

**Award Number:** P14AC00793

**Project Number**: UNM-101

**CFDA #:** 15.945

**Park/NPS Unit:** Carlsbad Caverns National Park

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

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

**CESU Partner:** The Regents of the University of New Mexico, for the Department of Biology (UNM)

**PROJECT CONTACTS:**

**Principal Investigator:** Diana E. Northup, Ph.D.; Visiting Associate Professor, Biology and Professor Emerita, College of University Libraries & Learning Sciences; Address: Biology, MSC03 2020, 1 University of New Mexico, Albuquerque, NM 87131-0001 USA; 505-277-5232, fax 505-277-6318; dnorthup@unm.edu.

**Researcher :** Debbie Buecher; Buecher Biological Consulting; 7050 E. Katchina Court, Tucson, AZ 85715; 520-722-1287 or 520-822-4726; dbuecher@comcast.net.

**Partner Administrative Contact*:***Timothy Wester; Contract and Grant Administrator; The University of New Mexico

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**FUNDING INFORMATION:**

**Amount Funded**: $70,009

**NPS Account Numbers (amounts in parentheses):** PPIMCAVE00 PPMRSNR1N.00000 144P103601

**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 1, 2014

***NOTE: This Task Agreement will become effective on the date of final signature or the effective date of the Award document, whichever is later.***

**End Date:** April 30, 2015

**NPS Administrative Contacts**

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

**Intermountain Region Administrative Contact:** Kelly Adams, Grants and Agreements Specialist, National Park Service, 12795 West Alameda Pkwy, Lakewood, CO 80228. Phone: 303-969-2303 Fax: 303-969-2992 Email: kelly\_adams@nps.gov

**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 1, 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)* – March 31, 2015

*Database, Collections/Specimens, Archives, and Maps provided to the NPS ATR or Technical Expert* – April 30, 2015

*Draft Final Report* – March 15, 2015

*Final Report* – April 30, 2015

*Project End Date* – April 30, 2015 (project reports/deliverables are due)

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

**PAYMENTS**

**2 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.

**2 CFR PART 215.25 (8)(e)(1):** Incur pre-award costs 90 calendar days prior to award or more than 90 calendar days with the prior approval of the Federal awarding agency. All pre-award costs are incurred at the recipient’s risk. (i.e. the Federal awarding agency is under no obligation to reimburse such costs if for any reason the recipient does not receive an award or if the award is less than anticipated and inadequate to cover such 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 toTodd Chaudhry, 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 2006-07, white-nose syndrome (WNS) has spread north, south and west from Albany, NY, killing hibernating bats as it continues to move westward. At present, *Pseudogymnoascus destructans*, a fungus likely from Europe and the causal agent of WNS, as well as 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*), but this record was withdrawn last week. In light of the westward movement of WNS, 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 Buecher has established that several caves in El Malpais National Monument, and other caves throughout 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 sites in Carlsbad Caverns National Park (CAVE) that test positive for *P. destructans* using Lorch primers (less precise testing), but are negative using the more accurate real time PCR. We hypothesize that these represent close relatives of *P. destructans*. Using these findings as a guide, Northup and 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. 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. Drs. Northup and Porras-Alfaro are currently testing *Actinobacteria* cultured from 12 different bat species against *P. destructans* to determine if these bat *Actinobacteria* may serve as a natural defense against *P. destructans*. 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 CAVE cave roosting bats. Additionally, this research provides an outstanding opportunity to conduct photodocumentation of the research to provide material to CAVE interpretive staff for exhibits.

**Scope of Work:**

 This study will address these objectives:

1: Capture bats in key study caves with roosting bats, and net bats at key surface locations;

2: Identify whether close relatives of *P. destructans* are present in CAVE caves, and if it is found on particular bat species that hibernate and share roosts with other species;

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

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

5: Perform metagenomics analysis on a subsample of bats;

6: Take a series of science-in-action photos of the field and laboratory work to provide the basis for an exhibit at CAVE on white-nose syndrome.

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

Bat sampling: All bats must be treated carefully, following an Institutional Animal Care and Use Committee (IACUC), issued to Northup by UNM and NPS. All bat work followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research. All personnel handling bats have pre-exposure immunizations for rabies and maintain an appropriate antibody titer.

We will strictly follow USFWS WNS decontamination protocol for bat researchers, 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 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 CAVE. The mist nets are standard 2-ply 50 denier, 38 mm mesh nylon nets, size as required, 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 and evaluate tooth wear. 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 *P. destructans*. Most bats will be easily identified using a key of standard morphological features. 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. 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).

Bat Swabbing for Objectives 2, 3, 4:

Microbiota will be sampled by swabbing. The skin (wing and tail membranes) and fur surfaces of the bats will be swabbed, 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) A*ctinobacteria* 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.

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 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 6-10oC incubator. Cultures will be monitored and subcultured as colonies develop.

Culture DNA extraction and sequencing: Because 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.

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

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.

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

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 100-200 actinobacterial pure cultures during 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 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*

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 at CAVE study caves; (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. destructans* 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 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).

Methods for addressing Objective 5: Perform metagenomics analysis on a subsample of 10 bats.

Metagenomic sequencing – Selected samples will undergo deep sequence metagenome analysis at MR DNA. Their methods include utilizing MiSeq sequencing (www.illumina.com) to generate DNA libraries prepared using Nextera DNA sample prep kits to create individual barcode indices. Samples will be analyzed utilizing MG-RAST metagenome analysis server using both assembled and unassembled data.

Metagenomics analysis – Processed unassembled sequences will be uploaded to the Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) server, v3.1.2, for annotation (Meyer et al., 2008). A taxonomic profile will be generated using sequence matches to the non-redundant protein database with a maximum e-value cutoff of 1e-5. Metabolic profiles will be determined from normalized abundances of SEED subsystems (with a minimum identity of 60%). For our 16S rRNA reads, amplicon sequencing data will be processed with MG-RAST using the RefSeq database (Pruitt et al., 2005; Meyer et al., 2008) and QIIME (Caporaso et al., 2010). Taxonomic assignment in Qiime will be carried out using the SILVA database (Pruesse et al., 2007). Functional assignment of the reads will performed using BLAST searches against the KEGG (Kanehisa et al., 2008) and COG (Tatusov et al., 2001) databases. The metagenomic sequence reads and contigs and the 16S rRNA amplicon sequences will be made publicly accessible through the MG-RAST server.

Methods for addressing Objective 6: Take a series of science-in-action photos of the field and laboratory work to provide the basis for an exhibit at CAVE on white-nose syndrome.

Kenneth Ingham will accompany the team to Carlsbad Caverns National Park and will take photos of the mist netting, handling, and swabbing of bats and culture inoculation by the researchers. All photos will be post-processed to enhance contrast and exposure, photo composition, and selection of a set of the best 100 photos for delivery to the CAVE NPS. Delivery will be via password-protected web page. Photos copyright Kenneth Ingham, but usable by NPS (and CAVE etc.) for any non-commercial use with attribution.

**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 will follow the local policy of the facility when federal facilities are closed due to early release for holidays, snow days, etc.

**PRODUCTS:**

1) Final analysis Excel files with metadata;

2) Digital photos;

3) Final report providing details on the monitoring set up and field methods, analysis, and discussion with management recommendations where appropriate.

**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.**