Characterization of Novel Protist Pathogen (Suspected *Ichthyosporea* / *Mesomycetozoea*) in American Toads *(Anaxyrus americanus)* in the Lamprey River Watershed

FINAL REPORT to the Lamprey River Advisory Committee (19 August 2025)

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Background

In April 2024, several American toads (*Anaxyrus americanus*) with severe skin lesions were found along the Lamprey River in the town of Durham, New Hampshire (Figure 1). Because amphibians are known to be a sentinel group for infectious diseases, environmental contamination, and even radiation, the emergence of a novel pathogen along the Lamprey River carries potential significance for the entire ecological community of the watershed. Based on what we have learned of emerging pathogens and the declining health of amphibians and reptiles across North America and worldwide, a proactive and robust response to any perceived emerging health threat to these species is vital to ensuring the resilience of the watershed as a whole. In that light and with the financial support of the Lamprey River Advisory Committee, the project described here sought to "[p]rotect and restore the ecological functions and resources of the Lamprey River that are critical to wildlife and humans" (2013 Lamprey River Management Plan) by meeting the following broad goals:

i) to definitively identify and characterize the cause of novel lesions in American toads along the Lamprey River, and
ii) to determine the geographic extent of infections in American toads (and other amphibians) within the Lamprey River watershed (and potentially others).

Only by learning the basics of a looming threat can we even begin to devise ways to deal with it.

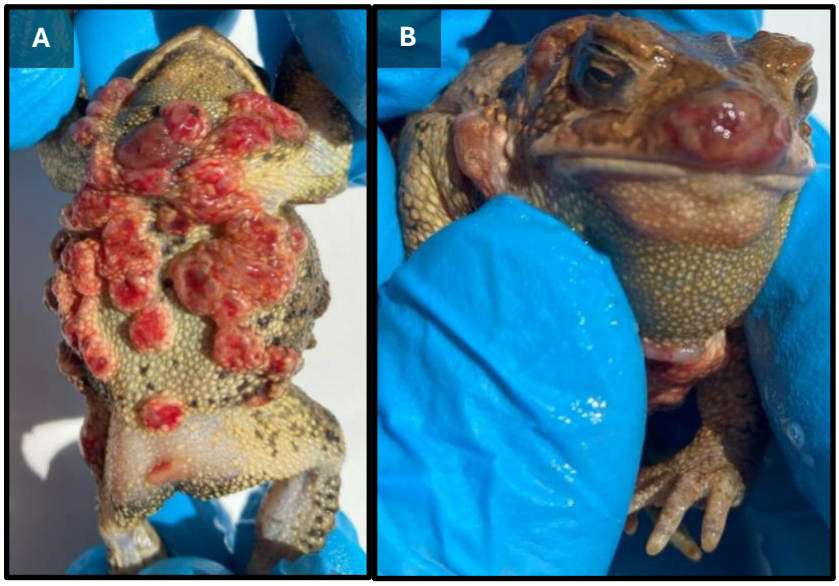

Figure 1. Two American toads (*Anaxyrus americanus*) captured in Durham, New Hampshire (April 2024). *A)* Individual presents with erythematous lesions and decreased girth and pelvic limb diameters, indicative of poor body condition. *B)* Individual presents with lesions around the mouth and nares, potentially limiting ability to feed and respire.

Results

Objective 1 – Characterize the pathogen

Following approval from the University of New Hampshire's Institutional Animal Care and Use Committee, ten adult American toads with lesions were collected and housed individually*. After anaesthetization with MS-222, animals were biopsied following American Veterinary Medical Association recommendations. Three individuals were euthanized due to the severity of infection, while

* One additional toad with lesions was collected as roadkill.

the others were allowed to recover before release[†]. The animals that recovered first shed their cysts, and their lesions later healed.

Lesions contained curvilinear, opaque, white, 3-6 x 2-4 mm, tapered parasitic cysts (sporangia). Samples were fixed in 10% neutral buffered formalin, fixed in 2% formaldehyde : 2.5 % glutaraldehyde in 0.1 M Sodium Cacodylate (pH 7.4), and frozen at -80ºC.

Histopathology (Figure 2) revealed sporangia composed of a loose basophilic lamellar ground substance enveloping monotypic eosinophilic, refractile, studded parasites consistent with an *Opisthokonta* (protist) in class *Mesomycetozoea*[1]. These unicellular organisms are related to fungi and animals and have been described in fish, amphibians, and mammals in Africa, Europe, and the Americas [2–4]. *Mesomycetozoea* can colonize hosts without disease, cause only cutaneous lesions, or infect many organs, with salamanders commonly colonized in the northeastern United States[5].

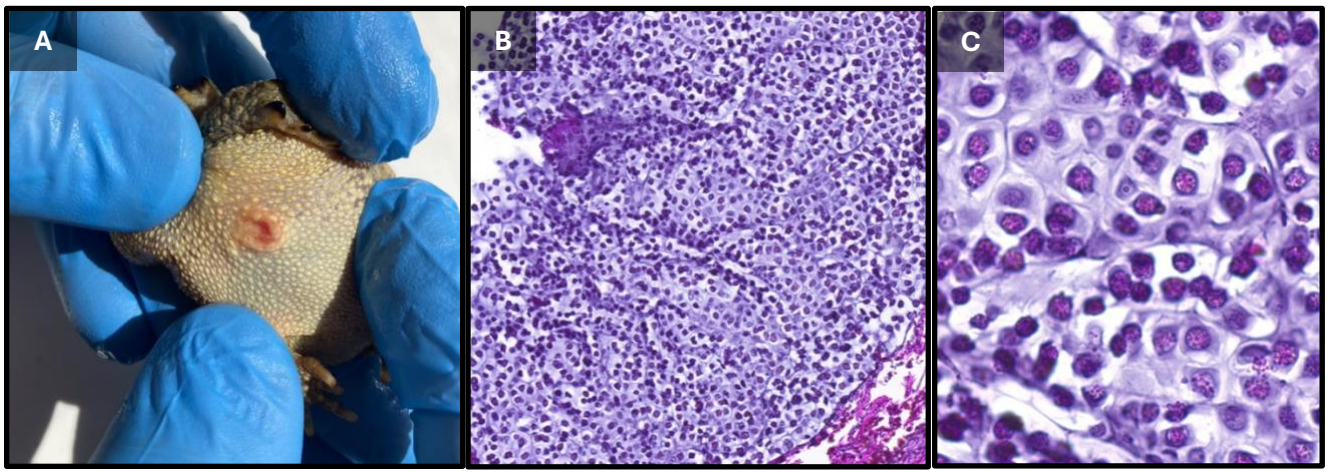

Figure 2. Lesion of an adult *Anaxyrus americanus* from Sanford, Maine (04/2024). *A)* Lesion on the ventral skin. *B)* Low magnification histopathology, hematoxylin and eosin (HE) 100x. *C)* Higher magnification of pathogen, HE 400x.

1.1 – Transmission Electron Microscopy (TEM): Transmission electron microscopy (Figure 3) revealed unicellular, extracellular organisms embedded in lamellar mildly electron-dense matrix. Organisms had electron dense walls formed by tightly apposed fibers, multiple membrane-bound organelles including nuclei, starch granules, mitochondria with short, orthogonally oriented cristae, and linearized rough endoplasmic reticulum.

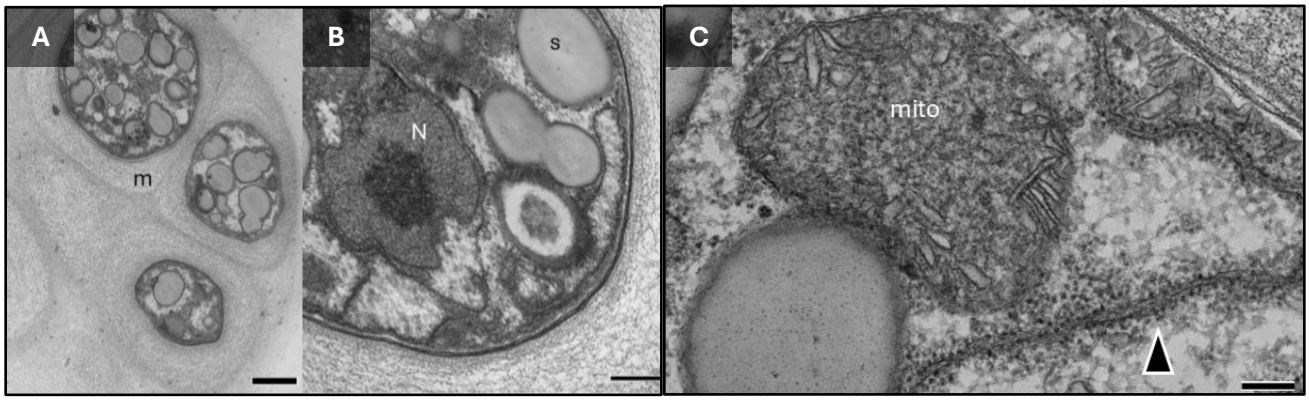

Figure 3. TEM of one lesion. *A)* 3,500x, bar = 2 µm. *B)* 18,000x, bar = 600 nm. *C)* 36,000x, bar = 200 nm. m = matrix; n = nucleus; s = starch granule; mito = mitochondria; arrowhead = rough endoplasmic reticulum.

[†] Two toads failed to recover.

1.2 – Metabarcoding & 1.3 Shotgun metagenomics: BLAST[6] 18S DNA sequence resulted in hits with ~99.7% alignment to *Amphibiothecum (Dermosporidium) penneri* (Figure 4), first morphologically described, but not sequenced, from an American toad in 1981 in southern CT[7], and genetically characterized as a single species in CT and VA in 2006[8]. We found no reports of this or similar pathogens in amphibians in NH, or in *Bufonidae* in northern New England. There was a report of a similar-looking agent in 1977-8 in toad tadpoles in Florida[9]. All pathogens from our study group were the same species, with identical 18S sequences. This finding is indicative of a single agent causing the noted lesions, but additional context in the form of comparison of more of the genomes of the pathogen would provide finer granularity to this initial observation.

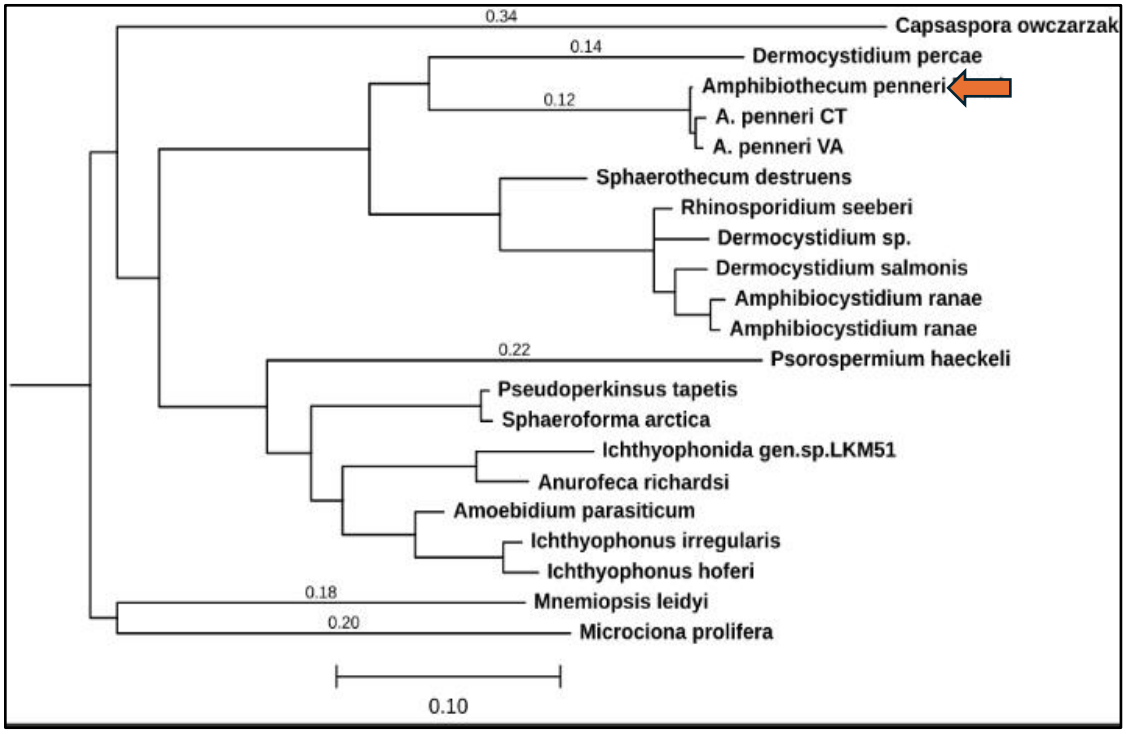

Figure 4. Phylogenetic tree including *Amphibiothecum penneri* (orange arrow) isolated from New Hampshire American toads (*Anaxyrus americanus*). 18S sequence was aligned to other *Mesomycetozoea* 18S and 2 outgroups to root the tree. Maximum likelihood phylogeny was performed with 1000 bootstrapped replicates. Scale = substitutions / site.

<u>Objective 2 – Investigate other amphibians and other portions of the watershed for presence of this pathogen</u>

2.1 – Expanded American toad surveillance in the Lamprey River watershed: We conducted periodic roadway surveys for American toads during humid or rainy nights in April, May, June, September, and October of 2024 and in April, May, and June 2025. These surveys took place on roads near the Lamprey River – lower (Pawtuckaway River to Newmarket), Little River, North River, Piscassic River, and Pawtuckaway River.

Briefly, we walked or drove slowly along roads until observing an animal. The animal was then captured by hand (gloved), inspected for lesions, and released unless needed for biopsy[‡]. Animals were not marked, which means that repeat captures (on separate nights) could theoretically inflate some values reported in Table 1. However, this is unlikely because different areas were sampled on different nights.

[‡] Roadkill animals are not included because they were often too mangled to inspect. The one freshly killed toad with obvious lesions that was recovered and used for histopathology is not included in Table 1.

In any case, we felt that the potential cost of re-encountering the same individual paled in comparison to the risk associated with marking animals (i.e., infection at PIT tag injection site or transmission).

The data in Table 1 can be misleading in many ways without further context. For example, an overall infection rate of 5.6% in the watershed seems alarmingly high. However, only a single infected animal was encountered in the watershed outside the lower Lamprey across two years of monitoring (Little River in Nottingham; 43.1202, -71.0458). In other words, the protist seems to be rare outside the lower Lamprey.

On the other hand, an infection rate of 10.8% along the lower Lamprey River might seem even more alarming. However, those infections are largely found along a high-infection (near 40%) stretch of river that runs near Bennett Road (Durham), Wiswall Road (Durham/Lee), York Lane (Lee), Little Hook Road (Lee), and Tall Pine Road (Durham), while the rest of the river seems little affected. This does not mean that the protist is not found outside the small geographic area shown in Figure 5. In fact, we were notified by colleagues of an infected toad in Harrisville, New Hampshire and one in Sanford, Maine in 2024. Why the protist seems to be thriving within the 'Wiswall Hotspot' and not elsewhere is an open question, as is the potential fate of the protist here and beyond. In the coming years, we will continue to monitor the infection rate of animals throughout the Lamprey River watershed, including expansion to the upper watershed.

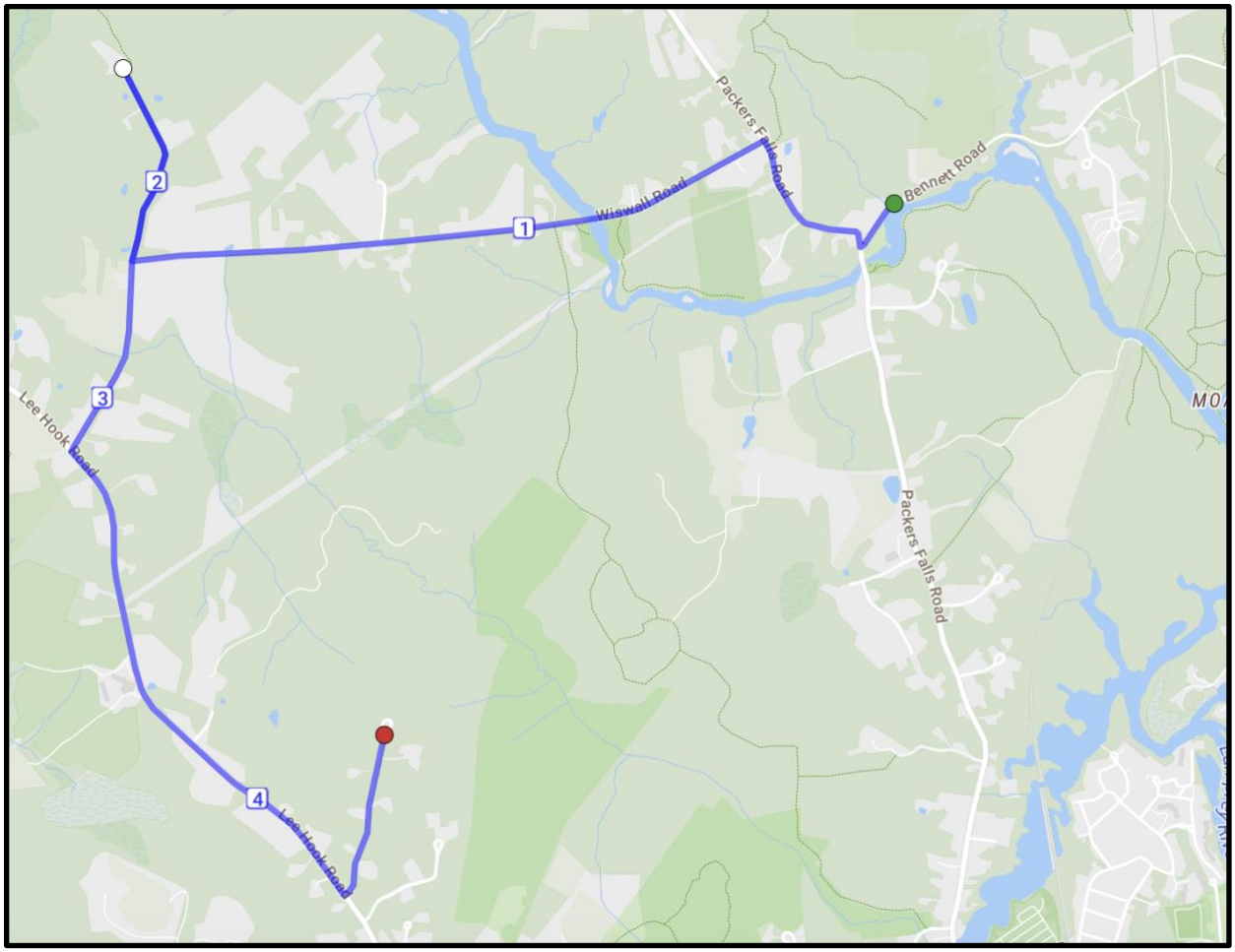

Figure 5. Map highlighting the *Amphibiothecum penneri* infection hotspot in Durham and Lee, New Hampshire.

Table 1. Number of American toad (*Anaxyrus americanus*) individuals observed in the Lamprey River watershed, by river and year and *Amphibiothecum penneri* infection status. Individuals with visible lesions are considered "infected," and those without visible lesions are considered "uninfected."

River	Number of individuals observed				Totals (Infected/Total)
	2024		2025		
	Infected	Uninfected	Infected	Uninfected	
Lamprey River - lower	12	62	13	143	25/230
Little River	1	33	0	68	1/102
North River	0	18	0	45	0/63
Piscassic River	0	7	0	48	0/55
Pawtuckaway River	0	0	0	12	0/12
Totals (Infected/Total)					26/462

2.2 – Expanded species surveillance: During the summer/fall of 2024 and the spring/summer of 2025, we did not encounter any other amphibian species (i.e., species other than *Anaxyrus americanus*) presenting with symptoms indicative of infection by *Amphibiothecum penneri* or a similar protist. Targeted periodic roadway surveys took place in the Lamprey River watershed during the months noted for objective 2.1 above (see data for the twelve species encountered in Table 2), while volunteers and collaborators in other parts of New Hampshire (e.g., Monadnock area) and Maine looked for infected animals while conducting other research or while engaged in citizen science monitoring projects (e.g., amphibian crossing brigades). While we don't have an exact number of animals surveyed by our collaborators outside the Lamprey River watershed, it was certainly well into the thousands and included additional species such as the Fowler's toad (*Anaxyrus fowleri)*. Herpetologists in the states of New Hampshire and Maine will continue to monitor for the occurrence of *Amphibiothecum penneri* in amphibians that are handled in the coming years.

Table 2. Number of individuals inspected for potential infection by *Amphibiothecum penneri* in the Lamprey River watershed, by species and year, excluding the American toad (*Anaxyrys americanus*). No animals appeared to be infected.

Species	Number of individuals observed		Total
	2024	2025	
Ambystoma maculatum (spotted salamander)	0	45	45
Ambystoma jeffersonianum x *laterale* (Jefferson x blue-spotted salamander complex)	0	4	4
Eurycea bislineata (northern two-lined salamander)	0	1	1
Hemidactylium scutatum (four-toed salamander)	1	8	9
Notophthalmus viridescens (eastern newt – eft stage)	2	11	13
Plethodon cinereus (northern redback salamander)	39	134	173
Hyla versicolor (gray tree frog)	9	36	45
Lithobates catesbeiana (bullfrog)	7	8	15
Lithobates clamitans (green frog)	11	14	25
Lithobates palustris (pickerel frog)	7	12	19
Lithobates sylvaticus (wood frog)	15	43	58
Pseudacris crucifer (spring peeper)	3	93	96
Total			503

Objective 3 – Apply to Morris Animal Foundation for additional funding

In late 2024 we applied for a $165,000 grant from the Morris Animal Foundation (an animal health charity that supports veterinary research) to support a two-year research study that would expand our understanding of the protist's ecology (i.e., where and how it lives and spreads in the environment) and the host's response (e.g., mortality rate, recrudescence rate, development of immunity). Unfortunately, our proposal [*Characterizing the Ecology, Genomics, and Host-Microbe Interactions of* Amphibiothecum penneri*, an Emerging Protist Pathogen of American Toads (*Anaxyrus americanus*) in New England*, prepared in collaboration with partners at the University of New Hampshire, the State of New Hampshire's Fish and Game Department, and the State of Maine's Department of Inland Fisheries and Wildlife] was ultimately not recommended for funding. In the future, we plan to continue to seek further financial support for this work from not-for-profit organizations and federal agencies.

Future work

We've identified the pathogen infecting American toads in New Hampshire as the protist *Amphibiothecum penneri*. This protist was identified in a small number of American toads in Connecticut nearly a half-century ago and in Virginia two decades ago. When and how the pathogen made its way to New Hampshire remains an open question, as does the protist's future impacts on our local toad populations and riverine communities. The samples from the Connecticut River watershed in southwestern NH and the Saco River watershed in ME had the same 18S sequence as those from the Lamprey River watershed. At this point, the final data we will generate is the whole genome of *Anaxyrus americanus*, and the whole genome of *Amphibiothecum penneri.* Neither of these genomes have been sequenced prior to our study, and we are in the midst of analyzing the data generated from OmniC and PacBio Sequel II sequencing of both of these specimens. These two genomes will have an immediate impact for basic and applied science, and will be foundational to further comparisons of the samples we have already collected, future infected toads, and potential investigations into the life cycle, prevalence, and infectious behavior of the protist in the natural environment of the Lamprey River watershed.

The work we've begun with LRAC's support is of immediate importance to the health and welfare of the American toad, and potentially to other amphibians and animals in the ecosystem. Severely impacted animals we describe were in decreased body condition, and while most recovered in captivity with readily available feed and after shedding cysts, the more significantly impacted animals were anorexic upon capture and their prospects for survival in the wild would likely be low. As this disease seems to be currently emerging (i.e., no earlier reports of this visually striking disease in northern New England), it is imperative that we build upon the identification of the disease's causative agent to elucidate its ecology, understand its impact on individual animal health in subsequent years, and characterize the molecular and behavioral response of the host to infection. Our team, which includes researchers in 3 academic units, veterinarians, and herpetologists from two state agencies, will continue to monitor the situation and pursue necessary funding when possible.

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