Detecting the Extent of Mortality Events from *Ranavirus* in Amphibians of the Northeastern U.S.

Report to the Northeast Association of Fish and Wildlife Agencies (NEAFWA) for Regional Conservation Needs (RCN) Grant #2012-01

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EXECUTIVE SUMMARY

In order to better understand the extent to which *Ranavirus* is impacting amphibian and reptile populations in the Northeast region of the U.S. and to develop and test a sampling protocol that could be used throughout the region, we conducted a survey of amphibian larvae at 122 randomly-selected wood frog (*Lithobates sylvaticus*) breeding ponds in a 142,286 km^2 study area encompassing parts of Delaware, Maryland, New Jersey, Pennsylvania, and Virginia. In 2013 and 2014, a total of 4,306 individual wood frog larvae (30 larvae per pond) were collected for quantitative PCR (qPCR) analysis by Montclair State University (New Jersey). Additionally, 158 individuals of seven amphibian species potentially involved in active die-offs were collected for analysis by the U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC). This study represents both the largest geographic area and the greatest sample size ever screened for *Ranavirus*.

Of the 122 ponds sampled, 36 ponds (29.5%) had at least one sample test positive for *Ranavirus* over the two-year study period. This resulted in 13 new county occurrence records for *Ranavirus* including all three counties in Delaware. Of the 30 ponds sampled in both years, 14 ponds (46.7%) tested positive in both years, suggesting the virus is persisting at these sites. This study was the first recorded incidence of a *Ranavirus* die-off in eastern spadefoot (*Scaphiopus holbrookii*) larvae. Lab results indicate that besides Frog Virus 3, some study ponds may have a genetic variant (different strain) of FV3. In ponds that tested positive in 2013, *Ranavirus* was detected in the first 10 samples for 83% of the ponds and within the first 20 samples for 100% of the ponds. Of the ponds that tested positive in 2014, *Ranavirus* was detected in the first 10 samples for 91% of the ponds and within the first 20 samples for 100% of the ponds. These findings suggest it is possible to detect *Ranavirus* in as little as 10 samples; however, 20 appears to be the optimal sample size for *Ranavirus* detection. A considerable number of ponds in 2013 and 2014 contained individuals that showed an exponential increase in fluorescence during qPCR but did not show the same melting temperature from the melting curve as the positive control. We refer to these individuals and populations as false positives; they underscore the importance of using exponential increase in fluorescence as well as melting temperature to confirm *Ranavirus* infections in individuals. The discovery of a high level of false positives was an important and unexpected outcome of this study, especially as so many current RT-PCR studies of *Ranavirus* use only exponential increase in fluorescence to determine *Ranavirus* infection.

Biologists from state natural resource agencies for all 13 northeastern U.S. states and the District of Columbia (D.C.) completed a questionnaire designed to determine if they were aware of lab-confirmed *Ranavirus* in their state, counties where it had been confirmed, presence or lack of a state wildlife veterinarian or similar in the state, a principal contact person for sick herpetofauna, and the process or lack thereof for both public reporting of sick herpetofauna and transporting animals to diagnostic laboratories. The questionnaire was augmented by diagnostic

reports from the NWHC and other labs, and published scientific literature on lab-confirmed *Ranavirus* in the northeastern U.S. *Ranavirus* has been lab-confirmed in 33 herpetofaunal species in at least 64 counties in the Northeast region. It is most commonly found in wood frog larvae (59 counties), eastern box turtles (*Terrapene c. carolina*) (20 counties), and the larvae of spotted salamanders (*Ambystoma maculatum*) (20 counties), green frogs (*L. clamitans*) (19 counties), and American bullfrogs (*L. catesbianus*; 14 counties). Half of the northeastern states have a state wildlife veterinarian, and 6 of 14 respondents also use other veterinarian resources. Only 3 states have a disease reporting process and only one state has online wildlife disease reporting. The majority of states (11 of 14) make use of the diagnostic services of the NWHC, but many northeastern states are also members of the Northeast Wildlife Disease Cooperative, while a few are members of the Southeast Wildlife Disease Cooperative. Scientists and conservation groups are now addressing the question of how to respond to the threat posed by *Ranavirus*. Disinfection protocols should become standard operating procedures for all land management agencies as they work with groups like Partners in Amphibian and Reptile Conservation to develop strategies to address the threat of emerging diseases.

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INTRODUCTION

Emerging infectious diseases are one of the most important factors contributing to global amphibian declines and have been implicated in local extinctions of several species (Daszak et al. 1999). Chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has received considerable and well-deserved attention over the last decade, as this fungus has been implicated in the decline and even the extinction of numerous species of amphibians (e.g., Lips et al. 2006). However, reports of significant mortality due to outbreaks of *Ranavirus* (Family Iridoviridae) are becoming increasingly common in the United States with the reported number of die-offs attributed to *Ranavirus* three to four times greater than those attributed to *Bd* (Green et al. 2002; Russell et al. 2011). *Ranaviruses* are considered a global threat to amphibian populations based on the lack of host specificity, high virulence, and global distribution (Daszak et al. 1999; Johnson et al. 2008). *Ranavirus* also differs from *Bd* in that the virus is known to affect both amphibians and reptiles.

Unfortunately, information on the timing, extent, and frequency of occurrence of *Ranavirus* outbreaks remain limited, partially due to lack of surveillance and partially due to the rapid onset and mortality caused by the disease. This is especially true for amphibian larvae; in many cases, only a few days elapse between the initial signs of the disease and the disappearance of tadpoles or larvae from the environment. Thus, unless observations are focused on detecting the outbreak of the disease, it would be easy to conclude that absence of tadpoles or larvae was the result of a rapid metamorphosis, instead of mass mortality from a disease outbreak (Harp and Petranka 2006; Petranka et al. 2007; Russell et al. 2011).

Green et al. (2009) recommended that states develop an amphibian disease surveillance process. Northeast Partners in Amphibian and Reptile Conservation (NEPARC) considered *Ranavirus* a major threat to northeastern herpetofauna at their 2011 annual meeting, such that NEPARC's Emerging Diseases Working Group formed at that meeting has begun specific conservation activities directed at this disease.

In order to better understand the extent to which *Ranavirus* is impacting amphibian and reptile populations in the Northeast region of the U.S. and to develop and test a sampling protocol that could be used throughout the region, we conducted a survey of amphibian larvae at a number of wood frog (*Lithobates sylvaticus*) breeding ponds in Delaware, Maryland, New Jersey, Pennsylvania, and Virginia. Wood frogs have the highest mortality and infection rates of northeastern amphibians and their breeding ponds (primarily vernal pools) may be the main source of the disease for other affected species (Harp and Petranka 2006; Gahl and Calhoun 2010; Haislip et al. 2011; Hoverman et al. 2011). Symptoms of ranaviral infection typically do not occur until a minimum of 1.5 – 2 months post-hatching (Petranka et al. 2007) at Gosner developmental stage 27 or greater (Gosner 1960; Warne et al. 2011). Mortality rates are 50-99% in the larval life stage compared to low mortality rates in adults (Harp and Petranka 2006; Green et al. 2009; Hoverman et al. 2011), thus sampling individuals in the larval stage increases the probability of detecting the disease. Our approach involved sampling 20-30 ponds per state over a two-year period, with samples spread over different watersheds and physiographic provinces to test the applicability of these methods to a diversity of regional conditions.

STUDY GOALS AND OBJECTIVES

The goals of this project were developed by the project Steering Committee with input from the NEAFWA Fish & Wildlife Diversity Technical Committee. Our study goals were to better understand the geographic distribution of *Ranavirus* in the northeastern U.S., as well as its potential effects on amphibian and reptile populations, and to develop and test a sampling protocol that could be used throughout the Northeast region. We accomplished these goals through the following objectives:

1. In consultation with the U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC), state wildlife health labs, state and federal fish and wildlife agencies, universities, local experts, and scientific literature, identify locations where *Ranavirus* had been confirmed or suspected in Delaware, Maryland, New Jersey, Pennsylvania, and Virginia.
2. In consultation with state and federal fish and wildlife agencies, universities, conservation and herpetological organizations, and local experts, identify locations of wood frog breeding ponds in the five study states, choose a random subset on public and private conservation lands to survey, gain permission to survey them, and secure state scientific collecting and endangered species permits to collect animals from these sites.
3. In consultation with state and federal fish and wildlife agencies, universities, and local experts, identify at least one qualified local individual in each state to be used as a seasonal technician for the field sampling portion of this study.
4. Adaptively manage a sampling protocol based on logistical challenges and input from field personnel.
5. In consultation with NWHC and Montclair State University (MSU) develop an efficient system for rapid shipment of animal samples to their respective labs for *Ranavirus* detection analysis.
6. Review pathology results from the NWHC (David Green) and MSU (Kirsten Monsen-Collar).
7. Prepare quarterly and final reports of our findings, including maps of study areas and sites of past and current *Ranavirus* outbreaks in the five-state study region.
8. Based on a questionnaire sent to states, review of scientific literature and consultation with the National Wildlife Health Center, develop a summary of all known and suspected *Ranavirus* events in the 13 northeastern states and determine which states are actively sampling for this disease.
9. Present study findings at regional and national professional scientific meetings and on appropriate websites.
10. Publish results in peer-reviewed scientific journals, with focus on disseminating recommended sampling protocol to be used throughout the region.
11. Disseminate results via website, such as the Global Ranavirus Consortium, to reach a broader audience.

The Pre-Study Landscape: *Ranavirus* Occurrence in the Five-State Study Area

The NWHC, state wildlife health labs, state and federal fish and wildlife agencies, universities, conservation and herpetological organizations, local experts, and the scientific literature were queried for records of lab-confirmed occurrences of *Ranavirus* prior to 2013 in the five-state study area. In most cases location information was only available at the county level so this report will present location information by county. Prior to 2013, *Ranavirus* had been lab-confirmed in five Maryland counties, one New Jersey county, four Pennsylvania counties, six Virginia counties, and the city of Virginia Beach (Table 1, Fig. 1). There were no lab-confirmed occurrences of *Ranavirus* in Delaware prior to this study. *Ranavirus* was reported in 25 herpetofaunal species in the study area with 15 of those species only reported from Virginia (Table 1), and the majority of these reports in the montane region of southwestern Virginia (Fig. 1; Hamed 2013, Hamed et al. 2013).

Table 1. History of lab-confirmed *Ranavirus* prior to 2013 in the five-state study area.

STATE	COUNTY	YEAR	SPECIES[a]	SOURCE(S)
MD	Prince George's	2001	AMMA, LICL, LICA	NWHC diagnostic lab
MD	Prince George's	2002	LICL, LISY, PSCR	NWHC diagnostic lab
MD	Prince George's	2003	LICL	NWHC diagnostic lab
MD	Montgomery	2005	AMMA, HYCH, LISY	NWHC diagnostic lab
MD	Montgomery	2008	TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2009	TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2010	AMMA, LISY, TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2011	TECA	NWHC, Farnsworth & Seigel 2013
MD	Anne Arundel	2008	TECA	NWHC diagnostic lab
MD	Frederick	2011	LICL	NWHC diagnostic lab
MD	Harford	2012	TECA	NWHC diagnostic lab
NJ	Ocean	2011	ANFO, LICL	Monsen-Collar et al. 2013
PA	Venango	2003	TECA	Johnson et al. 2008
PA	Northampton	2007	NOVI	Glenney et al. 2010
PA	Centre	2009	CHSE	NWHC diagnostic lab
PA	York	2012	AMMA	NWHC diagnostic lab
VA	City of Virginia Beach	2003	LISP	NWHC diagnostic lab
VA	Dickenson	2008/09	ANAE	Hamed 2013
VA	Grayson	2008/09	DEFU, DEMO, DEQU, DEOR, DEOG, PLMO, PLWE	Hamed et al. 2013, Hamed 2013
VA	Smyth	2008/09		
VA	Washington	2008/09		
VA	Wise	2010	DEFU, DEMO, DEQU, EUCI, EULO, EULU, LICA, LIPA, NOVI, PLGL	Davidson & Chambers 2011
VA	Prince Edward	2010	CHPI	Goodman et al. 2013

[a]see Appendix 1 for species acronyms

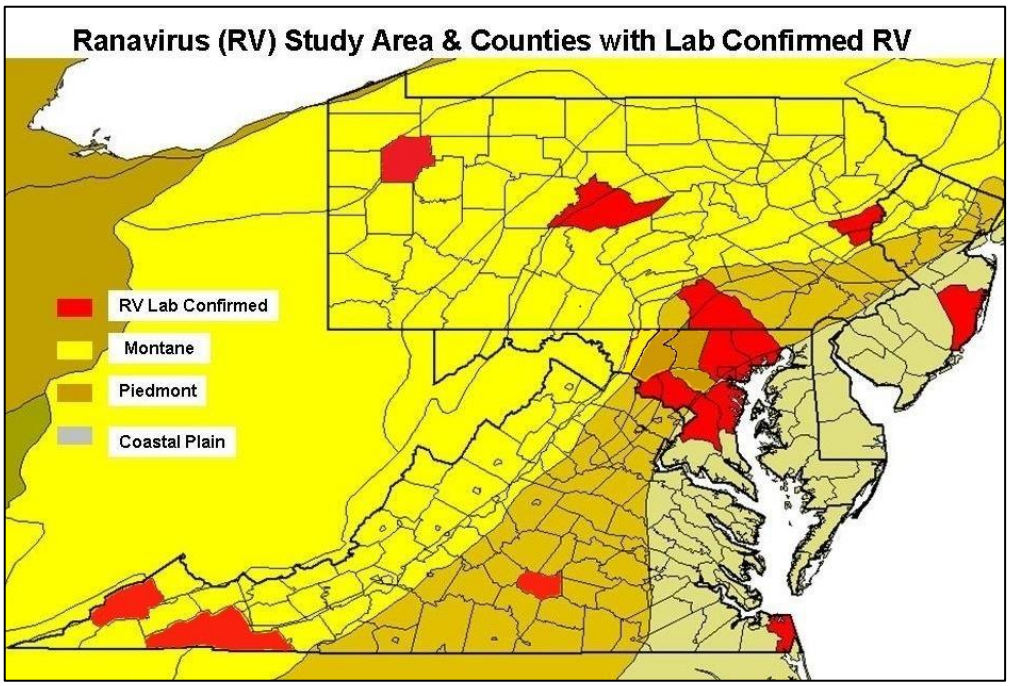

Figure 1. Counties with lab-confirmed *Ranavirus* prior to 2013 in the five-state study area.

METHODS

Study Pond Selection

The NWHC, state wildlife health labs, state and federal fish and wildlife agencies, universities, conservation and herpetological organizations, and local experts were contacted to obtain exact locations of wood frog breeding ponds in each state. In states where wood frog breeding pond location data was limited (primarily Delaware) frog chorus surveys were conducted to locate breeding sites. A database was developed for each state, and every breeding pond was given a unique sequential number to be used for random pond selection (the random selection was performed using the *Random* function in Microsoft Excel 2010). Study ponds had to meet several selection criteria, as follows. Ponds had to be at least 3 km apart to ensure independence (Berven and Grudzien 1990), have at least five wood frog egg masses (based on a field survey) to ensure an adequate source of larvae to sample, and be located on public or private conservation lands to allow the possibility of developing a conservation response to disease (Langwig et al. 2015). A random subsample of 30 primary ponds and 30 substitute ponds were selected for monitoring in each state to attempt to result in 20-30 ponds sampled per state, knowing that some ponds would fail to reach minimum criteria (at least five egg masses) or be found unusable later in the field season (e.g., total hatching failure, egg mass loss, or pond dry-up before metamorphosis). Ponds were selected to ensure representation of a diversity of physiographic provinces representative of the entire five-state region, and subdivided into three groups (Fig. 1): Coastal Plain, Piedmont, and Montane regions (the latter being a combination of Valley and Ridge, Blue Ridge, and Appalachian Plateau physiographic regions). Each state's technicians were given two years (2013 and 2014) to survey their ponds and collect a sample for *Ranavirus* testing from each pond, for a potential of 100-150 study ponds sampled over the two-year study. Delaware, Maryland, and New Jersey were able to mobilize study teams in time to collect data during the 2013 field season, while Pennsylvania and Virginia only collected data during 2014.

Sampling at Study Ponds

Starting in May 2013, a total of 30 wood frog larvae at Gosner stage 27 (Petranka et al. 2007) through metamorphosis (65-130 days post-hatching) were sampled by dip-net at each of the 20-30 study ponds in each state for qPCR analysis for *Ranavirus* at Montclair State University (MSU). This was called the ***Standard Sample***. Captured wood frog larvae were placed in a water-filled bucket after each sweep. Larvae of all species present were visually examined for indications of ranaviral infection (erratic swimming, reddening of ventral skin, especially around the base of the hind limbs and the vent opening, bloated abdomen). Larvae handling techniques that limited cross contamination of samples were used based on recommendations of experts (M. Gray pers. comm.) and are presented in detail in the Field Protocol document (Patterson and Smith 2013) in Appendix 2.

If *Ranavirus* symptoms were present, an additional sample of 10 larvae of each potentially affected species was collected for shipment to NWHC for full pathological screening (necropsies, histology of major organs, and viral, fungal and bacterial cultures where appropriate) regardless of Gosner stage, following procedures recommended in Green et al. (2009). This was called the ***Die-Off Sample***.

All amphibian larvae were euthanized in benzocaine hydrochloride water baths, regardless of sample type. This method of euthanasia is approved for amphibians by the American Veterinary Medical Association Panel on Euthanasia (AVMA 2013, p. 77). It was also approved specifically for this study through Institutional Animal Care and Use Committee (IACUC) review by the National Park Service and Smithsonian Conservation Biology Institute. Samples were organized, secured and labeled following either MSU or NWHC protocols depending on type of sample (*Standard* vs. *Die-off*), and all specimens were refrigerated (individually in 70% ethanol) or frozen and then shipped to the Department of Biology and Molecular Biology at MSU (Montclair, NJ) or to the USGS NWHC (Madison, WI), respectively. See Appendix 2 for shipping specifics.

All boots, equipment, and dip-nets were disinfected between sites in a 10% bleach solution to ensure no disease transmission between study sites following disinfection guidelines modified from Miller and Gray (2009). In 2014, an attempt was made to sample all study ponds where potential ranaviral infection was detected in 2013.

Laboratory Procedures

Standard Samples - MSU Lab

Necropsies were performed on all larval wood frogs with liver and kidneys removed for DNA extraction, as these are the organs most indicative of *Ranavirus* infection in individual specimens (Robert et al. 2007, 2011; Gray et al. 2012). Total genomic DNA was extracted from liver and kidney tissue and a quantitative real-time polymerase chain reaction (qPCR) screen was used with *Ranavirus*-specific primers targeted to amplify a portion of the *Ranavirus* major capsid protein gene for each sample. To be considered positive for *Ranavirus* DNA a sample needed to meet the following criteria:

1) Had an exponential increase in fluorescence during the qPCR (expected if double-stranded target DNA is amplified; Fig. 2).

2) Had a melting temperature within 2º C of the positive control run on the same RT-PCR plate (determined by a melting curve; Fig. 3).

3) The melting temperature peak had to be the prominent peak in the melting curve. Melting temperature is a function of the length and base pair composition of a DNA fragment and it was used to test the specificity of the product being amplified in the reaction, following Holopainen et al. (2011; Fig. 3).

4) A sample had to be positive on two separate independent plate runs on the qPCR.

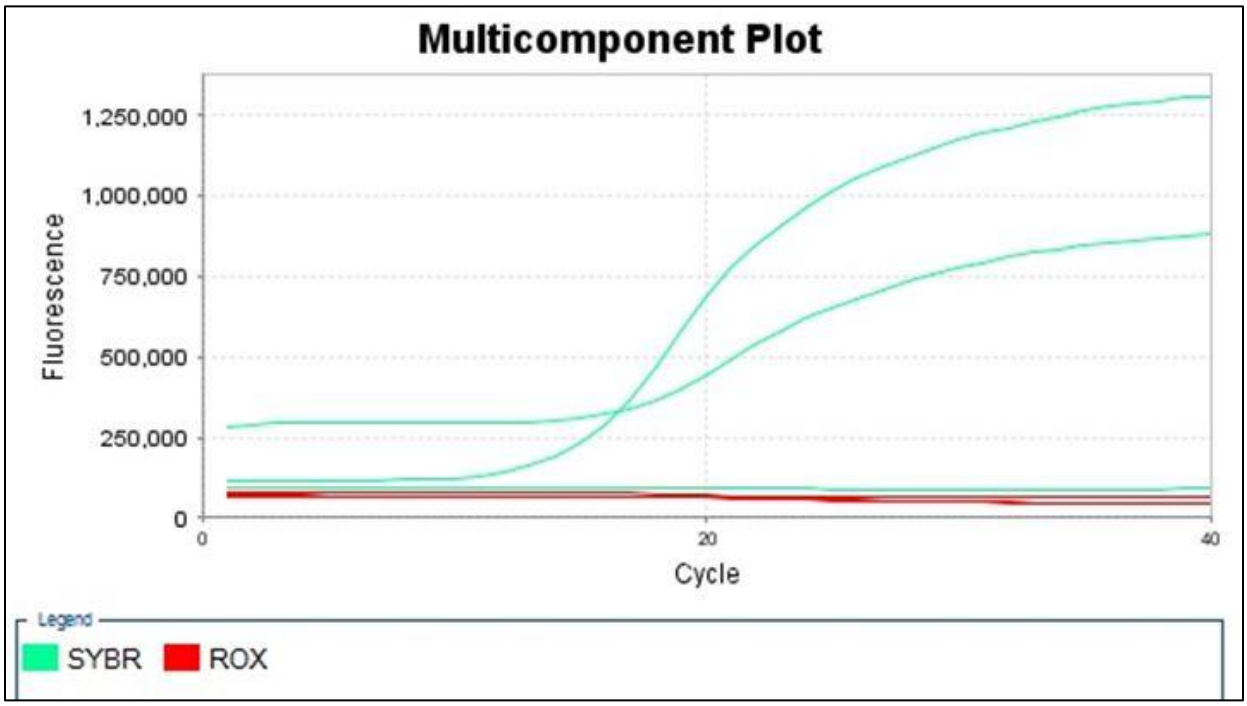

Figure 2. Positive fluorescence during qPCR. The top exponential blue curve is from a positive sample, the bottom exponential blue curve is the positive control, the bottom blue line is the negative control, and the red line is the fluorescence control (should remain straight line).

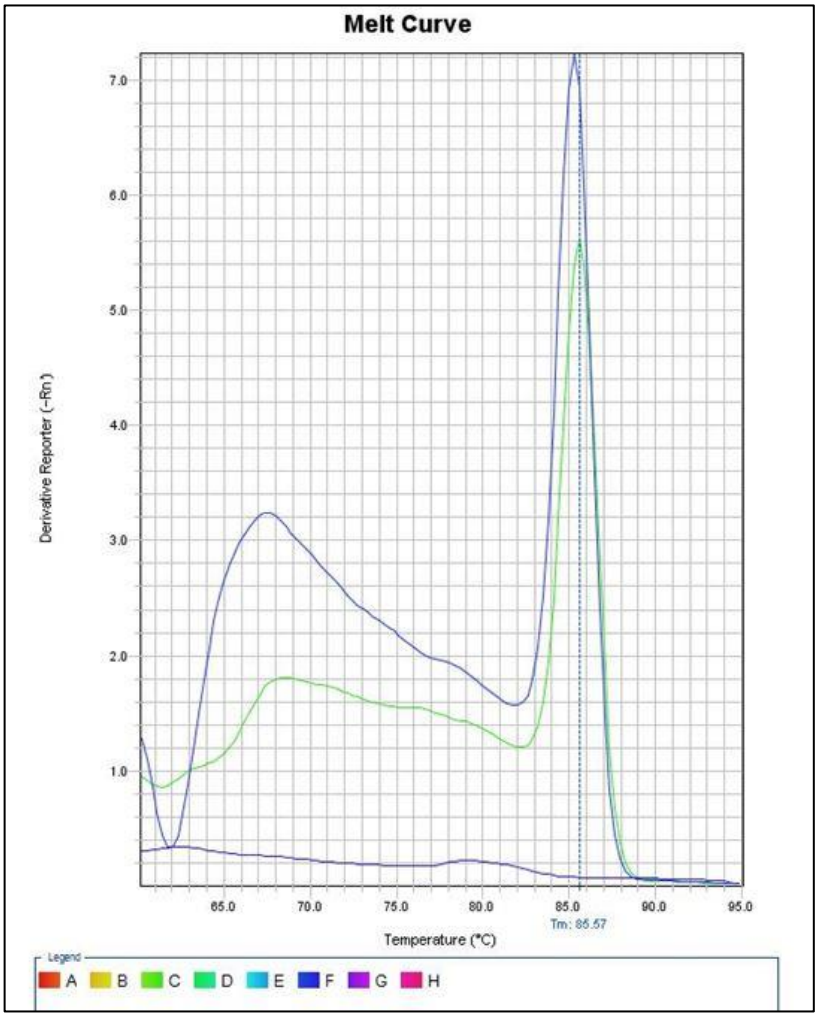

Figure 3. Positive melting curve during qPR. The top blue melt curve is from a positive sample, the middle green melt curve is from a positive control, and the bottom blue melt curve is from a negative control.

Die-off Samples – NWHC Lab

Necropsies were performed on a subsample of submitted specimens by species from each site. Full pathological screening was performed, including histology of major organs and viral cultures (also fungal and bacterial cultures where appropriate) following procedures in Green et al. (2009). Standard PCR was performed on cultures testing positive to determine type of *Ranavirus* (Frog Virus 3, etc.).

Questionnaire to Northeastern States and Summary of Ranavirus Events

A questionnaire (Appendix 3) was developed and sent to the NEPARC contact for the 13 northeastern states and the District of Columbia (comprising U.S. Fish and Wildlife Service Region 5). The questionnaire was designed to determine if state natural resources biologists with legal authority for reptiles and amphibians were aware of lab-confirmed *Ranavirus* in their state, counties where it had been confirmed, if their state had a state wildlife veterinarian or someone in a similar role, if there was a single contact person for sick herpetofauna, and if there was a process in place for both public reporting of sick herpetofauna and getting animals to diagnostic laboratories. The questionnaire was augmented by diagnostic reports from the NWHC and other labs, and published scientific literature on lab-confirmed *Ranavirus* in the northeastern U.S.

2013 RESULTS

Sampling at Study Ponds

A total of 65 ponds were sampled in 2013 (Delaware: 22 ponds; Maryland: 22; New Jersey: 21; Table 2, Fig. 4). This number was less than predicted because many potential study ponds dried up before Gosner stage 27 was reached, and a few had all wood frog larvae disappear before sampling could be conducted. Standard samples were collected at 54 ponds, die-off samples were collected at 5 ponds, and both standard samples and die-off samples were collected at 6 ponds. This latter allowed for comparison of results between different diagnostic laboratories. Total die-off samples were 2 ponds in Delaware, 6 ponds in Maryland, and 3 ponds in New Jersey.

Standard samples were collected from May 16–June 25 in Delaware, May 13–June 11 in Maryland, and May 29–June 25 in New Jersey. Gosner stage of wood frog larvae collected for standard samples ranged from stage 28–44 (mean=37.0±2.3 s.d.) in Delaware, stage 31–44 (mean=38.5±2.7 s.d.) in Maryland, and stage 26–46 (mean=37.6±4.1 s.d.) in New Jersey. Die-off samples were collected on May 3 and May 30 in Delaware, May 15–June 17 in Maryland, and June 5–July 2 in New Jersey. Gosner stages were not recorded for die-off samples.

Table 2. 2013 study ponds and types of samples collected in Delaware, Maryland, and New Jersey.

State	# Ponds with Standard Sample Only	# Ponds with Die-Off Sample Only	# Ponds with Both Sample Types	Total # of Study Ponds
DE	20	1	1	22
MD	16	3	3	22
NJ	18	1	2	21
Totals	54	5	6	65

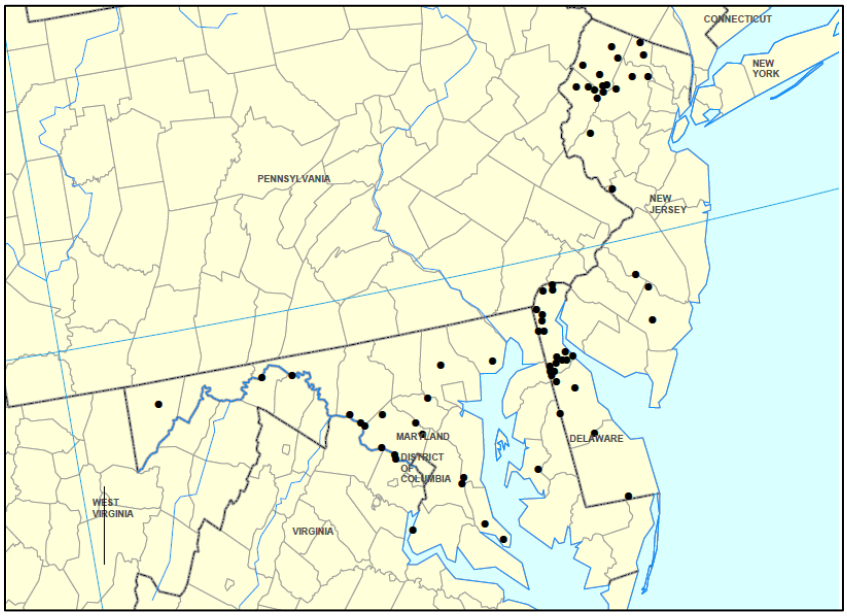

Figure 4. Study ponds in Delaware, Maryland and New Jersey in 2013 (n=65).

MSU Lab Results for 2013 Standard Samples
In 2013 the MSU lab screened 21 ponds from Delaware (630 individuals), 19 ponds from Maryland (568 individuals), and 20 ponds from New Jersey (600 individuals) for a total of 60 ponds and 1,798 individuals (only 28 specimens survived to sample collection at one Maryland pond, MD-GA-01). In Delaware 12 ponds (80 individuals) tested positive for *Ranavirus* (Table 3), in Maryland one pond (5 individuals) tested positive (Table 4), and in New Jersey 11 ponds (105 individuals) tested positive (Table 5).

NWHC Lab Results for 2013 Die-Off Samples
In 2013 the NWHC lab received die-off samples from 11 ponds, of which 9 tested positive for *Ranavirus* (DE: 2 ponds, 11 positive RV of 44 individuals, Table 3; MD: 6 ponds, 19 positive RV of 35 individuals, Table 4; NJ: 1 pond, 5 positive RV of 11 individuals). Six of the 11 ponds were solely wood frog larvae die-offs (MD: 4 ponds, 8 positive RV of 11 individuals, Table 4; NJ: 2 ponds, 5 positive RV of 11 individuals, Table 5), but the other five ponds were multiple species or a single species other than wood frog. This included ponds with both wood frog (9 positive RV of 26 individuals) and spring peeper larvae (*Pseudacris crucifer*; 2 positive RV of 18 individuals; 2 ponds) in Delaware (Table 3); wood frog (4/16 individuals) and spotted salamander larvae (*Ambystoma maculatum*; 1/1 individual; 1 pond) in Maryland (Table 4); spotted salamander (4/5 individuals) and eastern spadefoot larvae (*Scaphiopus holbrookii*; 2/4 individuals; 1 pond) in Maryland (Table 4); and just spotted salamander larvae (0/1 individual; 1 pond) in New Jersey (Table 5). Only one of the die-off samples collected in New Jersey tested positive for *Ranavirus* (of 3 ponds), while all die-off samples in Delaware and Maryland had at least one individual of each species test positive. In addition to wood frog, species that tested positive were spring peeper, spotted salamander, and eastern spadefoot. All die-off samples were larvae and all were determined to be Frog Virus 3 (FV3) by the NWHC lab.

Summary results for all ponds that tested positive for *Ranavirus* in 2013 from either the MSU or the NWHC labs (Fig. 5) were:

- **Delaware** – 13 of 22 ponds (59.1%) with a mean of 6.5±0.79 (95% CI) RV-positive individuals/RV pond.
- **Maryland –** 6 of 22 ponds (27.3%) with a mean of 3.4±0.37 (95% CI) RV-positive individuals/RV pond.
- **New Jersey –** 12 of 21 ponds (57.1%) with a mean of 9.2±4.77 (95% CI) RV-positive individuals/RV pond.
- **Total –** 31 of 65 ponds (47.7%) with a mean of 7.23±2.64 (95% CI) RV-positive individuals/RV pond.

All three Delaware counties (Kent, New Castle, Sussex) had ponds that tested positive for *Ranavirus*; these were all new county records. Maryland had 6 counties with ponds that tested positive with 3 new county records (Baltimore, Howard, and Talbot). Harford, Frederick, and Montgomery counties had previous records for *Ranavirus* (Table 1). New Jersey had 4 counties with ponds that tested positive; all were new county records (Morris, Passaic, Sussex, and Warren). The eastern spadefoot die-off in Maryland (at pond MD-TA-01 on June 17) was the first record of *Ranavirus* in that species.

Physiographic Region Results in 2013

During the 2013 field season 28 study ponds were located on the Coastal Plain (Table 6), 18 were on the Piedmont, and 19 were in Montane areas. All physiographic regions had over 30% of study ponds test positive for *Ranavirus* with Montane having the greatest frequency (63.2%), though that was biased by the large proportion of New Jersey ponds located in 5 Montane counties.

Table 3. 2013 *Ranavirus* (RV) lab results from MSU & NWHC - Delaware

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
DE-KT-01	0	30	Negative[a]			
DE-KT-02	2	28	Positive			
DE-KT-03	4	26	Positive			
DE-NC-01	2	28	Positive			
DE-NC-02	No Standard Sample Collected			5 LISY	12 LISY	Positive
DE-NC-02	No Standard Sample Collected			1 PSCR	7 PSCR	Positive
DE-NC-03	0	30	Negative[a]			
DE-NC-04	2	28	Positive			
DE-NC-06	0	30	Negative[a]			
DE-NC-07	3	27	Positive			
DE-NC-08	16	14	Positive			
DE-NC-09	3	27	Positive			
DE-NC-10	3	27	Positive			
DE-NC-11	25	5	Positive	4 LISY	5 LISY	Positive
DE-NC-11				1 PSCR	9 PSCR	Positive
DE-NC-12	0	30	Negative[a]			
DE-NC-13	0	30	Negative[a]			
DE-NC-14	6	24	Positive			
DE-NC-15	5	25	Positive			
DE-NC-16	0	30	Negative			
DE-NC-17	0	30	Negative[a]			
DE-NC-18	0	30	Negative[a]			
DE-SX-01	9	21	Positive			
DE-SX-02	0	30	Negative[a]			
Totals	**80/630 individuals**		**12/21 ponds**	**9/26 LISY 2/18 PSCR**		**2/2 ponds**

[a] Positive fluorescence but failed melting curve test.
LISY=Wood Frog (*Lithobates sylvaticus*)
PSCR=Spring Peeper (*Pseudacris crucifer*)

Table 4. 2013 *Ranavirus* (RV) lab results from MSU & NWHC - Maryland

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
MD-AA-01	0	30	Negative[a]			
MD-BA-01	No Standard Sample Collected			1 LISY	2 LISY	Positive
MD-CV-01	0	30	Negative			
MD-CV-07	0	30	Negative[a]			
MD-CL-01	0	30	Negative[a]			
MD-CH-01	0	30	Negative[a]			
MD-FR-02	0	30	Negative[a]			
MD-FR-04	No Standard Sample Collected			1 LISY		Positive
MD-GA-01	0	28	Negative[a]			
MD-HA-01	No Standard Sample Collected			1 LISY		Positive
MD-HO-03	0	30	Negative[a]	4 LISY	12 LISY	Positive
MD-HO-03				1 AMMA		Positive
MD-HO-11	0	30	Negative[a]			
MD-MO-01	0	30	Negative			
MD-MO-02	0	30	Negative[a]	5 LISY	1 LISY	Positive
MD-MO-03	0	30	Negative[a]			
MD-MO-04	0	30	Negative			
MD-MO-05	0	30	Negative			
MD-MO-06	0	30	Negative			
MD-PG-01	0	30	Negative[a]			
MD-TA-01	5	25	Positive	4 AMMA	1 AMMA	Positive
MD-TA-01				2 SCHO	2 SCHO	Positive
MD-WA-01	0	30	Negative[a]			
MD-WA-04	0	30	Negative[a]			
Totals	**5/568 Individuals**		**1/19 ponds**	**12/27 LISY 5/6 AMMA 2/4 SCHO**		**6/6 ponds**

[a] Positive fluorescence but failed melting curve test.
LISY=Wood Frog (*Lithobates sylvaticus*)
AMMA=Spotted Salamander (*Ambystoma maculatum*)
SCHO=Eastern Spadefoot (*Scaphiopus holbrookii*)

Table 5. 2013 *Ranavirus* (RV) lab results from MSU & NWHC - New Jersey

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
NJ-AT-01	0	30	Negative[a]			
NJ-AT-02	0	30	Negative[a]			
NJ-CA-02	0	30	Negative[a]			
NJ-HU-01	0	30	Negative[a]			
NJ-ME-01	0	30	Negative[a]			
NJ-MO-01	No Standard Sample Collected			5 LISY		Positive
NJ-MO-02	3	27	Positive			
NJ-MO-04	13	17	Positive		1 AMMA	Negative
NJ-MO-14	0	30	Negative[a]			
NJ-PA-05	3	27	Positive			
NJ-PA-06	3	27	Positive			
NJ-SU-04	0	30	Negative			
NJ-SU-05	13	17	Positive			
NJ-SU-06	5	25	Positive			
NJ-SU-07	0	30	Negative			
NJ-SU-08	0	30	Negative[a]			
NJ-SU-15	2	28	Positive			
NJ-WA-01	11	19	Positive			
NJ-WA-02	27	3	Positive			
NJ-WA-03	22	8	Positive		5 LISY	Negative
NJ-WA-04	3	27	Positive			
Totals	**105/600 individuals**		**11/20 ponds**	**5/10 LISY 0/1 AMMA**		**1/3 ponds**

[a] Positive fluorescence but failed melting curve test.
LISY=Wood Frog (*Lithobates sylvaticus*)
AMMA=Spotted Salamander (*Ambystoma maculatum*)

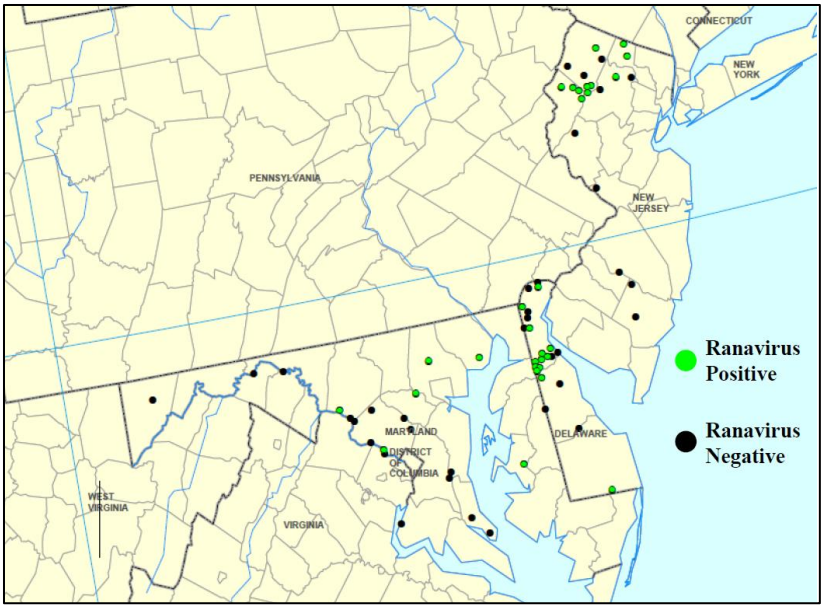

Figure 5. Study ponds with lab-confirmed *Ranavirus* in 2013.
(30 of 65 ponds (46.2%) with mean of 6.9±2.35 (95% CI) RV positive individuals/RV pond)

Table. 6. Ponds testing positive for Ranavirus in 2013 by Physiographic Region.

Physiographic Region	Delaware Ponds	Maryland Ponds	New Jersey Ponds	Total Ponds	% of Ponds
Coastal Plain	11/17	2/8	0/3	13/28	46.4
Piedmont	2/5	4/11	0/2	6/18	33.3
Montane	N/A	0/3	12/16	12/19	63.2
Total	13/22	6/22	12/21	31/65	47.7

2014 RESULTS

Sampling at Study Ponds

A total of 88 ponds were sampled in 2014 (Table 7, Fig. 6). This was the initial and only year of sampling in Pennsylvania (30 ponds, Table 8) and Virginia (25 ponds, Table 9). Surveys in Delaware, Maryland, and Virginia were primarily to resample ponds that were positive for *Ranavirus* in 2013 to assess persistence. Ponds sampled in 2014 included 13 ponds in Delaware (Table 10), 7 ponds in Maryland (Table 11), and 13 ponds in New Jersey (Table 12). One Maryland pond, MD-BA-02, was sampled in 2014 even though it had not been sampled in 2013, and it was not independent (within 3 km) of pond MD-BA-01. This was done to examine nearest-neighbor *Ranavirus* transmission as both ponds (447 m apart) had a history of use by environmental educators from a nearby nature center. Pond MD-BA-01 had only die-off samples collected in 2013 and 2014 (RV-positive both years), while pond MD-BA-02 had only a standard sample collected in 2014 (also RV-positive; Table 11). Similarly, pond NJ-SU-XX in New Jersey was not a 2013 RV-positive pond but was surveyed (both sample types) in 2014 to investigate an ongoing die-off event (Table 12). A Delaware pond that was RV-positive in

2013, DE-NC-14, was not sampled in 2014 because wood frogs did not use it. Conversely, a pond that was RV-negative in 2013, DE-NC-16, was sampled in 2014 due to an ongoing die-off (Table 10).

Standard samples were collected from May 19–June 23 in Pennsylvania, May 2–May 26 in Virginia, May 12–May 29 in Delaware, May 6–May 30 in Maryland, and May 30–June 12 in New Jersey. Gosner stage of wood frog larvae collected for standard samples ranged from stage 26–44 (mean=33.1±3.8 s.d.) in Pennsylvania, 24–41 (mean=29.7±3.1 s.d.) in Virginia, stage 27–41 (mean=33.2±3.8 s.d.) in Maryland, and stage 26–41 (mean=34.2±3.5 s.d.) in New Jersey. Gosner stage data for 2014 sampling in Delaware was not provided.

Die-off samples were collected from May 5–May 29 in Delaware, May 7–June 19 in Maryland, and June 4–June 30 in New Jersey. No die-off samples were collected in Pennsylvania. In Virginia some potential die-off specimens were collected from six ponds but supporting information did not suggest they were part of a die-off and collections were typically a single individual, so most were not sent to the NWHC lab for testing. Our study protocol required collection of 10 individuals per species when possible, though NWHC did not test all specimens provided. Thus samples from only two Virginia ponds were sent to the NWHC lab for testing. A solitary green frog (*L. clamitans*) larva was collected at pond VA-FC-13 on March 29, where many dead frog larvae were observed. Virus cultures at the NWHC lab did not find *Ranavirus*. An adult northern watersnake (*Nerodia s. sipedon*) from pond VA-AB-01 submitted to NWHC was determined to have died from trauma, likely from a predator. These two sites do not appear in Table 9 due to only one individual having been collected and the negative results. Gosner stages were not recorded for die-off samples in any state.

Table 7. 2014 study ponds and types of samples collected in Delaware, Maryland, and New Jersey.

State	# Ponds with Standard Sample Only	# Ponds with Die-Off Sample Only	# Ponds with Both Sample Types	Total # of Study Ponds
DE	8	3	2	13
MD	4[a]	1[a]	2	7[a]
NJ	9	N/A	4[b]	13[b]
PA	30	N/A	N/A	30
VA	25	N/A	N/A	25
Totals	76[a]	4[a]	8[b]	88[ab]

[a]Pond MD-BA-02 was not surveyed in 2013. It was not independent (<3 km apart) of MD-BA-01, which had only a die-off sample collected. Both had RV in 2014.
[b]Pond NJ-SU-XX was not surveyed in 2013. It was sampled in 2014 due to a reported die-off (i.e., not randomly selected) and accounted for 23 of the 45 RV-positive individuals in New Jersey.

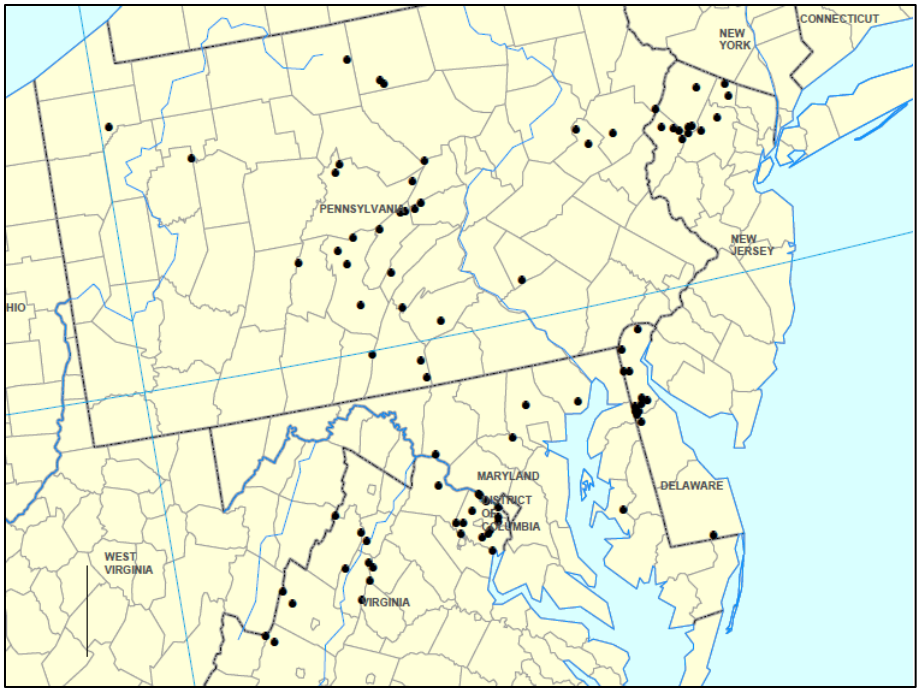

Figure 6. 2014 study ponds (n=88).

MSU Lab Results for 2014 Standard Samples

In 2014, the MSU lab screened standard samples from 30 Pennsylvania ponds (900 individuals; Table 8), 25 Virginia ponds (738 individuals; could only find 18 at pond VA-PA-02; Table 9), 10 Delaware ponds (300 individuals; Table 10), 6 Maryland ponds (180 individuals; Table 11), and 13 New Jersey ponds (390 individuals; Table 12) for a total of 84 ponds and 2,508 individuals. One Pennsylvania pond (16 individuals) tested positive for *Ranavirus*, one Virginia pond (3 individuals) tested positive, four Delaware ponds (6 individuals) tested positive (these four ponds were also positive in 2013), one Maryland pond (3 individuals) tested positive, and five New Jersey ponds (45 individuals) tested positive (4 ponds that were also positive in 2013 and one new pond, NJ-SU-XX, discussed above).

NWHC Lab Results for 2014 Die-Off Samples

In 2014, the NWHC lab received die-off samples from 12 ponds (Table 7), of which 8 tested positive for *Ranavirus* (DE: 4 ponds, 14 positive RV of 27 individuals, Table 10; MD: 2 ponds, 5 positive RV of 15 individuals, Table 11; NJ: 2 ponds, 6 positive RV of 18 individuals, Table 11). Six of the 8 RV-positive ponds were solely wood frog larvae die-offs (DE: 3 ponds, 10 positive RV of 14 individuals, Table10; MD: 2 ponds, 5 positive RV of 7 individuals, Table 11; NJ: 1 pond, 1 positive RV of 3 individuals, Table 12). The remaining two RV-positive ponds had multi-species die-offs, including a Delaware pond with wood frog larvae (2 RV-positive of 4 individuals) and southern leopard frog larvae (*L. sphenocephalus utricularius*; 2 of 2 individuals), and a New Jersey pond with wood frog larvae (3 of 6 individuals) and spotted salamander larvae (2 of 2 individuals). As in 2013, all die-off samples were determined to be FV3 by the NWHC lab.

Summary results for all ponds that tested positive for *Ranavirus* in 2014 from either the MSU or the NWHC labs (Fig. 5) were:

- **Pennsylvania –** 1 of 30 ponds (3.3 %) with 16 RV-positive individuals.
- **Virginia –** 1 of 25 ponds (4.0%) with 3 RV-positive individuals.
- **Delaware** – 8 of 13 ponds (61.5%) with a mean of 2.5±3.43 (95% CI) RV-positive individuals/RV pond.
- **Maryland –** 3 of 7 ponds (42.9%) with a mean of 2.7±1.6 (95% CI) RV-positive individuals/RV pond.
- **New Jersey –** 6 of 13 ponds (46.2%) with a mean of 8.8±2.83 (95% CI) RV-positive individuals/RV pond.
- **Total –** 19 of 88 ponds (21.6%) with a mean of 4.3±2.45 (95% CI) RV-positive individuals/RV pond.

Only 1 county each in Pennsylvania (Huntingdon) and Virginia (Shenandoah) had a pond that tested positive for *Ranavirus*. However, these were new county records for *Ranavirus*. All three Delaware counties (Kent, New Castle, Sussex) again had ponds that tested positive for *Ranavirus* and 7 of the 12 (58.3%) Delaware ponds that were RV-positive in 2013 that were sampled in 2014, were also positive in 2014. The significance of pond DE-NC-14 having no wood frogs to sample in 2014 after having a die-off in 2013 is hard to gauge without further monitoring. Maryland had 2 of 6 (33.3%) ponds that tested positive in 2013 also test positive in 2014. New Jersey had 5 of 12 (41.7%) ponds that tested positive in 2013 also test positive in 2014.

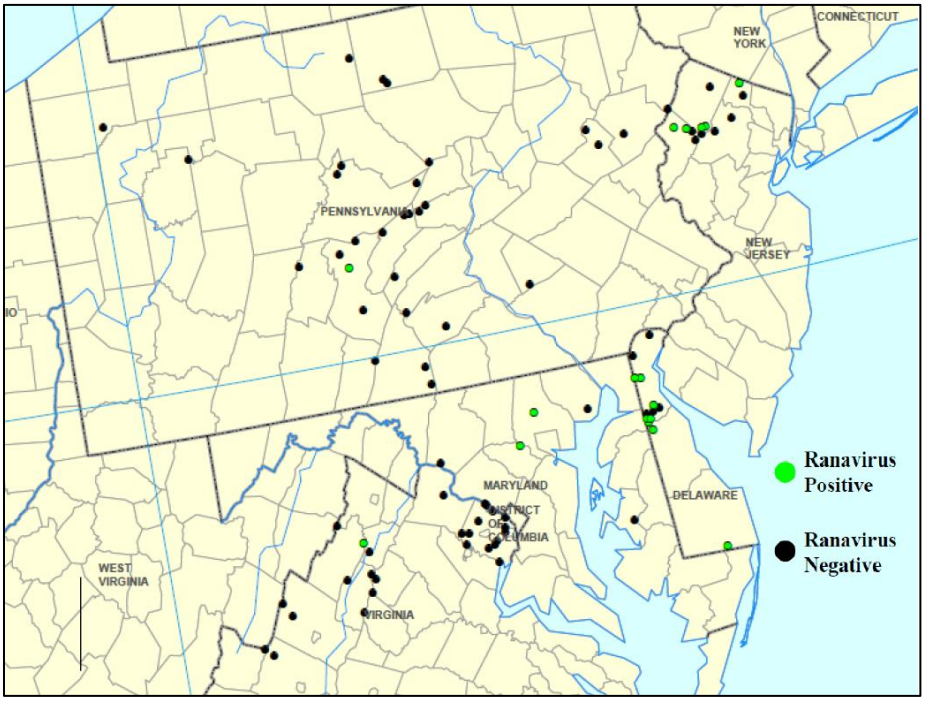

Figure 7. 2014 study ponds with lab-confirmed *Ranavirus*.

Table 8. 2014 Ranavirus lab results from MSU & NWHC - Pennsylvania

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
PA-BL-37	0	30	Negative			
PA-CA-53	0	30	Negative			
PA-CA-60	0	30	Negative			
PA-CE-12	0	30	Negative			
PA-CE-64	0	30	Negative			
PA-CE-68	0	30	Negative			
PA-CL-66	0	30	Negative			
PA-CR-25	0	30	Negative			
PA-CU-10	0	30	Negative			
PA-FR-02	0	30	Negative			
PA-FR-06	0	30	Negative			
PA-FU-43	0	30	Negative			
PA-HU-07	0	30	Negative			
PA-HU-19	16	14	Positive			
PA-HU-22	0	30	Negative[a]			
PA-HU-65	0	30	Negative			
PA-LE-03	0	30	Negative			
PA-MI-11	0	30	Negative			
PA-MI-29	0	30	Negative			
PA-MI-32	0	30	Negative			
PA-MI-49	0	30	Negative			
PA-MO-30	0	30	Negative			
PA-MO-67	0	30	Negative[a]			
PA-PE-27	0	30	Negative			
PA-PO-52	0	30	Negative			
PA-SN-01	0	30	Negative[a]			
PA-SN-62	0	30	Negative			
PA-TI-45	0	30	Negative[a]			
PA-TI-61	0	30	Negative			
PA-UN-35	0	30	Negative			
Totals	**16/900 individuals**		**1/30 ponds**			

[a] Positive fluorescence but failed melting curve test.

Table 9. 2014 Ranavirus lab results from MSU & NWHC – Virginia

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
VA-AL-01	0	30	Negative[a]			
VA-AR-01	0	30	Negative[a]			
VA-AU-01	0	30	Negative			
VA-AU-02	0	30	Negative			
VA-FC-01	0	30	Negative			
VA-FC-02	0	30	Negative			
VA-FC-03	0	30	Negative			
VA-FC-04	0	30	Negative[a]			
VA-FC-05	0	30	Negative			
VA-FC-06	0	30	Negative[a]			
VA-FC-07	0	30	Negative[a]			
VA-FC-08	0	30	Negative			
VA-FC-09	0	30	Negative[a]			
VA-FC-10	0	30	Negative[a]			
VA-FC-11	0	30	Negative			
VA-MA-01	0	30	Negative			
VA-PA-01	0	30	Negative			
VA-PA-02	0	18	Negative[a]			
VA-RA-01	0	30	Negative			
VA-RO-01	0	30	Negative			
VA-RO-02	0	30	Negative			
VA-SH-01	0	30	Negative			
VA-SH-02	0	30	Negative[a]			
VA-SH-03	3	27	Positive			
VA-WA-01	0	30	Negative[a]			
Totals	**3/738 individuals**		**1/25 ponds**			

[a] Positive fluorescence but failed melting curve test.

Table 10. 2014 Ranavirus lab results from MSU & NWHC – Delaware

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
DE-KT-02	0	30	Negative	5 LISY		Positive
DE-KT-03	1	29	Positive			
DE-NC-01	0	30	Negative[a]			
DE-NC-02	0	30	Negative		4 LISY 3 ANAM	Negative
DE-NC-04	0	30	Negative			
DE-NC-07	0	30	Negative[a]			
DE-NC-08	No Standard Sample Collected			2 LISY 2 LISP	2 LISY	Positive
DE-NC-09	0	30	Negative[a]			
DE-NC-10	1	29	Positive			
DE-NC-11	1	29	Positive			
DE-NC-15	3	27	Positive			
DE-NC-16	No Standard Sample Collected			1 LISY	4 LISY	Positive
DE-SX-01	No Standard Sample Collected			4 LISY		Positive
Totals	**6/300 individuals**		**4/13 ponds**	**12/22 LISY 0/3 ANAM 2/2 LISP**		**4/5 ponds**

[a] Positive fluorescence but failed melting curve test.
LISY=Wood Frog (*Lithobates sylvaticus*)
ANAM=Eastern American Toad (*Anaxyrus americanus*)
LISP=Southern Leopard Frog (*Lithobates sphenocephalus utricularius*)

Table 11. 2014 Ranavirus lab results from MSU & NWHC - Maryland

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
MD-BA-01	No Standard Sample Collected			2 LISY	1 LISY	Positive
MD-BA-01					2 PSCR, 3 AMMA, 2 LICL	
MD-BA-02[b]	3	27	Positive			
MD-FR-04	0	30	Negative[a]			
MD-HA-01	0	30	Negative[a]		1 AMMA	Negative
MD-HO-03	0	30	Negative[a]	3 LISY	1 LISY	Positive
MD-MO-02	0	30	Negative[a]			
MD-TA-01	0	30	Negative[a]			
Totals	**3/180 individuals**		**1/6 ponds**	**5/7 LISY 0/2 PSCR 0/4 AMMA 0/2 LICL**		**2/3 ponds**

[a] Positive fluorescence but failed melting curve test.
[b]This pond is 192 m from MD-BA-01 and was submitted to MSU to examine RV transmission to nearest-neighbor ponds (BA-01 had RV-positive die-offs in 2013 and 2014). It is not independent of MD-BA-01 (<3 km).
LISY=Wood Frog (*Lithobates sylvaticus*)
PSCR=Spring Peeper (*Pseudacris crucifer*)
AMMA=Spotted Salamander (*Ambystoma maculatum*)
LICL=Northern Greenfrog (*Lithobates clamitans*)

Table 12. 2014 Ranavirus lab results from MSU & NWHC - New Jersey

Pond Code	Standard Sample Result (MSU)			Die-Off Sample Result (NWHC)		
	# RV Positive	# RV Negative	Final Result	# RV Positive	# RV Negative	Final Result
NJ-MO-01	0	30	Negative[a]			
NJ-MO-02	0	30	Negative[a]			
NJ-MO-04	0	30	Negative[a]			
NJ-PA-05	1	29	Positive			
NJ-PA-06	0	30	Negative[a]			
NJ-SU-05	7	23	Positive			
NJ-SU-06	9	21	Positive		2 LISY 1 AMMA	Negative
NJ-SU-15	0	30	Negative[a]			
NJ-SU-XX[b]	23	7	Positive	1 LISY	2 LISY 1 PSCR	Positive
NJ-WA-01	0	30	Negative[a]			
NJ-WA-02	0	30	Negative[a]			
NJ-WA-03	0	30	Negative	3 LISY 2 AMMA	3 LISY	Positive
NJ-WA-04	5	25	Positive		3 LISY	Negative
Totals	**45/390 individuals**		**5/13 ponds**	**4/14 LISY 2/3 AMMA 0/1 PSCR**		**2/4 ponds**

[a] Positive fluorescence but failed melting curve test.
[b]This pond was not randomly chosen – a Standard Sample was submitted when only a die-off sample should have been submitted as ancillary data for other NJ die-offs. This was not one of the original 2013 study ponds.
LISY=Wood Frog (*Lithobates sylvaticus*)
AMMA=Spotted Salamander (*Ambystoma maculatum*)
PSCR=Spring Peeper (*Pseudacris crucifer*)

SUMMARY RESULTS & DISCUSSION

In 2013 and 2014, a total of 122 ponds were sampled for *Ranavirus* in the five-state study area (Fig. 8), including the collection of 4,306 individual wood frog larvae for analysis by the MSU lab and 158 individuals of 7 amphibian species for analysis by the NWHC lab. This study represents both the largest geographic (142,286 km^2) area and the greatest sample size ever screened for *Ranavirus*. Of the 122 ponds, 36 ponds (29.5%) had at least 1 sample test positive for *Ranavirus* over the two-year study period (Fig. 9). A total of 263 standard samples (6.1%) tested positive for *Ranavirus* as did 60 die-off samples (38.0%) of 5 species. Of the 30 ponds sampled in both years 14 ponds (46.7%) tested positive in both years, suggesting the virus is persisting at these sites. Whether the reservoir of *Ranavirus* continues to exist in the soil, water, animals at the site, or animals immigrating to the site between breeding seasons is speculative.

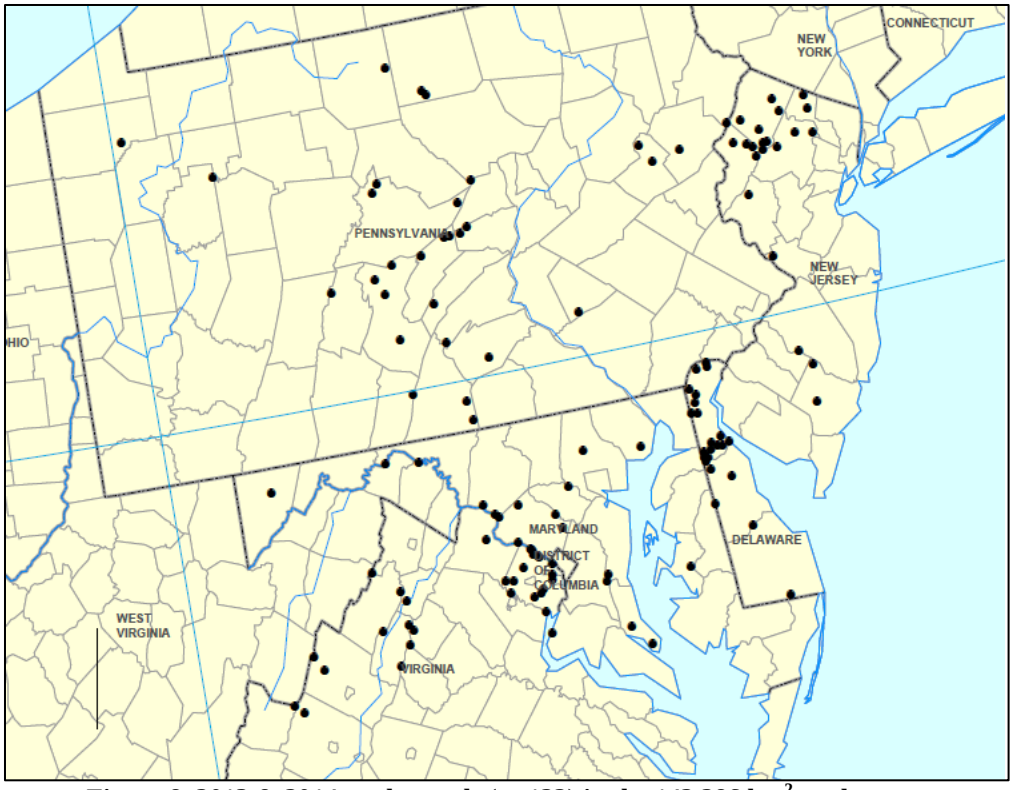

Figure 8. 2013 & 2014 study ponds (n=122) in the 142,286 km^2 study area.

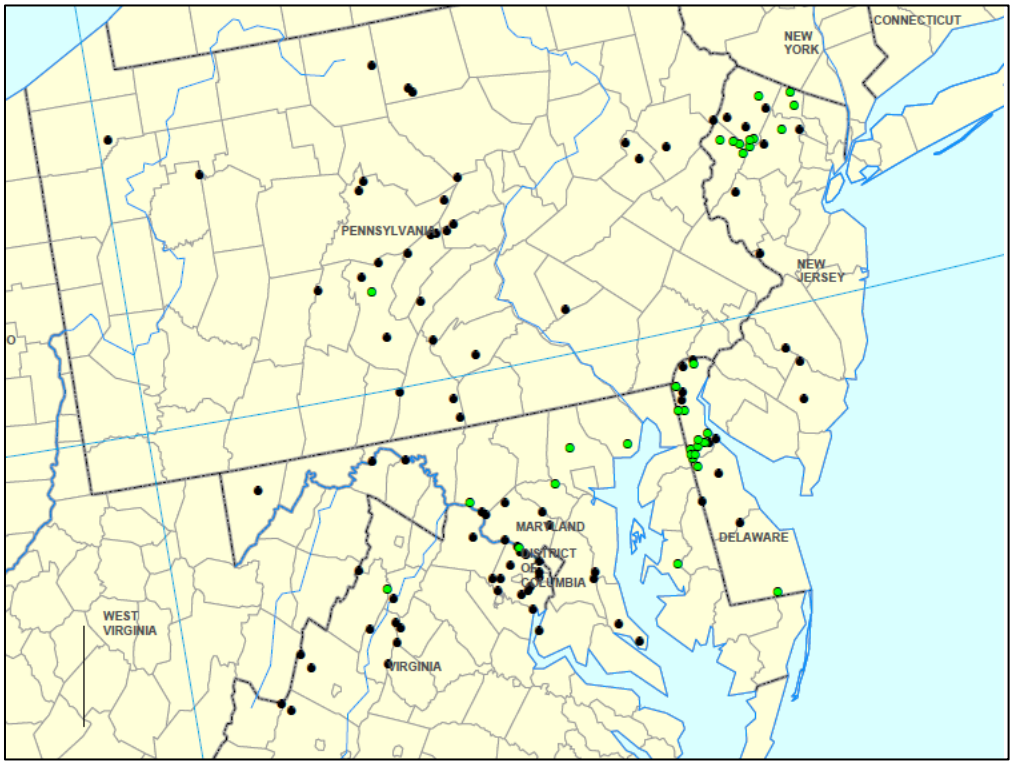

Figure 9. 2013 & 2014 study ponds with lab-confirmed *Ranavirus* (29.5%).

Differences in Lab Results

Some of the ponds screened by MSU in 2013 and 2014 were also independently screened for *Ranavirus* by NWHC. A surprising conflict of findings was that the NWHC lab was able to culture *Ranavirus* from two Maryland ponds (MD-HO-03 in 2013 and 2014, and MD-MO-02 in 2013), one Delaware pond (DE-KT-02 in 2014), and one Virginia pond (NJ-WA-03), but the MSU lab found these ponds to be negative for *Ranavirus*. Some individuals in these ponds displayed an exponential increase in fluorescence but failed to meet the melting curve criterion. However, some of these individuals displayed a single melting temperature on the melting curve, suggesting one double-stranded product was being amplified during PCR. The melting temperature for this product was approximately 5°C lower than the melting temperature of the positive control. It is possible these individuals are positive for *Ranavirus* but are infected with a strain that is genetically variable compared to the positive control.

To further investigate this the MSU lab examined 4 individuals from MD-MO-02 (collected in 2013) with traditional PCR and DNA sequencing in order to determine if these individuals were infected with a genetic variant. Two of the individuals had enough PCR product to sequence. The gene sequences were identical to each other and were a 99% match to FV3 *Ranavirus*. There was a single nucleic acid base pair difference between these samples and the FV3 samples that were used as the positive control. The Maryland samples contained an adenine base, while the control samples contained a guanine base. Because adenine bases in sequences lower the melting temperature, while guanine bases raise the melting temperature, this base pair difference may account for the difference in melting temperature in the nearly identical individuals. There may be other differences in the individuals' DNA sequences, but only the cleanest part of the sequence was used for this comparison. Thus we can conclude that MD-MO-02 is positive for RV but it contains a genetic variant (different strain) of FV3.

Minimum Number of Samples Needed to Detect Positives

One goal of this study was to develop a standardized *Ranavirus* screening protocol. To that end, the MSU lab determined success in detecting *Ranavirus* when screening 10, 20, and 30 animals. Although it is feasible to screen 30 animals per pond, and having more samples increases statistical power and confidence in results, the screening process is time-consuming and expensive. Additionally, the DNA extraction process requires a minimum of 10 microcentrifuge spins and a standard microcentrifuge rotor holds 24 samples. So, minimizing sample numbers whenever possible is beneficial for multiple reasons. The MSU lab screened 30 animals per pond for all ponds (except for one pond from Virginia with 18 individuals and another in Maryland with 28). Of the 24 ponds analyzed by MSU that tested positive in 2013, *Ranavirus* was detected in the first 10 samples for 83% of those ponds and we were able to detect it in the first 20 samples for 100% of those ponds. Of the 12 ponds that tested positive in 2014, the MSU lab was able to detect *Ranavirus* in the first 10 samples for 91% of those ponds and we were able to detect it in the first 20 samples for 100% of those ponds. These findings suggest it is possible to detect *Ranavirus* in as little as 10 samples; however, 20 appears to be the optimal sample size for *Ranavirus* detection.

Sample Preservation Issues for Both Years

The MSU lab encountered significant tissue perseveration issues in 2013 and 2014 that were largely attributed to the use of whirl packs as storage containers. In 2013, there were at least

seven New Jersey ponds (NJ-MO-02, NJ-SU-05, NJ-WA-02, NJ-PA-06, NJ-SU-015, NJ-SU-04, and NJ-SU-07) that appeared to be preserved in a substance other than ethanol (possibly the euthanasia solution), leading to advanced decay of the tadpoles. Despite the decay, MSU was able to isolate total genomic DNA from all individuals in these ponds and five individuals (NJ-MO-02, NJ-SU-05, NJ-WA-02, NJ-PA-06, and NJ-SU-15) tested positive for *Ranavirus*. Additionally, multiple ponds preserved in whirl packs in all states showed signs of decay and/or dehydration due to loss of ethanol in 2013 and 2014. Despite these preservation issues, there did not appear to be a negative impact on the isolation of DNA and the detection of ranaviral DNA if present. However, we strongly urge field researchers to use a different storage vessel, such as 5 or 15 ml snap cap or screw cap tubes, for tissue preservation in future studies. Regardless, our ability to detect ranaviral DNA in decayed tissue is encouraging as *Ranavirus* outbreaks are often associated with mass mortality events and in future studies it may be necessary to isolate DNA from dead or decaying tissue.

The NWHC lab also encountered issues with die-off samples.. Some die-off samples were either too crushed or decayed for necropsy and histology, or they had been exposed to freeze-thaw cycles such that they were difficult to use. Future researchers should handle, store, and ship samples with the care needed to ensure useable samples arrive at diagnostic laboratories.

False Positives

The fluorophore used at the MSU lab to detect amplified double-stranded DNA (SYBR Green) binds to any double-stranded DNA fragment, not just target DNA. Consequently, if random fragments of DNA are amplified because of false priming or primer-dimer formation, many non-target (non-ranaviral) double-stranded fragments of DNA will be amplified, leading to a corresponding increase in fluorescence. There were a considerable number of ponds in 2013 and 2014 with individuals that showed an exponential increase in fluorescence but did not show the same melting temperature from the melting curve as the positive control. We refer to these individuals and populations as false positives; they underscore the importance of using exponential increase in fluorescence as well as melting temperature to confirm ranaviral infection in individuals.. Specifically, in 2013 there were an additional eight ponds in Delaware (DE-NC-12, DE-NC-06, DE-KT-01, DE-NC-13, DE-NC-03, DE-NC-17, DE-NC-18, and DE-SX-02; Table 3), 13 ponds in Maryland (MD-MO-03, MD-WA-04, MD-FR-02, MD-GA-01, MD-WA-01, MD-PG-01, MD-MO-02, MD-CL-01, MD-AA-01, MD-SH-01, MD-HO-11, MD-CV-07, and MD-HO-03; Table 4), and seven ponds in New Jersey (NJ-AT-01, NJ-SU-08, NJ-AT-02, NJ-ME-01, NJ-CA-02, NJ-MO-14, and NJ-HU-01; Table 5) that met the exponential increase in fluorescence criterion but failed to meet the melting temperature criterion. In 2014, there were an additional three ponds in Delaware (DE-NC-07, DE-NC-01, and DE-NC-09; Table 10), five ponds in Maryland (MD-TA-01, MD-HO-03, MD-HA-01, MD-FR-04, and MD-MO-02; Table 11), seven ponds in New Jersey (NJ-WA-01, NJ-MO-01, NJ-MO-02, NJ-SU-15, NJ-PA-06, NJ-MO-04, and NJ-WA-02; Table 12), four ponds in Pennsylvania (PA-MO-67, PA-TI-45, PA-HU-22, and PA-SN-01; Table 8), and 10 ponds in Virginia (VA-FC-06, VA-AR-01, VA-FC-10, VA-WA-01, VA-FC-04, VA-FC-07, VA-FC-09, VA-SH-02, VA-PA-02, and VA-AL-01; Table 9) that were determined to be false positives. While it is possible some of these ponds are infected with a genetic variant of *Ranavirus*, and that is what accounts for differences in melting temperature, the overwhelming majority of false positives showed melting curves with multiple peaks suggesting multiple random double-stranded DNA

fragments were being amplified. The discovery of a high level of false positives was an important and unexpected outcome of this study, especially as so many current RT-PCR studies of *Ranavirus* use only exponential increase in fluorescence to determine *Ranavirus* infection.

Future Work

The MSU lab plans to use traditional PCR and DNA sequencing to determine if some of the false positive ponds are infected with a genetic variant of *Ranavirus*. This will be especially important to determine for the Maryland ponds that tested positive for *Ranavirus* at the USGS lab but negative at the MSU lab. This information will also be important to determine if there are unique genetic strains of this virus. Determining genetic relatedness among different strains, if present, will be instrumental in future disease outbreaks, allowing researchers to assess evolutionary relatedness and age amongst the viral strains and predict virulence of the virus and possible resistance in the host.

Questionnaire Results

Biologists from state natural resource agencies for all 13 northeastern U.S. states and the District of Columbia (D.C.) responded to the questionnaire. Results are summarized in Table 13 and Appendix 4. *Ranavirus* has been confirmed in every state in the Northeast region (including D.C.), except for Vermont. However, Brunner et al (2007) postulate that Clark et al. (1968) possibly discovered *Ranavirus* in Vermont in 1968; though Titus and Green (2013) state that die-offs associated with *Ranavirus* were first reported in the U.S. in the mid-1990s. Regardless, given the current known distribution of *Ranavirus* in the Northeast (Fig. 10), the virus is likely to be found in Vermont in the near future. *Ranavirus* has been lab-confirmed in 33 herpetofaunal species in at least 64 counties in the Northeast region (Appendix 1, Appendix 4). It is most commonly found in wood frog larvae (59 counties), eastern box turtles (*Terrapene c. carolina*) (20 counties), and the larvae of spotted salamanders (20 counties), green frogs (19 counties), and American bullfrogs (*L. catesbianus*; 14 counties) (Appendix 4). *Ranavirus* was first lab-confirmed in the northeastern U.S. in Maine in 1991 (Green et al. 2002). Wolf et al. (1968) reported on "tadpole edema virus" in American bullfrog larvae in 1968 in West Virginia, and Green et al. (2002) suggest this was *Iridovirus* (i.e., *Ranavirus*).

Half of the northeast states have a state wildlife veterinarian, and 6 of 14 respondents also use other veterinarian resources. Only three states have a disease reporting process and only one state has on-line wildlife disease reporting (although this question was not specifically asked, so may be a larger number). Surprisingly, only 10 of 14 state wildlife agencies receive lab reports on disease; however, a number of states have collaborative relationships with major universities which study and receive information on disease. The majority of states (11 of 14) make use of the diagnostic services of the NWHC, but many northeast states are also members of the Northeast Wildlife Disease Cooperative, while a few are members of the Southeast Wildlife Disease Cooperative. This figure is reflected in the finding that10 of 14 states also use diagnostic labs other than NWHC.

Table 13. State Questionnaire Results on Herpetofauna Disease Reporting

State	CT	DC	DE	MA	MD	ME	NH	NJ	NY	PA	RI	VA	VT	WV	Total
***Ranavirus* Confirmed in State?**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	13/14
# Counties RV Confirmed thru 2014	7	N/A	3	4+	9	5+	2+	5	8	5	2	10	0	4	64[a]
Year RV First Lab-Confirmed	2009	2014	2013	1999	2001	1991	1999	2011	2005	2003	2001	2003	N/A[b]	2012[b]	1991
Do You Have a State Veterinarian?	No	No	No	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	7/14
Use Other Veterinarian?	Yes	No	No	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	No	6/14
Have a Disease Reporting Process?	Yes	No	No	No	Yes	No	No	No	Yes	No	No	No	No	No	3/14
Reports Go To State Wildlife Agency?	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	10/14
Use NWHC Lab?	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	11/14
Use Other Diagnostic Lab?	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No	Yes	10/14

[a] Some lab reports lacked county location information (denoted by "+"), so this number is a minimum. *Ranavirus* was newly discovered in Calvert County, MD and Fairfax Co., VA in 2015, which is not represented in this table but is in Fig. 10.

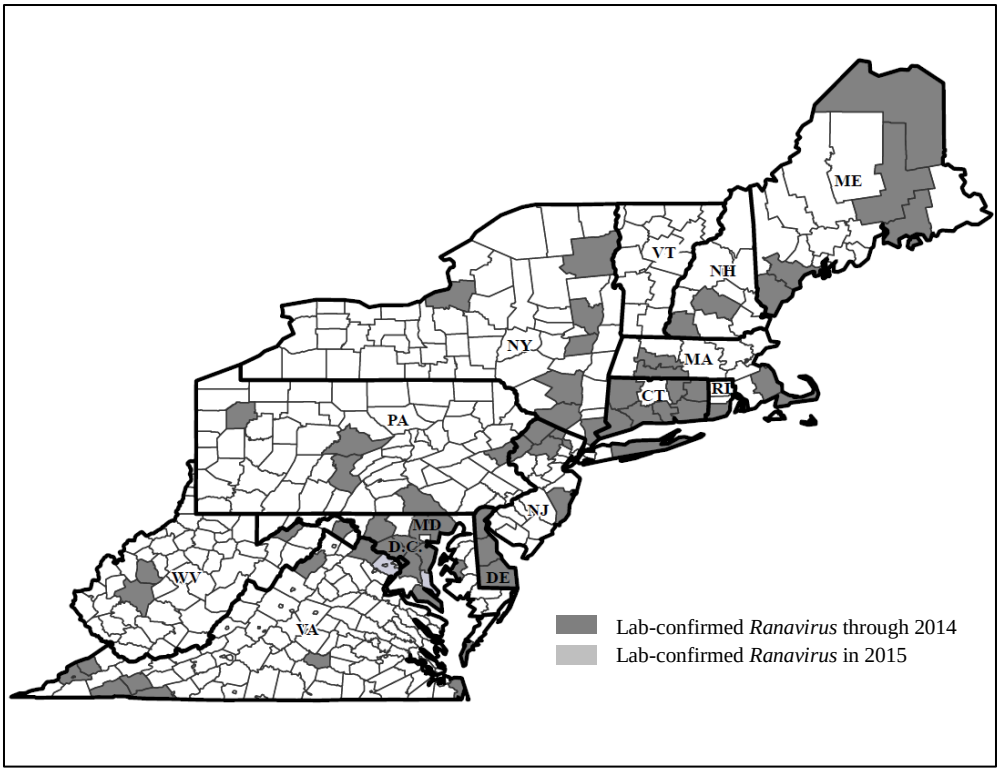

Figure 10. Counties with lab-confirmed *Ranavirus* in the northeast U.S.

CONCLUSIONS

We began this study with knowledge of the geographic distribution of *Ranavirus* within the five-state study area limited to four of the northeastern states (not in Delaware), 16 counties and the city of Virginia Beach, Virginia (Table 1, Fig. 1). Based on the results of this study and other subsequent lab-confirmed *Ranavirus* infections (including Calvert County, MD and Fairfax Co., VA in 2015) we now know viral infection occurs in all 5 states (including every county in Delaware) and in an additional 16 counties (Fig. 11); all numbers which unfortunately will expand as we look farther afield. It is possible that more than one strain of Frog Virus 3 exists in the Northeast region, which future analysis of samples collected for this study may aid in determining. As other researchers have noted, observing die-offs is very difficult because they happen so quickly – studies like this one are a challenging trade-off between attempting to monitor a large geographic area and monitoring individual wetlands often enough to observe and study die-offs. In many cases during the course of this study it appeared that we had missed die-offs by days at most.

This study has helped establish sampling protocols than can be used across large geographic areas (see Appendix 2).

Researchers in Connecticut (Rittenhouse et al. 2013), Maine (Gahl and Calhoun 2010), Maryland (Farnsworth and Seigel 2013), New York (Brunner et al. 2011, Titus and Green 2013), and Virginia (Davidson and Chambers 2011, Goodman et al. 2013, Hamid 2013, Hamid et al. 2013) have all added to our recent knowledge of the distribution of this emerging disease. As one respondent to the questionnaire, Phillip deMaynadier of Maine, stated with regard to

Ranavirus, "It's probably everywhere," and one look at the regional map in Figure 10 lends to the conclusion that the virus is already widespread in the northeastern U.S. As our data shows, die-offs occur in subsequent years, but the long-term consequences of these die-offs for our native herpetofauna is still an unanswered question (Daszak et al 1999, Earl & Gray 2014). Scientists and conservation groups are now addressing the question of how to respond to this threat (Langwig et al. 2015). Partners in Amphibian and Reptile Conservation (PARC) has been a leader in disseminating information on diseases of herpetofauna and potential conservation actions, such as developing a disinfection protocol for boots and field equipment. The Northeast PARC Emerging Disease Working Group is currently working on response strategies directed at land management agencies for use when *Ranavirus* is identified on their lands. It is hoped that this study's results will be an aid and catalyst for further research and conservation efforts.

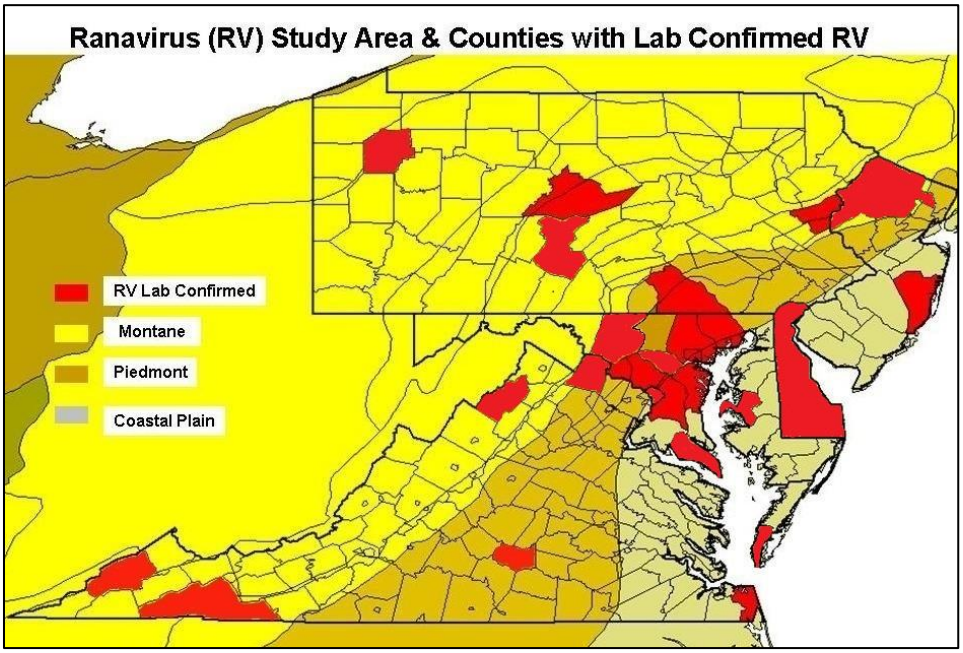

Figure 11. Counties with lab-confirmed *Ranavirus* through 2014.

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Appendix 1

Acronyms for Northeast Region Herpetofauna Species with Lab-confirmed *Ranavirus*

Appendix 1. Acronyms for Northeast Region Herpetofauna Species with Lab-confirmed *Ranavirus*[a]

Species Scientific Name	Species Common Name	Acronym
Turtles		
Chelydra serpentina	Snapping Turtle	CHSE
Chrysemys picta	Painted Turtle	CHPI
Glyptemys muhlenbergii	Bog Turtle	GLMU
Terrapene carolina	Eastern Box Turtle	TECA
Salamanders & Newts		
Aneides aeneus	Green Salamander	ANAE
Ambystoma jeffersonianum	Jefferson's Salamander	AMJE
Ambystoma laterale	Blue-spotted Salamander	AMLA
Ambystoma maculatum	Spotted Salamander	AMMA
Ambystoma opacum	Marbled Salamander	AMOP
Ambystoma tigrinum	Eastern Tiger Salamander	AMTI
Desmognathus fuscus	Northern Dusky Salamander	DEFU
Desmognathus monticola	Seal Salamander	DEMO
Desmognathus orestes	Blue Ridge Dusky Salamander	DEOR
Desmognathus organi	Northern Pygmy Salamander	DEOG
Desmognathus quadramaculatus	Blackbelly Salamander	DEQU
Eurycea bislineata	Northern Two-lined Salamander	EUBI
Eurycea cirrigera	Southern Two-lined Salamander	EUCI
Eurycea l. longicauda	Long-tailed Salamander	EULO
Eurycea lucifuga	Cave Salamander	EULU
Notophthalmus v. viridescens	Red-spotted Newt	NOVI
Plethodon glutinosus	Northern Slimy Salamander	PLGL
Plethodon montanus	Northern Gray-Cheeked Salamander	PLMO
Plethodon welleri	Weller's Salamander	PLWE
Frogs & Toads		
Anaxyrus fowleri	Fowler's Toad	ANFO
Hyla chrysoscelis	Cope's Gray Treefrog	HYCH
Lithobates catesbianus	American Bullfrog	LICA
Lithobates clamitans	Green Frog	LICL
Lithobates palustris	Pickerel Frog	LIPA
Lithobates pipiens	Northern Leopard Frog	LIPI
Lithobates sphenocephalus	Southern Leopard Frog	LISP
Lithobates sylvaticus	Wood Frog	LISY
Pseudacris crucifer	Spring Peeper	PSCR
Scaphiopus holbrookii	Eastern Spadefoot	SCHO

[a]33 species through 2014. Taxonomy based on Crother 2012.

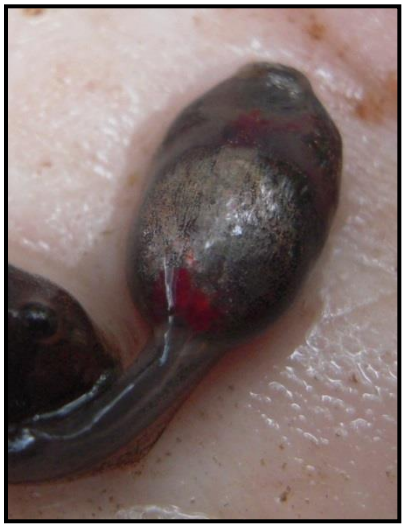
Appendix 2

Ranavirus Study Field Protocols

Ranavirus Study Field Protocols

for 2013-2014 RCN Grant

(DE, MD, NJ, PA, VA)



Craig A. Patterson & Scott A. Smith

April 19, 2013

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INTRODUCTION

Emerging infectious diseases are one of the most important factors contributing to global amphibian declines and have been implicated in local extinctions of several species. Amphibian declines due to the Chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), have received considerable and well-deserved attention over the last decade. However, reports of significant mortality due to outbreaks of *Ranavirus* (Family Iridoviridae) are becoming increasingly common in the U.S. with the reported number of die-offs 3-4X greater than for *Bd*. *Ranavirus* differs from *Bd* in that both amphibian and reptiles are known to be affected. Unfortunately, information on the timing, extent, and frequency of occurrence of outbreaks of *Ranavirus* remain limited, partially due to lack of surveillance and partially due to the rapid onset and mortality caused by the disease. This is especially true for amphibian larvae; in many cases, only a few days elapse between the initial signs of the disease and the disappearance of tadpoles from the environment. Thus, unless observations are directed at detecting the outbreak of the disease, it would be easy to conclude that absence of tadpoles was the result of a rapid metamorphosis, instead of mass mortality from a disease outbreak.

Ranavirus has been confirmed in six amphibian genera found in the Northeast U.S., including the following (with the number of RCN species in that genus listed by at least one Northeastern state in parentheses): *Bufo* (3), *Hyla* (4), *Rana* (7), *Pseudacris* (5), *Ambystoma* (9), and *Notopthlamus* (1). Northeast Partners in Amphibian and Reptile Conservation's (NEPARC) considered *Ranavirus* a major threat to northeastern herpetofauna at their 2011 Annual meeting. Ranaviruses likely represent the greatest pathogen threat to the biodiversity of amphibians in North America.

In order to begin to better understand the extent to which *Ranavirus* is impacting amphibian and reptile populations in the Northeast U.S. and to develop and test a sampling protocol that could be used throughout the region, we propose a survey of amphibian larvae at a number of wood frog (*Rana sylvatica*) breeding ponds in Maryland, Delaware, New Jersey, Pennsylvania, and Virginia. Wood frogs have the highest mortality and infection rates of northeast amphibians and their breeding ponds (primarily vernal pools) may be the main source of the disease for other affected species. Our approach involves sampling 20-30 ponds per state over a two-year period, with samples spread over different watersheds and physiographic provinces to test the applicability of these methods to a diversity of regional conditions.

Outcomes from this effort will include a standard regional *Ranavirus* sampling protocol, a relative frequency of prevalence of *Ranavirus* within the 5-state sampling area which can be extrapolated to a regional perspective, a summary of known or suspected *Ranavirus* events in the 13 northeastern states, and publication(s) in peer-reviewed scientific journals.

SUMMARY OF METHODOLOGY

Starting in 2013, a total of 30 wood frog larvae at Gosner stage 27 through metamorphosis (60-130 days post-hatching; Appendix A) will be sampled by dip-net at each of the 20-30 study ponds in each state and sent for PCR analysis at Montclair State University (MSU) for presence of Ranavirus. Analysis will be conducted in lots of 10 larvae per pond to help determine number needed to reach *Ranavirus* detection. No further PCR analysis will be needed on remaining lots of 10 if they are detected in the preceding lot. Remaining animals will be stored for future histological analysis. This will constitute a ***Standard Sample***.

Captured larvae of all species present in each study pond will be placed in a wet bucket or tray after each dip-net sweep and sorted by species. Special effort will be put into sampling any individuals exhibiting abnormal swimming behavior, morbidity or recently died. Larvae will be visually examined for indications of ranaviral infection (reddening of their ventral skin, especially around the base of the hind limbs and the vent opening). If Ranavirus symptoms are present an additional sample of 10 larvae of each affected species will be sent to the USGS National Wildlife Health Center (NWHC) for full pathological screening (necropsies, histology of major organs, and viral, fungal and bacterial cultures, where appropriate). This will constitute a ***Die-off Sample***.

Samples will be organized, secured and labeled following either MSU or NWHC protocol depending on type of sample (*Standard* vs. *Die-off*), placed in a cooler and then shipped within 24 hours to NWHC (Madison, WI) or to the Department of Biology and Molecular Biology at Montclair State University (Montclair, NJ).

All boots, equipment and dip-nets will be disinfected between sites in 10% bleach or 1% Nolvasan ® solution to ensure no disease transmission between study sites.

WOOD FROG LIFE HISTORY CHARACTERISTICS

Wood frogs (*Rana sylvatica*) are one of the earliest anuran species to breed within the five-state study area. They are an explosive breeder with synchronous breeding migrations typically occurring after the first warm rains of late winter or early spring. Timing of breeding varies by latitudinal and elevational gradients. Within the study area breeding begins at lower elevations in Virginia, Delaware and Maryland in February, while it may not be completed at the highest elevations in New Jersey and Pennsylvania until early May.

Breeding occurs in fish-free ephemeral and semi-permanent woodland pools or in isolated oxbow sections of former streams within or adjacent to woodlands. They will also use beaver-impounded wetlands. Breeding sites can also include man-made habitats such as borrow pits, ditches, stormwater management ponds, wildlife ponds, and even tire ruts.

The spherical egg masses are crystal-clear jelly about 75-100 mm in size with 300-1500 embryos per mass (Fig. 1). While wood frogs will lay single isolated egg masses, they are famous for laying large conspicuous communal "rafts" of egg masses (Fig. 2), typically in one or a few parts of the pond, often in shallower areas, and they tend to lay the rafts in the same location each year. Rafts can be up to 900+ individual egg masses. Eggs hatch out at varying rates depending on water temperature. Early season eggs develop slowly, when water temperatures are low, taking up to 1 month for larvae to hatch. Later laid eggs, when water temperatures are higher, can hatch in 9-10 days.

Wood frog larvae are usually very dark, appearing either black or dark green, with a high dorsal crest and the fins are clear or clear with dark spots (Fig. 3), though some individuals (usually females) are light (Fig. 4). The dorsum and sides are flecked with fine gold spots. They have a long acuminate tail. Larvae reach a maximum length of 50-66 mm prior to metamorphosis. Larval development varies depending on water temperature, depth and amount of shading, with a range of 60-130 days post-hatching, though the literature does have at least one report of 44 days from hatching to metamorphosis. In permanent and deep well-shaded pools development is slower, while in shallow temporary pools development is rapid, particularly as they dry out. Metamorphosis in our 5-state study area occurs from late April through July.

Figure 1. Solitary Wood Frog Egg Mass

Figure 2. Communal Raft of Wood Frog Egg Masses

Figure 3. Wood Frog Larvae – dark

Figure 4. Wood Frog larvae - light

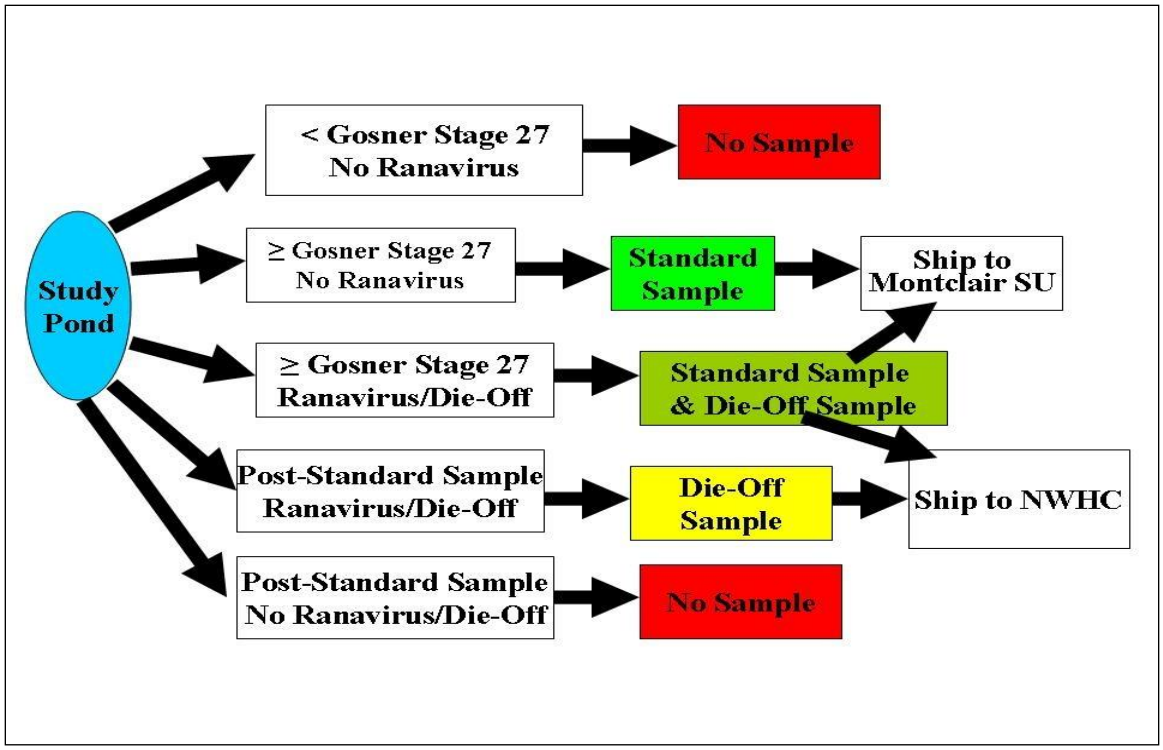

Figure 5. Flowchart of Sampling Decision Tree

STANDARD SAMPLING (30 wood frog larvae)

Field Methods

Standard Samples of 30 wood frog larvae at Gosner stage 27 through metamorphosis (60-130 days post-hatching) will be collected by dip-net at each study pond for PCR analysis at Montclair State University, regardless of whether symptoms of ranaviral infection are observed. Because field personnel will most likely be dip-netting live wood frog larvae, those tadpoles collected for each sample must be euthanized prior to being stored for shipment. However, since these samples will be limited to PCR analysis only, dead or decaying larvae may also be collected, particularly if a major die-off is observed on the day researchers visit a study pond to collect the Standard Sample. All boots, equipment and dip-nets must be disinfected between sites in 10% bleach or 1% Nolvasan ® solutions to ensure no disease transmission between study sites.

Within each state, wood frog larvae collected in **Standard Samples** will each be given a *unique* Sample Number and bagged individually. For example, if there are 30 study ponds in a state and 30 larvae are sampled at each pond, then Sample Numbers for individuals would range from 1 to 900. States should assign Sample Numbers (in groups of 30) to study ponds prior to collecting samples in the field. For example, Sample Numbers can be assigned to study ponds based on their order from initial site randomization exercises. It is important to keep detailed records of which sample numbers are used at each study pond, both on datasheets as well as in databases or spreadsheets (see Appendix B and C for pond and standard sample datasheets, resp.).

Instructions for Field Methods:

(1) Dipnet individuals and put wood frog larvae in a 5-gallon bucket. Disposable vinyl or nitrile gloves should be worn when handling amphibians and gloves should be changed between individuals.

(2) With small aquarium nets, dip each Gosner Stage 27+ tadpole from the bucket (one net per tadpole).

(3) Rinse each individual with sterile or dechlorinated water. This can be done while tadpoles are still in the small aquarium nets.

(4) Euthanasia: Place each tadpole in a separate small zip-lock bag (e.g. snack-size bags) with enough benzocaine hydrochloride to cover the tadpole (see **Euthanasia** section below for more details). The benzocaine hydrochloride solution can be poured into the zip-lock bags or transferred using disposable plastic pipets. Wait until respiration ceases (< 1 minute), and then keep tadpole in benzocaine solution for an additional 5 minutes.

Note: If tadpole is already dead when collected, skip ahead to step (5).

(5) Using the same aquarium net, transfer the individual to a Whirl-Pak bag containing 70% Ethanol (EtOH: just enough to cover the tadpole is sufficient). Place used nets in a bucket with disinfectant (see **Disinfection Guidelines** section). Remove the air from the Whirl-Pak bag and seal. Use a separate Whirl-Pak bag for each tadpole.

(6) Pour the used benzocaine hydrochloride solution from the zip-lock bags into a container (e.g., plastic bottle), and put used zip-lock bags into a larger garbage bag for disposal. The used solution can be poured down a sink drain after returning from the field.

(7) Write sample number (1-900), location (pond code: for example, MD-AL-01) and species on each bag with a permanent, water-resistant marker. Record this and other relevant information on a data sheet.

(8) Place Whirl-Pak bags with larvae in a cooler for transport from the field.

(9) Disinfect all field equipment and footwear between sites (see **Disinfection Guidelines** section).

Euthanasia

Amphibian larvae will be euthanized by benzocaine hydrochloride water baths. This method of euthanasia is approved by the American Veterinary Medical Association Panel on Euthanasia Guidelines on Euthanasia (AVMA, 2013, p. 77). The diluted solution of benzocaine hydrochloride that will be used for euthanasia can be prepared in the lab prior

to going out into the field and stored in a 1 L or larger container for field use. Caution should be taken when handling benzocaine, as it can cause skin and eye irritation. If contact occurs, flush skin or eyes with water for at least 15 minutes. Do not conduct euthanization in the wetland – move to an adjacent upland area.

Euthanization Instructions:

Note: Steps (1) and (2) below can be completed in the lab *before* going to a field site.

(1) Mix 5 g of benzocaine (chemical name = ethyl p-aminobenzoate) with 50 mL of 95% EtOH to create a concentrated solution of benzocaine hydrochloride.

(2) Add 2.5 mL of concentrated solution to 1 L of sterile or dechlorinated water.

(3) Add diluted solution to small zip-lock bag (enough to cover tadpole) and add tadpole. Disposable plastic pipets may be used to transfer the diluted solution into the small zip-lock bags.

(4) Remove tadpole 5 minutes after respiration ceases (which should occur in < 1 minute). Pour the used benzocaine hydrochloride solution from the bags into a container (e.g. plastic bottle), and put the used bags into a larger garbage bag for disposal. The used solution can be poured down a sink drain after returning from the field.

Labeling and Shipping Instructions for Standard Samples

(1) Place the already individually labeled 30 Whirl-Pak bags with wood frog larvae inside a larger zip-lock bag (e.g., gallon-sized, one larger bag per study pond) and seal closure with packing or duct tape. Place paper towels inside the larger zip-lock bag in case liquids leak during shipment.

(2) Label the outside of the larger bag with collector's name, date of collection, collection site (pond code), and number of specimens with a permanent, water-resistant marker.

(4) Fill out and include an **Amphibian Pathogen Sampling Datasheet** for each collection site. Seal the datasheets in a large zip-lock bag in case the specimen bags leak.

(6) Place specimens and completed datasheets in a Styrofoam container for shipment. Line the inside of the shipping container with paper towels incase bags leak. Specimens should be shipped by 1-day (overnight) service (e.g. FedEx, UPS), preferably on Monday, Tuesday, or Wednesday to ensure that specimens are received by Montclair State University by the end of the week. All packages containing Ethanol (<500 mL total) need to have a "Dangerous Good in Excepted Quantity" label on the outside of the package (Fig. 6), with the hazard class number

("**3"** for ethanol) written onto the label. Packages should also be labeled with the words "Exempt Animal Specimen" on the outside of the package.

Ship specimens to:

Kirsten Monsen-Collar
Dept. of Biology and Molecular Biology
Montclair State University
1 Normal Ave.
Montclair, NJ 07043.

(7) Contact Dr. Monsen-Collar the day that specimens are mailed and provide the tracking # so she knows to expect the samples and can trace the package if it does not arrive.

Figure 6. Example of "Dangerous Good in Excepted Quantity" Label

Equipment Needed

Many of the equipment items listed below can be obtained from grocery stores, pharmacies, or hardware stores. In some cases, we have included links to websites for ordering more obscure items. If **FS/BM** is included in the description, that means the item can be ordered from a Forestry Suppliers or Ben Meadows catalog.

General Field Equipment
Rubber boots, hip-waders, chest waders **(FS/BM)**
5-gallon buckets (at least one, for initial collection of dip-netted larvae)
Large Dip-nets (for initially capturing animals; **FS/BM)**
30 small aquarium nets (for working with individual larvae)
Disposable Gloves (vinyl or nitrile, ~ 2,000, **FS/BM**)

Bleach (for disinfection)
Nolvasan (if used for disinfection instead of bleach)
http://www.valleyvet.com/ct_detail.html?pgguid=30e0778a-7b6a-11d5-a192-00b0d0204ae5&ccd=IFF003&utm_source=google&utm_medium=cpc&utm_campaign=F%2BCat%2BLivestock%2BEquip%2BAnd%2BSupp%2B%286000%29%2BPLA&mr:trackingCode=2AF1A264-3C81-E211-BA78-001B21631C34&mr:referralID=NA&mr:adType=pla&mr:ad=15165184603&mr:keyword=&mr:match=&mr:filter=35193115603&gclid=CKqK5Kb71rYCFUOe4AodEiYAPA
Multi-Purpose Pump Sprayers (at least 2; one for bleach solution, one for water)
Disposable antibacterial wipes
Magnifying Glass to determine Gosner Stage (**FS/BM**)

Equipment for Processing, Labeling, and Shipment
Snack-size zip-lock bags (for euthanasia, ~ 1,500)
2 boxes of 1 oz. Whirl-Pak Bags (each box has 500 bags, for storing individual tadpoles in Ethanol) (http://www.enasco.com/product/B01067WA)
Gallon-size zip-lock bags (for collection and shipment, ~ 60)
Large garbage bags (for disposal of used bags for euthanasia and other waste, ~ 60)
Duct Tape or Packing Tape
Water-Resistant Marker (**FS/BM**)
Cooler(s) (for storing animals in the field)
Styrofoam Containers (for shipment)
Blue ice cold packs (for keeping specimens cool in the field when needed)
Paper Towels (absorbent packing material for shipping)
Benzocaine (Science Lab, http://www.sciencelab.com/page/S/PVAR/SLB3184)
Sterile or Dechlorinated Water
95% Ethanol (EtOH, for benzocaine solution for euthanasia)
70% Ethanol (EtOH, for fixing larvae for shipment to Montclair State)
Container (e.g., plastic bottle) for storing diluted benzocaine solution (**FS/BM)**
Container (e.g., plastic bottle) for storing used benzocaine solution for disposal (**FS/BM)**
Disposable pipets (for transfer of benzocaine solution or ethanol into bags, **FS/BM**)

DIE-OFF SAMPLING (All affected species – 10 animals)

Field Methods

Die-off Samples should be taken at a study pond in either of two situations: (1) Dip-netting yields amphibian larvae that are showing signs of ranaviral infection (e.g., reddening of their ventral skin, especially around the base of the hind limbs and the vent opening - Fig. 7; abnormal swimming behavior); and/or (2) a die-off has already occurred and dead tadpoles are observed in the breeding pond. Samples of 10 larvae of each infected species should be collected for shipment to the National Wildlife Health Center (NWHC). Attempts should be made, based on personnel availability, to visit infected ponds through metamorphosis to observe the length of time of the die-off and if any animals survive. Additional samples may be collected throughout the chronology of the disease event, based on consultation with the National Wildlife Health Center. Fill out Die-off datasheets when samples are collected (Appendix D). All boots, equipment and

dip-nets must be disinfected between sites in 10% bleach or 1% Nolvasan solutions to ensure no disease transmission between study sites.

Live sick tadpoles provide the best specimens for diagnostic evaluations. However, once tadpoles exhibit clinical signs of ranaviral infection, they will typically only live for another 12-48 hours. Therefore, if live sick larvae are sampled then they should be euthanized in the field. All Die-off Samples will involve shipment of dead larvae, but these may be from a combination of euthanized larvae and individuals already found dead. If dead tadpoles are observed at a site, it is very important that the collected tadpoles are freshly dead (i.e., not covered with watermold, body cavity is intact) and that they are promptly **frozen** before mailing. Chilled dead tadpoles should not be sent, since they will rot quickly in the overnight mail and likely be unsuitable for most tests and cultures.

For Die-Off Samples, the 10 individuals of a given species will be packaged together in a single bag. Each group of 10 individuals should be given a *unique* sample number, ordered sequentially as Die-Off Samples are taken. It is important to keep track of the species and study pond that each sample number is associated with, both on datasheets and in databases or spreadsheets.

Instructions for Field Methods:

(1) Disposable vinyl or nitrile gloves should be worn when handling live or dead amphibians.

(2) If signs of ranaviral infection are present in amphibian larvae or a die-off is observed, be sure to thoroughly sample all microhabitats of the breeding pond by dip-net to examine larvae of all species present. Ten individuals of each infected species should be collected for shipment to NWHC.

(3) For collection of **dead** larvae, the 10 individuals of one species can be placed in a single zip-lock bag (e.g., sandwich-size). If multiple species have been affected by the die-off, use separate bags for each species. Close and seal the zip-lock bags, and cover the zipper bag closure with packing or duct tape. Do **NOT** put water or any other liquid in the bags with the dead larvae. Using a water-resistant marker, write the date of collection, species, sample number, location (pond code), and collector name on the outside of each bag. Immediately place the bags in a cooler with ice packs in the field to chill the carcasses. Dead specimens should be frozen until they are shipped (preferably within 24 hours).

(4) Euthanasia: for collection of **live** larvae that are showing clinical signs of ranaviral infection, place each tadpole in a separate small zip-lock bag (e.g. snack-size bags) with enough benzocaine hydrochloride to cover the tadpole (see **Euthanasia** section below for more details). The benzocaine hydrochloride solution can be poured into the zip-lock bags or transferred using disposable plastic pipets. Wait until respiration ceases (< 1 minute), and then keep tadpole in benzocaine solution for an additional 5

minutes. The euthanized individuals of one species can be placed in a single zip-lock bag (sandwich-size). Close and seal the zip-lock bags, and cover the zipper bag closure with packing or duct tape. Do NOT put water or any other liquid in the bags with the dead larvae. Using a water-resistant marker, write the date of collection, species, sample number, location (pond code), and collector name on the outside of each bag. Immediately place the bags in a cooler with ice packs in the field to chill the carcasses. Dead specimens should be frozen until they are shipped (preferably within 24 hours).

Note: A combination of dead and euthanized larvae can be included in a sample to reach 10 individuals per species. If so, all 10 individuals of one species can still be placed in a single bag, as described above.

(5) If any animals were euthanized, pour used benzocaine hydrochloride into a container and put bags used for euthanasia into a garbage bag for disposal. The used benzocaine solution can be poured down a sink drain after returning from the field.

(6) Disinfect all field equipment and footwear between sites with 10% bleach or 1% Nolvasan solutions (see **Disinfection Guidelines** section).

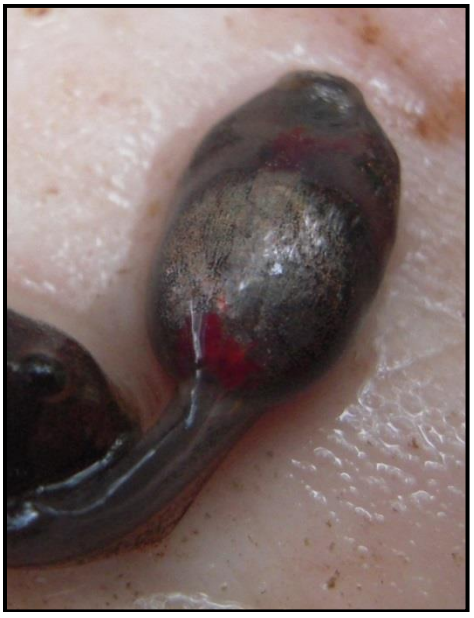

Figure 7. Wood Frog Larvae – RV Symptoms

Euthanasia

Amphibian larvae will be euthanized by benzocaine hydrochloride water baths. This method of euthanasia is approved by the American Veterinary Medical Association Panel on Euthanasia Guidelines on Euthanasia (AVMA, 2013, p. 77). The diluted solution of benzocaine hydrochloride that will be used for euthanasia can be prepared in the lab prior to going out into the field and stored in a 1 L or larger container for field use. Caution

should be taken when handling benzocaine, as it can cause skin and eye irritation. If contact occurs, flush skin or eyes with water for at least 15 minutes. Do not conduct euthanization in the wetland – move to an adjacent upland area.

Euthanization Instructions:

Note: Steps (1) and (2) below can be completed in the lab *before* going to a field site.

(1) Mix 5 g of benzocaine (chemical name = ethyl p-aminobenzoate) with 50 mL of 95% EtOH to create a concentrated solution of benzocaine hydrochloride.

(2) Add 2.5 mL of concentrated solution to 1 L of sterile or dechlorinated water.

(3) Add diluted solution to small zip-lock bag (enough to cover tadpole) and add tadpole. Disposable plastic pipets may be used to transfer the diluted solution into the small zip-lock bags.

(4) Remove tadpole 5 minutes after respiration ceases. Pour the used benzocaine hydrochloride solution from the bags into a container (e.g. plastic bottle), and put the used bags into a larger garbage bag for disposal. The used solution can be poured down a sink drain after returning from the field.

Labeling and Shipping Instructions for Die-off Samples

(1) If dead or euthanized larvae of multiple species were collected, place the bags of each species in a larger zip-lock bag (gallon-size, one large bag per site). Close the larger zip-lock bag and seal with packing or duct-tape.

(2) On the outside of the larger zip-lock bags, write the date collected, location, number of animals and species, and name of collector with a water-resistant lab marker.

(3) Use a hard-sided cooler in good condition for shipment. Close the drain plug of cooler and tape over inside. Line the cooler with a thick plastic bag (1 mil thickness).

(4) Place paper towels in the thick plastic bag to absorb any liquids that might leak during shipping.

(5) Pack the larger zip-lock bags (one per site) into the thick plastic bag lining the cooler with enough frozen blue ice packs or similar coolant to keep carcasses cold. Use enough coolant to keep samples chilled if there is a delay in delivery. Blue ice (unfrozen) can be obtained at hardware, sporting goods, or grocery stores. Wet ice can be used if frozen in a sealed plastic container (i.e., soda or water bottle). DO NOT USE DRY ICE.

(6) Seal the thick plastic bag by twisting it closed, folding it over on itself, and securing with packing or duct tape.

(7) Place the completed NWHC Specimen History Form and return shipping label in a zip-lock bag and tape to the inside lid of the cooler (if you want the cooler returned). NWHC CANNOT PAY FOR SHIPPING.

(8) Using packing or duct tape, tape the cooler shut around the lid and at each end using a continuous wrap around the cooler.

(9) Attach the shipping document (airbill) with the Department of Transportation information below to the outside of each cooler in a resealable pouch:

Address:
National Wildlife Health Center
Necropsy Loading Dock
6006 Schroeder Road
Madison, WI 53711
Emergency Contact:
NWHC FIT emergency
608-270-2400
Supplementary Labels**:**
Keep Cold

(10) The NWHC Shipping Instructions Form has a diamond-shaped symbol and the words **'Exempt Animal Specimens'** on the last page. These should be printed out and taped to the outside of the cooler.

(11) Contact Anne Ballmann (USGS Field Investigation Team Member, Eastern States, 608-270-2445) to get shipping approval and discuss shipping arrangements. Typically, specimens should be shipped by 1-day (overnight) service (e.g. Fedex, UPS), Monday through Wednesday, to guarantee arrival at NWHC before the weekend. If specimens are fresh and need to be shipped on Thursday or Friday, special arrangements can be made.

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(12) Note the tracking number in case packages are delayed.

(13) Email the completed **Specimen History Form** and tracking number to Anne Ballmann (**aballmann@usgs.gov**). This information must be received before the package arrives.

(14) Call David Green (608-270-2482) or Jenn Buckner (608-270-2443) on the day the tadpoles are mailed so that they know to expect the samples and can put a trace-back on the box if it does not arrive.

Equipment Needed

Many of the equipment items listed below can be obtained from grocery stores, pharmacies, or hardware stores. In some cases, we have included links to websites for ordering more obscure items. If **FS/BM** is included in the description, that means the item can be ordered from a Forestry Suppliers or Ben Meadows catalog.

General Field Equipment
Rubber boots, hip-waders, chest waders (**FS/BM**)
5-gallon buckets (at least one, for initial collection of dip-netted larvae)
Large Dip-nets (for initially capturing animals) (**FS/BM**)
Small Dip-nets (for working with individual larvae)
Disposable Gloves (vinyl or nitrile, ~ 2,000, **FS/BM**)
Bleach (for disinfection)
Nolvasan (if used for disinfection instead of bleach)
http://www.valleyvet.com/ct_detail.html?pgguid=30e0778a-7b6a-11d5-a192-00b0d0204ae5&ccd=IFF003&utm_source=google&utm_medium=cpc&utm_campaign=F%2BCat%2BLivestock%2BEquip%2BAnd%2BSupp%2B%286000%29%2BPLA&mr:trackingCode=2AF1A264-3C81-E211-BA78-001B21631C34&mr:referralID=NA&mr:adType=pla&mr:ad=15165184603&mr:keyword=&mr:match=&mr:filter=35193115603&gclid=CKqK5Kb71rYCFUOe4AodEiYAPA
Multi-Purpose Pump Sprayers (at least 2; one for bleach solution, one for water)
Disposable antibacterial wipes
Magnifying Glass to determine Gosner Stage (**FS/BM**)

Equipment for Processing, Labeling, and Shipment
Snack-size zip-lock bags (for euthanasia, ~ 1,500)
Sandwich-size zip-lock bags (for storing lots of 10 larvae for die-off samples, ~ 100)
Gallon-size zip-lock bags (for collection and shipment, ~ 60)
Thick (1 mil) plastic bags (30, for lining inside of cooler when shipping Die-Off Samples, e.g. Glad Force Flex bags)
Large garbage bags (for disposal of used bags for euthanasia and other waste, ~ 60)
Duct Tape or Packing Tape
Water-Resistant Marker (**FS/BM**)
Cooler(s) (for storing animals in the field)
Hard-Sided Coolers (for shipment to NWHC)
Blue ice cold packs (for chilling specimens in the field and for shipment to NWHC)
Paper Towels (absorbent packing material for shipping)
Benzocaine (Science Lab, http://www.sciencelab.com/page/S/PVAR/SLB3184)
Sterile or Dechlorinated Water
95% Ethanol (EtOH, for benzocaine solution for euthanasia)
Container (e.g., plastic bottle) for storing diluted benzocaine solution (**FS/BM)**
Container (e.g., plastic bottle) for storing used benzocaine solution for disposal (**FS/BM)**
Disposable pipets (for transfer of benzocaine solution or ethanol into bags, **FS/BM**)

DISINFECTION GUIDELINES (modified from Miller and Gray, 2009)

Anthropogenic spread of pathogens is a potential threat to amphibian and reptile populations, and in some cases field researchers may have contributed to this threat. In the case of this study, disease transmission between sites from field researchers may not only negatively impact breeding populations but could also severely bias study results. Although additional research is warranted, it is likely that pathogens such as Ranaviruses and *Bd* can persist outside the host for months in aquatic environments. Therefore, it is critical that field personnel follow basic disinfecting procedures to prevent disease transmission between study sites, particularly when multiple sites are visited in the same day.

After sampling or monitoring activities are completed at a study pond, all personal gear (e.g., waders) and field equipment (e.g., buckets, dip-nets) should be cleaned and disinfected BEFORE traveling to a different site or returning from the field. First, equipment should be rinsed with water and all debris and mud removed, as organic matter and soil can reduce the effectiveness of disinfectants. Scrubbing brushes may be used to help remove mud and debris. Disinfection can be accomplished using either a 10% bleach solution or a 1% Nolvasan solution, as these concentrations have been shown to kill both *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses. The disinfectant should be in contact with the equipment for a minimum of 5 minutes for bleach and 1 minute for Nolvasan. Equipment should then be rinsed with water to remove any residual bleach/Nolvasan and avoid negatively impacting aquatic organisms. We recommend that all field personnel obtain two pump sprayers (one for bleach, one for rinse water) to have out in the field. Because bleach breaks down with exposure to air and sunlight, bleach solutions should be discarded if not used up within 5 days after mixing. After returning from the field, equipment and personal gear should be thoroughly washed and disinfected again before being hung in order to dry completely. Do not forget to thoroughly wash your hands, even though you were wearing nitrile gloves.

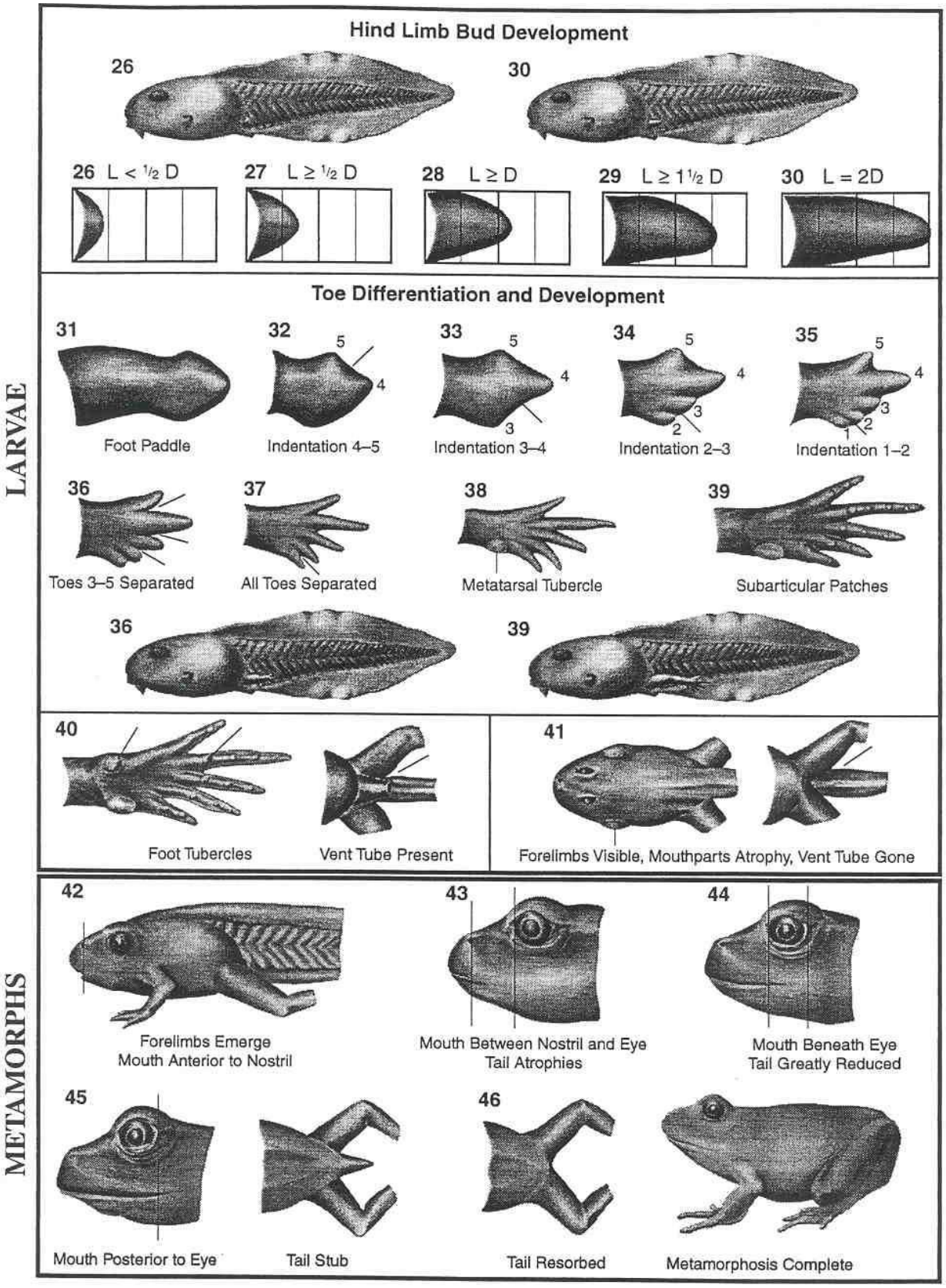
SAFETY GUIDELINES

Personal safety is the main priority during any field activities for this study. In general, field researchers should wear waterproof footwear (e.g., rubber boots, waders) and disposable gloves when conducting standard monitoring or collecting samples. If a die-off is observed at a study pond, first note if there are dead or morbid animals of other vertebrate taxa (e.g. fish, birds) in the vicinity. If so, then it is more likely that the animal mortalities may be due to toxins, which could pose significant human health risks. Contact the nearest public health department and wildlife agency if a die-off of several different wildlife taxa is observed prior to collecting specimens. If a die-off of many vertebrate taxa is observed, field personnel should wash and disinfect all equipment and change clothes before getting into a vehicle to leave the site.

Infectious amphibian diseases are rarely contagious to humans. However, amphibians may be carriers of pathogens that can be transmitted and cause disease in humans (e.g. *Salmonella* spp., mycobacteria). Therefore, disposable gloves should be worn whenever

handling amphibians. Field personnel should wash their hands or clean them with disposable antibacterial wipes after removing gloves. Avoid touching your eyes or mouth until you have washed your hands if you were handling amphibians. If any soaps or disinfectants are used for cleaning your hands in the field, do not expose these substances to the surface water of the breeding pond, as they could have negative impacts on aquatic organisms.

Appendix A – Gosner Stage Chart (Gosner 1960)



Appendix B – Study Pond Datasheet

RCN 2013-2014 RANAVIRUS STUDY: POND DATA SHEET

GENERAL SITE INFORMATION

State:_____ County:_____ Pond Code (e.g. MD-MO-01):_____

Ownership:_____ Latitude:_____ Longitude:_____

General Location Description:_____

POND CHARACTERISTICS

Natural/Manmade?:_____ Distance to Nearest Paved Road:_____ Road Type:_____

Pond Type (e.g. vernal pool, stormwater pond, beaver pond, etc.):_____

Canopy (open, closed, mixed):_____ Pond Dimensions (at max capacity): _____m x _____m

General Comments:_____

_____

POND VISIT SUMMARY (wood frog larvae observations and sampling)						
Date	Observer(s)	Max Water Depth (cm)	Gosner Stage(s)	Disease Signs? (Y/N)	Standard Sample Taken?	Die-Off Sample Taken?

ADDITIONAL HERPETOFAUNA OBSERVED							
	check appropriate boxes below						Die-Off
Species	Adult	Juv.	Meta.	Larvae	Call	Eggs	Sample Taken?

Appendix C – Standard Sampling Datasheet

RCN 2013-2014 RANAVIRUS STUDY: STANDARD SAMPLE DATA SHEET

GENERAL INFORMATION

State:_____ County:_____ Pond Code (e.g. MD-MO-01):_____

Date Collected:_____ Date Shipped:_____ WOFR Sample Numbers for Pond (e.g. 31 – 60):_____

Names of Field Personnel:_____

STANDARD SAMPLE INFORMATION

Dipnetting/Sampling: Start Time:_____ End Time:_____ # of Personnel_____

Euthanization/Processing: Start Time:_____ End Time:_____ # of Personnel_____

WOFR Larvae Exhibiting Disease Signs? (Y/N):_____ Gosner Stage(s) of Larvae Sampled (e.g. 36-38):_____

General Comments:_____

_____

_____

SUMMARY TABLE OF STANDARD SAMPLE OF 30 WOOD FROG LARVAE			
Sample Number	Disease Signs? (Y/N)	Collection (Euthanized, Found Dead)	Describe Any Disease Signs/ Abnormalities Observed

Appendix D – Die-off Datasheet

RCN 2013-2014 RANAVIRUS STUDY: DIE-OFF SAMPLE DATA SHEET

GENERAL INFORMATION

State:_____ County:_____ Pond Code (e.g. MD-MO-01):_____

Date Collected:_____ Date Shipped:_____

Names of Field Personnel:_____

DIE-OFF SAMPLE INFORMATION

Dipnetting/Sampling: Start Time_____ End Time_____ # of Personnel_____

Euthanization/Processing: Start Time_____ End Time_____ # of Personnel_____

General Comments:_____

_____

_____

_____

GENERAL SUMMARY OF LARVAL AMPHIBIANS OBSERVED DURING DIE-OFF				
Species	Sick/Dead Larvae? (Y/N)	Estimated # Sick	Estimated # Dead	Describe Any Disease Signs/ Abnormalities Observed

SUMMARY OF DIE-OFF SAMPLES COLLECTED						
Sample Number[a]	Species	Gosner Stage(s)	Collection Method			Comments
			# Euthanized	# Found Dead	Total	

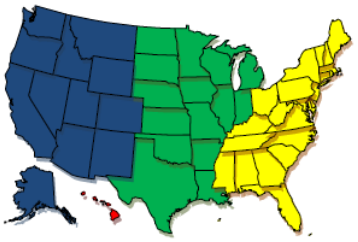
[a] Sample Number used for lots of 10 larvae of one species collected in Die-Off Sample

Appendix E – NWHC Shipping Instructions

November 2012

USGS – National Wildlife Health Center

INSTRUCTIONS FOR COLLECTION AND SHIPMENT OF AVIAN AND MAMMALIAN CARCASSES



Contact your USGS Field Investigation Team (FIT) member first!

Western States: Barb Bodenstein bbodenstein@usgs.gov, 608-270-2447
Central States: LeAnn White clwhite@usgs.gov, 608-270-2491
Eastern States: Anne Ballmann aballmann@usgs.gov, 608-270-2445
Hawaii, Pacific Islands: Thierry Work thierry_work@usgs.gov, 808-792-9520

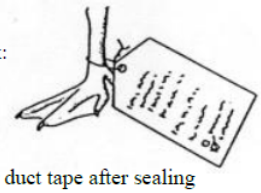
For single animal cases
Nationwide: Jennifer Buckner jbuckner@usgs.gov, 608-270-2443

Emergency Contact Number 608-270-2400

The following instructions should be used for collecting and shipping wildlife carcasses, carcass parts, and samples extracted from animals to the National Wildlife Health Center (NWHC) in Madison, Wisconsin, to insure adequate and well preserved specimens. For shipping instructions to the Honolulu Field Station, please contact Thierry Work (please see contact information above).

Freezing/thawing impedes isolation of some pathogens and damages tissues. NWHC prefers unfrozen specimens if they can be sent within 24-36 hours of collection or death. We will provide guidance on freezing samples on a case-by-case basis. As a general guideline: if you cannot call or ship within 24-36 hours, freeze the animal(s).

- ☐ Contact FIT to get shipping approval and discuss shipping arrangements. Typically, ship specimens by 1-day (overnight) service, Monday through Wednesday, to guarantee arrival at NWHC before the weekend. If specimens are fresh and need to be shipped on Thursday or Friday, special arrangements can be made.
- ☐ Email/fax history and tracking number to FIT. Packages will not be opened if history does not arrive first!
- ☐ Use rubber, vinyl, or nitrile gloves when picking up sick or dead animals. If you do not have gloves, insert your hand into a plastic bag.
- ☐ More than one disease may be affecting the population simultaneously. When possible, collect both sick and dead animals. Note behavior of sick animals before euthanizing.
- ☐ Collect specimens that are representative of all species affected and geographic areas.
- ☐ Collect the freshest dead specimens. Decomposed or scavenged carcasses are usually of limited diagnostic value. If you plan to collect animals in the field, take along a cooler containing ice to immediately chill carcasses.
- ☐ Contact NWHC for assistance when collecting samples from animals that are too large to ship.
- ☐ Collect animals under the assumption that an infectious disease or toxin is involved and other animals may be at risk. Protect yourself as some diseases and toxins are hazardous to humans.
- ☐ Immediately attach a leg tag to each animal with the following information in pencil or waterproof ink:
  - Date collected
  - Location (specific site, town, county, state)
  - Collector (name/address/phone)
  - Species
  - Found dead or euthanized
  - Your reference #



- ☐ Place each animal in a plastic bag, close, and seal the bag. Cover zipper bag closure with strapping or duct tape after sealing zipper.
  Twist non-zipper bags closed, fold over on itself, and secure with package strapping or duct tape.

- ☐ Place 1st bag inside a 2nd bag, close and seal. More than one individually bagged animal can be placed in the 2nd bag. This prevents cross-contamination of individual specimens and leaking shipping containers.

☐ Tag the outside of 2nd bag and number of animals and type, date collected, location, and name of collector. Reminder order: TAG, BAG, BAG, TAG.

☐ Use a hard-sided cooler in good condition for shipment. Close the drain plug of cooler and tape over inside. Line cooler with a thick bag (1 mil thickness, 3rd layer of bags).

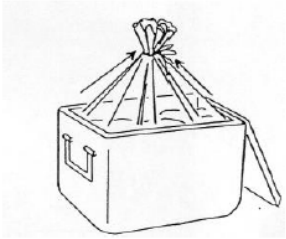
☐ Place absorbent material in the 3rd plastic bag to absorb any liquids that might leak during shipping.
See appendix for examples of bags and absorbent materials.

☐ Pack the individually bagged animal(s) that are contained within the 2nd sealed bag into the 3rd bag with enough FROZEN BLUE ICE PACKS or similar coolant to keep carcasses cold. Use enough coolant to keep samples chilled if there is a delay in delivery.

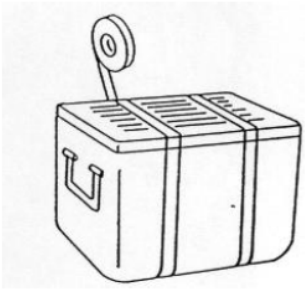
- Blue ice (unfrozen) can be obtained at hardware, sporting goods, or grocery stores.
- Wet ice can be used if frozen in a sealed plastic container (i.e., soda or water bottle).
- DO NOT USE DRY ICE.

☐ Seal the 3rd bag with methods described for 1st bag.

☐ Place the completed specimen history and return shipping label to the inside lid of the cooler (if you want the cooler returned). FOR SHIPPING.



in a ziplock bag and tape NWHC CANNOT PAY



☐ Using packing or duct tape, tape the cooler shut around the lid and at each end using a continuous wrap around the cooler.

☐ Attach the shipping document (airbill) with the DOT information below to the outside of each cooler in a resealable pouch:

Address:
National Wildlife Health Center
Necropsy Loading Dock
6006 Schroeder Road
Madison, WI 53711

Emergency Contact:
NWHC FIT emergency
608-270-2400

Supplementary Labels:
Keep Cold

☐ Mark the cooler with the appropriate information:
(See Pg. 3 for printable marking labels)

- Carcasses of animals that died of unknown causes:
  BIOLOGICAL SUBSTANCE, CATEGORY B and **UN 3373.**
- Blood and tissue samples from apparently healthy animals (hunter-killed, live captured):
  EXEMPT ANIMAL SPECIMENS.
- Blood and tissue samples from dead or sick animals:
  BIOLOGICAL SUBSTANCE, CATEGORY B and **UN 3373**.

☐ Note the tracking number in case packages are delayed.

☐ These instructions cover federal shipping regulations for commercial carriers.

Appendix:
Example of bags available at large supermarkets (list not all inclusive):

Inner and second layer bags:

Hefty Big Bag – 22 gal
Hefty Freezer – 1 gal
Hefty Jumbo – 2.5 gal
Ziplock Freezer – 1 gallon
Ziplock Big Bag – 20 gallon
Glad Freezer – 1 qt, 2 qt, 1 gal

Third layer for cooler liner:

Hefty Cinch Sak (1.1 mil) – 33 and 39 gal	Glad Force Flex (1.05 mil) – 25 gal
Hefty Lawn and Leaf (1.1 mil) – 33 and 39 gal	Hefty Ultra Flex (1.3 mil) – 30 gal
House brand large trash (1.1 mil) – 30 gal	House Lawn - Leaf (1.2 mil) – 39 gal

Absorbent material:

Super absorbent packet or pads for water	Cellulose wadding
Paper towels	Cotton batting or cotton balls
Do not use packing peanuts or shredded paper.	

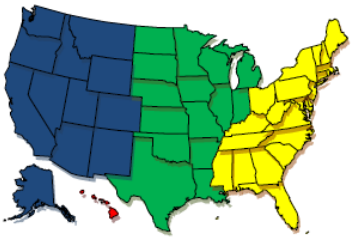
BIOLOGICAL SUBSTANCES, CATEGORY B

EXEMPT ANIMAL SPECIMENS

Appendix F – NWHC Specimen History Form

USGS
science for a changing world

National Wildlife Health Center
6006 Schroeder Road
Madison, WI 53711
Phone: 608.270.2400
FAX: 608.270.2415



SPECIMEN HISTORY FORM

For mortality events please e-mail a USGS Field Investigation Team member before shipping
Western States: Barb Bodenstein bbodenstein@usgs.gov, 608-270-2447
Central States: Dr. LeAnn White clwhite@usgs.gov, 608-270-2491
Eastern States: Dr. Anne Ballmann aballmann@usgs.gov, 608-270-2445
Hawaii, Pacific Islands: Dr. Thierry Work thierry_work@usgs.gov, 808-792-9520

For single animal cases,
Nationwide: Jennifer Buckner jbuckner@usgs.gov, 608-270-2443

Submitter's name: **Telephone:**

Address:

E-mail:

Collector's Name: **Affiliation:**

Telephone:

E-mail:

Date collected:

Method of animal collection: ☐ **Found Dead,** ☐ **Died in Hand,** ☐ **Euthanized**
Method of euthanization:

Species:

Number Submitted: **Condition:** ☐ **Chilled,** ☐ **Frozen,** ☐ **Preserved Tissues**

Specific die-off location (refuge unit, pond, address, intersection, park, etc):

State: **County:** **Nearest City:**

Latitude/longitude (Decimal degree in WGS 84): **Zone:**

Disease onset date: (Best estimate) **Disease end date:** (best estimate)

Species affected: (The diversity of species affected may provide clues to the disease involved.)

Age/sex: (Any pattern noticed that is related to age and sex?)

Known dead: (Actual number counted) **Known sick:**

Estimated dead: **Estimated sick:**
(Consider removal by scavengers or other means, density of vegetation, etc.)

Clinical signs: (Any unusual behavior and physical appearance.)

Population at risk: (Number of animals in the area that could be exposed to the disease.)

Population movement: (Recent changes in number of animals on area and their source or destination, if known.)

Problem area description: (Land use, habitat types, and other distinctive features.)

Environmental factors: (Record conditions such as storms, precipitation, temperature changes, or other changes that may contribute to stress.)

Comments: (Additional information/observations of value such as past occurrences of disease in area, photographs and videos are great additions.)

Appendix 3

Ranavirus Questionnaire to the States

Ranavirus (RV) Questionnaire to the States

This questionnaire is aimed at the agencies and persons who have legal authority over reptiles and amphibians in each State and their health (which may not be the same). This information is being used to compile data for the U.S. on *Ranavirus* prevalence, and people and processes in each state for dealing with this infectious disease.

STATE: **Your name:**
Agency:
Title:
Phone:
E-mail:

1) **Has *Ranavirus* (RV) in reptiles and/or amphibians been confirmed in your state? Suspected?**

2) **Was RV confirmation from one or more of the following sources:**

a) **National Wildlife Health Center (WI)?**
b) **State Animal Health Lab?**
c) **University? What school/lab?**
d) **Other? Please elaborate:**

3) **How many counties in your state has RV been confirmed? Suspected?**

4) **Does your state have a State Wildlife Veterinarian?**

If so please provide – Name:
Address:
Phone:
e-mail:

5) **Is there a single contact person in your state for sick herpetofauna?**

Other?

If not State Vet please provide name and contact info:
Name:
Address:
Phone:
e-mail:

6) **Is there a process in place in your state for reporting sick herpetofauna and getting them to labs for diagnosis?**

If yes, what is that process?

Appendix 4

Summary of lab-confirmed *Ranavirus* in northeastern U.S.

Appendix 4. Summary[a] of lab-confirmed *Ranavirus* in northeastern U.S. by state, county & year.

STATE	COUNTY	YEAR	SPECIES[b]	SOURCE(S)
CT	Fairfield	2009	LISY	NWHC Diagnostic Lab
CT	Fairfield	2013	LISY	Rittenhouse et al. 2013/WSU Lab
CT	New London	2011	AMMA, LISY	NWHC Diagnostic Lab
CT	New London	2012	LISY, LICL	Rittenhouse et al. 2013/WSU Lab
CT	New London	2013	LISY	Rittenhouse et al. 2013/WSU Lab
CT	Windham	2012	LISY, LICL	Rittenhouse et al. 2013/WSU Lab
CT	Windham	2013	LISY	Rittenhouse et al. 2013/WSU Lab
CT	Litchfield	2013	LISY	Rittenhouse et al. 2013/WSU Lab
CT	Middlesex	2013	LISY	Rittenhouse et al. 2013/WSU Lab
CT	New Haven	2009	AMMA, LISY	NWHC Diagnostic Lab
CT	New Haven	2010	LISY	NWHC Diagnostic Lab
CT	New Haven	2013	LISY, LICL	Rittenhouse et al. 2013/WSU Lab
CT	Tolland	2013	LISY, LICL	Rittenhouse et al. 2013/WSU Lab
DC	N/A	2014	TECA	NWHC Diagnostic Lab
DE	Kent	2013	LISY	This study/MSU Lab
DE	Kent	2014	LISY	This study/MSU&NWHC labs
DE	New Castle	2013	LISY, PSCR, GLMU, TECA, ESTU	This study/MSU&NWHC labs; WCS/Bronx Zoo lab
DE	New Castle	2014	LISY, LISP	This study/MSU&NWHC labs
DE	Sussex	2013	LISY	This study/MSU Lab
DE	Sussex	2013	LISY	This study/NWHC Lab
MA	Not given	1999	LICA	Green et al. 2002
MA	Hampshire	2000	LISY, AMMA	Green et al. 2002/NWHC Lab
MA	Barnstable	2008	TECA	UCONN Vet. Med. Diagnostic Lab
MA	Plymouth	2009	TECA	UCONN Vet. Med. Diagnostic Lab
MA	Hampden	2009	TECA	UCONN Vet. Med. Diagnostic Lab
MD	Prince Georges	2001	AMMA, LICL, LICA	NWHC Diagnostic Lab
MD	Prince Georges	2002	LICL, LISY, PSCR	NWHC Diagnostic Lab
MD	Prince Georges	2003	LICL	NWHC Diagnostic Lab
MD	Montgomery	2005	AMMA, HYCH, LISY	NWHC Diagnostic Lab
MD	Montgomery	2008	TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2009	TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2010	AMMA, LISY, TECA,	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2011	TECA	NWHC, Farnsworth & Seigel 2013
MD	Montgomery	2013	LISY	This study/NWHC Lab
MD	Anne Arundel	2008	TECA	NWHC Diagnostic Lab
MD	Frederick	2011	LICL	NWHC Diagnostic Lab
MD	Frederick	2013	LISY	This study/NWHC Lab

[a] This table includes results of this study, the information presented in Table 1, and all lab-confirmed RV for the other 9 states and DC.
[b] 33 species in total. See Appendix 1 for species acronyms.

STATE	COUNTY	YEAR	SPECIES[b]	SOURCE(S)
MD	Harford	2012	TECA	NWHC Diagnostic Lab
MD	Harford	2013	LISY	This study/NWHC Lab
MD	Harford	2014	TECA	NWHC Diagnostic Lab
MD	Baltimore	2013	LISY	This study/NWHC Lab
MD	Baltimore	2014	LISY	This study/MSU&NWHC labs
MD	Howard	2013	LISY	This study/NWHC Lab
MD	Howard	2013	LICA, CHPI	NWHC Diagnostic Lab
MD	Howard	2014	LISY	This study/NWHC Lab
MD	Talbot	2013	AMMA, LISY, SCHO	This study/MSU&NWHC labs
MD	St. Mary's	2014	TECA	NWHC Diagnostic Lab
ME	Not given	1991	LISY	Green et al. 2002
ME	Aroostook	1998	AMMA	Green et al. 2002/NWHC diagnostic lab
ME	Aroostook	2003	LICA, LICL	NWHC Diagnostic Lab
ME	Not given	2000	LIPA	Green et al. 2002
ME	Hancock	1999	LISY	Green et al. 2002/Gahl and Calhoun 2010
ME	Hancock	2000	AMMA, PSCR, LICL, LISY	Green et al. 2002/Gahl and Calhoun 2010
ME	Hancock	2001	AMMA, LICA, LICL, LISY	Gahl and Calhoun 2010/NWHC diagnostic lab
ME	Hancock	2002	PSCR, LISY	Gahl and Calhoun 2010/NWHC diagnostic lab
ME	Hancock	2003	LICA, LICL	Gahl and Calhoun 2010/NWHC diagnostic lab
ME	Hancock	2004	LICA, LICL, LISY	Gahl and Calhoun 2010/NWHC diagnostic lab
ME	Hancock	2005	LICL, LISY	Gahl and Calhoun 2010/NWHC diagnostic lab
ME	Penobscot	2000	LICA	Green et al. 2002/NWHC diagnostic lab
ME	Penobscot	2013	AMMA	NWHC Diagnostic Lab
ME	Penobscot	2014	AMLA, LISY	NWHC Diagnostic Lab
ME	Cumberland	2013	LISY	Wheelwright et al. 2014
ME	York	2014	LISY	NWHC Diagnostic Lab
NH	Not given	1999	LIPA	Green et al. 2002
NH	Cheshire	2000	LICA	Green et al. 2002/NWHC diagnostic lab
NH	Merrimack	2014	LISY	NH Vet. Diagnostic Lab
NJ	Ocean	2011	ANFO, LICL	Monsen-Collar et al. 2013
NJ	Morris	2013	LISY	This study/MSU Lab
NJ	Passaic	2013	LISY	This study/MSU Lab
NJ	Passaic	2014	LISY	This study/MSU Lab
NJ	Sussex	2013	LISY	This study/MSU Lab
NJ	Sussex	2013	CHPI	MSU Lab
NJ	Sussex	2014	LISY	This study/MSU&NWHC labs
NJ	Warren	2013	LISY	This study/MSU Lab
NJ	Warren	2014	AMMA, LISY	This study/MSU&NWHC labs
NY	Suffolk	2005	TECA	Johnson et al. 2008
NY	Suffolk	2007	AMTI	Titus and Green 2013
NY	Suffolk	2008	AMTI, LICL, LISP	Titus and Green 2013/NWHC Lab

STATE	COUNTY	YEAR	SPECIES[b]	SOURCE(S)
NY	Suffolk	2009	LICA, LISP	NWHC diagnostic lab
NY	Albany	2008	LISY	Brunner et al. 2011.
NY	Essex	2008	AMMA, LICL, LISY	Brunner et al. 2011.
NY	Ulster	2008	AMJE, AMMA	Brunner et al. 2011.
NY	Ulster	2013	AMMA	NWHC/San Diego Zoo Amph. Dis. Lab
NY	Westchester	2008	LISY, Ambystoma spp.	Brunner et al. 2011.
NY	Oswego	2012	EUBI, LICA, LICL, LIPI, PSCR	SUNY Oswego Diagnostic Lab
NY	Oswego	2013	EUBI, LICA, LICL, LIPI, LISY	SUNY Oswego Diagnostic Lab
NY	Orange	2013	AMMA	San Diego Zoo Amphibian Disease Laboratory
NY	Saratoga	2013	LICA	San Diego Zoo Amphibian Disease Laboratory
PA	Venango	2003	TECA	Johnson et al. 2008
PA	Northampton	2007	NOVI	Glenney et al. 2010
PA	Centre	2009	CHSE	NWHC Diagnostic Lab
PA	York	2012	AMMA	NWHC Diagnostic Lab
PA	Huntingdon	2014	LISY	This study/MSU Lab
RI	Washington	2001	LISY	NWHC Diagnostic Lab
RI	Washington	2002	LISY	NWHC Diagnostic Lab
RI	Washington	2005	AMMA, CHPI, LISY	NWHC Diagnostic Lab
RI	Washington	2007	LISY	NWHC Diagnostic Lab
RI	Washington	2008	AMMA, AMOP, LISY	NWHC Diagnostic Lab
RI	Newport	2004	LIPI	NWHC Diagnostic Lab
VA	City of Virginia Beach	2003	LISP	NWHC Diagnostic Lab
VA	Dickenson	2008/09	ANAE	Hamed 2013
VA	Grayson	2008/09	DEFU, DEMO, DEQU, DEOR, DEOG, PLMO, PLWE	Hamed et al. 2013, Hamed 2013
VA	Smyth	2008/09		Hamed et al. 2013, Hamed 2013
VA	Washington	2008/09		Hamed et al. 2013, Hamed 2013
VA	Wise	2010	DEFU, DEMO, DEQU, EUCI, EULO,	Davidson & Chambers 2011
VA	Wise	2010	EULU, LICA, LIPA, NOVI, PLGL	Davidson & Chambers 2011
VA	Prince Edward	2010	CHPI	Goodman et al. 2013/Univ. of GA Diagnostic Lab
VA	Loudon	2013	TECA	Univ. of TN Diagnostic Lab
VA	Northampton	2013	TECA	NWHC Diagnostic Lab
VA	Shenandoah	2014	LISY	This study/MSU Lab
WV	Kanawha	2012	TECA	NWHC Diagnostic Lab
WV	Berkeley	2014	LICL	NWHC Diagnostic Lab
WV	Mineral	2014	TECA	USGS Fish Health Lab
WV	Roane	2014	TECA	USGS Fish Health Lab