An investigation of co-infection by *Batrachochytrium dendrobatidis* and *Ranavirus* (FV3) in anurans of two natural areas in Anne Arundel County, Maryland and Fairfax County, Virginia, USA.

Lauren D. Fuchs[1], Todd A. Tupper[2*], Christine A. Bozarth[2], David Fernandez[2], Robert Aguilar[3]

[1]George Mason University
Department of Systems Biology
10900 University Blvd
Manassas, VA 20110

[2]Northern Virginia Community College
Department of Math, Science, and Engineering
5000 Dawes Avenue
Alexandria, Virginia 22311

[3]Smithsonian Environmental Research Center
Fish and Invertebrate Ecology Lab
647 Contees Wharf Road
Edgewater, Maryland 21037

*Corresponding author: ttupper@nvcc.edu

Introduction

Since the 1980s, amphibian populations have experienced global population declines and extinctions (Skerratt et al., 2007; Robert, 2010). Enigmatic events, including the emergence and spread of infectious diseases, are associated with many of these recent declines (Daszak et al., 2003; Stuart et al., 2004; Bielby et al., 2008; Olson et al., 2013). Globally-occurring mass mortality events of amphibians have been associated with two pathogens in particular: the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), and ranaviruses (specifically *Ranavirus* type species Frog Virus 3 [hereafter FV3]; Chinchar, 2002; Fox et al., 2006; Lips et al., 2006; Haislip et al., 2011; Echaubard et al., 2016). Both *Bd* and the ranaviruses are listed as notifiable pathogens by the World Organization for Animal Health (OIE, 2008; Echaubard et al., 2016).

Bd and ranaviruses are both known to occur globally, and across broad geographic and host ranges (Schock et al., 2010; Bancroft et al., 2011; Miller et al., 2011). Data indicate that *Bd* is widespread and often highly prevalent in the mid-Atlantic United States and that it often occurs without concomitant population declines (Longcore et al., 2007; Grant et al., 2008; Rothermel et al., 2008; Pullen et al., 2010; Lannoo et al., 2011; Petersen et al., 2016; Fuchs et al., 2017; Tupper et al., 2017). Conversely, data on FV3 in mid-Atlantic amphibians are limited. However, FV3 infections in this region have been documented, and have also been associated with localized mortality events and declines (Petranka et al., 2003; Harp and Petranka, 2006; Schock et al., 2009; Davidson & Chambers, 2011; Hoverman et al., 2012; Fairfax County Park

Authority, 2015; Duffus et al., 2015). Although deficiencies in FV3 data may be due to sparse sampling, it may also reflect the disease's biology. FV3 is highly virulent and can cause > 90% mortality in some cases (Green et al., 2002). This virulence can make sub-lethal infections difficult to detect, as deceased frogs may be less conspicuous and/or available for sampling (Harp and Petranka, 2006).

Bd and *Ranavirus* FV3 have been found to co-occur and co-infect individual hosts in various habitats (Whitfield et al., 2013; Warne et al., 2016; Rosa et al., 2017). Throughout North America, co-occurrence of *Bd* and FV3 has been reported in a number of aquatic communities. However, concurrent infections within an individual host has not yet been demonstrated *in situ* (Fox et al., 2006; Hoverman et al., 2012; Souza et al., 2012; Whitfield et al., 2013). Although data on concurrent infection are limited, it is suspected that the pathogens interact synergistically, promoting greater infection intensity and disease progression within the host due to the initial immunocompromising-effects from the primary invading pathogen (Garyfer et al., 2012; Warne et al., 2016). The objective of our study was to determine whether infection by these pathogens (both *Bd* and FV3) was occurring concurrently in anurans at two natural areas in Virginia and Maryland where *Bd* is known to occur (*see* Fuchs et al., 2017; Tupper et al., 2017). We also aimed to provide baseline data on rates of FV3 infection across anuran species of the mid-Atlantic that could be incorporated into a growing body of literature on FV3 infection.

Methods

We collected samples at the Smithsonian Environmental Research Center (hereafter SERC; 38°53'17.41"N; 76°33'15.52 W) in Anne Arundel County, MD (for more about SERC, *see* Tupper et al., 2016), and at Huntley Meadows Park (hereafter HMP; 38°45'36.57" N; 77°05'44.13" W) in Fairfax County, Virginia (for more about HMP, *see* Tupper et al., 2017) between 13 March and 25 September 2016. Following Virginia Herpetological Society biosecurity protocols (VHS, 2016), we opportunistically hand-captured and sampled anurans at various locations throughout each study site. To sample for FV3, we chose a minimally-invasive method that has previously been used to detect the virus (Driskell et al., 2009; Gray et al., 2009; Pessier and Mendelson, 2010; Miller et al., 2015). This method consisted of collecting epithelial cells of the oropharyngeal region by circling the swab along the tongue, roof and sides of the mouth, and the pharynx (San Diego Zoo ICR, 2016). All swabs were stored in 1.5 mL microcentrifuge tubes and kept frozen until molecular analyses could be performed. To sample for *Bd*, we swabbed several skin surfaces (following methods of Hyatt et al., 2007) using a sterile dry swab (no. MW113, Medical Wire and Equipment Company, Durham, NC).

DNA was eluted from each swab using the Purification of Total DNA from Animal Tissues protocol (Qiagen®, Valencia, CA). To assay for FV3, we prepared a PCR master mix which contained 10µL SSo Advanced™ universal probes supermix (Bio-Rad, Hercules, CA), 2µL eluted DNA, 0.6 µL forward primer, 1.8µL reverse primer (Mao et al., 1996), 0.5 µL MGB probe, and 5.1µL sterile water, for a 20µL reaction total (Brunner and Collins, 2009). We

included DNA elusion and amplification positive and negative controls with each PCR run. We amplified the DNA using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). We ran samples at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 54°C for 30 seconds, and 72°C for 15 seconds. We performed at least two rounds of PCR on each sample; samples with inconsistent results were run through a third cycle. We considered any sample that fluoresced prior to the 40th cycle of the PCR reaction on at least two occasions to be positive. The *Bd* PCR techniques followed methods described by Boyle et al. (2004). For a more detailed description of these methods, *see* Fuchs et al. (2017) and Tupper et al. (2017).

We chose multiple logistic regression analysis with stepwise variable selection (Zar, 2009) to determine if *Bd* was associated with FV3 infection. To determine the total rates of infection across both sites, we calculated the proportion of *Bd* and FV3 positive samples for five grouping variables: anuran species, sampling month, ecological guild, and sex and age classes (*see* Tupper et al., 2017). Statistical analyses were completed in Minitab version 18 (www.minitab.com).

Results

We collected samples from a total of 100 anurans at HMP, and 88 anurans at SERC between 13 March and 25 September 2016. We sampled for FV3 in 170 anurans; all samples tested negative for the virus. Of the 186 testable *Bd* samples, 59 tested positive for an overall infection rate of 31.7% (Table 1). We found that the highest *Bd* infection rates occur in March and April (59.3% and 59.1%, respectively; Table 2), in male adults (43.7%; Table 3), and in Southern Leopard (*Lithobates sphenocephalus*; 66.7%) and Pickerel Frogs (*Lithobates palustris*; 63.6%; Table 1). Anurans sampled in the aquatic guild resulted in a higher percent of *Bd* positive individuals (40.3%) than in the terrestrial/arboreal guild (17.9%; Table 3). Logistic regression could not be completed due to the paucity of FV3 positive results.

Table 1. Proportion of *Bd* and *Ranavirus* FV3 positive samples by species. AMTO = Eastern American Toad (*Anaxyrus americanus*); BUFR = American Bull Frog (*Lithobates catesbeianus*); CGTF = Cope's Gray Tree Frog (*Hyla chrysoscelis*); CRFR=Eastern Cricket Frog (*Acris crepitans*); GRFR = Green Frog (*Lithobates clamitans*); GTFR = Green Tree Frog (*Hyla cinerea*); PIFR = Pickerel Frog (*Lithobates palustris*); SLFR = Southern Leopard Frog (*Lithobates sphenocephalus*); SPPE = Spring Peeper (*Pseudacris crucifer*); WOFR = Wood Frog (*Lithobates sylvaticus*). * = Aquatic guild. Species without an asterisk indicates terrestrial/arboreal guild.

Species	N (FV3)	% FV3 Positive	N (*Bd*)	% *Bd* Positive
AMTO	26	0	26	26.9
BUFR*	26	0	25	24
CRFR*	13	0	17	5.9
CGTF	12	0	12	16.7
GRFR*	34	0	33	36.4
GTFR	12	0	12	8.3
PIFR*	10	0	11	63.6
SLFR*	31	0	33	66.7
SPPE	5	0	16	6.3
WOFR*	1	0	1	0
Total	170	0	186	31.7

Table 2. Proportion of FV3 and *Bd* positive individuals grouped by sampling month.

Month	N (FV3)	% FV3 Positive	N (*Bd*)	% *Bd* Positive
MARCH	22	0	27	59.3
APRIL	22	0	22	59.1
MAY	75	0	74	27.0
JUNE	12	0	24	12.5
JULY	5	0	5	20.0
AUG	5	0	5	0.0
SEPT	29	0	29	24.1

Table 3. Proportion of FV3 and *Bd* positive individuals grouped by sex (and by default, age class) and ecological guild.

Sex/Guild	N (FV3)	% FV3 Positive	N (*Bd*)	% *Bd* Positive
Female	66	0	64	26.6
Juvenile	34	0	51	23.5
Male	70	0	71	43.7
Aquatic	114	0	119	40.3
Terrestrial/arboreal	56	0	67	17.9

Discussion

Ranavirus infections, including FV3, have been documented in Maryland and Virginia (Davidson and Chambers, 2011; Hamed et al., 2013; Scott et al., 2016), however, FV3 was not detected in any of our samples. Our results reflect one of two possibilities, either FV3 was not present at either location, or the pathogen was present, but we were unsuccessful in detecting it due to our sampling techniques. In order to limit stress to the animal, we chose the least intrusive sampling method shown to detect FV3, (oropharyngeal swabbing; *see* Driskell et al., 2009; Pessier and Mendelson, 2010; Miller et al., 2015; San Diego Zoo ICR, 2016). Despite being less invasive, swabbing is also less reliable than lethal techniques, such as liver tissue sampling, and may produce more false negatives than other non-lethal techniques, such as toe and tail clips (Miller et al., 2008; Gray et al., 2012; Forzán et al., 2017). Therefore, it is possible that even if an anuran was carrying the virus, our sampling method may not have been sensitive enough to detect it.

Though *Ranavirus* has low host specificity, larvae and metamorphs of certain species show far greater susceptibility to infection than others (Daszak et al., 1999; Brunner et al., 2004; Robert et al., 2005; Robert, 2010; Lesbarréres et al., 2012; North et al., 2015). For instance, larval and recently metamorphosed Wood Frog (*Lithobates sylvaticus*), Gopher Frog (*Lithobates capito*) and Eastern Spadefoot Toad (*Scaphiopus holbrookii*) are among the most susceptible to FV3 (Goodman and Araraso, 2009; Haislip et al., 2011; Hoverman et al., 2011; Miller et al., 2011; Lesbarréres et al., 2012; Earl and Gray, 2014; Forzán et al., 2017). Because anurans in our study were captured opportunistically, we were unable to adequately sample the most susceptible species. While oropharyngeal swabbing can effectively detect *Ranavirus* in tadpoles (Gray et al., 2012; Kolby et al., 2015), this method would likely damage oropharyngeal tissues in smaller anurans. Therefore, we chose to restrict our sampling to metamorphosed anurans.

Although we did not detect FV3 at either of our sampling sites, *Ranavirus* has been documented in both Virginia and Maryland (Davidson and Chambers, 2011; Hamed et al., 2013; Smith et al., 2016). Additionally, FV3 has been confirmed as the likely cause of a recent Wood Frog tadpoles die-off at the nearby Old Colchester Park and Preserve in Fairfax County, VA (Fairfax County Park Authority, 2015). We therefore suggest continued FV3 monitoring at HMP and SERC and recommend that future studies focus sampling efforts on larval Wood Frog and Eastern Spadefoot Toad (while minimizing harm to anurans). We also suggest continued monitoring for *Bd*, which is already known to be prevalent at both locations (Fuchs et al., 2017; Tupper et al., 2017). Our study confirms that *Bd* remains prevalent at both SERC and HMP, with an overall infection rate that is among the highest in the region (31.72%; *see also* Hughey et al., 2014). Notably, a post-hoc analysis of data collected over a three-year period (2014-2016) revealed a nearly-20% increase in the infection rate of *Bd* at SERC (Tupper and Fuchs, unpublished data; Fuchs et al., 2017). Continued monitoring of both pathogens will facilitate more informed management decisions, and will allows us to better understand the effects of their interactions within anuran hosts.

Acknowledgements

Caitlin O'Connor Love was essential to completion of fieldwork, and Michael Rutherford was essential to the completion of lab work. David Lawlor, Resource Manager for Huntley Meadows Park, and Susan Williams, Lab Manager at Northern Virginia Community College provided a great deal of logistical support. Lab work was completed at the Alexandria Campus of Northern Virginia Community College (Department of Math, Science, and Engineering). The Northern Virginia Community College Educational Foundation provided financial support. Sampling was approved by the Maryland Department of Natural Resources and the Virginia Department of Game and Inland Fisheries.

Literature Cited

Bancroft, B. A., B. A. Han, C. L. Searle, L. M. Biga, D. H. Olson, L. B. Kats, J. J. Lawler, and A. R. Blaustein. 2011. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the Unites States. Biodiversity and Conservation 20: 1911-1920.

Bielby, J., N. Cooper, A. A. Cunningham, T. W. J. Garner, and A. Purvis. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters 1: 82–90.

Brunner, J. L., D. M. Schock, E. W. Davidson, and J. P. Collins. 2004. Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560–566.

Chinchar, V. G. 2002. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. Archives of Virology 147: 447-470.

Daszak, P., L. Berger, A. A. Cunningham A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735-748.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9: 141–150.

Davidson, S. R. A. and D. L. Chambers. 2011. Ranavirus prevalence in amphibian populations of Wise County, Virginia, USA. Herpetological Review 42: 214–215.

Driskell E. A., D. L. Miller, S. L. Swist, and Z. Z. Gyimesi. (2009) PCR detection of ranavirus in adult anurans from the Louisville Zoological Gardens. Journal of Zoo and Wildlife Medicine 40:559–563.

Duffus ALJ, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, Hick P, Hines MK, Marschang RE. 2015. Distribution and host range of ranaviruses, p. 9–57. In Gray MJ, Chinchar VG (ed), *Ranaviruses*: lethal pathogens of ectothermic vertebrates. Springer, New York. doi:10.1007/978-3-319-13755-1_2.

Earl, J. E. and M. J. Gray. 2014. Introduction of ranavirus to isolated wood frog populations could cause local extinction. EcoHealth 11: 581-592.

Echaubard, P., B. D. Pauli, V. L. Trudeau, and D. Lesbarrères. 2016. Ranavirus infection in northern leopard frogs: the timing and number of exposures matter. Journal of Zoology 298: 30-36.

Fairfax County Park Authority. 2015. Ranavirus, amphibians, and how to keep them apart. https://ourstoriesandperspectives.com/2015/09/14/ranavirus-amphibians-and-how-to-keep-them-apart/ (Accessed March 2018).

Forzán, M. J., K. M. Jones, E. Ariel, R. J. Whittington, J. Wood, J. J. F. Markham, and P. Y. Daoust. 2017. Pathogenesis of Frog Virus 3 (*Ranavirus*, *Iridoviridae*) infection in wood frogs (*Rana sylvatica*). Veterinary Pathology 54: 531-548.

Fox, S. F., A. L. Greer, R. Torres-Cervantes, and J. P. Collins. 2006. First case of ranavirus associated morbidity and mortality in natural populations of a South American frog, *Atelognathus patagonicus*. Diseases of Aquatic Organisms 72: 87–92. PLoS ONE 6(7): e22307.

Fuchs, L. F., T. A. Tupper, T. Sepapur, R. Aguilar, and C. A. Bozarth. 2017. A Survey of the Pathogenic Fungus, *Batrachochytrium dendrobatidis*, at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland. Catesbeiana 37: 32-39.

Grant, E. H. C., L. L. Bailey, J. L. Ware, and K. L. Duncan. 2008. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in stream and wetland populations in Maryland, USA. Applied Herpetology 5: 233–241.

Grayfer, L., F. D. J. Andino, G. Chen, G. V. Chinchar, and J. Robert. 2012. Immune evasion strategies of ranaviruses and innate immune responses to these emerging pathogens. Viruses 4: 1075-1092.

Gray M. J., D. L. Miller, J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87: 243–266.

Gray, M. J., D. M. Miller, and J. T. Hoverman. 2012. Reliability of non-lethal surveillance methods for detecting ranavirus infection. Diseases of Aquatic Organisms 99: 1-6.

Green D. E., K. A. Converse, and A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Annals of the New York Academy of Sciences 969: 323–339.

Haislip, N. A., M. J. Gray, J. T. Hoverman, and D. L. Miller. 2011. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PLoS ONE6(7): e22307. doi:10.1371/journal.pone.0022307.t001.

Hamed, M. K., M. J. Gray, and D. L. Miller DL. 2013. First report of ranavirus in plethodontid salamanders from the Mount Rogers National Recreation Area, Virginia, USA. Herpetological Review 44: 455–457.

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, N. A. Haislip, and D. L. Miller. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8: 301-319.

Hoverman, J. T., M. J. Gray, D. L. Miller, and N. A. Haislip. 2012. Widespread occurrence of ranavirus in pond-breeding amphibian populations. EcoHealth 9: 36-48.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R.Campbell, G. Marantelli, F. Gleason, and A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73:175–192.

Kolby, J. E., K. M. Smith, S. D. Ramirez, F. Rabemananjara, A. P. Pessier, J. L. Brunner, C. S. Goldberd, L. Berger, L. F. Skerratt. 2015. Rapid response to evaluate the presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in wild amphibian populations in Madagascar. PLoS ONE10(6): e0125330.doi:10.1371/journal.pone.0125330.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. Do frogs get their kicks on Route 66? Continental U.S. Transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLoS ONE 6: e22211. doi: 10.1371/journal.pone.0022211.

Lesbarréres, D., A. Blaseiro, J. Brunner, V. G. Chinchar, A. Duffus, J. Kerby, D. L. Miller, J. Robert, D. M. Schock, T. Waltzek, and M. J. Gray. 2012. Ranavirus: past, present and future. Biology Letters 8: 481-483.

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 of the United States of America 103: 3165–3170.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71: 435–444.

Miller D. L., S. Rajeev, M. Brookins, J. Cook, L. Whittington, and C. A. Baldwin. 2008. Concurrent infection with Ranavirus, *Batrachochytrium dendrobatidis*, and Aeromonas in a captive amphibian colony. Journal of Zoo and Wildlife Medicine 39: 445–449.

Miller, D., M. Gray, and A. Storfer. 2011. Ecopathology of ranaviruses infecting amphibians. Viruses 3: 2351-2373.

Miller D. L., A. P. Pessier, P. Hick, and R. J. Whittington. 2015. Comparative pathology of ranaviruses and diagnostic techniques. In: Gray MJ, Chinchar VG (eds) Ranaviruses: lethal pathogens of ectothermic vertebrates. Springer, New York.

North, A. C., D. J. Hodgson, S. J. Price, A. G. F. Griffith. 2015. Anthropogenic and ecological drivers of amphibian disease (Ranavirosis). PLoS ONE 10(6): e0127037.doi:10.1371/journal.pone.0127037.

OIE (2008) OIE Aquatic Animal Health Code, Chapter 2.4.2. OIE, Paris. www.oie.int/eng/normes/fcode/en_chapitre_ 2.4.2.htm. Accessed November 2018.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Gerner, G. Weaver, The *Bd* Mapping Group, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS ONE 8(2): e56802. doi: 10.1371/journal.pone.0056802.

Pessier A. P. and J. R. Mendelson. 2010. A manual for control of infectious diseases in amphibian survival assurance colonies and reintroduction programs. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN.

Petersen, C. E., R. E. Lovich, C. A. Phillips, M. J. Dreslik, and M. J. Lannoo. 2016. Prevalence and seasonality of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* along widely separated longitudes across the United States. EcoHealth pp 1-15. DOI 10.1007/ s10393-016-1101–4.

Petranka, J. W., S. S. Murray, and C. A. Kennedy. 2003. Responses of amphibians to restoration of a southern Appalachian wetland: perturbations confound post-restoration assessment. Wetlands 23: 278–290.

Pullen, K. D., A. M. Best, and J. L. Ware. 2010. Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia. Diseases of Aquatic Organisms 91: 9–16.

Robert, K., H. Morales, W. Buck, N. Cohen, S. Marr, and J. Gantress. 2005. Adaptive immunity and histopathology in frog virus 3-infected Xenopus. Virology 332: 667–675.

Robert, J. 2010. Emerging ranaviral infectious diseases and amphibian decline. Diversity 2:314-330.

Rosa, G. M., J. Sabino-Pinto, T. G. Laurentino, F. Pasman, R. Rebelo, R. A. Griffiths, A. C. Stöhr, R. E. Marschang, S. J. Price, T. W. J. Garner, and J. Bosch. 2017. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. Scientific Reports 7:43260.doi: 10.1038/srep43260.

Rothermel, B. B., S. C. Walls, J. C. Mitchell, C. K. Dodd Jr., L. K. Irwin, D. E. Green, V. M. Vasquez, J. W. Petranka, and D. J. Stevenson. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium deondrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82: 3–18.

San Diego Zoo Institute for Conservation Research. 2016. Sampling protocol for ranaviruses. http://admininstitute.sandiegozoo.org/sites/default/files/Ranavirus%20Guidelines%202016.pdf. Accessed February 2018.

Schock, D. M., T. K. Bollinger, and J. P. Collins. 2009. Mortality rates differ among amphibian populations exposed to three strains of a lethal ranavirus. EcoHealth 6: 438-448.

Schock, D. M., G. R. Ruthig, J. P. Collins, S. J. Kutz, S. Carriere, R. J. Gau, A. M. Veitch, N. C. Larter, D. P. Tate, G. Guthrie, D. G. Allaire, and R. A. Popko. 2010. Amphibian chytrid fungus and ranaviruses in the northwest territories, Canada. Diseases of Aquatic Organisms 92: 231-240.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A.D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134.

Smith, S. A., K. J. Monsen-Collar, D. E. Green, H. S. Niederriter, M. L. Hall, K. A. Terrell, K. D. Gipe, C. A. Urban, C. A. Patterson, R. A. Siegel, B. Zarate, J. D. Kleopfer, E. H. Campbell-Grant, and C. P. Driscoll. 2016. 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 for Regional Conservation Needs.

Souza, M. J., M. J. Gray, P. Colclough, and D. L. Miller. 2012. Prevalence of infection by *Batrachochytrium dendrobatidis* and *Ranavirus* in eastern Hellbenders (*Cryptobranchus alleganeiensis alleganeiensis*). Journal of Wildlife Diseases 48:560–566.

Stuart, S. N, J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306: 1783−1786

Tupper, T. A., L. D. Fuchs, C. O'Connor-Love, R. Aguilar, C. Bozarth, and D. Fernandez. 2017. Detection of the pathogenic fungus, *Batrachochytrium dendrobatidis*, in anurans of Huntley Meadows Park, Fairfax County, Virginia. Catesbeiana 37: 109-120.

VHS (Virginia Herpetological Society). 2016. Infectious diseases of our native herps and disinfection protocols. http://virginiaherpetologicalsociety.com/disease/index.htm. Accessed May 2016.

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): e0145864.doi:10.1371/journal.pone.0145864.

Whitfield, S. M., E. Geerdes, I. Chacon, E. B. Rodriguez, R. J. Jimenez, M. A. Donnelly, and K. L. Kerby. 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. Diseases of Aquatic Organisms 104: 173-178.

Zar, J.H. 2009. Biostatistical Analysis. 5th edition. Pearson, Upper Saddle River, N.J. 960 pp.