COVER PAGE

Title of Project:	Assessment and Evaluation of Prevalence of Fungal Dermatitis in New England Timber Rattlesnake (*Crotalus horridus*) Populations
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RCN Funds Requested:	$81,148.71

Project Description:

Historically, the Timber Rattlesnake (Crotalus horridus) occurred in all six New England states. The species is now extirpated from Maine and Rhode Island, and is close to extirpation in New Hampshire and Vermont with only one and two remaining populations, respectively. Since 2009, Timber Rattlesnakes from separate populations in eastern, central and western Massachusetts have been found to have significant disease identified as fungal dermatitis. Fungal dermatitis has been previously documented as a cause of morbidity and mortality in both captive and free-ranging Viperidae snakes (Cheatwood et al., 2003, Jessup and Seely, 1981, McAllister et al., 1993).

Wildlife disease studies in natural populations are becoming increasingly important in aiding wildlife management and conservation. Many infectious agents pose a threat to wildlife populations, and there is a growing body of literature documenting disease outbreaks which have affected populations and even extirpated species. This proposal describes a comprehensive evaluation to provide a baseline health assessment of multiple New England populations as well as to provide scientific support for future policy and wildlife management decisions for this species. The study will provide evidence of the extent of fungal dermatitis among multiple rattlesnake populations and evaluate potential underlying stressors or factors predisposing the species to fungal disease. The data gathered will attempt to elucidate whether the fungal disease seen is a primary pathogen or a secondary opportunistic invader, in addition to providing insight as to whether these are isolated cases or if they are indicative of wider health concerns within Timber Rattlesnake populations and will provide a geographical map of fungal infections identified in New England populations. The study will also evaluate presence of heavy metals and toxins as potential immune system stressors thus utilizing the Timber Rattlesnake as an indicator of potential environmental pollutants as well as overall ecosystem health.

A. PRIORITY RCN TOPICS ADDRESSED:

RCN Topic 5: Design and Implement Conservation Strategies for NE Species of Greatest Conservation Need

Utilizing methodologies previously developed by the Northeast Partners in Reptile Conservation, the collaborative workgroup developing this proposal will work to identify the most effective conservation strategies for the endangered timber rattlesnake.

RCN Topic 6: Design and Implement Monitoring Protocols, Measures, and Indicators for NE Species of Greatest Conservation Need

A comprehensive health assessment is needed to establish a clear baseline for data collection. As these data are gathered and studied, effective monitoring protocols will be developed.

RCN Topic 7: Identify and Assess Threats to NE Species of Greatest Conservation Need

An important component of the proposed study is to identify the specific nature of the threat to timber rattlesnakes in the Northeast. Fungal dermatitis has been identified, but it is not yet known whether the disease is the result of opportunistic infections affecting animals with compromised immune function, or whether the fungal dermatitis represents the primary pathogen. The incidence of fungal disease has been particularly high in the only remaining NH population and in the only remaining populations in eastern MA. Some infected snakes have been blinded and others have died. Fungal disease has also been observed in central and western MA and in CT, but at lower frequencies. The fear is that the incidence and distribution of fungal disease in Timber Rattlesnakes may increase.

B. PROJECT AREA:

Massachusetts, Vermont, Connecticut and New Hampshire

C. PROJECT TIMEFRAME:

The proposed work is scheduled to begin on or about January 15, 2013 and will conclude 24 months later.

D. PROJECT GOALS AND OBJECTIVES:

Timber Rattlesnakes were identified as of 'Severe Concern' by the Northeast Partners for Amphibian and Reptile Conservation (NEPARC, 2010) and the species is listed as a Species of Greatest Conservation Need in 12 Northeast states. It is believed to be extant in only 10 of those states. In 2009, three Timber Rattlesnakes from three separate regions of Massachusetts (eastern, central, and western) were identified with significant disease identified as fungal dermatitis with or without stomatitis. All three snakes subsequently died. In spring of 2010, a fourth Timber Rattlesnake (western Massachusetts population) was diagnosed with fungal dermatitis and was released back to the wild with a transmitter for continued observation. That fall, a fifth Timber Rattlesnake (eastern population) was identified with similar lesions and transferred to the Roger Williams Park Zoo for workup and medical care. The skin lesions in this fifth snake were also identified as a fungal dermatitis. Isolates from four of the five snakes were identified as *Beauveria bassiana, Chrysosporium ophiodiicola,* and *Trichosporon*. Since this time there have been 5 additional snakes presented to Roger Williams Park Zoo for evaluation of fungal dermatitis from Massachusetts and one snake from New Hampshire. Additionally, there have been numerous anecdotal reports of facial dermatitis in the Blue Hills Timber Rattlesnake population which have not received further diagnostic workup. Fungal dermatitis has also been confirmed in several of the last known existing Timber Rattlesnake populations in New Hampshire, although prevalence estimate data has not been generated (Clark et al., 2011).

It is very possible that the effects of fungal dermatitis are not restricted to Timber Rattlesnakes. Lesions have recently been described in eastern Massasuagas and one ringneck snake was captured at the New Hampshire rattlesnake site with lesions confirmed to be fungal dermatitis, specifically *Chrysosporium ophidiicola.* Therefore, this issue may have more far reaching impacts to other snake species and in other states. In fact, anecdotal reports of lesions have been described in several other states in the northeast but prevalence and effects have not yet been quantified. This proposal describes a

focused study of Timber Rattlesnakes in New England because its 'severely threatened' conservation status, documentation of the lesions in these areas, and ongoing monitoring efforts. However, the results of this study will be directly relevant to all other states in the region.

Fungal dermatitis with and without stomatitis have been previously documented as a cause of morbidity and mortality in both captive and free-ranging Viperidae snakes (Cheatwood et al., 2003, Jessup and Seely, 1981, McAllister et al., 1993). Fungal pathogens have become increasingly important in free-ranging wildlife study during the past decade due to the well-documented effects of *Batrachochytrium dendrobatidis* on global amphibian population declines (Berger et al., 1998) and white-nose syndrome on North American bat populations (Frick et al., 2010). In most instances, fungal disease, especially in reptiles, tends to occur as opportunistic infections affecting animals with compromised immune function. However, both of the aforementioned diseases can cause widespread morbidity and mortality in apparently healthy individuals in free-ranging populations. Additionally, there is evidence of primary fungal pathogens causing disease in reptiles such as that caused by the emerging disease, *Chrysosporium* anamorph of *Nannizziopsis viresii* (CANV). Experimental studies have shown CANV can serve as a primary pathogen and the disease has been associated with mortality in numerous reptile species (Nichols et al., 1999, Pare et al., 2006, Thomas, et al., 2002). While we have documented fungal dermatitis in free-ranging New England Timber Rattlesnakes, the extent of the condition in New England Timber Rattlesnake populations is unknown. It is also unknown whether these previous cases represent isolated disease in compromised individuals or a more expansive syndrome similar to outbreaks described in other species.

Numerous intrinsic and extrinsic factors can lead to immune system suppression in reptiles. Commonly in free-ranging settings, these factors include environmental contamination or habitat alteration (degradation, climatic change, etc.) which can then lead to organ dysfunction through complex pathways or secondary diseases (i.e. bacterial or parasitic infections). Environmental toxins including heavy metals have been associated with disease syndromes and immunosuppression in wildlife (Courtin et al., 2010, Rocke and Samuel, 1991) and could potentially serve as a cause of immune compromise in free-ranging reptiles. Underlying infectious disease can also potentially cause immune system compromise in reptiles. Ophidian paramyxovirus has been previously documented as a cause of epizootic mortality in captive snakes (Jacobson et al., 1992). Paramyxoviruses in mammals are known to have immunosuppressive effects and the effect of ophidian paramyxovirus most likely results in immune system compromise in snakes, as infected snakes often succumb to secondary bacterial pathogens. However, there has only been a limited assessment of ophidian paramyxovirus in free-ranging snake populations (Allender et al., 2006). Research focusing on several of these areas has not been undertaken for New England populations of Timber Rattlesnakes and is warranted to determine the impact of these factors on both the Timber Rattlesnake population and overall ecosystem health.

This proposed study will allow for the determination of the prevalence of fungal dermatitis and its overall impact on Timber Rattlesnake health in several New England populations. The study will compare changes in hematology, plasma biochemistry, and blood parasites that could signify deteriorating overall health. Furthermore, as the status of ophidian paramyxovirus and heavy metals have not been determined in New England Timber Rattlesnakes, the proposed study will assess these factors in determining causality of the disease.

Objectives:

1) Obtain an overall health assessment of multiple New England Timber Rattlesnake populations and determine the prevalence of skin lesions within each population
2) Determine the prevalence of fungal dermatitis via biopsy of any lesions identified
3) Determine the identification of any fungal organisms on biopsy by polymerase chain reaction testing with ITS2 sequencing
4) Obtain baseline hematological values and compare values between individuals with or without the presence of fungal dermatitis
5) Obtain baseline plasma biochemical values and compare values between individuals with or without the presence of fungal dermatitis
6) Determine prevalence of ophidian paramyxovirus in New England Timber Rattlesnake populations using polymerase chain reaction and association with the presence of skin lesions
7) Determine presence of heavy metals or toxins in individuals and association with the presence of skin lesions

E. METHODS:

The project director and other principal investigators on this proposed project will employ the following research methods:

Clinical Survey: Clinical signs will be determined by a biologist and/or veterinarian and recorded on a standard data sheet of all free-ranging Timber Rattlesnakes captured during the survey. The survey proposes sampling 20 snakes from each populations for a total of approximately 200 samples. A population of snakes is determined by the biologists overseeing rattlesnakes in a specific area and using their understanding of physical barriers likely to prevent movement of snakes between groups (i.e. highways, rivers, urban areas, etc).

The presence or absence of clinical signs compatible with those previously described for fungal dermatitis will be documented. Specific clinical signs include cellulitis, cutaneous abscessation, ocular swelling, oral swelling, and respiratory distress. Each sign will be scored as absent (0) or present (1) and total clinical signs score will be tabulated for each animal. Evaluation of representative sample of both healthy and diseased Timber Rattlesnakes is necessary to determine total overall population health and ascertain the extent and effect of fungal dermatitis on populations.

If the snake has no evidence of dermatitis on visual examination by the biologist, the remainder of the sampling will be done in the field. If the snake has evidence of dermatitis, the snake will be placed in a secure, rattlesnake appropriate transport carrier and transported to either the hospital at Roger Williams Park Zoo, or another facility. The snake will be maintained in captivity for a short period of time (a few days) until someone with enough veterinary training can come up to obtain biopsies. Snakes will be maintained by population and cared for under strict in-house quarantine procedures.

The investigators are aware that biopsies can often be performed in the field, but our experience to date has shown that lesions are usually restricted to the head at clinical presentation. This poses two problems for obtaining biopsies in the field:

1) Working in close on a venomous snake to obtain skin biopsies poses some serious safety challenges for the people restraing the snake and those who are attempting to obtain a biopsy.

2) In order to protect important vital structures on the face (eyes, labial pits, mucocutaneous junctions of the mouth, etc) careful dissection of abnormal tissues from normal tissues is often needed.

For these reasons, we believe it is in the better interest of both the snakes and those working with these animals to perform biopsies under anesthesia. We also believe it is in the snakes best interest to perform this work in a more controlled environment (indoors, with appropriate heat support) to ensure maximum survival of the individual snakes while collecting biopsies, We also plan to keep the snakes for two (2) days after anesthesia to make sure animals are completely recovered from anesthesia prior to release at their site of capture. Release dates may be shifted one or two days to avoid releasing the snakes in weather that would be detrimental to their health.

Blood Collection: Individual animals will be restrained in dorsal recumbency and a 3 ml blood sample (or up to 0.8% body weight) will be collected via cardiocentesis (if anesthetized for biopsy or facial lesions) or ventral tail vein (if restrained via snake tube) and placed in lithium heparin and EDTA microtainers.

Hematology: A complete blood count will be performed on all individual snakes handled during the clinical survey. The Eosinophil Unopette system will be used to determine the white blood cell count using a hemocytometer and a white blood cell differential will be determined from review of a slide preparation. Packed cell volumes will be determined using a microhematocrit centrifuge. Plasma collected from the hematocrit tubes will be used to determine total protein levels. Prevalence of intraerythrocytic parasites will be quantified per 400 cells.

Plasma Biochemistries: A series of biochemistries will be analyzed on all individual snakes handled during the clinical survey, including calcium, phosphorus, sodium, potassium, chloride, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatinine kinase, uric acid, bile acid, glucose, and total protein.

Biopsies: A representative number of skin biopsy samples from any individuals identified with skin lesions during the clinical survey will be obtained using local anesthesia (2% lidocaine) or under isoflurane gas anesthesia (necessary to safely obtain biopsy lesions on the face). Biopsy samples will be evaluated by a veterinary pathologist (Kimbro) to determine the cause of lesions.

Identification and PCR of Fungi (University of Florida): Biopsy samples from any individuals displaying skin lesions will be submitted for fungal culture and identification of isolates using polymerase chain reaction testing with sequencing of the internal transcribed spacer 2 (ITS2) region. ITS2 has been observed to be more variable than the D2 region and is therefore a better target for accurate identification of fungal species.

Toxic Elements Screen (Michigan State University): An extended toxic elements screen of whole blood will be performed through the Diagnostic Center for Population and Animal Health at Michigan State University. The elements tested will include antimony, arsenic, beryllium, cadmium, chromium, lead, mercury, nickel, selenium, thallium, and vanadium.

Paramyxovirus PCR (University of Florida): Whole blood will be screened for presence of ophidian paramyxovirus by polymerase chain reaction through the University of Florida Clinical Diagnostic Laboratory.

Statistical Analysis: The mean, median, standard deviation and range will be determined for each hematologic, biochemical parameter, and toxicologic analyte. A Shapiro-Wilk test will be used to determine if the data is normally distributed. A 95% confidence interval will be calculated for those parameters with a normal distribution. An ANOVA test will be used to assess between group differences for normally distributed data. Any between group differences will be further evaluated using a Tukey's test to determine within-group differences. For non-normally distributed data, a Wilcoxen signed rank test will be used to assess between group differences. The observed power will be calculated for those parameters where no statistical difference is identified.

F. OUTCOMES:

The Timber Rattlesnake is currently listed as State Endangered in all of the New England states with extant populations, as well as several other New England and Mid-Atlantic states. This comprehensive health evaluation will provide a baseline health assessment of multiple New England populations as well as provide scientific support for future policy and wildlife management decisions in the species. The study will provide evidence of the extent of fungal dermatitis amongst multiple rattlesnake populations (i.e. whether these are isolated cases or whether this is reflective of more wide scale health problems within New England timber rattlesnake populations) and evaluate potential underlying stressors or factors predisposing to fungal disease to help elucidate whether disease seen is due to a primary pathogen or secondary opportunistic invaders. The study will also evaluate presence of heavy metals and toxins as potential immune system stressors thus utilizing the Timber Rattlesnake as an indicator of potential environmental pollutants as well as overall ecosystem health.

PROGRAM BUDGET

Budget Item	Total 2 year Program Expenses		RCN Funds Requested	Non-Federal Match	Notes
Personnel					
Veterinary Technician / Staff Time	To run Complete Blood Counts (200 samples, 1 hour each)	$ 4,390.00		$ 7,384.75	The City of Providence funds RWPZ's veterinary technician salaries.
	Run Chemistry Panel (200 samples, 15 min each)	$ 1,097.50			
	Educational Sessions to teach biologists blood draw and blood smear techniques (5 sessions, 4 hours each)	$ 439.00			
	Assisting veterinarians to obtain biopsies (25 hours)	$ 548.75			
	Shipping samples (10 hours total)	$ 219.50			
	Keeper Time (0.5 hours/day, 60 days)	$ 690.00			
	Total	$ 7,384.75			
Project Management	Telephone coordination (1 hour/week @ $25/hour)	$ 2,600.00		$ 8,100.00	Lou Perroti's Salary is funded by the RI Zoological Society
	Field work coordination (220 hours @ $25/hour)	$ 5,500.00			
	Total	$ 8,100.00			
Veterinarian Costs	This is an estimate of the time that a RWPZ veterinarian will spend on the project. This number is an estimate and is largely dependant on the dermatitis incidence rate.	$ 24,000.00		$ 24,000.00	RWPZ veterinarian salaries are funded by the RI Zoological Society
Fringe					
Est. @ 20% of total personnel	This includes health insurance and benefits that are not included in the hourly wages of Veterinary and Project Management time.	$ 5,706.95		$ 5,706.95	RWPZ animal care staff salaries are funded by the RI Zoological Society

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Direct Program Expenses

Blood work[1]	Complete Blood Counts (200 @ $30.79)	$ 6,158.00	$ 17,158.00		
	Biochemistry Profile (200 @ $55.00)	$ 11,000.00			
	Total	$ 17,158.00			
Fungal Sample Testing[2]	Biopsy (50 @ $62.29)	$ 3,114.50	$ 18,339.50		
	Fungal Identification and PCR (50 @ $149.50)	$ 7,475.00			
	Fungal Senstitivity Testing (50 @ $100.00)	$ 5,000.00			
	Sample Shipping (50 @ $55.00)	$ 2,750.00			
	Total	$ 18,339.50			
Ophidian Paramyxovirus Testing[1]	Paramyxovirus Testing (200 @ $130.00)	$ 26,000.00	$ 26,000.00		
Extended Toxin Screen[1]	Extended Toxicology Screen (200 @ $92.40)	$ 18,480.00	$ 18,480.00		
Sampling Supplies	Blood tubes, swabs, etc	$ 1,171.21	$ 1,171.21		

Indirect / Overhead

Space to perform veterinary procedures and hold snakes during processing	32' x 20' space @ $12/SF/month for a total of 2 months of occupancy during the project	$ 15,360.00		$ 15,360.00	Existing space will be shared with this program.

Supplies / Materials

Caging		$ 7,500.00		$ 7,500.00	
Animal Care Supplies		$ 2,000.00		$ 2,000.00	Animal Care Supplies are funded by a grant from the RI Foundation.
Crotalid Antivenin	12 vials as required by the Association of Zoos and Aquariums	$ 9300.00		$ 9300.00	

Travel

Est. Travel Costs to Sample Sites		$ 2,022.42		$ 2,022.42	RWPZ staff travel expenses are funded by grants & individual donations.
	Totals	$ 162,522.83	$ 81,148.71	$ 81,374.12	

[1] Note: These costs are based on 200 sampled snakes. If less than 200 snakes are sampled, the amount requested to reimburse lab costs will be reduced.
[2] Note: This cost was based on a 25% incidence rate of dermatitis in sampled snakes. If the incidence rate is lower than 25%, the amount requested to reimburse lab costs will also be reduced.

PROGRAM PERSONNEL

Louis Perrotti – Director of Conservation and Research, Roger Williams Park Zoo
Perrotti serves as Roger Williams Park Zoo (RWPZ)'s representative on local and national conservation collaborations, including those at the AZA level, and manages the Zoo's ongoing conservation initiatives. Perrotti is RWPZ's Institutional Representative for Invertebrates, acting as liaison and providing the most up-to-date invertebrate information on husbandry, exhibit information, education opportunities, conservation and research. Perrotti has been appointed by the American Zoo and Aquarium Association's Terrestrial Invertebrate Taxon Advisory group to initiate invertebrate-based conservation programs in AZA institutions nationwide.

Mike McBride, D.V.M. - Director of Veterinary Services, Roger Williams Park Zoo
Dr. McBride has been RWPZ's Director of Veterinary Care since 2008. He supervises the veterinary staff, has expanded the keeper education program, manages the department's budget, and contributed significantly to the design of the Zoo's state-of-the art Veterinary Hospital. Prior to becoming the Director, Dr. McBride spent four years as a staff veterinarian at the Zoo, designing and implementing animal care protocols that are still in place today.

Kim Wojick, D.V.M.- Associate Veterinarian, Roger Williams Park Zoo
Dr. Wojick has been with RWPZ since September 5, 2011. She graduated from Tufts University School of Veterinary Medicine in 2006. She spent three years in Chicago completing a zoological medicine residency at Brookfield Zoo, Shedd Aquarium, and the Lincoln Park Zoo.

Jason Kimbro, D.V.M. - Anatomic Pathologist, IDEXX Laboratories, MA
Dr. Kimbro is a pathologist for a commercial laboratory (IDEXX). He specializes in anatomic pathology, particularly regarding diseases of wild and exotic animals. He completed his specialty training at the University of Florida and spent his final year jointly with the university and the veterinary services department of Walt Disney World.

Maureen Murray, D.V.M., Dipl. A.B.V.P. (Avian) - Wildlife Clinic, Tufts University School of Veterinary Medicine
Dr. Murray is a Clinical Assistant Professor in the Department of Environmental and Population Health at Tufts University. She specializes in wildlife medicine and surgery and the treatment of traumatic injuries in wildlife.

Tom French, Ph.D., Assistant Director, MA Division of Fisheries and Wildlife
Dr. French's educational background includes a B.S. in Biology from Georgia State University, an M.S. in Zoology from Auburn University, a Ph.D. in Ecology and Systematics from Indiana State University, and a post-doctoral position at Cornell University. He was a zoologist with The Nature Conservancy and an instructor-naturalist and field biologist with the National Audubon Society. Since 1984 he has been an Assistant Director of the Massachusetts Division of Fisheries and Wildlife, where he serves as Director of the Natural Heritage and Endangered Species Program. In 2007, Tom spent four months as the acting Commissioner of the Department of Fish and Game. He has written over forty papers on small mammals, birds and herptiles, and works frequently with the media to foster greater public interest in conservation.

Mike Marchand, Wildlife Biologist, NH Fish and Game Department
Marchand leads species recovery efforts in New Hampshire for freshwater mussels and all amphibians and reptiles. He is the New Hampshire representative for the Northeast Partners for Amphibian and Reptile Conservation (NEPARC) and is currently the Grant Coordinator for a Nationally Competive SWG investigating Blanding's turtles in the Northeast. He has been the timber rattlesnake recovery leader in New Hampshire since 2005 and recently published on the status of the state's population including observations of disease impacting the population (Clark et al., 2011).

Doug Blodgett, Wildlife Biologist, VT Fish and Wildlife Department
Blodgett has been employed as a wildlife biologist with the Vermont Fish and Wildlife Department for 30 years. Throughout his career, Doug has worked extensively on game and non-game management programs as well as public and private land management programs. Most recently, Doug's professional interest has focused on reptiles and specifically, rare snake research in Vermont.

Jenny Dixon, Supervising Wildlife Biologist, CT DEP Wildlife Division
Dickson is a wildlife biologist employed by the Connecticut Department of Energy & Environmental Protection. Dixon has worked as a research biologist for the federal Environmental Protection Agency and as a naturalist at the Kellogg Environmental Center in Derby, Connecticut. She has served as vice-chair of the Northeast Partners in Flight Working Group, president of the New England Chapter of The Wildlife Society, and is on the executive committee of the Northeast Bat Working Group. Current projects include Connecticut's Grassland Habitat Conservation Initiative, conservation of wading bird colonies on Connecticut's offshore islands, implementation of Connecticut's Wildlife Action Plan—including providing conservation information to local land managers—and addressing the devastating impacts of white-nose syndrome in bats.