information on occurrence, habitat use, and movement, as well as a baseline of naturally occurring microbiota of ELMA bats that can be compared to eastern species and insight on the dynamics of *P. destructans*. Our efforts to address Objective 4 will provide new information on the dynamics of antifungal treatments to existing or preventing infections from *P. destructans*)

*Methods for Objective 4:*

In our effort to target *Actinobacteria* and because these bacteria have the highest G-C content of all bacteria, we will use the medium humic acid-vitamin agar developed by Hayakawa and Nonomura (1987) for its ability to select high G-C content bacteria. We are also in contact with Paul Lawson at the University of Oklahoma, who has done extensive new species characterization. He has supplied several suggestions for additional media to use to isolate novel *Actinobacteria*. Inoculated cultures will be grown in incubators in the Northup lab and after sufficient growth is obtained, we will sub-culture to obtain pure isolates on ½ R2A medium. Our target will be to obtain 200-300 actinobacterial pure cultures in each year of the project.

*Verification of Actinobacteria Presence*: DNA from pure cultures will be extracted using the MoBio UltraClean Microbial DNA Isolation kit. DNA will then be amplified with 8F and 1492R (universal bacterial specific) primers using the polymerase chain reaction. Amplicons will be purified with an ExoSap cleanup step and will then be sequenced in Big Dye 1.1 reactions, using 46F and 1409R primers in separate reactions to provide a nearly full-length sequence of the 16S SSU gene. Sequences will be assembled and edited in Sequencher 4.9 and will then be run in the national database BLAST (NCBI) to ascertain whether they are Actinobacteria based on their closest relatives. These methods parallel those used in several of Dr. Northup’s recent publications (see Northup CV). Final sequences will be clustered using the results of an identity matrix generated in BioEdit, using the cutoff of 99%, a level that was ascertained to be the appropriate level in our preliminary 200 actinobacterial cultures. A representative culture from each cluster will be picked for *P. destructans* testing.

*Testing of* Actinobacterial *Cultures Against* P. destructans *(Spring 2015 and 2016):* To investigate the possibility that some bats host bacteria on their skin or fur that produce anti-fungal compounds effective against *P. destructans*, we will: (1) capture and sample bats that are emerging from hibernation at BLM study caves 45 and 55 and at netting sites in the northeast corridor of NM; (2) inoculate cultures of media that target Actinobacteria, incubate in the laboratory, and sub-culture to obtain individual isolates; (3) sequence to verify the isolates are indeed *Actinobacteria*; and, (4) test actinobacterial isolates against cultures of *P. destrucans* to determine if any anti-fungal compounds produced are effective against *P. destructans*. Positive results from these tests will address our hypothesis that *Actinobacteria* isolated from bats produce antifungals that could be a potential natural defense against *P. destructans*. These tests will be conducted in the lab of Dr. Andrea Porras-Alfaro at Western Illinois University. She is currently conducting research on *P. destructans* in Illinois and her lab is situated in a state that already has *P. destructans* present in its caves and on its bats. Because of the danger of spreading this very infective fungus to bats, none of these tests will be performed in New Mexico. During a preliminary test conducted in December 2013, we determined that the most effective method of testing is to initially grow the actinobacterial isolate on R2A medium as a single inoculation streak across the middle of the plate. This is followed by the pouring of a fungal-appropriate medium overlap onto which a lawn *of P. destructans* is plated. If the *Actinobacteria* are producing antifungals, a zone of inhibition develops around the actinobacterial isolate and no *P. destructans* grows in that region. These methods were modified from those used in Northup’s lab for the testing of cave actinobacterial antibiotic production (Montano and Henderson 2012).

**Stakeholder Coordination/Involvement:**

*Dr. Diana Northup* (PI) provides expertise in fungi and bacteria related to bats, caves/hibernacula, and *P. destructans.* Diana has been research life in caves since 1984, first focusing on cave invertebrates and then shifting to cave microorganisms in 1994. She has conducted extensive field investigations in caves and some extremophile surface environments for the last 30 years, including research into *P. destructans* and WNS since 2010. Her laboratory skills include microbiological culturing of fungi and bacteria, DNA extraction, PCR amplification, DNA sequencing, sequence analysis, and scanning electron microscopy. She will oversee the microbiota and culturing efforts in close collaboration with Valdez and Buecher and the genetic sequence analysis. See CV for additional and pertinent information.

*UNM responsibilities*: Conduct field culturing and microbiota sample acquisition, culture of microbiota, genetic sequencing, analyses of data; write up of results.

*Buecher Biological Consulting responsibilities*: Conducts in-cave bat captures and handling.

*Western Illinois University*: Testing of actinobacterial antifungal production against *P. destructans*

**Permitting**:

* New Mexico state scientific collecting permit #3350;
* El Malpais National Monument Research Permit ELMA-2014-SCI-001
* Animal Care and Use: Capture and handling of bats will follow the Fort Collins Science Center Standard Operating Procedure (SOP) SOP#: 2013-01 2001-01 (Ellison et al. 2013). Buecher and Valdez have the permits from New Mexico Game and Fish that allow us to conduct these activities and Northup holds a University of New Mexico IACUC Protocol, as well as an NPS IACUC permit recognizing and approving the UNM IACUC permit. We will follow current decontamination protocols established by the U.S. Fish and Wildlife Service to prevent any potential spread of *P. destructans* should it already be in New Mexico caves undetected (www.fws.gov/whitenosesyndrome/pdf/WNSDecon\_Researchers\_
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**COOPERATIVE AGREEMENTS OR TASK AGREEMENTS INVOLVING COOPERATORS WORKING ON-SITE**

**Background**

In cooperative agreements or task agreements with universities where the university utilizes interns, student employees, research associates (RAs) or cooperators on-site (hereafter called “cooperator personnel”), these cooperator personnel sometimes work on government sites in close proximity to federal employees. It is illegal (without specific statutory authority) for federal employees to directly supervise the cooperator personnel or any university employees or for the students or other university employees to supervise federal employees. When cooperator personnel are working on an NPS site, it is important that there is a clear distinction between students and federal employees.

**Office Environment and Vehicles**

* The office space of the cooperator personnel and NPS personnel should be clearly labeled (Name and NPS or University affiliation on office or cubicle space).
* Cooperator personnel should be listed separately from NPS personnel in telephone lists, other identification or organizational rosters, and publication credits.
* Cooperator personnel should not receive “all-employee” e-mail or other communications intended for NPS personnel (unless it relates directly to the work the cooperator is doing for the NPS). When the e-mail does relate to the work being done, a copy of the same e-mail message should be sent to the University or cooperator’s supervisor.
* Cooperator personnel may use NPS e-mail systems when the communication relates directly to the work the cooperator is doing for the NPS. The e-mail addresses of the cooperator personnel must include a label associated with their NPS e-mail address that identifies the cooperator’s status (i.e., “Linda Webb, Cooperator” would be the label associated with the e-mail address, linda\_webb@contractor.nps.gov). Doing so clearly identifies this individual each time they send an e-mail message using the NPS system, and it identifies their status as a research associate, student intern or student employee in the e-mail directory.
* Unless stipulated in the agreement, cooperator personnel should not drive government vehicles.
* Unless stipulated in the agreement, cooperator personnel should not ride as a passenger in a government vehicle. When this is planned as part of the agreement, an appropriate amount of liability insurance should be negotiated.
* Prior written approval by the Park Superintendent or Center Manager must be obtained in order for a task to allow cooperator personnel to drive or ride in government vehicles.

**Supervision and Scheduling**

* Each task must specify the university’s/cooperator’s supervisor for the cooperator personnel.
* Unless stipulated in the agreement, NPS staff should not set hours for cooperator personnel, specify where the work should be done, or conduct performance appraisals. National Park Service staff may give performance feedback to the cooperator personnel supervisor.
* Cooperator personnel should report leave, scheduling, and other related issues to the university or cooperator’s supervisor, not to NPS employees. The supervisor of the cooperator personnel should then communicate with the NPS. National Park Service employees cannot directly supervise cooperator personnel on a day-to-day basis. Work should be given to the cooperator personnel (via the cooperator’s supervisor) on a “task basis.” Cooperators should work without NPS supervision to accomplish each task, although technical consultations and cooperation is permissible.
* The Cooperator will be responsible for any disciplinary action needed to correct student employee conduct or performance problems. The NPS agreements technical representative will inform the university/cooperator’s supervisor of any conduct or performance problems.
* The Cooperator will remove student employees from their positions if they fail to improve performance or address conduct issues.
* The NPS will review and provide feedback to students or interns regarding work assignments.
* The NPS will inform the cooperator of conduct or performance problems with cooperator personnel so that the university can counsel employees and correct the performance problems.
* The NPS will recommend to the cooperator dismissal of cooperator personnel based on conduct or performance issues.
* The Cooperator will hire students, interns or RAs to work on NPS tasks identified in the agreement. Hiring will be conducted in consultation with the NPS Agreements Technical Representative (ATR).
* The Cooperator will: pay students, interns or RAs for hours they have worked in support of the agreement.

**Representation and Communication**

* Cooperator personnel cannot in any way represent themselves to the public as NPS employees.
* Cooperator personnel are required to wear visible identification at all times.

**Other Issues**

* Cooperator personnel should not list an NPS affiliation on publications, but rather should list the cooperative agreement under which the work was performed.
* Cooperator personnel should not be invited to official NPS “social” events.
* Cooperator personnel are not authorized to purchase property and supplies with government funds.
* Cooperator personnel will follow the local policy of the facility when federal facilities are closed due to early release for holidays, snow days, etc.

**PRODUCTS:**

* **I**nvestigators will provide two hard copies and an electronic version (in PDF file format) of the final report. The final report will include the following components:
	+ Locations of sample sites plotted on 1:24,000 USGS topographic maps and with GPS coordinates.
	+ A review of pertinent literature on current information on *P. destructans*;
	+ Insight into bat species and their microbial skin and fur fauna, and how they might influence the bat’s risk from *P*. *destructans*.

* Recommendations for:
	+ Minimizing and identifying the risk and spread of *P. destructans*
	+ Maintaining bats as a part of the natural ecosystem
	+ Minimizing visitor health exposure risks; and
	+ Addressing potential impacts to habitats by ongoing and proposed park management actions.
* At least one interpretive guest lecture on results will be given to staff.
* The results will also be prepared for submission to a refereed scientific journal. Copies of the article, once published, will be supplied to ELMA staff.

**BUDGET:***(You may create your budget in a spreadsheet and attach it as a separate document when you submit your project coversheet and Form 4.9.)*

[See Attached Budget Justification]