MD-16-SHA-TU-1-04

Maryland Department of Transportation
STATE HIGHWAY ADMINISTRATION

RESEARCH REPORT

Are Outbreaks of Emerging Pathogens Correlated with Construction of Wetlands?

Report 2: Amphibian Breeding and Disease Outbreaks During 2014 2015 and Possible Correlates with Environmental Variables

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TOWSON UNIVERSITY

FINAL REPORT
October 2016

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Technical Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
MD-15-SHA-TU-1-04		

Are Outbreaks of Emerging Pathogens Correlated With Construction of Wetlands? Report 2: Amphibian Breeding and Disease Outbreaks During 2014-2015 And Possible Correlates With Environmental Variables	5. Report Date October, 2016
	6. Performing Organization Code
7. Author/s R. A. Seigel, W. Saffell, C. Patterson, B. Durkin, S. Martin, M. Lawrance, and A. Savage	8. Performing Organization Report No.
9. Performing Organization Name and Address Towson University 8000 York Road Towson, MD 21252	10. Work Unit No. (TRAIS)
	11. Contract or Grant No. SP509B4N
12. Sponsoring Organization Name and Address Maryland State Highway Administration Office of Policy & Research 707 North Calvert Street Baltimore MD 21202	13. Type of Report and Period Covered Final Report
	14. Sponsoring Agency Code (7120) STMD - MDOT/SHA

15. Supplementary Notes

16. Abstract

A study of wetlands near the Intercounty Connector construction site (now a toll facility – MD 200) in Maryland, found that an emerging pathogen known as *Ranavirus* was having a significant impact on at least two species of amphibians as well as on Box Turtle populations. Of special interest was the finding that *Ranavirus* outbreaks were found in two wetlands constructed by Montgomery County Parks as part of habitat restoration procedures. This supports the findings of earlier researchers who suggested that *Ranavirus* outbreaks might be associated with the construction of wetlands. Such newly constructed wetlands sites may be a focal point for infection due to rapid colonization by susceptible species, such as Wood Frogs or Spotted Salamanders. These sites might also be used as foraging sites by species thought to be carriers of *Ranavirus,* such as Bullfrogs or Green Frogs.

In order to understand how *Ranavirus* is affecting amphibian and reptile populations in the Northeast U.S. and to determine the link, if any, between construction of wetlands and *Ranavirus* outbreaks, Towson University examined 60 Maryland Department of Transportation State Highway Administration (SHA) created wetlands. Ultimately, 16 sites in 2014 and 22 in 2015 were selected for focal monitoring. Six sites (37.5%) had some level of die offs in 2014 compared with eight sites (36.3%) in 2015. These rates were slightly higher than those seen in a study of non-SHA wetlands in Maryland. Due to quality control issues, Polymerase Chain Reaction (PCR) testing was considered reliable only in 2015. PCR testing in 2015 found the presence of *Ranavirus* in all die-off sites sampled and 54% of SHA wetlands sampled. Levels of *Ranavirus* in die off sites were extremely high, on the order of hundreds of millions of copies of the virus. These data suggest a possible link between environmental variables and presence of *Ranavirus* infection, but a larger sample size of ponds would provide a more robust test of this association.

17. Key Words	18. Distribution Statement: No restrictions		
Amphibians, emerging pathogens, *Ranavirus*, wetlands, disease	This document is available from the Research Division upon request.		
19. Security Classification (of this report) None	20. Security Classification (of this page) None	21. No. Of Pages 35	22. Price

Form DOT F 1700.7 (8-72) Reproduction of form and completed page is authorized.

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EXECUTIVE SUMMARY

A study conducted by Towson University (TU) at wetlands near the Intercounty Connector (ICC) construction site (now a toll facility – MD 200) in Montgomery County Maryland, found that an emerging pathogen known as *Ranavirus* was having a significant impact on at least two species of amphibians as well as on Box Turtle populations. Reports of outbreaks of disease caused by this virus are becoming increasingly common in the United States and worldwide. This disease is now thought to be a significant threat to a number of species of fish, amphibians, and reptiles. Of special interest was the finding by Towson University found that *Ranavirus* outbreaks at the ICC were present in two wetlands constructed by Montgomery County Parks as part of habitat restoration procedures. These data support the findings of earlier researchers that *Ranavirus* outbreaks may be associated with the construction of wetlands. Rapid colonization by highly susceptible species such as Wood Frogs or Spotted Salamanders may be a focal point for infection at such newly constructed wetlands sites. Also, these wetlands may be used as foraging sites by species thought to be carriers of *Ranavirus,* such as Bullfrogs or Green Frogs, or may have high infection rates due to unknown reasons.

To better understand the extent to which *Ranavirus* is impacting amphibian and reptile populations in the Northeast U.S. and to determine the link, if any, between construction of wetlands and *Ranavirus* outbreaks, the U.S. Fish & Wildlife Service funded a regional study from 2013-2015 designed to examine the prevalence of *Ranavirus* infections in Maryland, Virginia, Delaware, Pennsylvania, and New Jersey. Their study focused primarily on natural wetlands, especially vernal pools (i.e. temporary wetlands created when a depression fills with water), although some constructed wetlands were surveyed as well.

To address the question of the role (if any) between constructed wetlands and disease outbreak, the Maryland Department of Transportation State Highway Administration (SHA) funded a study by TU from 2014-2015. The study was designed to do the following:

a) examine whether the prevalence of *Ranavirus* is associated with wetlands built as part of highway construction projects,
b) examine whether such rates of prevalence are higher than for natural wetlands,
c) complete an assessment of how these rates differ among several states, and
d) examine the relationship between selected habitat variables and the prevalence of *Ranavirus*.

Combined with additional data from current and future studies, these data could eventually be used in a "best practices" set of recommendations to SHA to minimize chances that constructed or restored wetlands are contaminated with the disease.

Research Findings

The frequency of amphibian die-offs in SHA wetland sites in 2014 was 37.5% (6 of 16 ponds) compared with 36.3% in 2015 (8 of 22 ponds), with no significant differences between years. The composite rate of die-offs at SHA sites was slightly (but not significantly) higher than the 27.3% rate of die-offs seen at non-SHA sites in Maryland. However, differences in die-off rates need to be interpreted cautiously, as die-offs can be easily missed if field sampling is not conducted at least twice per week. Thus, the die-off rates noted here need to be taken as

minimum estimates and not exact values. In addition, die-off rates may be species-dependent, and the composition of species breeding at sampling sites in this study varied between years. For example, despite the fact that wetlands for the SHA study were selected to focus on sites where Wood Frogs were likely to breed, TU found successful Wood Frog breeding at only one of the 16 wetlands surveyed in 2014. Instead, TU found Pickerel Frogs or Leopard Frogs breeding at all of these wetlands.. By contrast, Wood Frogs bred at 17 of 22 SHA wetlands in 2015, likely due to a milder winter that year.

Samples of 435 tadpoles from all 16 SHA wetlands were tested via PCR to confirm the presence of *Ranavirus* in 2014. Unfortunately, the laboratory that did the processing apparently used a less sensitive method of detecting the virus, so the 2014 data are not considered reliable.

Samples of 719 tadpoles from all 22 SHA wetlands were tested via PCR to confirm the presence of *Ranavirus* in 2015 at the University of Central Florida. Of the 22 wetlands sampled, *Ranavirus* was isolated at 11 sites and inferred at 12 sites (54.5%). This value is comparable to the percentage of sites with positive tests for *Ranavirus* in Delaware (57.1%) and New Jersey (48.4%), but much higher than estimates from the non-SHA sites in Maryland (28.6%), Virginia (4.2%), and Pennsylvania (3.3%). Differences among years in the composition of amphibian communities may affect these results.

In addition to higher frequency of infections in the SHA wetlands, the levels of virus ("infection intensity") seen in affected specimens from the SHA sites were extremely high. In the six SHA-wetlands where samples of dying tadpoles were collected and used for DNA analysis, infection intensities ranged from the tens of millions to the billions of copies of the virus, levels several orders of magnitude higher than those seen in some other recent studies. No explanation for these differences are immediately apparent.

TU used an "information-theoretic" approach to analyze the 2015 data for prevalence of *Ranavirus*. This approach uses a maximum-likelihood analysis to determine which of several competing models best explain the observed data. This analysis showed that while models using the likelihood of wetlands drying and distance from nearest stream could be important variables in determining the occurrence of *Ranavirus* infections, models using these variables could not be separated from a "null" model. The same was true of models used to explain the occurrence of die-offs at specific sites. These models suggested that die-offs were related mainly to the presence of the virus, but had wide confidence intervals. Thus, there is, at present, insufficient data to indicate that environmental variables such as wetlands age or distance to road are associated with higher rates of prevalence of *Ranavirus*. A larger number of wetlands need to be sampled for more years to test this association in a rigorous manner.

The complexities of how this emerging pathogen spreads and affects amphibian populations are high and research on this pathogen remain in the early stages (e.g., Paull et al., 2012). Yearly differences in weather that affect the breeding populations of frogs and salamanders, different levels of wetlands depth, species composition, temperature during the breeding season can all play roles in determining how this virus affects amphibian communities. Additional studies using a large data set are needed to better understand how this pathogen spreads and affects wetlands in Maryland and elsewhere.

Table of Contents

List of Figures

Page No.

List of Tables

Page No.

List of Acronyms and Abbreviations

1) **FV3**: Frog Virus 3, the most common strain of *Ranavirus* that infects amphibians.

2) **GPS**: Global Positioning System

3) **JMP**: Statistical software used for data analysis

4) **MD-DNR**: Maryland Department of Natural Resources

5) **PCR**: Polymerase Chain Reaction.

6) **R**: Statistical software used for data analysis

7) **SHA**: Maryland Department of Transportation State Highway Administration

8) **SYSTAT**: Statistical software used for data analysis

9) **TU**: Towson University

10) **UCF**: University of Central Florida

ACKNOWLEDGEMENTS

Thanks go to the Maryland Department of Transportation State Highway Administration (SHA) for arranging funding and granting permission to conduct this study. Not many state agencies and municipalities are willing to spend their limited funds and time to better understand how to protect wildlife, and TU is most grateful to SHA for their willingness to support our work. Special thanks go to Bill Buettner, Allison Hardt, Cheryl Jordon, and Sharon Hawkins for their input and guidance. Special appreciation also goes to Scott Smith of MD-DNR, who has been a constant source of support during our work on Ranavirus, including collaboration with the RCN study. Thanks also go to Cindy Driscoll of MD-DNR for her support, as well as to Kirsten Monsen of Montclair State University for processing the DNA samples from 2014. Scott Farnsworth and Craig Patterson, former TU graduate students, who were close collaborators in the early portion of this study, as well as TU graduate student Scott Martin for his contribution in the statistical analysis. Special thanks to TU-Northeast (TUNE) for providing office space for the completion of this report in 2016.

A very large number of individuals assisted us with fieldwork or other aspects of the study. Among those providing field help, special thanks goes to Brian Durkin, Matt Gutt, Kaytlyn Gilliam, Caitlin Principe, Tuana Phillips, Eileen Ball, Marion Clement, and Michael Lagua.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Emerging infectious diseases are one of the most important factors contributing to global wildlife declines (Daszak et al 2000; Walker et al. 2008; Pavlin et al 2009). Severe declines of amphibian species worldwide are associated with several such emerging pathogens, especially the so-called "Chytrid fungus", *Batrachochytrium dendrobatidis* (*Bd*), which has received considerable and well-deserved attention over the last decade (e.g., Lips et al. 2006). Reports of significant mortality due to outbreaks of *Ranavirus* (Family Iridoviridae) are increasingly common in the United States and worldwide, with the reported number of die-offs 3-4 times greater than for *Bd*. *Ranavirus* differs from *Bd* in that both amphibian and reptiles are known to be affected (Gray et al. 2009; Hoverman et al. 2011; Brenes et al. 2014).

Unfortunately, information on the timing, extent, and frequency of occurrence of outbreaks of *Ranavirus*[1] 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 3-4 days elapse between the initial signs of the disease and the disappearance of tadpoles from the environment (Farnsworth and Seigel, 2013). Thus, unless frequent observations (twice per week) 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.

A study conducted at wetlands near the Intercounty Connector (ICC) construction site (now a toll facility – MD 200) in Montgomery County Maryland, found that *Ranavirus* was having a significant impact on at least two species of amphibians as well as on Box Turtle populations (Farnsworth and Seigel, 2013). Of special interest was the finding that *Ranavirus* outbreaks were found in two wetlands that were constructed by Montgomery County Parks as part of habitat restoration procedures. These data support the findings of Harp and Petranka (2006), Petranka et al. (2007), and Richter et al. (2013) that *Ranavirus* outbreaks may be associated with the construction of wetlands sites. Such newly constructed wetlands sites may be a focal point for infection due to rapid colonization by highly susceptible species, such as Wood Frogs or Spotted Salamanders. These sites may also be used as foraging sites by species thought to be carriers of *Ranavirus* such as Bullfrogs, or may have high infection rates due to unknown reasons.

This project had two primary goals (a) to gain a better understanding of the extent to which *Ranavirus* has affected amphibian and reptile populations in the Northeast U.S. and (b) to determine the link, if any, between construction of wetlands sites and *Ranavirus* outbreaks. To accomplish these goals, Towson University sampled amphibian larvae at a series of constructed or restored wetlands sites in Maryland in order to determine the prevalence of *Ranavirus* at these sites. The U.S. Fish & Wildlife Service, funded parallel studies to examine the prevalence of Ranavirus infections on a regional basis, in Maryland, Virginia, Delaware, Pennsylvania, and New Jersey. This study focused primarily on natural wetlands, especially vernal pools, although some constructed wetlands were also surveyed.

[1] Ranavirus: Genus of virus known to cause disease outbreaks in fish, amphibians, and reptiles

The study was designed to do the following:

(a) examine whether the prevalence of *Ranavirus* is associated with wetlands built as part of highway construction projects,

(b) examine whether such rates are higher than for natural wetlands, and

(c) conduct an assessment of how these rates differ among several states, and

(d) examine the relationship between a variety of habitat variables and the prevalence of *Ranavirus*.

Combined with additional data from current and future studies, these data can eventually be used in a "best practices" set of recommendations to SHA to minimize chances that constructed or restored wetlands are contaminated with the disease. For example, there is preliminary evidence that pond persistence (hydro-period) plays a major role in determining probability of infection, with permanent ponds more likely to have infections than ephemeral wetlands. Thus, changing the drainage and depth of constructed wetlands to make the constructed wetlands dry periodically can be an important means of reducing the likelihood of *Ranavirus* infections. In addition, it is important for SHA to understand the correlation (if any) between site maturity (time since construction) and probability of *Ranavirus* infection. Finally, this research helped test the hypothesis that both natural and constructed wetlands have the same rate of infection; in that case, no alterations of current construction guidelines would be necessary.

CHAPTER 2: RESEARCH OBJECTIVES AND METHODOLOGY

2.1 Determine the number and spatial distribution of potential wetland sampling sites

In consultation with SHA, the research team first determined the number and spatial distribution of possible or "candidate" sampling sites for this study. The goal was to use a stratified random approach to select an appropriate number of sites for potential sampling during late winter and spring 2014 and during the same periods in 2015. The number of sites selected was dependent on the spatial distribution of the available wetlands, as logistical constraints meant that sampling would likely occur in some combination of the following counties; Anne Arundel, Baltimore, Carroll, Cecil, Harford, Howard, Montgomery, and Prince Georges.

The research team worked with SHA to review the available SHA database of constructed wetlands. Sites constructed by SHA or modified as part of SHA projects were included in the database. Based on meeting with SHA and an examination of this database on constructed wetlands, the research team compiled a catalog of potential field sites, using wetlands age, habitat type, and proximity of roads as major variables. On-site inspection of sixty suitable sites began in February and March 2014-2015. This was the maximum number of potentially suitable sites available within a logistically feasible geographic area.

2.2 Conduct an on-site evaluation of potential wetland sampling sites

The research team conducted on-site evaluations of candidate wetlands and eliminated, for future use, ponds that did not meet established criteria (e.g., were no longer functional wetlands, had become polluted, heavily used by humans, lack of amphibian populations, etc.). Some sites that had acceptable physical characteristics were deleted from the sampling size due to either lack of water during the period February-May or the lack of any significant amphibian breeding activity.

During February and March 2014, the research team visited all 60-candidate wetlands sites. Site inspections consisted of an assessment of suitability of the sites using specific criteria, including presence/absence of amphibian breeding, proximity to roads, proximity to natural habitats for amphibian dispersal, water quality, and a preliminary assessment of wetland depth and likely hydro-period.

2.3 Determine the timing and impact of Ranavirus on amphibians breeding at SHA wetlands

The wetlands sites were sampled at the onset of the breeding season for Spotted Salamander and Wood Frogs egg masses and larvae to document the presence of active amphibian use. Return visits to these sites were conducted starting about 60 days after initial identification to determine degree of larval development. More frequent visits (normally two times per week) were used to determine whether there was a *Ranavirus*-caused mortality event, which is characterized by such easily-observed signs of disease as hemorrhaging from the ventral surface and abnormal swimming behavior (see Farnsworth and Seigel 2013 for details). Such mortality events typically cause complete loss of all larvae and tadpoles in the wetlands.

The research team made repeated sampling trips to the selected wetlands starting in mid-February 2014 and 2015, with visits continuing until July 2015 (Fig. 2.1).

Figure 2.1. Photograph of TU staff conducting routine field sampling of an SHA wetland

The primary goal during the early part of each season in 2014 and 2015 (February-early April) was to document the extent of breeding by amphibians, especially Wood Frogs and Spotted Salamanders. Breeding of both species was easy to document considering that they lay large, easily identified egg masses near the surface of the water, often in large clusters. The presence of other amphibian species (e.g., Pickerel Frogs, Leopard Frogs, American Toads, and Green Frogs) was assessed by either listening for distinctive vocalizations during field surveys, by direct observations of adults at the field sites, or by identification of tadpoles under a dissecting scope. In addition, automated recording devices (so-called "Frog Loggers) were used to record anuran (tailless amphibian) vocalizations at selected ponds in 2015 (Fig. 2.2).

Figure 2.2. Photograph of "frog logger" used to record calls of anurans during field sampling of an SHA wetland

Once breeding by at least one target amphibian species was documented, that wetland was marked for continuing surveys for *Ranavirus* and disease outbreaks.

Because of concerns for spreading infections among sampling sites, all boots, equipment and dip-nets were disinfected between sites in a bleach or Novalson solution to ensure no disease transmission between study sites. The Towson University Animal Care Committee approved all animal handling protocols.

2.4 Collect samples of amphibian larvae for DNA analysis

Because other diseases may cause sudden morality events in the field, it is essential that the causative agent is correctly identified. This is done via DNA samples using PCR[2]. The research team provided samples of tadpoles possibly infected with *Ranavirus* from Maryland to two

[2] Polymerase Chain Reaction. A technique for genetic confirmation of the presence of *Ranavirus*. The specific method of "qPCR" was used in this study

research laboratories to allow PCR testing. The first lab (at Montclair State University in New Jersey) was selected because it was also being used for the regional study of *Ranavirus* led by MD-DNR. The laboratory at the University of Central Florida was used for 2015 due to the availability of better and more sophisticated assays.

Starting about 60 days after the onset of breeding, the research team began to sample wetlands on a more frequent basis, at least once to twice per week. Once tadpoles or larvae were an appropriate size for collection (Gosner Stage 27-38) and neared metamorphosis, a sample of up to 30 individuals was taken for later examination for DNA markers for *Ranavirus*. Sampling involved 5-meter linear dip-net sweeps along the pond bottom around the pond perimeter in each of the cardinal directions (4) and two 5-meter sweeps in the central (deeper) pond area. The research team placed captured larvae in a wet bucket or tray after each sweep and sorted by species. Larvae were examined visually for indications of infection (reddening of their ventral skin, especially around the base of the hind limbs and the vent opening) (see Fig. 2.3).

Figure 2.3. Photo showing wood frog tadpoles infected with *Ranavirus*

Larvae specimens were killed humanely, preserved in either ethanol or frozen, and brought back to the research lab at Towson University for later DNA analysis (see Fig. 2.4).

Figure 2.4. Photograph of TU staff preparing tadpoles in 2014 for eventual DNA testing at Montclair State University.

DNA sampling was done at either Montclair State University (in 2014) or at the University of Central Florida (in 2015). The following general procedures were followed in 2015:

- Approximately 5mg of liver tissue was excised from each sample.
- Total genomic DNA was extracted via Qiagen DNeasy kits, with a final elution volume of 200µL.
- Genomic DNA was pooled based on species and collection site, with a 5µL of elution from each sample.
- Samples were stored at -20 C until qPCR runs.

TaqMan assays[3] were performed on a BioRad CFX96[4] real-time system. The target of amplification was FV3 – *Ranavirus* major capsid protein. Samples were amplified in tandem with standards of known concentration. UCF staff synthesized GeneBlock™[5] fragments matching the FV3 major capsid protein. Serial dilutions ranging from 2 x 109 to 2 x 10 gene copies were utilized to quantify viral load in sample reactions.

[3] TaqMan assay: a quantitative polymerase chain reaction (qPCR) procedure that uses a fluorescent DNA probe that is specific to the gene target of interest (the alternative is a non-specific SYBR green probe, which is less accurate). In this study, a TaqMan probe was custom built to bind only to *Ranavirus* and *Bd*.

[4] BioRad CFX96: the make and model of the qPCR machine we used to quantify *Ranavirus* infections

[5] GeneBlock: a custom-built synthetic gene fragment composed of an exact copy number of up to 500 DNA base pairs available through Integrated DNA Technologies (IDT). UCF staff built a GeneBlock consisting of the fragment of FV3 that the TaqMan probe and primers bind to, enabling them to use serial dilutions of precise quantities of this gene fragment to generate a standard curve and infer the FV3 quantity in each of the amphibian tissue samples.

In 2014, the laboratory procedures at Montclair State University were as follows:

- Necropsies were performed on all larval wood frogs with liver and kidneys removed for DNA extraction,
- The 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.
- A DNA sample, considered positive for *Ranavirus*, met the following criteria:
    - 1) Had an exponential increase in fluorescence during the qPCR (expected if double-stranded target DNA is amplified;
    - 2) Had a melting temperature within 2º C of the positive control run on the same RT-PCR plate;
    - 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. It was used to test the specificity of the product being amplified in the reaction; and
    - 4) A sample had to be positive on two separate independent plate runs on the qPCR.

2.5 Determine the environmental correlates of disease outbreaks

The research team determined the correlation, if any, between the physical, hydrological, chemical and biological parameters of the wetlands where *Ranavirus* occurs in an attempt to create a predictive model that shows what environmental factors are most closely associated with disease outbreaks.

Several variables were measured, at monitored wetlands, including:

- age of wetland in years, length of hydro-period (defined at the period selected ponds hold water from January 1st through the end of July),
- maximum observed pond depth, pond size,
- wetland type, source of water (ground water versus surface water),
- distance to perennial stream, distance to roadways, and
- distance to other sources of disturbance (houses, agricultural fields).

Water quality data were documented, with a hand-held YSI-85 meter[6], recording information on pH, salinity, conductivity, and temperature.

Following Otto et al. (2007), an information-theoretic approach was used to determine key habitat variables correlated with the occurrence (or lack of occurrence) of *Ranavirus* infections. The team constructed a series of explanatory models, then used AIC values to determine which models were the most parsimonious, i.e., which had the greatest explanatory power with the fewest number of steps (Otto et al., 2007).

[6] YSI-85 meter: Field instrument used to measure such water quality variables such as conductivity, pH, and dissolved oxygen.

CHAPTER 3: RESEARCH FINDINGS AND DISCUSSION

3.1 Number and spatial distribution of wetland sampling sites

Of the 60 wetlands sites initially identified via the SHA database as candidate sites for sampling, 16 were chosen to be monitored actively for amphibian breeding during 2014 and 22 for sampling in 2015. The composite table, of all 26 wetlands sites sampled, is in Table 3-1 below.

Table 3-1: List of wetland sites sampled for Ranavirus during 2014-2015, years sampled, and key habitat variables. *County abbreviations; AA (Anne Arundel), Balt (Baltimore), Harf (Harford), How (Howard), Mont (Montgomery), PG (Prince Georges).*

Pond ID	Co.	Sampled 2014?	Sampled 2015?	Pond type	Pond size full (m^2)	Mean pond depth (cm)	Wetland Dry up? (Y/N)	Dist to Stream (m)	Dist to Road (m)	Pond age (yr)
AA-01	AA	No	Yes	POW, PEM	700	25	N	1299	158	3
B-1	Balt	Yes	No	PEM	985	23	Yes-July	72	230	22
B-11	Balt	Yes	Yes	PEM	4933	19	N	156	195	10
B-GB	Balt	No	Yes	PEM	1478	6	N	5	7	23
HA-1	Har	No	Yes	PEM	6817	25	N	45	386	12
H-E-1	Har	Yes	Yes	POW, PFO	448	43	N	120	72	23
HO-05	How	Yes	No	PEM	334	30	Y-July	37	26	20
HO-06	How	Yes	Yes	PEM	918	18	N	25	11	21
HO-07	How	Yes	No	PEM	1361	26	N	69	140	21
HO-09	How	No	Yes	Vernal, PFO	116	12	N	10	78	14
HO-10	How	Yes	Yes	PEM	5461	11	Y	5	20	18
HO-15	How.	No	Yes	PEM	520	34	N	70	90	9
HO-16	How	Yes	Yes	POW, PEM	440	21	Y	101	296	5
HO-2-A	How	Yes	Yes	POW, stormwater	946	37	N	20	34	23
ICC-01	Mont	Yes	Yes	POW, PEM	1423	25	Y	12	20	8
ICC-05	Mont	No	Yes	POW, PFO	3411	11	N	5	15	3
ICC-08	Mont	Yes	Yes	POW, PEM	2136	34	N	170	279	3
ICC-16	Mont	No	Yes	PEM	57	21	N	15	353	4
M-11	Mont	Yes	No	POW, PEM	54	35	N	237	220	9
M-1-M2	Mont	Yes	Yes	Vernal, PFO	228	31	N	27	24	22
M-3	Mont	Yes	Yes	POW, PEM	1559	28	Y	12	8	22
M-8	Mont	No	Yes	POW, PEM	230	15	Y	15	5	21

MD-200	Mont	No	Yes	Vernal, PFO	60	25	N	111	92	7
SC-19	Mont.	No	Yes	PEM	137	22	N	30	193	2
P-4-A	P.G.	Yes	Yes	POW, mixed	1886	30	N	53	322	22
P-4-B	P.G.	Yes	Yes	POW, PFO	1942	91	N	249	227	23

These sites were located in six counties; Anne Arundel, Baltimore, Harford, Howard, Montgomery, and Prince Georges (see Fig. 3.1).

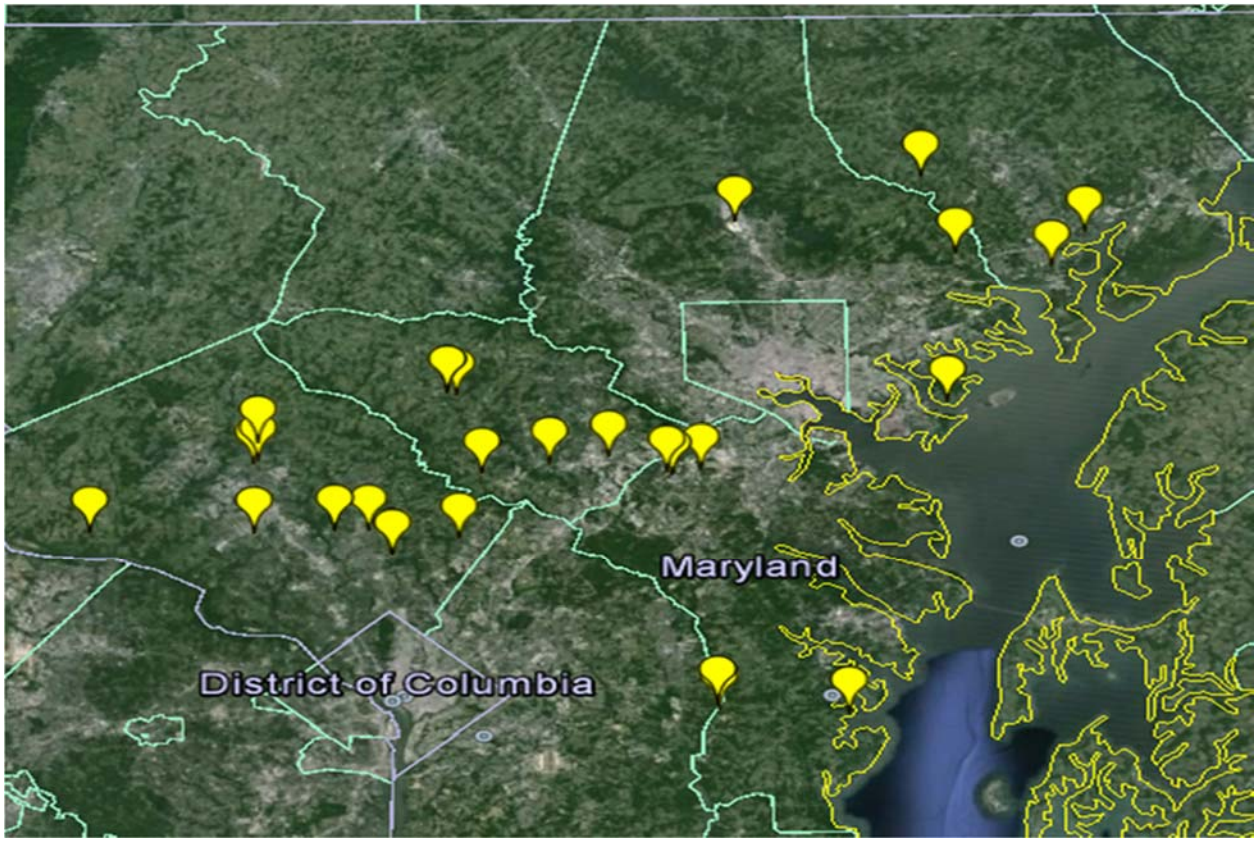

Figure 3.1. Map showing geographic distribution of selected sampling sites.

3.2 On-site evaluation of wetland sampling sites

On-site evaluations started in February 2014 and in March 2015. Twenty sites were actively monitored for amphibian breeding in 2014 and all 20 sites had confirmed breeding of at least one species of amphibian. However, only 16 sites were used for repeated sampling in 2014 due either to limited breeding at the site (<5 eggs masses) or accelerated metamorphosis attributed to rapid pond drying. In 2015, 22 sites were actively monitored for amphibian breeding and all 22 sites had confirmed breeding of at least one species of amphibian during the 2015 breeding season.

Table 3-1 described the 26 wetland sites sampled for Ranavirus during 2014 and 2015, as well as key habitat variables associated with those sites, the occurrence of amphibian die-offs, and presence of *Ranavirus*. Of the 26 sites, 12 were classified as Palustrine Open Water Wetlands[7] (POW), 11 as Palustrine Emergent Wetlands (PEM), and three as Vernal Pools[8].
Most sites were within 200 m (656 feet) from a road (mean = 134 m [439 feet]), with a range of 5-386 m (16-1266 feet). Ages of wetlands ranged from 2-23 years and most wetlands were fairly shallow, with only two ponds frequently being over one meter in depth (>40 inches) and only three exceeding 0.70 m (2.3 feet) at any point. Despite the shallow nature of these ponds, most had fairly long hydro-periods; only 7 sites dried at all during our study and only five of these dried before late June of 2014 and 2015. Thus, in most cases, there was ample time for amphibians to complete metamorphosis before the onset of pond drying.

3.3 Timing and impact of *Ranavirus* on amphibians

3.3.1. Frequency of amphibian die-offs at SHA sites

The distribution of any noted amphibian die-offs (regardless of numbers of affected amphibians) among these wetlands shown in Table 3-2 for 2014-2015. The overall frequency of die-offs in SHA-wetland sites was 37.5% (6 of 16 ponds) in 2014 and 36.4% in 2015 (8 of 22 ponds). There was no significant difference in the frequency of observed die-offs between years (Fisher Exact Test, P = 0.74). The overall rate of die-offs for 2014 and 2015 combined was 36.8% (14 of 38 sites). Of the 12 sampled sites in both 2014 and 2015, five had no die-offs in either year, five had die-offs in one year only, and only two sites had die-offs in both 2014 and 2015 (see Table 3-2).

[7] Palustrine Wetlands: any inland wetland, which lacks flowing water, contains ocean-derived salts in concentrations of less than 0.5 parts per thousand, and is non-tidal. The U.S. Fish and Wildlife Service (USFWS) to refer to wetlands that are vegetated - dominated by trees, shrubs, herbaceous plants, mosses or lichens, also use the term palustrine in the wetlands classification system.

[8] Vernal Pools: classified as (1) Wetlands that occur in shallow basins that are generally underlain by an impervious subsoil layer (e.g., a clay-pan or hard-pan) or bedrock outcrop, which produces a seasonally perched water table. (2) A type of Wetland in which water is present for only part of the year, usually during the wet or rainy seasons (e.g., spring). Also referred to as Temporary Wetland.

Table 3-2: List of wetland sites sampled for Ranavirus during 2014-2015, showing which sites were positive for Ranavirus via PCR testing and which sites had observed or inferred die-offs. PCR testing only valid for 2015. *County abbreviations; AA (Anne Arundel), Balt (Baltimore), Harf (Harford, How. (Howard), Mont (Montgomery), PG (Prince Georges).*

Pond ID	Co.	Sampled 2014?	Sampled 2015?	Die-off 2014?	Die-off 2015?	Rv Positive?	Notes
AA-01	AA	No	Yes	Not sampled	No	No	
B-1	Balt.	Yes	No	No	Not sampled	Not sampled	
B-11	Balt.	Yes	Yes	No	**Yes**	**Yes**	
B-GB	Balt	No	Yes	Not sampled	No	No	
HA-1	Hart.	No	Yes	Not sampled	**Yes**	**Yes**	
H-1-E	Harf	Yes	Yes	No	No	No	
HO-2-A	How.	Yes	Yes	No	**Yes**	**Yes**	
HO-5	How.	Yes	No	**Yes**	Not sampled	Not sampled	
HO-6	How.	Yes	Yes	No	No	No	
HO-7	How.	Yes	No	No	Not sampled	Not sampled	
HO-9	How.	No	Yes	Not sampled	**Yes**	**Yes**	
HO-10	How.	Yes	Yes	No	No	No	
HO-15	How.	No	Yes	Not sampled	**Yes**	**Yes**	
HO-16	How.	Yes	Yes	No	No	**Yes**	
ICC-01	Mont.	Yes	Yes	**Yes**	**Yes**	**Yes**	
ICC-05	Mont.	No	Yes	Not sampled	No	**Yes**	
ICC-08	Mont.	Yes	Yes	**Yes**	No	No	
ICC-16	Mont.	No	Yes	Not sampled	No	No	
M-1-M2	Mont.	Yes	Yes	No	No	No	
M-3	Mont.	Yes	Yes	**Yes**	No	**Yes**	
M-8	Mont.	No	Yes	Not sampled	**Yes**	**Yes**	
M-11	Mont.	Yes	No	No	Not sampled	Not sampled	
M-200	Mont.	No	Yes	Not sampled	No	**Yes**	
SC-19	Mont.	No	Yes	Not sampled	No	No	
P-4-A	P.G.	Yes	Yes	**Yes**	No	No	
P-4-B	P.G.	Yes	Yes	**Yes**	**Yes (inferred)**	**Yes (inferred)**	No die-off sample taken

However, it is important to note that the exact frequency of *Ranavirus*-related die-offs must be treated as a minimum estimate instead as of exact value, as even minor delays in sampling wetlands can results in equivocal results. For example, in 2015, a delay due to equipment issues allowed an 11-day gap between sampling periods for wetland P-4-B. During this interval, there was a massive reduction of abundance of larval amphibians that is unlikely to be the result of metamorphosis, as the larvae were in a fairly early stage of development. Thus, a die-off was inferred to have occurred at this site, but cannot be positively determined.

3.3.2. Comparisons with regional study

The regional study recorded the widespread occurrence of *Ranavirus*, with die-offs and/or *Ranavirus* infections documented in Maryland, New Jersey, Delaware, Virginia, and Pennsylvania. Shown in Figure 3.2, is a comparison of die-off rates between SHA sites and other sites. The frequency of die-offs was higher at SHA wetlands in Maryland (36.8%) compared with non-SHA sites in Maryland (27.3%), but these differences were not statistically significant (Fisher's exact test, P = 0.57). The frequency of die-offs in SHA sites (36.8%) was substantially higher than the frequency of the overall regional sample of non-SHA sites (12.3%) and these differences are statistically significant (Fisher's exact test, P = 0.006).
No die-offs were recorded in Pennsylvania or Virginia, but this could be related to low sampling frequency (S. Smith, MD-DNR, personal communication), so comparisons with these states are not possible.

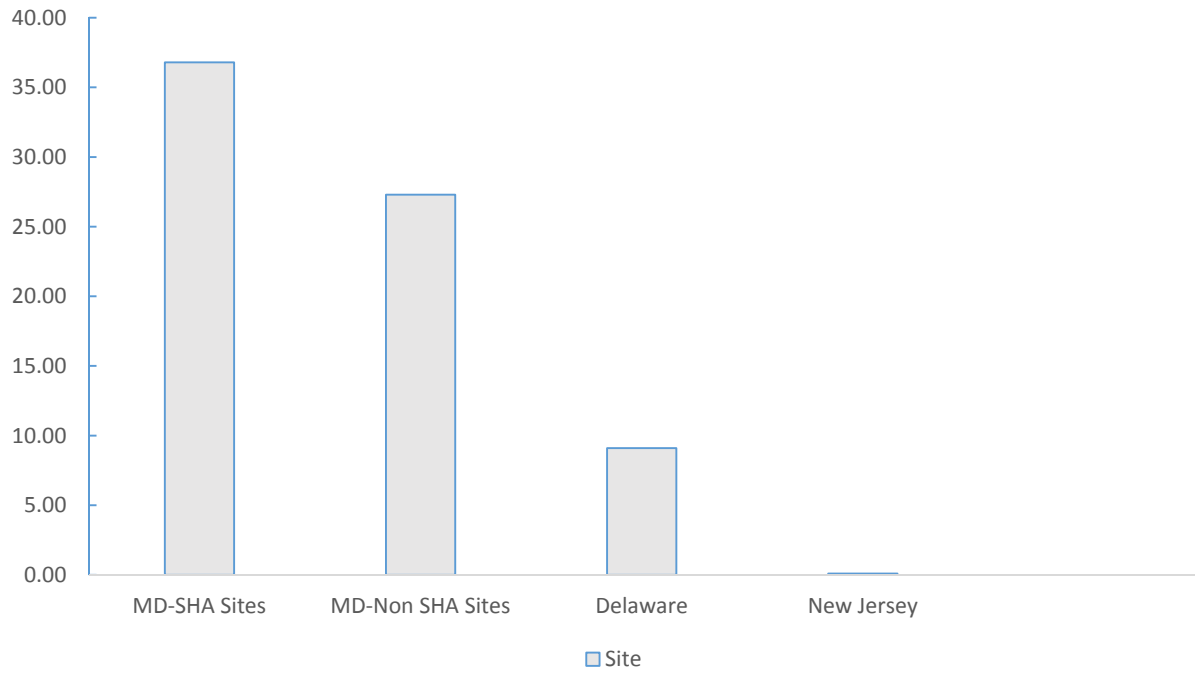

Figure 3.2. Comparison of the percentage of sites with known "die-offs" at Maryland SHA sites compared with non-SHA sites in Maryland, Delaware, and New Jersey

However, as noted in Section 4.3.1, interpreting these apparent differences must be done cautiously. First, the fact that the research team sampled most ponds at shorter intervals than was the case with the regional sample (S. Smith, personal communication), means that the rate of detection of die-offs was more likely at SHA sites than in the regional sample. Second, differences in the species of amphibians present at a site may have a strong impact on the occurrence of die-offs (S. Richter, personal communication), so regional differences in amphibian community composition may confound these comparisons as well.

3.4 Samples of amphibian larvae for DNA analysis

3.4.1. 2014 PCR Data

Samples of 435 tadpoles from all 16 wetlands in 2014 were sent to Montclair State University for PCR testing to confirm the presence of *Ranavirus*. Of these, only 15 tadpoles tested positive for the presence of the virus (3.4%). Of the six wetlands that had die-offs (see above), the presence of *Ranavirus* was confirmed by PCR testing at only three sites. In addition, PCR testing found the presence of the virus at three sites where the team did not find any apparent die-offs. An additional 78 tadpoles from sites with known die-offs were submitted for PCR testing. Surprisingly, none of these tadpoles tested positive for the presence of *Ranavirus*.

After consultation with directors of other labs involved in PCR analysis for *Ranavirus*, the researchers considered the 2014 results to be unreliable. Specifically, the percent of positive results appeared to be unnaturally low. Based on consultations with other experts, the research team felt that any comparisons using the 2014 PCR data gave a biased result, so these data can not be analyzed further at this point.

3.4.2. 2015 PCR Data

Tadpole samples, of both die-off sites and "standard" samples, were delivered to the University of Central Florida in January 2016. The sample analysis was completed by June 2016 and the results are summarized in Table 3-2. Overall, 12 of 22 SHA sites (54.5%) had positive qPCR results for *Ranavirus*. Of six sites that had die-offs as well as collection of larval samples taken during those die-offs, all (100%) tested positive for *Ranavirus*. The percentages of the amphibian larvae within die-off sites that were positive for *Ranavirus* were shown in Figure 3.3 and ranged from 33-100%

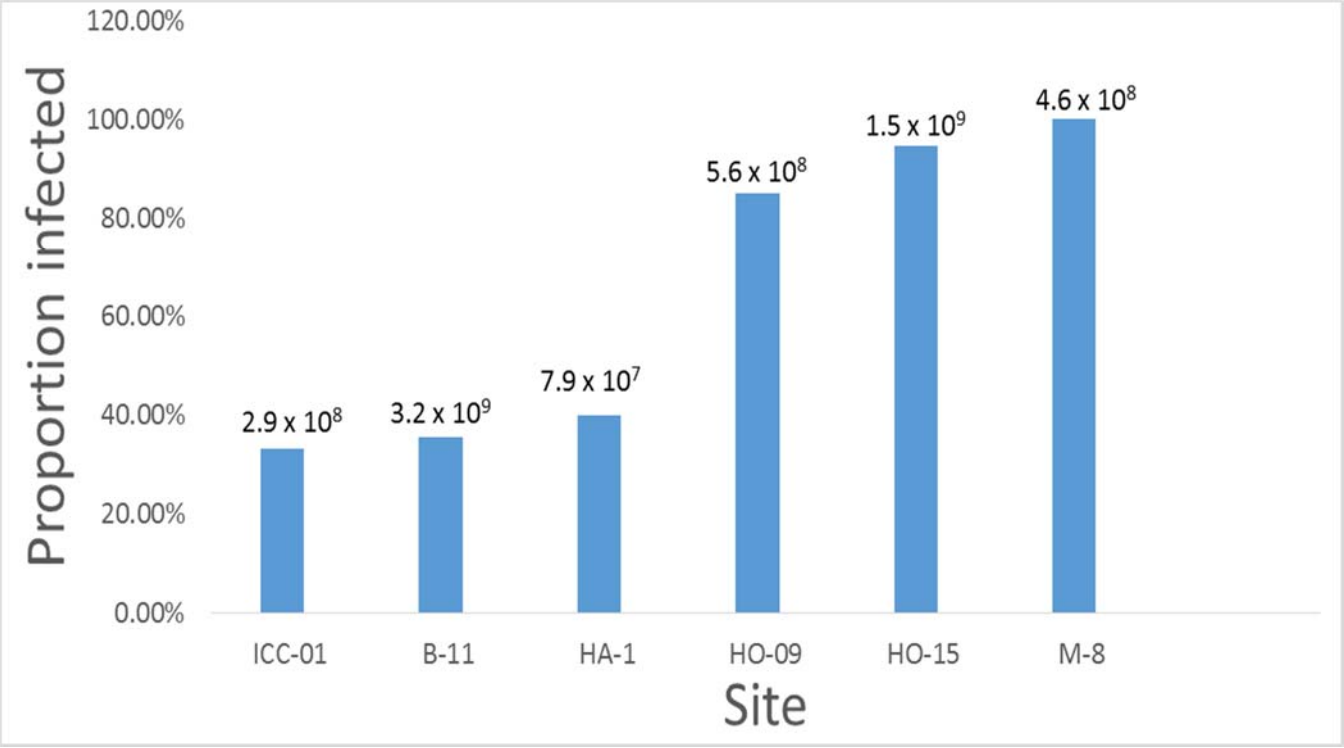

Figure 3.3. Illustration of percent of tadpoles or larvae with positive qPCR results from SHA sites in 2015 (vertical bars) and the "infection intensity" or viral load, shown as numbers above the vertical bars.

Figure 3.3 also shows the infection intensity, for each site, i.e., the average "viral load" measured as numbers of copies of the virus. These ranged from tens of millions of copies to literally billions of copies of the virus at sites B-11 and HO-15, levels that are considerably higher than levels seen at other sites reported in the literature (e.g., Hall et al., 2016; Warne et al. 2016). The possible importance of these high values cannot be determined at this time.

3.4.3. Comparisons of 2015 data with regional study

Figure 3.4 shows the prevalence of *Ranavirus* infection via PCR analysis compared among SHA sites in Maryland with sites across the mid-Atlantic region. Prevalence rates at SHA sites were comparable to rates shown in Delaware and New Jersey, but were much higher than the rates seen at non-SHA sites in Maryland and sites in both Pennsylvania and Virginia.

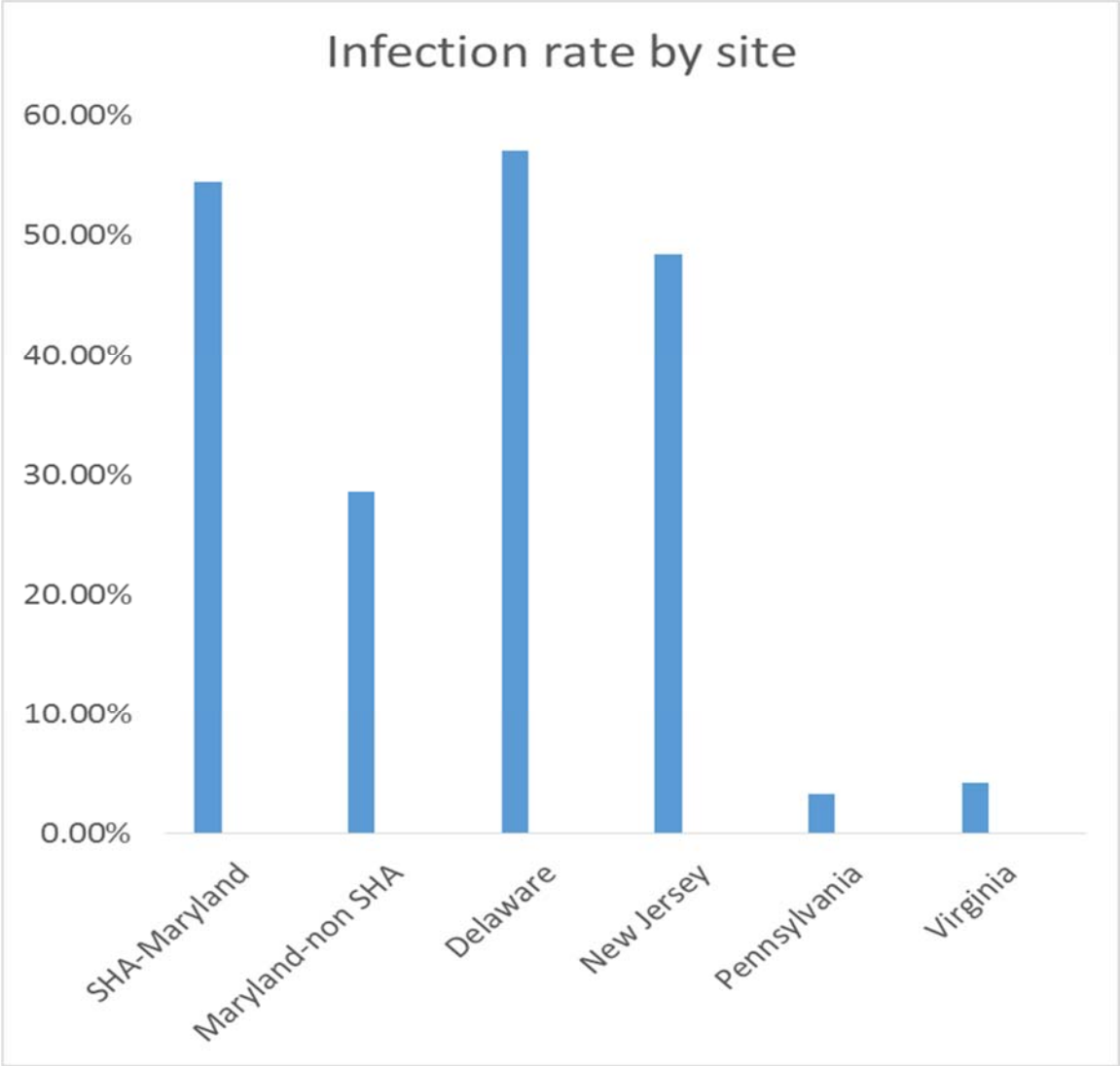

Figure 3.4. Differences in prevelance of *Ranavirus* infection via PCR analysis compared among SHA sites in Maryland with sites across the mid-Atlantic region

The much higher rate of *Ranavirus* infection, at SHA sites compared with non-SHA sites, supports both the initial hypothesis and previous work (Richter et al. 2013) that constructed wetlands may have higher rates of infection then do non-constructed sites. Unfortunately, the lack of understanding in the scientific community about the detectability of *Ranavirus* in biotic samples, how such detection varies among sites and seasons, make it difficult to draw firm conclusions from these data. As noted in Section 3.3.2, differences in amphibian communities among sites could easily confound these results. Only additional data from these and other wetlands sites will allow robust comparisons to be made.

3.5 Environmental and habitat correlates of presence of Ranavirus and disease outbreaks

3.5.1. Presence of Ranavirus

Seven competing models evaluated and explained the variation in the presence of *Ranavirus* at the 22 sites examined in 2015. Three of the models were narrowly differentiated by AIC[9] values; a null model, a model based on whether wetland dried or not, and a model based on distance from the nearest stream (see Table 3-3).

Table 3-3: Results of model selection analysis for environmental correlates of Ranavirus infection as determined via qPCR. Note that the standard errors for the covariates are extremely high, suggesting broad confidence limits. * = significant at P = 0.05. ** = significant at P = 0.01

Model	Intercept (SE)	Covariate (SE)
Null	0.54 (0.61)	N/A
Wetland Dry	0.47 (0.62)	0.82 (0.77)*
Distance to stream	0.59 (0.62)	0.50 (0.50)**

The second model showed that if a wetlands does not dry up, the risk of *Ranavirus* infection is 50%, rising to 82% if the wetlands does dry (i.e., a 32% increase in risk if wetlands dries). The third model showed that there was approximately a 5% reduction in risk of infection per each 100 m (109 yards) and with increasing distances from streams.

Although these models are suggestive of a link between risk of *Ranavirus* infection and two key environmental variables, two cautionary notes are important. First, due to issues with the 2014 PCR analysis, these results were based on a single year of data, and must be interpreted in that light. Second, the 95% confidence intervals around the estimates are very high, a constraint from the single year of data and only a moderate number of sites examined. A longer-term study using more wetlands sites is needed for comprehensive conclusions.

3.5.2. Disease Outbreaks (die-offs)

Results of the model selection analysis showed that the best-supported model was one that used the presence or absence of *Ranavirus* from PCR results as a covariate. No other models were supported. In other words, the presence of *Ranavirus* at a site was linked to whether that site experienced a die-off (Table 3-4). However, as with the models for risk of *Ranavirus* infection,

[9] AICc values: Statistical tool used to separate possible models explaining the cause of some event, such as infection by *Ranavirus*. Specifically, the method used differences in AICc values known as "ΔAICc" to determine which models are superior to others

95% confidence levels for these models are extremely wide, again indicating the need for a longer-term, more comprehensive study of these relationships.

Table 3-4: Results of model selection analysis for environmental correlates of Ranavirus infection as determined via presence of absence of die-offs. Models separated by < 2 ΔAICc values cannot be statistically distinguished from each other.

Die-off covariates	ΔAICc	AICc Weight	Cumulative Weight	Neg-log likelihoods
Ranavirus (Y/N)	0	0.33	0.33	-8.32
Ranavirus + Distance to road	1.92	0.13	0.46	-7.91
Ranavirus + Year constructed	2.13	0.11	0.57	-8.01
Ranavirus + distance to road	2.28	0.11	0.67	-8.09
Ranavirus + mean depth	2.31	0.10	0.78	-8.10
Ranavirus + wetland dry up	2.75	0.08	0.86	-8.32
Ranavirus + distance to road + year constructed	4.01	0.04	0.91	-7.41
Ranavirus + Mean depth + distance to road	4.77	0.03	0.94	-7.78
Ranavirus + mean depth + year constructed	5.12	0.03	0.96	-7.96
Ranavirus + wetland dry + distance to stream	5.32	0.02	0.98	-8.06
Null model	6.04	0.02	1	-12.56

CHAPTER 4: CONCLUSIONS

The primary goals of this study were to determine (a) whether constructed or rehabilitated wetlands had the same rate of observed die-offs, as did other, non-constructed wetlands and (b) whether there were clear environmental correlates between die-offs, the presence of *Ranavirus*, and habitat variables. Based on two years of data, there are suggestive, but unconfirmed trends that hint that constructed or rehabilitated wetlands do indeed have a higher rate of both amphibian die-offs and of the presence of *Ranavirus* than do other wetlands in Maryland. In addition, data from this study showed that at least two environmental variables (whether a wetlands dries or not during the amphibian breeding season and distance from a stream) may be associated with the likelihood of the occurrence of *Ranavirus* in a given wetland.

Unfortunately, given the highly stochastic nature of the amphibian populations and the equally high stochastic occurrence of *Ranavirus* (Richter et al., 2003; Harp and Petranka, 2006; Petranka et al., 2007), any robust conclusions from the current research study must be considered preliminary and in need of additional confirmation. First, data from this study showed strong differences in the composition of amphibian communities between years, with breeding at SHA sites in 2014 being primarily Pickerel Frogs (*Rana* [*Lithobates*] *paulustris*), rather than the Wood Frogs (*Rana [Lithobates]sylvatica*) seen in 2015. Some researchers see wood frogs as "magnifiers" of *Ranavirus*, so their presence can be an important trigger for a disease outbreak (S. Richter, personal communication). Because recent studies have indicated that the composition of breeding communities can both differ between constructed and natural wetlands (Drayer and Richter 2016) and affect the probability of *Ranavirus* infection (S. Richter, personal communication), annual (weather-related) differences in amphibian breeding can be a confounding factor in understanding how *Ranavirus* impacts amphibian populations. Second, the models used in this study to show a possible relationship between the occurrence of *Ranavirus* and environmental variables had very broad confidence intervals, meaning that these conclusions must be regarded as preliminary.

Studies of *Ranavirus,* in other states, further supported the hypothesis that *Ranavirus* has the potential to affect amphibian and reptile populations in extremely serious ways (e.g., Sutton et al., 2014). A better understanding of how this disease spreads as a function of wetland mitigation projects represents an urgent conservation and management need.

REFERENCES

Brenes, R., M. J. Gray, T. B. Waltzek, R. P. Wilkes, and D. L. Miller. 2014. Transmission of *Ranavirus* between ectothermic vertebrate hosts. PLoS ONE 2014;9(3):e92476.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443-449.

Drayer, A. N., and S. C. Richter. 2016. Physical wetland characteristics influence amphibian community composition differently in constructed wetlands and natural wetlands. Ecological Engineering 93:166-174.

Farnsworth, S. D., and R. A. Seigel. 2013. Responses, movements, and survival of relocated box turtles during construction of the Intercounty Connector Highway in Maryland. Transportation Research Record 2362:1-8.

Gray, M. J., J. T. Hoverman, and D. L. Miller. 2009. Amphibian Ranaviruses in the Southeastern United States. Disease, Pathogens and Parasites Task Team, Information Sheet #1. Southeastern Partners in Amphibian and Reptile Conservation. Available from: http://www.separc.org/products/diseases-and-parasites-of-herpetofauna

Hall, E. M., J. C. E, C. S. Goldberg, and J. L. Brunner. 2016. Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations. Molecular Ecology Resources 16:423-433

Harp, E.M., and J.W. Petranka. 2006. *Ranavirus* in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. Journal of Wildlife Diseases 42:307-318.

Hoverman, J. T., M. J. Gray, D. L. Miller, and N. A. Haislip. 2011. Widespread occurrence of Ranavirus in pond-breeding amphibian populations. *EcoHealth* DOI: 10.1007/s10393-011-0731-9.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences 103:3165-3170.

Otto, C. R. V., D. C. Forester, and J. W. Snodgrass. 2007. Influences of wetland and landscape characteristics on the distribution of Carpenter Frogs. Wetlands 27:261-269.

Pavlin B. I., L. M. Schloegel L.M., and P. Daszak. 2009. Risk of importing zoonotic diseases through wildlife trade, United States. Emerging Infectious Diseases. Available from http://wwwnc.cdc.gov/eid/article/15/11/09-0467.htm

Petranka, J.W., E.M. Harp, C.T. Holbrook, and J.A. Hamel. 2007. Long-term persistence of amphibian populations in a restored wetland complex. Biological Conservation 138: 371-380.

Paull, S. H., S. Song, K. M. McClure, L. C. Sackett, A. M. Kilpatrick, and P. T. J. Johnson. 2012. From superspreaders to disease hotspots: linking transmission across hosts and space. Frontiers of Ecology and the Environment 10:75–82.

Richter, S. C., A. N. Drayer, J. R. Strong, C. S. Kross, D. L. Miller, and M. J. Gray. 2013. High prevalence of ranavirus infection in permanent constructed wetlands in Eastern Kentucky, USA. Herpetological Review **44**:464-466.

Richter, S. C., J. E. Young, G. N. Johnson, and R. A. Seigel. 2003. Stochastic variation in reproductive success of a rare frog, *Rana sevosa*: implications for conservation and for monitoring amphibian populations. Biological Conservation 111:171-177.

Sutton, W. B., M. J. Gray, R. H. Hardman, R. P. Wilkes, A. J. Kouba, and D. L. Miller. 2014. High susceptibility of the endangered dusky gopher frog to ranavirus. Diseases of Aquatic Organisms 112:9-16.

Walker, S. F., J. Bosch, T. Y. James, A. P. Litvintseva, J. A. Oliver Valls, S. Piña, G. García, G. A. Rosa, A. A. Cunningham, S. Hole, R. Griffiths, and M. C. Fisher. 2008. Invasive pathogens threaten species recovery programs. Current Biology 18:R853-R854.

Warne, R. W., B. LaBumbard, S. LaGrange, V. T. Vredenburg, and A. Catenazzi. 2016. Co-Infection by Chytrid Fungus and Ranaviruses in wild and harvested frogs in the tropical Andes. PLoS ONE 11:1-15.

Appendix I. List of all SHA wetlands intially screened for inclusion in this study.

Year Constructed	Site I. D.	Mitigation Site	County	Latitude	Longitude
1992	AA-1	I-195/MD 295 INTERCHANGE	Anne Arundel	39.195880	-76.692476
1994	AA-12	MD 162 (AVIATION BLVD.)	Anne Arundel	39.176618	-76.650017
1991	AA-14-B	MD 32/MD 175 INTERCHANGE	Anne Arundel	39.097968	-76.710399
1995	AA-16	OSPREY SITE	Anne Arundel	39.153714	-76.661579
1996	AA-17	BUCKINGHAM SITE B	Anne Arundel	39.146433	-76.695878
1992	AA-18	PRICE CLUB	Anne Arundel	39.194638	-76.601296
1997	AA-21	PINEY RUN	Anne Arundel	39.167615	-76.722348
1996	AA-22	BUCKINGHAM SITE A	Anne Arundel	39.149278	-76.694293
1998	AA-24	648/3 SITE	Anne Arundel	39.175947	-76.637439
1991	AA-3-C	CAPE SAINT CLAIRE	Anne Arundel	39.031535	-76.445123
1992	AA-6	MD 100 MEDIAN	Anne Arundel	39.121913	-76.571168
1991	AA-8	MD 648, CATTAIL CREEK	Anne Arundel	39.087971	-76.553054
2002	AA-CB	CHESAPEAKE BAPTIST CHURCH	Anne Arundel	39.112900	-76.662870
1993	AA-S	SANDS ROAD	Anne Arundel	38.861760	-76.685030
1994	AA-19	I-195	Baltimore	39.217064	-76.703755
1993	B-1	WARREN ROAD WETLAND/RARE PLANT SITE	Baltimore	39.475179	-76.658364
2005	B-11	HOLLYNECK	Baltimore	39.275556	-76.417778
1993	B-5	BROADMEAD SITE	Baltimore	39.500680	-76.649110
1988	B-8-A	MD 140/I-795	Baltimore	39.471913	-76.838324
1992	B-GB	GLENBAUER SITE/ BUCKHILL	Baltimore	39.458680	-76.404690
1992	H-1-A	I-95 SOUTHBOUND ENTRANCE RAMP	Harford	39.484790	-76.255090
1992	H-1-B	SOUTH CORNER OF MD 543/MD 7 INTERSECTION	Harford	39.480580	-76.250550
1992	H-1-C	0.25 MILES NE OF MD 543/MD 7 INTERSECTION	Harford	39.481320	-76.244742

1992	H-1-D	0.2 MILES S OF MD 543/MD 7 INTERSECTION	Harford	39.480370	-76.254380
1992	H-1-E	MD 543/I-95 SOUTHBOUND ENT. RAMP	Harford	39.483410	-76.259900
1994	H-2	KMS SITE	Harford	39.494736	-76.195187
1994	H-3	MD 146 @ HESS RD	Harford	39.554783	-76.525944
1996	H-4	MD 24/ US 40 J.V.	Harford	39.441687	-76.297810
2003	HA-1	RAHLL SITE	Harford	39.553530	-76.440930
2008	HA-4	MAGNESS FARM	Harford	39.661015	-76.522157
1996	AA-20	DEEP RUN SITE	Howard	39.181688	-76.741435
1997	HO-10	UNIVERSITY OF MD HORSE FARM	Howard	39.212173	-76.794352
2006	HO-15	WEST FRIENDSHIP	Howard	39.293430	-76.971870
2010	HO-16	NIXON FARM	Howard	39.288888	-76.961413
1992	HO-2-A	US 29 CORRIDOR	Howard	39.204860	-76.859900
1994	HO-4	US 29/MD 103	Howard	39.246631	-76.828215
1995	HO-5	MD 97/I-70	Howard	39.322869	-77.018985
1994	HO-6	BEEHIVE SITE	Howard	39.193830	-76.730556
1994	HO-7	SCHULTZ FARM	Howard	39.189579	-76.724293
1995	HO-8	BRAMPTON HILLS	Howard	39.245410	-76.821570
1996	HO-9	MD 32/MD 108 (Guilford Rd.)	Howard	39.193180	-76.934920
2012	HO-17	DORSEY RUN	Howard	39.148280	-76.786182
2007	ICC-01	NW-128	Montgomery	39.094407	-77.036051
2006	M-11	CLARKSBURG SITE	Montgomery	39.212240	-77.270703
1993	M-1-M2	I-370 W.B.	Montgomery	39.121230	-77.184410
1993	M-1-M3	I-370 W.B. (RAMP D)	Montgomery	39.123920	-77.188100
1993	M-1-M6	I-270/370, RAMP D	Montgomery	39.123512	-77.196454
1993	M-3	MD 107 OVER DRY SENECA CREEK	Montgomery	39.124520	-77.369320
1993	M-5	MD 124/MD 108	Montgomery	39.289872	-77.200507
1994	M-8	HAWKINS	Montgomery	39.211550	-77.182270
2012	ICC-05	SC-2	Montgomery	39.206748	-77.187338
2012	ICC-08	PB-1	Montgomery	39.113679	-76.961288
2011	ICC-16	NW-69	Montgomery	39.124357	-77.061249

1991	P-10-A	MD 214/MD 193	Prince Georges	38.900840	-76.792710
1990	P-15-B, Basin 1	MD 197/I 595	Prince Georges	38.955692	-76.743920
1990	P-15-B, Basin 4	MD 197/I-595 INTERCHANGE	Prince Georges	38.951964	-76.746421
1990	P-15-B, Basin 6	MD 197/US 301 INTERSECTION	Prince Georges	38.943356	-76.718699
1993	P-4-A	ENTZIAN SITE	Prince Georges	38.909692	-76.679515
1992	P-4-B	GLAZIER SITE	Prince Georges	38.902428	-76.678035