Determining the prevalence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis)* and *Ranavirus* at Long Branch Nature Center in Arlington, Virginia

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Introduction:

Emerging diseases are one of the factors responsible for population declines in both reptiles and amphibians worldwide. *Batrachochytrium dendrobatidis (Bd)* is a fungus that causes an infectious disease called chytridiomycosis. Amphibian chytrid fungus affects the epidermal cells of amphibians and causes electrolyte loss (Voyels et al., 2007), hyperkeratosis (Brem et al., 2007) and death in susceptible species (James et al., 2009). Some species, such as *Plethodon cinereus* (Red-backed Salamander), have anti-fungal bacteria on their skin that inhibit the growth of *Bd* (Brucker et al., 2008) while others, such as *Lithobates catesbeianus* (American Bullfrog), are asymptomatic carriers of this disease (Garner et al., 2006). *Lithobates catesbeianus* have been introduced in the western United States and South America (Daszak et al., 1999) and could be vectors for this deadly fungus. The spread of this highly virulent disease is causing rapid amphibian declines on several continents (Skerratt et al., 2007).

Ranavirus, a genus of Iridoviruses, is also a highly transmissible disease (Cinchar, 2002) primarily infecting amphibian species that breed in standing water (Harp and Petranka, 2006). This pathogen affects multiple amphibian hosts, both larval and adult, and may persist outside a host for several weeks or longer (Gray et al., 2009). *Ranavirus* appears as swelling in the limbs or body, erythema, and susceptible amphibians usually succumb to chronic cell death in their organs (Gray et al., 2009). Transmission of this pathogen occurs through direct contact with infected individuals, ingestion of infected tissue, and indirectly by contact with infected water or soil (Gray et al., 2009). This virus also affects reptiles and has been seen in wild populations of *Gopher polyphemus* (Gopher Tortoise) in Florida (Westhouse et al., 1996), *Chrysemys picta picta* (Eastern Painted Turtle) in Virginia (Goodman et al., 2013), and *Terrapene carolina carolina* (Eastern Box Turtle) in Tennessee (Allender et al., 2011) and Pennsylvania (Johnson et al., 2008). The effects of this disease are less clear than that of *Bd*, but infections are being identified in new populations and *Ranavirus* is more geographically widespread than previously thought.

Amphibian chytrid and Ranavirus are known to occur in Virginia (Olson, http://www.bd-maps.net/). A plethora of studies conducted in South-western Virginia show amphibian chytrid fungus and Ranavirus have been detected in a number of amphibian species such as: *Desmognathus fuscus* (Northern Dusky Salamander), *Desmognathus monticola* (Seal Salamander), *Desmognathus orestes* (Blue Ridge Dusky Salamander), *Desmognathus organi* (Northern Pygmy Salamander), *Desmognathus quadramaculatus* (Black-bellied Salamander), *Plethodon montanus* (Northern Gray-checked Salamander), *Plethodon welleri* (Weller's Salamander) (Hamed et al., 2013),

Aneides aenus (Green Salamander) (Blackburn et al., 2015), *Notophthalmus viridescens* (Eastern Red-Spotted Newt) (Bletz and Harris, 2013), *Pseudacris crucifer* (Spring Peeper), *Lithobates catesbeianus* (Bullfrog) (Hughey et al., 2014), and *Cryptobranchus alleganiensis alleganiensis* (Eastern Hellbender) (Eskew et al., 2014). However, none of the listed studies occurred in the heavily urganized Northern Virginia, and Long Branch Nature center (LBNC) has not been tested for both diseases. The goal of this study was to determine the presence or absence of amphibian chytrid fungus and Ranavirus in as many species as possible at LBNC. This information will be valuable to the park to influence land management decisions.

Methods:

Long Branch Nature Center is an urban park located in Arlington, Virginia and is home to an abundance of wildlife, herpetofauna in particular. Long Branch sees 12,000 visitors walk through their doors annually and the 6.9 hectare park joins with Glencarlyn Park for a continuous 49.4 hectares. The nature center is passionate about educating their guests and committed to preserving its wild lands. Long Branch is the only nature center in Northern Virginia that is permitted through the Virginia Department of Game and Inland Fisheries (VDGIF) to take in wild, injured reptiles for their rehabilitation program. This park has never been surveyed for emerging diseases and acknowledges the value in this type of research.

Long Branch Nature Center was surveyed nine times between August 2014 and June 2015. Swabbing protocols provided by the San Diego Global Disease Lab (Pessier, 2014) were used. Fine tip swabs and screw-top tubes were provided by the San Diego Zoo Disease Lab. For amphibian chytrid testing the protocols were as follows: The ventral surface of each amphibians' skin was swabbed for approximately 30 passes which included the pelvic patch (5 passes with the swab), ventral thighs (5 passes with the swab) and toe webbing of each foot (5 passes with the swab). For *Ranavirus* testing the protocols were as follows: The mouth of each amphibian was swabbed in a gentle circular motion including the tongue, roof and sides of the oral cavity. After both sampling techniques the swab was then individually placed and sealed in a provided screw-top tube. Each sample was labeled with species, date, and location collected. Samples were then stored in a freezer until it was time to submit all samples. All samples were sent to the San Diego Amphibian Disease Lab for analysis; both tests used TaqMan PCR assay techniques to test for amphibian chytrid fungus and *Ranavirus* respectively.

The following protocols were followed to avoid the potential spread of pathogens between individuals and sites visited: gloves were worn while handling individuals and were changed between specimens. A 1:10 solution of bleach to water was used to disinfect footwear before entering a new habitat.

Four different habitats were sampled: Poplar Pond (a man-made pond approximately three meters deep), Willow Pond (a vernal pool), Long Branch stream, and Salamander Creek (a tributary of Long Branch stream). We attempted to swab individuals from eight species of amphibian and *Terrapene carolina carolina* (Eastern Box Turtles) that occur in LBNC: *Lithobates catesbeianus* (American Bullfrog)*, Lithobates sylvaticus* (Wood Frog)*, Lithobates clamitans* (Green Frog), *Pseudacris crucifer*

(Spring Peeper), *Plethodon cinereus* (Red-backed Salamander), *Eurycea bislineata* (Northern Two-lined Salamander), *Eurycea guttolineata* (Three-lined salamander), and *Ambystoma maculatum* (Spotted Salamander). Swabbing occurred over a period of 10 months to maximize the number of species sampled. For example, species such as *A. maculatum*, *L. sylvaticus*, and *P. crucifer* breed in late winter to early spring and are not frequently encountered during other times of the year.

Results:

A total of 53 animals were swabbed, 52 amphibians and 1 *T. c. carolina*. All targeted amphibians were swabbed for both *Bd* and *Ranavirus* except *E. guttolineata* and *L. clamitans* because neither species was found in the park during sampling. A total of 25 samples were submitted for amphibian chytrid fungus analysis; all but two swabs returned negative results. Two samples were positive for chytrid fungus, both were *E. bislineata* in Salamander Creek from August 27, 2014 (Table 1). A total of 28 samples were submitted for *Ranavirus*; all were negative (Table 2).

Table 1: Results of amphibian chytrid fungus testing by species over a 10 month period at Long Branch Nature Center

Common Name	Species	Chytrid Result	Date Collected
Spotted Salamander	*Ambystoma maculatum*	Negative	7/14/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	3/27/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	4/2/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	5/26/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	5/26/2015
Two-lined Salamander	*Eurycea bislineata*	Negative	7/14/2015
Two-lined Salamander	*Eurycea bislineata*	Negative	8/27/2014
Two-lined Salamander	*Eurycea bislineata*	Positive	8/27/2014
Two-lined Salamander	*Eurycea bislineata*	Positive	8/27/2014
Two-lined Salamander	*Eurycea bislineata*	Negative	8/27/2014
American Bullfrog	*Lithobates catesbeianus*	Negative	7/14/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	5/26/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015

Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Spring Peeper	*Pseudacris crucifer*	Negative	3/17/2015
Spring Peeper	*Pseudacris crucifer*	Negative	3/17/2015
Spring Peeper	*Pseudacris crucifer*	Negative	3/27/2015
Spring Peeper	*Pseudacris crucifer*	Negative	4/2/2015
Spring Peeper	*Pseudacris crucifer*	Negative	4/2/2015

Table 2: Results of *Ranavirus* testing by species over a 10 month period at Long Branch Nature Center

Common Name	Species	*Ranavirus* Result	Date Collected
Spotted Salamander	*Ambystoma maculatum*	Negative	3/27/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	5/26/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	5/26/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	3/17/2015
Spotted Salamander	*Ambystoma maculatum*	Negative	10/1/2014
Spotted Salamander	*Ambystoma maculatum*	Negative	10/1/2014
Two-lined Salamander	*Eurycea bislineata*	Negative	10/1/2014
Two-lined Salamander	*Eurycea bislineata*	Negative	10/1/2014
American Bullfrog	*Lithobates catesbeianus*	Negative	7/14/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	7/14/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	5/26/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	5/26/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	5/26/2015
American Bullfrog	*Lithobates catesbeianus*	Negative	10/1/2014
American Bullfrog	*Lithobates catesbeianus*	Negative	10/1/2014
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Wood Frog	*Lithobates sylvaticus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Red-backed Salamander	*Plethodon cinereus*	Negative	3/17/2015
Spring Peeper	*Pseudacris crucifer*	Negative	3/27/2015
Spring Peeper	*Pseudacris crucifer*	Negative	4/2/2015

Spring Peeper	*Pseudacris crucifer*	Negative	4/2/2015
Spring Peeper	*Pseudacris crucifer*	Negative	4/2/2015
Eastern Box Turtle	*Terrapene carolina carolina*	Negative	8/27/2014

Discussion:

This study demonstrates the importance of conducting disease screening at a local level. Having baseline data on the presence and absence of emerging infectious diseases is an important conservation measure. Although our results failed to detect *Ranavirus* and only detected chytrid fungus in two individuals, it is best to take precautions that minimize disease transmission. The two chytrid-positive *E. bislineata* appeared to be healthy and did not exhibit any outward signs of chytrid infection such as ventral redness, poor righting ability, or abnormal body postures (Pessier and Mendelson, 2010). It is worth noting that both individuals were from Salamander Creek, a headwater stream. Past studies have indicated that *Bd* was less likely to be found in headwater streams. *Batrachochytrium dendrobatidis* was detected in less than 1% of individuals sampled in headwater streams and when combined with other studies found that *Bd* had a prevalence of 3% (Hossack et al., 2010). However, it should be noted that of the 3% of individuals that were found to be chytrid positive from the combined studies, 67% of them were *E. bislineata* and *E. cirrigera* (Northern and Southern Two-Lined Salamanders). Furthermore, changing sampling techniques could increase the scope of the results; for instance, assessing amphibian chytrid fungus zoospore loads similar to the study Hossack (2010) completed. Perhaps *E. bislineata* have bacterial flora that inhibit the growth of chytrid, such as those Brucker observed in *P. cinereus* (2008). Conversely, if these individuals had a detectable zoospore count that could tell us they probably do not have a bacteria or peptide limiting the growth of chytrid on their skin. Also, they may serve as asymptomatic carriers of this disease.

Ranavirus was not detected during this study. This could be a result of the sampling methods used, as swabbing for the disease has a 22% false-negative and 12% false-positive rate when compared to other methods such as tail-clip sampling or necropsy of deceased animals (Gray et al., 2009). Oral swabbing was selected for this study because it is still a reliable way to detect *Ranavirus* and is the least invasive method compared to the aforementioned (Goodman et al., 2013). The results from this study will contribute to a larger study being conducted by Smithsonian scientists and the Virginia Department of Game and Inland Fisheries on *L. sylvaticus* tadpoles at LBNC as well as sites across the state of Virginia.

Although there was no to low presences of *Ranavirus* and amphibian chytrid fungus respectively, steps should be taken to minimize disease transmission at LBNC. Continued monitoring of these diseases in LBNC would help track the prevalence over time. The distribution and spread of these diseases is an important aspect of disease ecology and can aid in future studies and preventative methods. Protocols to reduce the spread of chytrid fungus and *Ranavirus* should also be followed. Similar to the protocols used in this study, equipment should be disinfected between sites and staff should educate visitors about the importance of disinfecting hiking equipment to prevent the spread of diseases within and outside the park. In the future, continued disease screening of both the wild animals and the captive specimens kept at LBNC would contribute to the overall knowledge of these two highly virulent diseases.

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