INVESTIGATIONS INTO THE DISEASE ECOLOGY OF VIRAL HEMORRHAGIC
SEPTICEMIA VIRUS (VHSV) GENOTYPE IVB IN BUDD LAKE, MICHIGAN

By

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ABSTRACT

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Since 2005, fish health in the Laurentian Great Lakes region of North America has been affected by a new strain of viral hemorrhagic septicemia virus (VHSV IVb). Uncertainty about persistence of VHSV IVb in infected systems arose when it caused a large fish die-off in Budd Lake, Michigan, in 2007 without subsequent mortality events or isolations several years after. One purpose of this thesis was to determine if VHSV IVb still existed in Budd Lake (i.e. active infections, production of antibodies, or reservoir sources). Additionally, the consequences of stocking naïve fish into systems that have experienced VHSV IVb outbreaks were explored by performing a mock stocking event with age-0 sentinel Largemouth Bass. Despite four years of negative surveillance results, the virus was isolated from resident Muskellunge and Largemouth Bass and stocked sentinel fish in Budd Lake in spring 2011, and Muskellunge, Largemouth Bass, Bluegills, and Pumpkinseeds tested positive for antibody production using the 50% plaque neutralization test, the competitive enzyme-linked immunosorbent assay, or both. The virus was not detected in leech, mussel, water, or sediment samples; however, the virus was detected in amphipods in both 2011 and 2012. Based on the study results, it is concluded that VHSV IVb has likely persisted in Budd Lake since the initial outbreak without causing additional mortality events, and that amphipods may be an incidental reservoir for the virus. In systems where VHSV IVb outbreaks have occurred, managers should be aware that the virus may be circulating in populations even though surveillance efforts may fail to find evidence of active infections.

To Mom, Dad, Bailey, Gretel, Teeny, Coco, Lillie, Monte, and Ruby.

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INTRODUCTION

The recent isolations of the viral hemorrhagic septicemia virus (VHSV) in the Great Lakes

basin have been a matter of concern for fisheries managers in the region because of its

pathogenicity and virulence. Since it was first discovered in Europe, VHSV has emerged in

waterbodies in Asia and North America in both freshwater and marine systems. Of particular

concern to managers is the wide host range of strain IVb of the virus; 28 species of fish are

acknowledged as susceptible to this strain (USOFR 2008). It is important to understand how this

virus interacts with its environment for scientists and managers to improve preventative efforts to

reduce the effects of outbreaks on fish populations and fisheries in infected systems.

1.1. Historical accounts

In 1938, farmed freshwater Rainbow Trout *Oncorhyncus mykiss* began dying of an

unidentified disease in Germany (Schäperclaus 1938). The etiological agent was identified 24

years later as a virus when it was isolated from Rainbow Trout in Egtved, Denmark, hence the

early name "Egtved virus" (Jensen 1965). The pathogen was later named viral hemorrhagic

septicemia virus because systemic infection in fish often resulted in hemorrhaging throughout the

body. In 1979, VHSV was isolated from an Atlantic Cod *Gadus morhua* population in the Baltic

Sea (Jensen et al. 1979). Thereafter, the virus quickly spread from Europe to the Pacific

Northwest of North America and to Asia. In the late 1980s and early 1990s, the virus was

isolated from hatchery-raised Coho Salmon *Oncorhynchus kisutch* (Eaton et al. 1991), Chinook

Salmon *Oncorhynchus tshawytscha* (Hopper 1989), and Pacific Cod *Gadus macrocephalus*

(Meyers et al. 1992) in Washington state. In 1993, it was discovered in wild Pacific Herring

Clupea pallasii (Meyers and Winton 1995), Pacific Sardines *Sardinops sagax*, Pacific Mackerel

Scomber japonicas, Pacific Smelt *Thaleichthys pacificus*, and Surf Smelt *Hypomesus pretiosus* in the northeast Pacific Ocean (Hedrick et al. 2003). VHSV was then found in wild Olive Flounder *Paralichthys olivaceus* in coastal Japan in 1999 (Takano et al. 2000), and shortly thereafter in farmed juvenile Olive Flounder in Korea (Kim et al. 2003). In the early 2000s, the virus was found in Mummichog *Fundulus heteroclitus* and Striped Bass *Morone saxatilis* populations in New Brunswick, Canada (Gagne et al. 2007).

In 2005, VHSV IVb was isolated from Freshwater Drum *Aplodinotus grunniens,* Round Goby *Neogobius melanostomus,* and Muskellunge *Esox masquinongy* in Lake Ontario, Canada, following the occurrence of a large fish die-off (Lumsden et al. 2007). This was the first discovery of VHSV in the Great Lakes or in any wild freshwater fish population (Faisal et al. 2012). Up until it was isolated in Lake Ontario, VHSV had been considered a marine pathogen that had only adapted to farmed freshwater conditions. In response to this discovery, archived Muskellunge samples that had been collected from Lake St. Clair in 2003 were tested and found positive for presence of VHSV IVb. Additionally, several fish populations in Lake St. Clair, including Gizzard Shad *Dorosoma cepedianum*, Yellow Perch *Perca flavescens*, Freshwater Drum, and Rock Bass *Ambloplites rupestris*, experienced mortality events attributed to outbreaks of VHSV IVb from March to July 2006. To date, the virus has been found in all 5 of the Great Lakes (Faisal et al. 2012). Additionally, VHSV IVb has since been discovered in small inland lakes in New York, Ontario, Wisconsin, Michigan, and Ohio (Thompson et al. 2011, Faisal et al. 2012). The rapid spread of the virus throughout the Great Lakes region, including inland systems and the St. Lawrence River, suggest that natural resource managers should clearly be concerned about the potential for continued spread of the virus perhaps even to regions outside the Great Lakes basin.

1.2 VHSV biology

VHSV is a single-stranded, negative-sense, RNA virus (Wagner 1990) in the genus *Novirhabdovirus*, family Rhabdoviridae, and order Mononegavirales (OIE 2009). Other rhabdoviruses include infectious hematopoietic necrosis virus (IHNV) and the rabies virus. Four genotypes of VHSV have been described, designated I-IV, as reviewed in Faisal et al. (2012). Genotype I consists of five sublineages (a-e) and is mainly found in farmed rainbow trout and some wild marine species in Europe. Genotype II exists in Atlantic Herring *Clupea harengus*, European Sprat *Sprattus sprattus*, and Atlantic Cod *Gadus morhua* in the Baltic Sea (Snow et al. 2004). Genotype III occurs in several marine species and is found in coastal waters of the North Sea (Stone et al. 1997). Genotype IV, which consists of two sublineages (a and b), is found in Korea (Kim et al. 2003), Japan, and North America (Nishizawa et al. 2002). The isolates from the Great Lakes region in North America have been identified as sublineage IVb (Elsayed et al. 2006).

VHSV IVb is extremely virulent, causing a range of clinical signs in infected fish. Although these clinical signs can vary by species, location, and strain, they include anemia (resulting in gill and hepatic pallor), abdominal ascites, petechial hemorrhaging, exophthalmia, splenomegaly, discoloration, lethargy, and erratic swimming behaviors (Kim and Faisal 2011). Morbidity and mortality rates of fish infected with VHSV vary with factors including host characteristics (e.g. naivety to the virus and age) and environmental conditions (e.g. water temperature) (OIE 2009).

1.3 Disease ecology

The frequency or severity of disease in wild populations is a function of the interacting effects of pathogens, hosts, and the environment (Hedrick 1998). Rarely does a single factor determine the outcome of a disease event; rather, according to Beaulaurier et al. (2012), episodes of epizootic events are generally a result of multiple host and environmental factors. Stressors may include chemical toxicants, management actions, predators, non-native species introductions, host immune response, host population density, and climate change (Riley et al. 2008). More than 70 years ago, Strong (1935) considered climate to be an important predisposing agent to disease and the physical environment to severely alter host resistance. It is now accepted that the susceptibility of an ecosystem to disease is largely governed by the health of that ecosystem, and that maintaining ecosystem function, as it is currently understood, may be one way to reduce the negative effects of disease events in natural systems (Hedrick 1998, Riley et al. 2008).

1.4 Disease ecology of fish

Although it is important to thoroughly understand the pathogen of concern through laboratory analyses and experimental challenges, it is also important to examine the effects of disease in fish at the population, community, and ecosystem levels as this combination of scales will yield the most useful information for management (Power 2002). Three outcomes are possible after a pathogen, particularly a virus, has been introduced to an aquatic system. First, exposure to the pathogen may result in no infection in the population. Second, the pathogen may become established in only a small proportion of the population causing some mortalities, after which it may become quickly diluted in a large area or a system with high flushing rates,

preventing a disease epizootic. Lastly, the pathogen may become fully established in a small portion of the population, after which it is amplified and shed at high concentrations into the surrounding environment, resulting in an epizootic (Hershberger et al. 2010). The outcome of a pathogen introduction depends on many of the factors listed above, and decreased immunity or increased susceptibility to infectious diseases may occur when various stressors are acting on fish populations (Antychowicz and Kozinska 2011).

At least 80 marine and freshwater fish species in the northern hemisphere are known to be susceptible to VHSV in general (OIE 2009); 28 species are considered susceptible to VHSV IVb and are regulated by the Viral Hemorrhagic Septicemia Interim Rule, implemented by the USDA in 2008, which establishes restrictions for interstate movement of these species in the United States (USOFR 2008). Kim and Faisal (2010a) experimentally infected several Great Lakes fish species with VHSV IVb to determine their relative susceptibilities. Muskellunge and Largemouth Bass experienced the highest mortality rates, followed by Yellow Perch and salmonids. Based on tests conducted to date, VHSV IVb appears to be more virulent in cool water species than cold water species, similar to the pathogenicity data obtained for genotype IVa in the Pacific Northwest of North America (Winton et al. 1991, Follet et al. 1997, Kocan et al. 1997, Arkush et al. 2006, Hershberger et al. 2007).

Screening for neutralizing antibody presence can provide information on past exposure to the virus even if the sample is negative for VHSV IVb (Millard and Faisal 2012a). In a comparative study, Millard and Faisal (2012b) found that Muskellunge yielded the highest percentage of antibodies against VHSV IVb, followed by Northern Pike, Freshwater Drum, and Smallmouth Bass *Micropterus dolomieu*. Species in which antibodies were not detected included Channel Catfish *Ictalurus punctatus*, Lake Sturgeon *Acipenser fulvescens*, Quillback *Carpiodes*

cyprinus, Rock Bass, Shorthead Redhorse *Moxostoma macrolepidotum*, Silver Redhorse *Moxostoma anisurum*, Walleye *Sander vitreus*, White Perch *Morone Americana*, and Yellow Perch.

To fully understand fish health and disease in wild populations, Riley et al. (2008) proposed that research incorporate an integrated, multi-species, and ecosystem-level approach to improve understanding of population-level risks of disease in fishes. The approach proposed by Riley et al. (2008) is logistically difficult to execute on systems as large as the Great Lakes because of the large number of variables that would need to be accounted for as well as the difficulties associated with sampling. To improve the feasibility of the holistic approach proposed by Riley et al. (2008), studying the virus in one of the inland lakes from which it has been isolated would be far better for characterizing aspects of the virus such as virulence, transmissibility, vectors, and reservoirs than in one of the Great Lakes by virtue of sampling facilitation and ensuring sampling representativeness.

VHSV IVb has been isolated in two Michigan inland lakes: Budd Lake in 2007 and Baseline Lake in 2009. Between these two lakes, Budd Lake, located in Clare County, Michigan, is arguably a better model system for studying VHSV IVb dynamics because it is a closed system (no inlets or outlets), whereas Baseline Lake has three small inlets and one outlet (Dexter 1991). Budd Lake is also appropriately sized (71 ha) for this research as it is large enough to reflect a fairly complex fish community, while still being small enough to ensure a high probability of locating the virus if it is still circulating.

1.5. VHSV IVb in Budd Lake, MI

In May of 2007, a large mortality event occurred in Budd Lake. Of the five species involved in the die-off, four tested positive for VHSV IVb: Black Crappie, Bluegill, Pumpkinseed, and Largemouth Bass. One month later over 300 fish were collected from the lake and tested again for the virus, but all were negative. Testing conducted in 2008 and 2010 also failed to turn up evidence of the virus. These negative surveillance results spurred an array of questions regarding the status of the virus in the lake (i.e. was it still circulating in fishes or invertebrates or was it absent from the system?) It was also unknown what components of the system might harbor the virus – fish, invertebrates, or environmental reservoirs such as water or sediment. The event also triggered inquiry into whether indirect evidence of virus activity existed through antibody presence in susceptible fish populations. Without a more thorough understanding of VHSV IVb activity in general, optimal fisheries management decisions will be difficult to make. Through further examination of these unknowns and a better understanding of VHSV IVb dynamics in aquatic systems, fisheries managers will be more prepared to make confident and informed decisions regarding stocking and legislation.

1.6. Thesis purpose and objectives

The purpose of this research was to determine whether VHSV IVb still occurred in Budd Lake and if so in what manifestations (i.e. active infections, production of antibodies), or whether infection could be induced in fish due to a stressed environment. An additional goal was to try and determine what the reservoirs for the virus might be if it was still detectable in the lake. Specific objectives of Chapter 1 were to:

1. Assess the current status of viral presence in game fishes by use of direct methods.

2. Determine current and past VHSV IVb infection in fish by means of indirect assays.

The specific objectives of Chapter 2 were to:

1. Assess the current status of viral presence in prey fish, invertebrates, water, and sediment.

2. Assess the risk of future infections by exposing a sentinel species to density-induced stress. If VHSV IVb is still viable in the system, these sentinel fish should succumb to viral infection.

CHAPTER 1

Persistence of VHSV IVb in an infected water body: a case study from Budd Lake, MI, USA

Abstract

In May of 2007, viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) was isolated in several fish species in Budd lake, a 71-ha inland lake in central Michigan. Because of VHSV IVb's virulence and pathogenicity, follow-up testing was conducted in 2007, 2008, and 2010 to evaluate infection status in the lake. This testing failed to find additional evidence of VHSV IVb infections, which led to several questions regarding status of the virus (e.g. was it still circulating at low prevalences?) and the implications that it could have for fisheries management (e.g. could stocking lead to additional epizootic occurrences?). This study was conducted to better gage the status of the VHSV IVb infection in Budd Lake, including active viral infection and antibody production, by intensively sampling across multiple seasons, locations, and size groups of important game fishes: Muskellunge *Esox masquinongy*, Northern Pike *Esox Lucius*, and Largemouth Bass *Micropterus salmoides*. Active virus was isolated in esocid and Largemouth Bass samples collected in the spring of 2011. Infection prevalences ranged from 6 to 10% for esocids depending on tissue type, and 30% for Largemouth Bass. In terms of antibody production, between 60 and 100% of collected esocids were found to produce neutralizing antibodies by the PNT depending on the sampling season. Conversely, neutralizing VHSV IVb antibody production in Largemouth Bass ranged from 0 to 11%. Given these results, we conclude that despite several years of negative surveillance results, VHSV IVb has likely persisted in Budd Lake and the system can be considered enzootic for the virus. Based on our results, we recommend vigilance in the management of VHSV IVb-positive systems to limit the

risk of virus spread as it is perhaps questionable whether previously infected systems will ever be

virus free.

Introduction

A serious finfish pathogen known as viral hemorrhagic septicemia virus (VHSV), the

causative agent of viral hemorrhagic septicemia (VHS), has affected fisheries in the Laurentian

Great Lakes region of North America since at least 2005. Fishes infected with VHSV experience

varying levels of internal and external hemorrhaging, exophthalmia, anemia, and ascites (OIE

2009) depending on the course of infection, which can take acute, sub-acute, or chronic forms

(Kim and Faisal 2010a). VHSV was first identified in Europe in 1938 (Schäperclaus 1938) and

was later detected in the Pacific Northwest of North America and subsequently Asia in the late

1900s (Hopper 1989, Eaton et al. 1991, Meyers et al. 1992).

Shortly after the discovery of VHSV as the etiological agent of a 2005 Freshwater Drum

Aplodinotus grunniens die-off in Canadian waters of Lake Ontario (Faisal et al. 2012), isolates

were classified as VHSV genotype IVb (VHSV IVb), a unique sublineage of the strains found in

other areas of the globe (Elsayed et al. 2006, Faisal et al. 2012). To date, the virus has been

detected in all 5 of the Great Lakes (Faisal et al. 2012). VHSV IVb has also been discovered in

small inland lakes in New York, Ontario, Wisconsin, Michigan, and Ohio, as well as in the St.

Lawrence River (Thompson et al. 2011, Faisal et al. 2012). Between 2005 and 2010, a total of 19

fish species tested positive for VHSV IVb in the Great Lakes region (Faisal et al. 2012).

The frequency or severity of diseases in wild populations is a function of interactions

among pathogens, hosts, and the environment (Hedrick 1998). Rarely does a single factor

determine the outcome of a disease event; rather, episodes of epizootics are generally a result of

multiple factors (Beaulaurier et al. 2012) including stressors, which may include chemical

toxicants, management actions, predators, non-native species introductions, host immune

response, host population density, and climate change (Riley et al. 2008). More than 70 years ago, Strong (1935) considered climate to be an important predisposing agent to disease and the physical environment to severely alter host resistance.

Host susceptibility of VHSV IVb was assessed by Kim and Faisal (2010a), who experimentally infected several common Great Lakes species with VHSV IVb to determine their relative susceptibilities. Muskellunge and Largemouth Bass experienced the highest mortality rates, followed by Yellow Perch *Perca flavescens* and salmonids. Based on tests conducted to date, VHSV IVb appears to be more virulent in coolwater species than coldwater species, similar to the pathogenicity data obtained for genotype IVa in the Pacific Northwest of North America (Winton et al. 1991, Follet et al. 1997, Kocan et al. 1997, Arkush et al. 2006, Hershberger et al. 2007). Additionally, the comparative antibody production from various Great Lakes species was assessed by Millard and Faisal (2012b). The species that had the highest percentage of antibodies was Muskellunge, followed by Northern Pike, Freshwater Drum, and Smallmouth bass *Micropterus dolomieu*.

Despite extensive research having been conducted on VHSV IVb, there are several aspects of the virus that are not well understood. One major area of uncertainty regards the persistency of the virus in infected systems. As is common with endemic or enzootic diseases, VHSV is often detectable for several years within a system following an initial outbreak (Faisal et al. 2012, Garver et al. 2013). Yet in other cases, follow-up testing for the virus after an initial "debut" mortality event has failed to identify evidence of additional infections. This raises questions as to what the viability of the virus is in enzootic regions where VHSV IVb-associated mortality events have not occurred for several consecutive years. As well, it raises a question as to what point an area can be considered VHSV IVb-free. From a fisheries management

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standpoint, the uncertainty as to whether the virus still exists in a lake despite the inability to detect it raises numerous questions. Can management activities such as stocking proceed as normal or do managers need to be concerned that stocking could lead to further outbreaks due to either stressing the system or introducing a number of naïve individuals? Do fishery managers still need to be concerned about the spread of the virus from a previously infected system?

In the present study, we aimed to clarify some issues regarding persistency of VHSV IVb in systems that have experienced a VHSV IVb outbreak by intensively studying a lake (Budd Lake located in central Michigan) that experienced a VHSV IVb-related mortality event in May 2007 and then tested negative for VHSV IVb from June 2007 to 2010. Precisely, we wanted to determine the current infection status of Muskellunge, Northern Pike, and Largemouth Bass in Budd Lake by conventional isolation methods and to seek evidence of past infection by detecting specialized antibodies using two distinct serological assays. If VHSV IVb was truly absent from Budd Lake, negative virus and antibody results from all fish would be expected. If there was an active outbreak in the lake, we would anticipate finding tissues or blood sera that contain viable virus. If a recent outbreak had occurred in the system, we would anticipate finding blood sera containing VHSV IVb-specific antibodies.

Materials and Methods

Study site

Budd Lake is a 71-ha inland lake located in Clare County, Michigan, which is in the central region of the state's lower peninsula. The lake is within the watershed for Lake Huron. It is an important recreational fishery that is regularly stocked with Muskellunge by the Michigan Department of Natural Resources (MDNR 2012) and borders Wilson State Park to the northwest. Since it is a morainic, calcareous seepage lake (Coffey and McNabb 1974), there are no inlets or outlets so the main water sources are precipitation and runoff supplemented by groundwater.

Fish and sample collection

Pulsed DC boat electrofishing at low amperage was conducted in spring (May), summer (July), and fall (September and November) of 2011. Sampling of esocids primarily targeted Muskellunge, but Northern Pike were also collected if they were seen. Sampling for Largemouth Bass was also conducted in spring 2012 using pulsed-DC boat electrofishing based on the results from the spring 2011 sampling. The spring 2012 sampling was conducted in mid-April., approximately one month earlier than the spring 2011 sampling as a result of warmer temperature earlier in the year. For the purpose of sampling Largemouth Bass, the lake was stratified into two areas (North and South regions) and approximately equal sample sizes from both areas were targeted to help ensure collections were distributed throughout the lake. The target sample size for Largemouth Bass for each sampling event was 60. Sampling of esocids was more opportunistic given the lower densities in the lake and difficulties associated with sampling Muskellunge and Northern Pike.

For all captured individuals, length, weight, and sex (if possible) were recorded. Scales for aging individuals were removed from the mid-length and ventral to the lateral line region by scraping with a knife. Scales were placed directly into a coin envelope until processing. Aging of Muskellunge by scales is generally only reliable for younger age fish with there being some disagreement as to ages for which accurate estimates can be obtained (Johnson 1971, Fitzgerald et al. 2007). Fitzgerald et al. (2007) found that aging Muskellunge by scales was only reliable for fish age-3 and younger, with age-estimates for older fish on average deviating from known-age by one year. Our purpose for aging fish was to ensure that at least some collected individuals recruited to Budd Lake after the initial VHS outbreak so error associated with aging Muskellunge by scales was considered acceptable. Scales were examined using the image analysis software Optimas (Media Cybernetics, Inc. 1999) and total number of annuli were counted as an estimate of fish age. For the analyses, fish were grouped into size intervals as an index of fish age. Muskellunge were grouped in 20 cm increments; length groups 1 through 6 represented fish 0-20 cm in length, 20.1-40 cm, 40.1-60 cm, 60.1-80, 80.1-100 cm, and 100.1-120 cm, respectively. Largemouth Bass were grouped into 10 cm increments; length groups 1 through 6 corresponded to fish 0-10 cm, 10.1-20 cm, 20.1-30 cm, 30.1-40 cm, 40.1-50 cm, and 50.1-60 cm, respectively.

For esocids, tissue and blood samples for virus and antibody testing was obtained non-lethally. Captured individuals were anesthetized with 0.1 g/L of methanetricaine sulfonate (Fenquel) buffered with 0.2 g/L of sodium bicarbonate. Approximately 5 mL of blood was collected from the caudal artery of each individual if possible. A small gill biopsy, fin biopsy, and mucus sample (<1 gram each) were collected from each individual as well. Fish were then placed into fresh lake water until equilibrium was restored and then released. For sampling

conducted in 2011, Largemouth Bass collected from Budd Lake were transported to the

Michigan State University Research Containment Facility (MSURCF) and held live until sample

collection. For sampling conducted in 2012, Largemouth Bass samples were processed on-site

within a few hours of capture.

Largemouth Bass were euthanized with an overdose of MS-222 at a concentration of 0.25

g/L buffered with 0.5 g/L of sodium bicarbonate. Blood was collected from the caudal artery.

Kidney, spleen, and heart tissues were collected and stored in 4 oz. bags. All blood and tissue

samples were stored at 4°C until processing. Both tissue and serum was collected to more

accurately assess the state of infection; for example, if serum is positive for virus but tissue is

not, this could indicate that it was a recent infection that has only had enough time to invade the

bloodstream. If both tissue and sera are positive for virus, this may indicate an older infection

that has been given enough time to penetrate the internal tissues.

Tissue sample processing

Tissues were processed within 12 hours of collection. The weight of each tissue sample

was multiplied by four to obtain the volume of diluent to be added to each sample. Diluent

consisted of primarily Earle's salt-based minimum essential medium (MEM) and tryptose

phosphate broth, in addition to 1 M tris buffer (Trizma base and Trizma hydrochloride, Sigma),

gentamycin sulfate (Sigma), penicillin and streptomycin (Invitrogen), and 250 ug/mg

Fungizone® (Fisher Scientific). Tissues were then homogenized with a Stomacher®80

Biomaster laboratory paddle blender for four minutes at full speed. Homogenized samples were

centrifuged at 5,000 rpm for 30 minutes at 4°C, and supernatant was stored at 4°C until cell

culture inoculation. Blood was centrifuged at 5000 rpm for 10 minutes to separate the serum.

Serum was stored at -80°C until serology assays were performed.

Active VHSV IVb infection

We applied cell culture methods to propagate and isolate virus in tissue and serum

samples. The cell-cultured samples were confirmed positive or negative by extraction of total

RNA from the cells, followed by conversion of viral RNA to complementary DNA (cDNA) by

reverse transcription, and amplification of the cDNA by polymerase chain reaction (PCR).

Serum samples were additionally tested for antibodies with two separate serological assays.

Epithelioma papulosum cyprinii (EPC) cells were seeded onto flat-bottom 96-well plates

(Corning® Costar®) supplemented with a media consisting of primarily Earle's salt-based

MEM and tryptose phosphate broth, in addition to heat-inactivated fetal bovine serum (Gemini),

200 mM L-glutamine (Invitrogen), 1 M tris buffer (Sigma), penicillin and streptomycin

(Invitrogen), gentamycin sulfate (Sigma), and 250 μg/mg Fungizone® (Fisher-Scientific). A

confluent monolayer of cells developed for 24-48 hours before inoculation of tissue or serum

homogenate. Thirty μl of homogenate supernatant was dispensed into each well at six replicates

per sample. Cytopathic effect (CPE) was observed at 14 days post infection (dpi). All samples

underwent a second passage for an additional 14 days, allowing for a total incubation period of

28 days. CPE was recorded and samples were stored at -80°C until RNA extraction. To assess

viremia in sera, samples were diluted 1:10 and 30 μl was inoculated into each well at six

replicates per sample. If there was not enough serum for viremia testing on cell culture, samples

were saved for serological assays. Results were observed and samples were stored until RNA extraction.

Positive cell culture samples were confirmed with polymerase chain reaction (PCR) to amplify a region of the VHSV IVb G-protein gene, which was preceded by RNA extraction from cells and reverse transcription (RT) to convert RNA to complementary DNA. RNA was extracted from all tissue and serum cell culture supernatant using the QIAamp© Viral RNA Mini Kit and following the manufacturer's instructions. Viral RNA was translated into complementary DNA via reverse transcription using AffinityScript Multiple Temperature Reverse Transcriptase (Agilent© Technologies) by following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a conserved region of the VHSV IVb genome. Primers contained the following sequences: 5' GGG GAC CCC AGA CTG T 3' (forward) and 5' TCT CTG TCA CCT TGA TCC 3' (reverse). The 25 μl reaction consisted of 2.5 μl of each primer, 12.5 μl of master mix (Promega GoTaq Green), 5 μl of nuclease-free water (Promega), and 2.5 μl of template. PCR began with 1 cycle of polymerase activation at 94°C for 2 minutes, followed by 35 cycles of annealing at 94°C for 30 seconds, elongation at 52°C for 30 seconds, and denaturation at 68°C for 60 seconds, and finished with 1 cycle of elongation at 68°C for 7 minutes. Nucleic acid was stained with SYBR® Green II (Lonza) and results were observed through gel electrophoresis with a 1.5% Ultrapure^{TM} agarose gel (Invitrogen). Size of cDNA fragments were compared to a 2-log (0.1-10.0 kb) ladder (New England BioLabs). Bands at 811 kb were considered positive for VHSV IVb.

VHSV IVb antibody production

To measure the presence of neutralizing antibodies, or specific antibodies that bind to regions of the G protein on the outside of the virus that facilitate neutralization of the virus, a complement-dependent 50% plaque neutralization test (PNT) was performed for all serum samples. The PNT was created from a modification of the protocols by Olesen and Jorgensen (1986) and LaPatra et al. (1993), as described in Millard and Faisal (2012a). This antibody titer is conveyed as the inverse of the highest serum dilution that is still able to neutralize at least 50% of the average number of viral plaques calculated in the negative control sera (taken from naïve lake trout housed at the MSURCF). A titer of 160 or above is considered positive for neutralizing antibodies by the PNT based on experimental studies with Muskellunge (Millard and Faisal 2012b).

The competitive enzyme-linked immunosorbent assay (cELISA) detects antibodies that bind to any available proteins on the outside of the virus. A cELISA specialized for VHSV IVb has recently been developed and optimized by Millard et al. (in review) and was used for this study. In cases of only enough serum being present from an individual for one assay, it was used for cELISA as this assay detects a broader range of VHSV IVb antibodies. The level of antibodies in an individual detected by the cELISA is measured in percent inhibition; an inhibition of 14.53% is considered positive for antibodies by the cELISA based on data from Muskellunge (Millard et al., in review).

Data analysis

Prevalence of active VHSV IVb infections and antibody production by sampling group (esocids, Largemouth Bass) and collection season were estimated by dividing the number of

positive samples by total number of samples. Differences in prevalence in active VHSV IVb infections within species between tissue type and within tissue type between species were compared using odds ratios. Similarly, differences in antibody production within species between antibody type, within antibody type between species, and within antibody type between sampling season were compared using odds ratios. Confidence intervals (95%) were calculated for all odds ratios as a means of assessing the uncertainty associated with the measures. Odds ratios were used to compare prevalences rather than statistical tests of significance since they are considered more valuable for disease studies as a result of their emphasizing the biological significance of the results as opposed to statistical significance (Rutledge and Loh 2004). In addition, the length categories designated for esocids and Largemouth Bass were used to assess the distribution of antibody presence in different taxonomic and size classifications of fish.

Results

       Sample sizes for evaluating VHSV IVb infection in Muskellunge ranged from 0 to 23 depending on the sampling season and whether serum or tissue was used to evaluate infection status (Table 1-1). For evaluating antibody production, sample sizes in Muskellunge ranged from 0 to 19 depending on sampling season and type of assay (Table 1-3). For the spring season, 23 Muskellunge and 0 Northern Pike were captured. For the summer season, one Northern Pike was captured. For the fall season, 7 Muskellunge and 5 Northern Pike were captured. For Largemouth Bass, sample sizes for evaluating VHSV IVb infection ranged from 27 to 71 depending on the sampling season and whether serum or tissue was used to evaluate infection status (Table 1-1 and Table 1-2). The low sample size of 27 was due to high mortality during the summer season after fish were transported to the MSURCF; consequently, blood was not able to be collected from several fish in this group. For evaluating antibody production, sample sizes in Largemouth Bass ranged from 18 to 67 depending on sampling season and serum assay (Table 1-3).

1. *Detection of VHSV IVb in fish*

1.1.*Isolation on EPC cell line*

       In spring 2011, VHSV IVb was isolated from both esocids and Largemouth Bass collected from Budd Lake, which was the first time that the virus had been detected in the system since the initial outbreak in early May 2011. For esocids, VHSV IVb prevalence in non-lethal tissues was 13% (3 of 23 positive) and prevalence in serum was 6% (1 of 17 positive) (Table 1-1). Of the esocids, one individual (a Muskellunge) presented virus in fin and gill tissues, one in the gill only, and one in the fin and serum. For Largemouth Bass, prevalence of VHSV IVb

infection in visceral tissue was 30% (19 of 63 positive) and in serum was 26% (13 of 50 positive). Prevalence of VHSV IVb infection on the cell line in summer and fall 2011 for esocids and Largemouth Bass was 0% in tissue and serum (only one Northern Pike was collected in summer 2011). For spring 2012, 0% of Largemouth Bass tested positive for VHSV IVb infection in both tissue and serum samples and this sampling was specifically conducted because of the positive findings from the 2011 spring sampling.

On cell culture, positives were observed in esocids ranging from 48-100 cm in length and in Largemouth Bass ranging from 15.4-42.2 cm in length. Virus was isolated from a total of 3 esocids and 21 Largemouth Bass during this study by the cell culture method.

1.2.Confirmation of VHSV IVb isolates by RT-PCR

Results of cell culture isolation were confirmed with VHSV IVb by the RT-PCR. RNA was first isolated from all cells inoculated with tissue or serum samples before being tested by the RT-PCR. For spring 2011, prevalence in VHSV IVb infection in esocids was 9% (2 of 23 positive) based on tissue and 12% (2 of 17 positive) based on serum (Table 1-2). Of these, one individual contained virus in its mucus and gill tissues, one in its fin, and two in serum only Prevalence in Largemouth Bass was identical to the cell culture results (19 of 63 positive in tissue and 13 of 50 positive in serum). For summer 2011, prevalence in esocids was 0% based on both tissue and serum. For Largemouth Bass in summer 2011, prevalence was again 0% for based on tissue, but was 4% (1 of 27 positive) based on serum. For fall 2011, prevalence in esocids was 8% based on tissue (1 of 12 positive) and 0% based on serum. For Largemouth Bass in fall 2011, prevalence was again 0% based on tissue but 4% based on serum (3 of 69 positive).

For the Largemouth Bass collected in spring 2012, results were identical to the cell culture results (prevalence of 0% based on both tissue and serum).

By RT-PCR, positives were observed in esocids ranging from 48-100 cm in length and in Largemouth Bass ranging from 15.4-46.2 cm in length. Virus was detected from a total of 5 esocids and 25 Largemouth Bass during the study by this method.

1.3.Prevalence comparisons

Because active VHSV IVb infection was primarily detected in the spring 2011 samples, comparisons of prevalence were limited to this season. Depicted in Table 1-5 are the odds ratios for VHSV IVb prevalence in esocids and Largemouth Bass by both cell culture and PCR methods. On cell culture, esocid tissue was 2.35 times more likely to be positive for VHSV IVb than esocid serum (the odds ratio was 2.35). In addition, the odds ratio for Largemouth Bass tissue versus serum was 1.23, for Largemouth Bass tissue versus esocid tissue was 2.85, and for Largemouth Bass serum versus esocid serum was 4.36. With the RT-PCR method, the odds ratio for esocid serum versus tissue was 1.39. The odds ratio for Largemouth Bass tissue versus serum was 1.23, for Largemouth Bass tissue versus esocid tissue was 4.47, and for Largemouth Bass serum versus esocid serum was 2.6. Cases where virus was more likely to be detected in serum versus tissue may indicate recent infections where virus has not yet been cleared from the bloodstream. Small sample sizes resulted in wide confidence intervals for all the calculated odds ratios, which limited our ability to make conclusions regarding the statistical significance of observed differences in prevalences.

2. *Detection of antibodies against VHSV IVb*

2.1. *PNT*

Prevalence of neutralizing antibody production in collected esocids was 100% in spring 2011 and 60% in fall 2011 (no samples were available for summer 2011) (Table 1-3). These antibodies were observed in Muskellunge lengths ranging from 47.2-106 cm for all seasons combined (Fig 1-1). Despite the large number of Largemouth Bass infected with VHSV IVb in the spring of 2011, only one individual contained neutralizing antibodies. No Largemouth Bass produced antibodies detected by the PNT in summer 2011, fall 2011, or spring 2012.

2.2. cELISA

Prevalence of antibodies in esocids detected by the cELISA was 74% in spring 2011 and 80% in fall 2011 (Table 1-3). These were observed in Muskellunge lengths ranging from 47.2-106 cm for all seasons combined (Fig 1-2). In Largemouth Bass, prevalence of antibodies detected by the cELISA was 7% in spring 2011, 11% in summer 2011, 8% in fall 2011, and 5% in spring 2012. These were observed in Largemouth Bass lengths ranging from 16.3-42.2 cm for all seasons combined (Fig 1-3).

2.3. Prevalence comparisons

The odds ratios for esocids and Largemouth Bass based on antibodies detected by the PNT or cELISA in different seasons tested are displayed in Table 1-6. The odds ratios of comparisons were as follows: esocid antibodies detected by the cELISA versus the PNT (2.41); Largemouth Bass antibodies detected by the cELISA versus the PNT (3.52); antibodies detected by the cELISA in esocids versus Largemouth Bass (35.16); Largemouth Bass antibodies detected by the cELISA in summer 2011 versus spring 2011 (1.71), fall 2011 versus spring 2011 (1.38), summer 2011 versus fall 2011 (1.52), spring 2011 versus spring 2012 (1.35), summer 2011 versus spring 2012 (2.31), and fall 2011 versus spring 2012 (1.52). There were two cases where

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an input value of zero resulted in an output odds ratio value of infinity. These comparisons were

esocid antibodies detected by the PNT versus the cELISA in spring 2011 and antibodies detected

by the PNT in esocids versus Largemouth Bass in spring 2011. Again, for these comparisons the

small sample sizes resulted in large confidence intervals which made it difficult to ascertain

statistical significance.

2.4.Correspondence between VHSV IVb infection and antibody production

Overall, in spring 2011, there was a higher percentage of esocids producing only

antibodies (82%), compared to the percentage of esocids exhibiting both virus and antibodies

(18%) in spring 2011 (Table 1-4). In contrast, there were few Largemouth Bass producing only

antibodies (13%) or exhibiting both virus and antibodies (9%) in spring 2011, while there was a

large percentage exhibiting only virus (78%). In the fall, both esocids and Largemouth Bass only

presented antibodies.

Discussion

After the initial outbreak of VHSV IVb in Budd Lake in May 2007, periodic sampling of fishes and testing for the virus was conducted to determine if the virus was still circulating in the fish community. In 2007, one month after the initial detection of the virus, 60 Bluegills *Lepomis macrochirus*, 60 Pumpkinseeds *Lepomis gibbosus*, 60 Largemouth Bass, 60 Bluntnose Minnows *Pimephales notatus*, 60 Sand Shiners *Notropis stramineus* and 9 Golden Shiners *Notemigonus crysoleucas* were collected and all tested negative for the virus. In late April 2008, 60 Bluegills, 60 Black Crappies *Pomoxis nigromaculatus*, and 60 Pumpkinseeds were collected and tested and again all individuals were negative for the virus. In 2010, 60 Bluegills, 60 Pumpkinseeds and 30 Yellow Perch were collected and tested negative for the virus. Based on these testing results, a widely held belief prior to this study was that the virus no longer existed in the lake, and that it had been eliminated from the system perhaps because of warm summer temperatures, which is not conducive to productivity of the virus (Goodwin and Merry 2011). Thus, our finding of active infection in esocids and Largemouth Bass in 2011 was somewhat unanticipated. Further, our finding of active infection immediately raised the question regarding whether the virus had been reintroduced to the system or whether it had remained in the system but had just gone undetected.

Based on previous findings of viral infections in fishes in both wild and aquaculture settings, we are of the opinion that the virus has remained in the system and that fish in Budd Lake have been coping with an enzootic infection for several consecutive years. Although there is some evidence of VHSV IVb isolates being eliminated from a system after an initial outbreak (Kahns et al. 2012), the bulk of evidence suggests that eradication of viruses is rare. In VHSV IVb-enzootic Lake St. Clair, Michigan, the same variant of VHSV was identified in Muskellunge

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collected in 2003, several species in 2006, and again in 2009 (Faisal et al. 2012). This pattern of outbreaks suggests that the virus becomes active enough to cause visible mortalities every few years, but in other years may not be detectable. This activity is probably caused by several interacting factors, including stress levels and environmental conditions. Additionally, antibodies against VHSV IVb were detected from several fishes in Lake St. Clair in 2004, 2006-2007, and 2009-2010 (Millard and Faisal 2012b), indicating the possibility that fish are being continually exposed to the virus and mounting immunity rather than the coincidental repeated introduction of VHSV IVb from outside sources.

In terms of aquaculture, Sahoo (2012) found that eradication of fish viruses is difficult because many viruses remain latent until stressors (e.g. high densities, poor water quality, water temperature) trigger another viral epidemic. In VHSV IVb studies conducted on Muskellunge, Kim and Faisal (2012) found that an individual can shed VHSV IVb up to 15 weeks after initial exposure, but when subjected to handling stress after shedding had ceased individuals can begin shedding the virus again and continue for an additional 15 weeks (Kim and Faisal 2012). Findings in the studies mentioned above contribute to the statement made by Hershberger et al. (2010) that shedding from infected individuals, combined with environmental stressors, may cause epizootics in wild fish populations. Understandably, physiological stressors (i.e. spawning) are also significant in prompting viral infection. Many fishes spawn during the spring including the three species of interest in this study, and spring is when VHSV is most likely to be detected, as observed for VHSV IVb in the present study as well as others (Kane-Sutton et al. 2010, Eckerlin et al. 2011, Faisal et al. 2012).

A plausible explanation for the failure to detect VHSV IVb from Budd Lake after the initial outbreak is that the virus remained in the lake below detectable levels. This theory is

supported by experimental data from Kocan et al. (2001) showing that VHSV was detected in only 5% of survivors that underwent a second infection, indicating that they had produced a protective immune response. Thus, even though the virus was at low prevalences, it was not entirely eliminated. It has also been observed that asymptomatic carriers are integral in sustaining viable virus in fish populations (Hershberger et al. 2010), and that customary diagnostic techniques may have a minimum threshold that is still above the lowest effective exposure level in a population, possibly leading to false negative results (Hershberger et al. 2011). This could explain why no virus was detected in any Largemouth Bass collected in the spring of 2012 in this study. However, these fish were not transported a long distance or confined in raceways before they were dissected as the 2011 fish were, so the absence of these stressors may have contributed to the lack of VHSV IVb-positive fish from this group.

As mentioned earlier, past research has shown that Muskellunge are more susceptible to VHSV IVb than Largemouth Bass (Kim and Faisal 2010a) but Largemouth Bass are nevertheless considerably susceptible to the virus. Paradoxically, there is also evidence that Muskellunge are capable of producing more antibodies to VHSV IVb (titer levels in excess of 12,000) compared to several other species, including Smallmouth Bass (titer levels around 200) (Millard and Faisal 2012b). Since Smallmouth and Largemouth Bass are closely related (therefore physiologically similar), we assume their antibody response to VHSV IVb would be similar. Consequently, because Muskellunge are highly susceptible and can develop a high antibody response to the virus, the assumption is made that Largemouth Bass develop a moderate antibody response (compared to Muskellunge) since they are moderately susceptible.

Based on antibody levels measured in this study, it appears that esocids are mounting effective protection to VHSV IVb in Budd Lake. The antibody titers observed in some

individuals were very high, which perhaps was a consequence of the size of Budd Lake and it resulting in the virus being highly concentrated (Hershberger et al. 1999). Alternatively, the high titer levels could indicate a relatively recent exposure to the virus since lower titers were observed in Lake St. Clair several years after the initial detection of VHSV IVb, which was attributed to diminishing exposure to the virus over time (Elena Millard, personal communication).

Considering what we know about the higher virulence of VHSV IVb in the spring versus other times of the year for many fishes, it is understandable that direct and indirect evidence of the virus was primarily detected from esocids in the spring. Furthermore, some individuals exhibited virus in non-lethal tissues but were apparently healthy, indicating that these fish could be asymptomatic carriers that are shedding the virus into the system. Given the noticeable immunity of esocids in this lake in the absence of severe clinical signs of VHS, the apparent ability to respond rapidly may be due to accumulated antibody production from continual exposure throughout the years. We suggest that low levels of shed virus from previously infected individuals in this lake may boost the humoral immune response of naïve fish, resulting in a higher proportion of protected fish and ultimately reducing the risk of future large-scale mortality events.

This same concept cannot be applied to the Largemouth Bass, and interpreting their role in this system is slightly more challenging because very little is known about this species' relationship with VHSV IVb. Positive virus-only fish were solely detected in the spring; in addition, few fish were detected with antibodies throughout the seasons. Considering interspecific variations between Largemouth Bass and esocids in their opportunities for virus exposure (Millard and Faisal 2012b), spawning temperatures, condition factors, trophic level,

habitat preferences, and susceptibility to the virus, it is not surprising that we see this species respond differently. Water temperature on the day of capture in 2011 was approximately 14°C, thus conditions were nearly ideal for Largemouth Bass spawning. In contrast, 2012 Largemouth Bass were captured earlier in the spring at a lower temperature thus reducing our likelihood of obtaining individuals experiencing peak spawning stress, which could also explain why similar infection prevalence as the 2011 Largemouth Bass was not observed. Largemouth Bass do not appear to be as competent in producing antibodies to the virus, but antibodies were nonetheless detected in a few individuals during each of four seasons tested (Table 1-3) despite the lack of positive virus results from most of these fish. Thus, it appears that Largemouth Bass are producing sufficient immunity to survive and they have had time to acquire this response with the passing of each season.

Management implications

The isolation of VHSV IVb in Budd Lake has been an issue of concern for fishery managers not simply from the standpoint of the effect the virus could have on the lake's fish community and fishery but also on potential risk of the virus to be spread to other inland lakes in the region. In response to VHSV IVb detection in the Laurentian Great Lakes region of North America, regulations were put in place to limit intra- and inter-state movement of fishes. Given that there have been no additional VHSV IVb outbreaks in systems close to Budd Lake, regulations that were implemented appear to have prevented the spread of the virus into nearby systems. As previously mentioned, prior to this study being conducted it was uncertain whether VHSV IVb was still present in Budd Lake and thus whether managers still needed to be concerned about possible spread of the virus or whether management actions (e.g. stocking)

might result in another disease outbreak. Based on the results of this study, VHSV IVb still very much appears to be circulating in the system and thus managers need to be concerned about the possibility of virus spread and future outbreaks. Esocid and Largemouth Bass populations appear to be recuperating from the VHSV IVb epidemic that occurred in Budd Lake in 2007. As indicated by Riley et al. (2008), pathogens can still be present in an ecosystem in the absence of disease because hosts and their pathogens evolve in response to one another and to the ecosystem. However, this does not necessarily mean that at some point there may not be additional fish mortality events that could be caused by VHSV IVb. Both natural and anthropogenic stressors could easily trigger another viral outbreak and additional fish kills, similar to what has been observed on Lake St. Clair.

To better understand how VHSV IVb in enzootic areas affects fishes from a population standpoint, it would be useful to monitor population abundances and dynamics of different susceptible species and compare this information with virus prevalence over time, particularly because the population effects of a disease will be more valuable to resource managers than a disease that severely affects just a few individuals (Riley et al. 2008). We also recommend that additional research be conducted to identify potential reservoirs for the virus in the system. Given the size of the lake and it being a closed system, we reiterate that in our opinion Budd Lake would be a model system for trying to clarify potential reservoir sources that may be responsible for persistence of the virus in water bodies. For example, the virus has been isolated from leeches in Lake St. Clair and amphipods in several of the Great Lakes (Faisal and Schulz 2009, Faisal and Winters 2011). Other than fish and invertebrates, rhabdoviruses are capable of replicating in other vertebrates as well as plants (Lyles et al. 2007). Identifying reservoirs for the virus may help in the development of efforts to limit spread or future outbreaks of the virus.

APPENDIX

Table 1-1. VHSV IVb isolation on the epithelioma papulosum cyprinii (EPC) cell line from Budd Lake fish. Results are displayed as the number of positive individuals/total number tested by cell culture.

		2011			2012	Total Positive Fish
		Spring	Summer	Fall	Spring	
Esocids						
Muskellunge						3/30 (10%)
	Mucus	0/23	0/0	0/7	NA	
	Fin	2/23	0/0	0/7	NA	
	Gill	2/23	0/0	0/7	NA	
	Serum	1/17	0/0	0/2	NA	
Northern Pike						0/6 (0%)
	Mucus	0/0	0/1	0/5	NA	
	Fin	0/0	0/1	0/5	NA	
	Gill	0/0	0/1	0/5	NA	
	Serum	0/0	0/0	0/3	NA	
Largemouth Bass						21/252 (8%)
	K/S/H	19/63	0/58	0/71	0/60	
	Serum	13/50	0/27	0/64	0/60	

NA: Esocids were not collected in the spring of 2012.

Table 1-2. VHSV IVb detection by RT-PCR in epithelioma papulosum cyprinii (EPC) cells inoculated with tissues and serum from Budd Lake fish. Results are displayed as number of positive individuals/total number tested by RT-PCR.

| | | | 2011 | | 2012 | Total # of |
		Spring	Summer	Fall	Spring	Positive Fish
Esocids						
Muskellunge						5/30 (17%)
	Mucus	1/23	0/0	0/7	NA	
	Fin	1/23	0/0	1/7	NA	
	Gill	1/23	0/0	0/7	NA	
	Serum	2/17	0/0	0/2	NA	
Northern Pike						0/6 (0%)
	Mucus	0/0	0/1	0/5	NA	
	Fin	0/0	0/1	0/5	NA	
	Gill	0/0	0/1	0/5	NA	
	Serum	0/0	0/0	0/3	NA	
Largemouth Bass						25/252 (10%)
	K/S/H	19/63	0/58	0/71	0/60	
	Serum	13/50	1/27	3/69	0/60	

NA: Esocids were not collected in the spring of 2012.

Table 1-3. Detection of serum antibodies against VHSV IVb in Budd Lake fish as measured by the 50% plaque neutralization test (PNT) and the competitive enzyme-linked immunosorbent assay (cELISA). Results are displayed as number of positive individuals/total number tested by each assay.

			2011		2012
		Spring	Summer	Fall	Spring
Esocids	PNT	18*/18	0/0	3/5	NA
	cELISA	14**/19	0/0	4/5	NA
Largemouth Bass	PNT	1/50	0/18	0/67	0/59
	cELISA	4/59	3/27	5/66	3/59

* Positive PNT: titer above 80

**Positive cELISA: percent inhibition above 14.53 with 95% confidence

NA: Esocids were not collected in the spring of 2012.

Table 1-4. Comparison of the tests for VHSV IVb (tissue and serum) and for antibodies (Ab) against VHSV IVb. Results are displayed as number of positive individuals/total number tested for each season.

	Esocids		LMB		
	Spring	Fall	Spring	Summer	Fall
VHSV IVb(+)/Ab(-)	0/17	0/4	18/23	0/3	0/5
VHSV IVb(-)/Ab(+)	14/17	4/4	3/23	3/3	5/5
VHSV IVb(+)/Ab(+)	3/17	0/4	2/23	0/3	0/5

Table 1-5. Testing of differences between esocid and Largemouth Bass (LMB) tissue and serum

in spring 2011 as assessed by cell culture and polymerase chain reaction (PCR).

Method	Scenario			Odds Ratio	Confidence Interval
Cell culture	Esocid tissue	vs	Esocid serum	2.3522	0.17-133.64
Cell culture	LMB tissue	vs	LMB serum	1.2268	0.50-3.1
Cell culture	LMB tissue	vs	Esocid tissue	2.847916	0.71-16.73
Cell culture	LMB serum	vs	Esocid serum	4.363489	0.57-199.47
PCR	Esocid serum	vs	Esocid tissue	1.3881	0.09-21.18
PCR	LMB tissue	vs	LMB serum	1.2268	0.50-3.1
PCR	LMB tissue	vs	Esocid tissue	4.470214	0.93-43.13
PCR	LMB serum	vs	Esocid serum	2.602472	0.49-26.55

Table 1-6. Testing of differences between esocid and Largemouth Bass (LMB) antibodies (Ab) detected by the plaque neutralization tests (PNT) and the competitive enzyme-linked immunosorbent assay (cELISA).

Scenario			Common Factor	Odds Ratio	Confidence Interval
Esocid PNT Ab	vs	Esocid cELISA Ab	Spring 11	Infinity	0.97-infinity
Esocid cELISA Ab	vs	Esocid PNT Ab	Fall 11	2.414224	0.08-195.65
LMB cELISA Ab	vs	LMB PNT Ab	Spring 11	3.527511	0.33-178.85
Esocid PNT Ab	vs	LMB PNT Ab	Spring 11	Infinity	24.18-infinity
Esocid cELISA Ab	vs	LMB cELISA Ab	Spring 11	35.16179	7.7-210.99
LMB Summer 11	vs	LMB Spring 11	cELISA Ab	1.707163	0.23-10.96
LMB Fall 11	vs	LMB Spring 11	cELISA Ab	1.382734	0.28-7.34
LMB Summer 11	vs	LMB Fall 11	cELISA Ab	1.517616	0.22-8.52
LMB Spring 11	vs	LMB Spring 12	cELISA Ab	1.354076	0.22-9.67
LMB Summer 11	vs	LMB Spring 12	cELISA Ab	2.307987	0.29-18.5
LMB Fall 11	vs	LMB Spring 12	cELISA Ab	1.524954	0.28-10.27

Figure 1-1. Esocid antibody titers detected by the plaque neutralization test (PNT). Titers of ≥160 were considered positive by this assay (N=23).
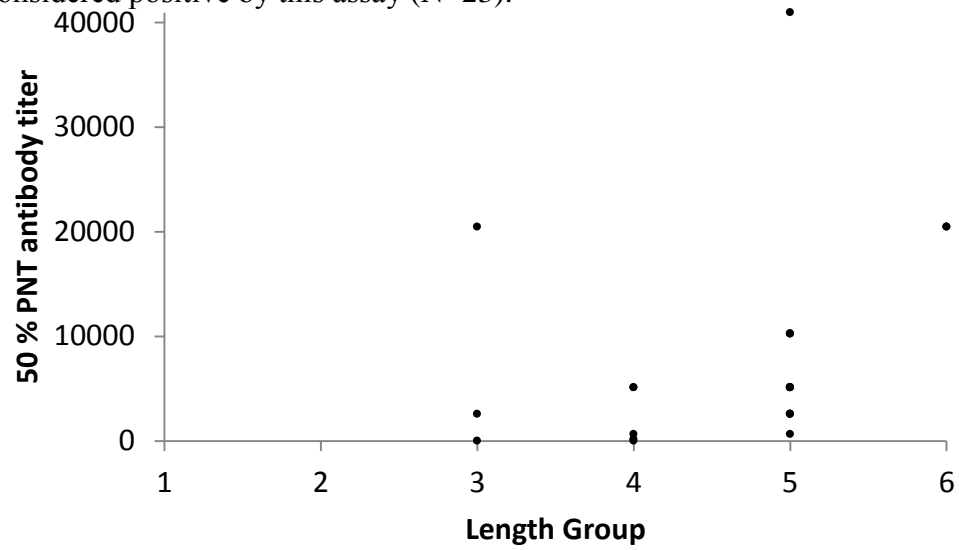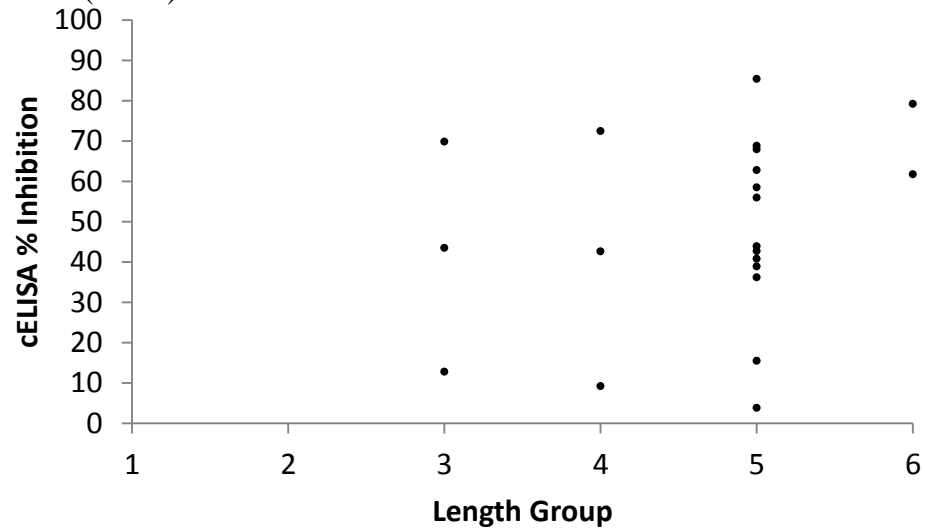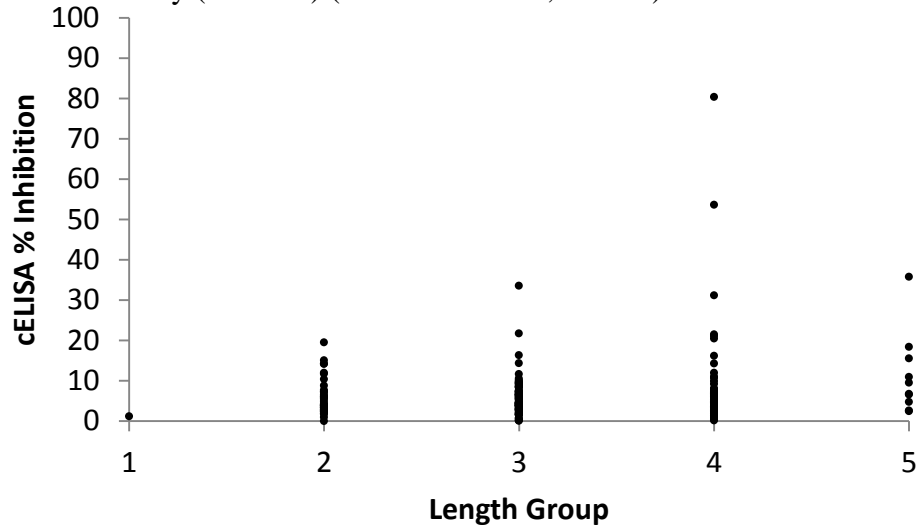
Figure 1-2. Esocid antibody titers detected by the competitive enzyme-linked immunosorbent assay (cELISA). With 95% confidence, inhibition that was above 14.53% was considered positive for antibodies (N=21).

Figure 1-3. 2011 and 2012 Largemouth Bass antibody titers detected by the competitive enzyme-linked immunosorbent assay (cELISA) (95% confidence, N=196).

CHAPTER 2

A comprehensive assessment of potential reservoirs and risk factors for VHSV IVb in an enzootic system

Abstract

Viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) has caused large fish die-offs in the Great Lakes region since 2005. Although studies have been conducted exploring VHSV IVb host range and susceptibilities, other characteristics of the virus remain unknown. One characteristic for which better understanding would assist in formulating management decisions for systems that have experienced a viral outbreak are factors affecting persistence of the virus. It is not uncommon for the virus to go undetected in systems for several years after an outbreak has occurred before it is again isolated or it causes additional mortality events. While such infection patterns could result from reintroduction of the virus, they could also result from reemergence of the virus from an environmental reservoir. The aim of this study was to identify potential reservoirs of VHSV IVb in Budd Lake, Michigan, which experienced an initial outbreak in 2007 and then went undetected until 2011. Additionally, we explored the risk of future epizootics by conducting a mock stocking event using juvenile Largemouth Bass *Micropterus salmoides* as sentinels. Minnow (*Notropis spp.*), panfish (*Lepomis spp.*), mussel, leech, sediment, and water samples collected from the lake tested negative for VHSV IVb. However, the virus was isolated in pools of amphipods (Hyalellidae) collected from Budd Lake in both 2011 and 2012. Only one Largemouth Bass from the mock stocking event tested positive for VHSV IVb. Our results suggest that amphipods could possibly serve as a reservoir or vector for VHSV IVb infected systems. Based on our surveillance testing and the results from the mock stocking event, we conclude that if there are environmental reservoirs for VHSV IVb in infected

systems, they are likely patchily, rather than uniformly, distributed throughout a system and we

encourage additional research into factors that allow VHSV IVb to persist in infected systems.

Introduction

Within the last decade, viral hemorrhagic septicemia virus (VHSV) has emerged in the Laurentian Great Lakes region of North America. The particular strain of the virus that has been found in the Great Lakes region has been classified as genotype IVb, which is similar to genotype IVa in the Pacific Northwest of North America but phylogenetically different enough to be designated as a separate sublineage. VHSV is a serious finfish pathogen that has caused fish kills in marine and freshwater systems as well as in aquaculture settings. There has been a fair amount of research conducted on this pathogen, including its molecular characteristics, host range, and geographical range. As well, research has been conducted identifying vulnerable hosts and host susceptibility to the virus. A far less studied, but no less important, feature of VHSV IVb is the environmental characteristics of the virus, such as the biological and ecological factors affecting the dynamics of the disease between the host and pathogen (Hedrick 1998). The lack of attention to the environmental characteristics of the virus is somewhat surprising considering that the course a disease takes in a system depends upon interactions between the environment, host, and pathogen (Martin et al. 1987). Although, according to Hedrick (1998), it is not uncommon for host and environmental aspects to receive less attention relative to aspects of the pathogen itself.

One environmental aspect of VHSV IVb that poses some challenge to those responsible for managing systems where outbreaks have occurred is the periodic and unpredictable reemergence of the virus even though surveillance efforts in intervening years often fail to find evidence of the virus. For example, in Lake St. Clair, VHSV IVb was identified in fish populations in 2003, 2006, and 2009 (Faisal et al. 2012). In Budd Lake, Michigan, VHSV IVb was detected in 2007 and 2011, but sampling conducted in 2008 and 2010 failed to isolate the

virus in several different fish species. Past research has found that eradication of viruses in natural systems is a rare occurrence; thus, this sporadic reemergence of VHSV IVb suggests that perhaps there are environmental reservoirs that could be facilitating transmission and management of the virus in enzootic systems.

Reservoirs can be classified as incidental or natural (Ashford 2003). An incidental reservoir is defined as a species that may or may not become infected by a pathogen, and has the possibility of transmitting it but is not a part of its normal maintenance cycle. This is opposed to a "natural reservoir", for which fish would play the role in the case of VHSV IVb since fish are required for maintenance of the virus. Knowing the full range of reservoirs for a particular pathogen is an important tool for pathogen control and management; Eckerlin et al. (2011) recommended an expansion of traditional views of incidental reservoirs to unusual abiotic and biotic components such as invertebrates and environmental substrates.

The primary goal of this study was to identify incidental reservoirs of VHSV IVb in Budd Lake, which based on isolation of the virus in 2007 and 2011, is considered endemic for the virus despite the virus going undetected in 2008 and 2010. Incidental reservoirs considered in this research included prey fish of the *Lepomis* and *Notropis* genera, and invertebrates including Cylindrical Papershell mussels (*Anodontoides ferussacianus*), amphipods of the Gammaridae and Hyalellidae families, and various freshwater leeches. Abiotic matter (water and sediment) was also tested for virus presence. Presently, there is evidence of VHSV IVb susceptibility in the genera *Lepomis* (Kim and Faisal 2010a) and *Notropis* (Frattini et al. 2011), as well as in amphipods of the genus *Diporieia* (Faisal and Winters 2011) and the *Myzobdella lugubris* leech (Faisal and Schulz 2009). Mussels were included since they are filter-feeding bivalves, which have the ability to harbor many viruses such as hepatitis A and norovirus (Li-ping et al. 2013,

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Pavoni et al. 2013, Roldán et al. 2013). Pertaining to abiotic matter, it has been established that VHSV is stable outside of the host for a number of days depending on the temperature and strain (Hawley and Garver 2008), and this has been corroborated for VHSV IVb by Bain et al. (2010). Furthermore, Pham et al. (2012) concluded that fomites, which are inanimate objects that can retain and transfer infectious organisms, may exist for VHSV IVb. Based on these findings, we thought it was possible for lake sediments to retain VHSV IVb particles or for the virus to simply circulate in water in a suspended state.

Another aspect of this study was to perform a mock stocking event using age-0 Largemouth Bass as sentinels for the purpose of assessing risks of epizootics stemming from fish stocking. Fishery managers commonly use stocking to supplement or enhance recruitment of fish populations. In the case of systems that have experienced a disease outbreak, stocking may be viewed as a particularly attractive management intervention tool particularly if younger age classes experience high mortality rates. The stocking of naïve fish into a previously infected system has the potential to be very problematic as these individuals could be highly susceptible to the virus and have high rates of infection. Because infected individuals will be interacting with other potentially susceptible individuals as well as shedding virus particles into the water, this has the potential to trigger an epizootic (Reno 2011). Our mock stocking event was intended to study the risk (or lack thereof) associated with stocking susceptible fish into systems that have experienced a VHSV IVb outbreak. .

Materials and Methods

Study site

Budd Lake is a 71-ha inland lake located in Clare County, Michigan. It is a morainic, calcareous seepage lake (Coffey and McNabb 1974) so there are no inlets or outlets and the main water sources are precipitation and runoff supplemented by groundwater.

The initial outbreak of VHSV IVb in Budd Lake occurred in May 2007. Black Crappies *Pomoxis nigromaculatus*, Bluegills *Lepomis macrochirus*, Pumpkinseeds *Lepomis gibbosus*, and Largemouth Bass were involved in the initial mortality event. In June 2007, 60 Bluegills, 60 Pumpkinseeds, 60 Largemouth Bass, 60 Bluntnose Minnows *Pimephales notatus*, 60 Sand Shiners *Notropis stramineus*, and 9 Golden Shiners *Notemigonus crysoleucas* were collected and all tested negative for the virus. In late April 2008, 60 Bluegills, 60 Black Crappies, and 60 Pumpkinseeds were collected and tested and again all individuals were negative for the virus. In 2010, 60 Bluegills, 60 Pumpkinseed, and 30 Yellow Perch *Perca flavescens* were collected and tested negative for the virus. In spring 2011, active infections were detected in Muskellunge *Esox masquinongy* and Largemouth Bass at prevalences of 17 and 10% respectively.

Prey Fish

Fish collection

Sunfish (*Lepomis* spp.) and cyprinids (*Notropis* spp.) were collected by pulsed-DC boat electrofishing (sunfish) or seining (cyprinids) in spring (May), summer (July), and fall (September) 2011. The lake was divided into northern and southern sections and approximately equal numbers of individuals were collected from both regions to help ensure samples were

distributed from throughout the lake. Fish were brought to the Michigan State University Research Containment Facility (MSURCF) and held live until tissue collection.

Tissue collection

Fish were euthanized with an overdose of methanetricaine sulfonate (Fenquel) (0.25 g/L) buffered with sodium bicarbonate (0.5 g/L). Length and weight was recorded for all sunfish, and for the first five cyprinids from each season because of the homogeneity in sizes of individuals of the same school. Blood was collected from *Lepomis* for antibody testing. Kidney, spleen, and heart were pooled for each individual sunfish and for every five cyprinids and tissues were placed into 4 oz. bags and stored at 4°C until processing.

Tissue processing

Within 12 hours of tissue collection, samples were processed for cell culture. Tissue weight was multiplied by four to obtain the volume of diluent to be added, which contains Earle's salt-based minimum essential medium (MEM) and tryptose phosphate broth as well as 1 M tris buffer (Trizma base and Trizma hydrochloride, Sigma), gentamycin sulfate (Sigma), penicillin and streptomycin (Invitrogen), and 250 μg/mg Fungizone (Fisher Scientific). Samples were homogenized for 4 minutes with a paddle blender (Stomacher 80 Biomaster) at full speed. About 1 mL of the mixed product was then centrifuged for 30 minutes at 5,000 RPM, and supernatant was stored at 4°C until inoculation onto cells. Sera from Bluegills and Pumpkinseeds were extracted by centrifugation of blood for 10 minutes at 5,000 RPM within 12 hours of collection, followed by storage at -80°C until use in cell culture and serological assays. Serum for use in cell culture was diluted 1:10 in diluent.

Cell culture isolation

Tissue homogenate and sera supernatants were inoculated onto epithelioma papulosum cyprinii (EPC) cells, which were grown on flat-bottom 96-well plates in media containing Earle's salt-based minimal essential medium (MEM), tryptose phosphate broth, heat-inactivated fetal bovine serum (Gemini), 200 mM L-glutamine (Invitrogen), 1 M tris buffer (Sigma), penicillin/streptomycin (Invitrogen), gentamycin sulfate (Sigma), and 250 μg/mg Fungizone (Fisher-Scientific). Cells were propagated for 24 to 48 hours to develop a confluent monolayer, after which six replicate wells were inoculated with 30 μl per well for each sample. At 14 days post-infection (dpi), samples were passed onto fresh cells for an additional 14 days, and the resulting CPE was recorded after the 28-day period. Samples were stored at -80°C until molecular assays. Volumes of sera that were too small bypassed cell culture testing and were used for serological assays only.

Serological assays

Short-lived, rapidly recruited antibodies were detected with the PNT, following modifications described in Millard and Faisal (2012a) from the protocols by Olesen and Jorgensen (1986) and LaPatra et al. (1993). For the PNT, the titer of antibodies in the serum is depicted as the inverse of the maximum serum dilution that still neutralizes at least 50% of the average number of viral plaques (which is determined from the negative control). Serum for the negative control was collected from naïve lake trout located at the URCF. If only enough serum existed for one assay, it was used for the cELISA. Antibodies that bind to any VHSV IVb antigenic epitopes are detected by the cELISA, which was recently optimized by Millard et al.

(in review). Inhibition levels of 14.53% or above were considered positive for VHSV IVb antibodies by the cELISA.

Molecular assays

RNA was extracted from cell culture supernatant with a viral RNA kit (QIAamp) following the manufacturer's instructions. One-step reverse transcription real-time PCR was completed with the Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems), following the manufacturer's protocol. The program was as follows: 1 cycle of reverse transcription at 50°C for 30 minutes, 1 cycle of activation and denaturation at 95°C for 15 minutes, and 40 cycles of denaturation at 94°C for 15 seconds, annealing and amplification at 60°C for 40 seconds, and polishing at 72°C for 20 seconds.

Invertebrate collection and testing

Leeches and amphipods were manually collected from leaf matter near the northern and southern shores of the lake in 2011, and amphipods were also collected in the spring of 2012 from 20 locations around the perimeter of the lake. Individuals of the same group were pooled into lots of five and placed into 1.5 mL microcentrifuge tubes. The weight of organisms in each tube was multiplied by four to obtain volume of diluent to be added, followed by homogenization with a mortar and pestle. Mussels were manually collected from the shoreline surrounding the island in the middle of the lake and stored live at the URCF until dissection. They were euthanized with carbon dioxide that was pumped through an aerator into the water. All internal viscera was collected and grouped into pools of five. Tissue weight was multiplied by four to obtain volume of diluent to be added. Samples were homogenized at full speed for 4

minutes with a Stomacher®80 Biomaster laboratory paddle blender. Approximately 1 mL was centrifuged at 5,000 RPM for 30 minutes, inoculated onto cells, and tested for VHSV IVb by real-time PCR as described above.

Abiotic matter collection and testing

Water and sediment samples were manually collected from various areas around the perimeter of the lake in 2011. Each substance was collected from 10 locations (five from the north half of the lake, five from the south half) during each season (spring, summer, and fall), yielding a total of 30 water samples and 30 sediment samples in 2011 (approximately 100 mL or 100 g per sample). The five samples from each location in each season were combined, yielding six pools of water and six pools of sediment. In 2012, methods of isolating virus from water and sediment were intensified. Each substance was collected from 20 locations throughout the lake; 10 liters of water was pumped from 10 feet below the surface, and sediment was collected from the same site using an Ekman grab. Water samples were supplemented with 0.5% fetal bovine serum to aid in stabilization of the virus. After each water sample was obtained, the pump and tubing was disinfected, de-chlorinated, and rinsed by circulating 10% bleach, 10% sodium thiosulfate, and lake water, respectively, for 10 minutes each through the pump before collecting the next sample. Samples were stored at -20°C until processing. A tangential flow filtration (TFF) system was used to filter virus from each substance with a 500 kDa hollow fiber filter (Spectrum Labs) after pre-filtration through a 20 μl and 2.5 μl filter. Tubing and filter were pre-cooled by pumping cold ultra-pure water through the system. Pump speed was 200 mL/Min, pressure was held between 1.5 and 2 psi, and the system was back-flushed every 5 minutes or less. Retentate (approximately 120 mL) was further concentrated via ultracentrifugation at

25,000 RPM for 2.5 hours at 4°C, and the resulting pellet was stored at -80°C until RNA extraction, RT, and real-time PCR as described previously. The protocol for sediment was similar, except for the addition of chemical buffers to disrupt the electrostatic and hydrophobic interactions between the virus and sediment particles (Gerba 1984). This addition to the protocol was adopted from Wommack et al. 2009. For each 100 g sediment sample, 400 mL of 10 mM sodium pyrophosphate and 40 µl of 5 mM EDTA was added. This mixture was placed on a mixing plate for 20 minutes. After samples were gravity-filtered through cheesecloth followed by vacuum filtration through 20 and 2.4 µm filters, sediment filtrate underwent TFF and centrifugation as described for the water samples.

Mock stocking event

In May 2011, age-0 Largemouth Bass from Jones Fish Hatcheries in Newton, OH, and certified VHSV-free, were placed into 54 enclosures that were split between the northern and southern shores of the lake. Enclosures were stocked with 5, 10, or 15 fish per enclosure (18 enclosures per density). Each enclosure was approximately 0.3 cubic meters and blocks of cages (each block included cages with each of the three stocking densities) were distributed at various locations throughout the lake. Enclosures were removed every four weeks throughout the summer of 2011; 15 (5 per density) were removed in May, 15 (5 per density) in June, 12 (4 per density) in July, and 12 (4 per density) in August. Fish were brought back to URCF and held live until dissection. Depending on the mortality in each enclosure, tissues and sera were pooled into groups of 5, yielding a maximum of 1, 2, and 3 pools from the 5, 10, and 15 density enclosures, respectively. Some pools contained less than 5 individuals due to mortalities, and some pools

contained one extra individual so that it was not in a separate pool on its own. Tissues and sera were collected and processed as described above for sunfish.

Results

Samples from which VHSV IVb was isolated in 2011 and 2012 are depicted in Table 2-1. Of the 85 total pools of fish tissues, VHSV IVb was not detected in any of the Bluegills, Pumpkinseeds, or members of the genus *Notropis* collected in 2011. However, one Bluegill and one Pumpkinseed were positive for VHSV IVb antibodies based on cELISA testing. The Bluegill was collected in the fall, the Pumpkinseed was collected in the summer, and both individuals were collected at the north end of the lake (Fig 2-1). It is important to note that the percent inhibition threshold determined for a positive result from the cELISA (14.53 %) is based on Muskellunge serological studies. Thus at this point, we cannot define the different threshold values for species other than Muskellunge.

As with the fish samples, there was no evidence of active VHSV IVb infection in any of the mussel or leech samples. Additionally, VHSV IVb was not detected from any water or sediment samples collected in 2011 or 2012. However, two pools of amphipods (of unidentified genera) from 2011 displayed amplification of VHSV IVb through real-time PCR. One pool was collected in the summer, and one in the fall. Both pools were collected from the north end of the lake. Because of these findings, amphipods were more intensively sampled in 2012 in an effort to confirm these results. During the 2012 sampling, three additional pools of amphipods tested positive for the virus. The amphipods collected in 2012 that tested positive for VHSV IVb were identified as members of the genus *Hyalellidae*.

Of the 85 pools of kidney/spleen/heart samples from the mock stocking event, one pool tested positive for VHSV IVb by real-time PCR. This pool of individuals was from an enclosure containing 15 Largemouth Bass that was located near the south end of Budd Lake and was removed on June 28. In addition, a pool of serum yielded a low positive indicating a low level of

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viremia in a different group, which was also removed from the south end of the lake during the

July 26 collection from an enclosure containing 10 individuals.

Discussion

Although VHSV IVb was not detected in prey fishes, leeches, mussels, or abiotic samples, this does not entirely exclude the possibility that some of the individuals were harboring low amounts of virus that were below detectable levels. In Atlantic herring in Scotland, populations have tested positive for VHSV IVb in the past, but even sensitive real-time PCR assays have not been able to detect the virus in recent sampling events although this species still may be a possible reservoir that transfers VHSV IVb from wild to captive populations of fish (Matejusova et al. 2010). Given that Bluegills and Pumpkinseeds tested positive for VHSV IVb in Budd Lake in May 2007 but were negative when additional samples were collected only one month later (Faisal et al. 2012), it is clear that detection of VHSV IVb in these species is highly dependent upon the time of collection. Therefore, the absence of positive results from these species does not necessarily designate them as risk-free in terms of sustaining VHSV IVb in the system. Additionally, with the evidence of oral transmission of VHSV in rainbow trout (Schönherz et al. 2012), it is not unlikely that if there are infected Bluegills or Pumpkinseeds in Budd Lake, they may be able to transmit VHSV IVb orally to predator species such as Largemouth Bass, Muskellunge, or Northern Pike (*Esox Lucius*). It is also possible that the virus could be harbored in younger (i.e. larval stages) (Hershberger et al. 2007), which were not collected during this research.

Initially, bivalves, such as mussels, were identified as potential reservoirs for VHSV IVb based on their filter-feeding capabilities. Research conducted on other viruses, such as hepatitis A virus and norovirus, have found that mussels can concentrate viral pathogens through their filter feeding capacity. Pathogens are thought to build up in the digestive system of bivalves, and as viruses are dispersed throughout the water, they may be circulated into the feeding pathway of

bivalves, which can then become a source for continued infections (Roldán et al. 2013). However, Faust et al. (2009) found that some bivalves have the ability to not only remove the avian influenza virus from water, but they can reduce its infectivity as well. This could be what mussels are able to do with VHSV IVb, and this potential phenomenon deserves further investigation as bivalves are currently being used worldwide as a management tool for reducing chemical and pathogenic contamination. Ultimately, the positive or negative contribution of bivalves to pathogen control is dependent upon whether the bivalve maintains or diminishes infection and development of the parasite (in this case, a virus) (Faust et al. 2009).

The detection of VHSV IVb from five total pools of amphipods collected in 2011 and 2012 is consistent with the findings of Faisal and Winters (2011), but only the sensitive real-time RT-PCR could detect the low amount of nucleic acid as our samples were not initially positive by cell culture. This is not unusual; for example, Atlantic Herring *Clupea harengus* in UK waters were repeatedly detected by PCR without initial isolation by cell culture (Dixon et al. 1997). The exact taxonomic identification of the positive pools from 2011 is unknown, but based on the two primary families collected in 2012, Hyalellidae and Gammaridae, it is likely that they fall under one or both of these categories. The positive amphipods from 2012 were all identified as belonging to the Hyalellidae family. Given this crustacean's role at the base of the food chain, from where juvenile fish normally feed, it is possible that they are a noteworthy contributor to viral persistence. The risk is further increased since juvenile fish have shown to be less immune to VHSV infection than more immunocompetent adult cohorts (Kocan et al. 1997, Kocan et al. 2001, Marty et al. 2003, Hershberger et al. 2007, Kim and Faisal 2010b). If amphipods do indeed facilitate transmission, it will be important to recognize the various thermal niches of different fishes, as spatial segregation allows for utilization of different food resources (Coutant 1987);

thus, amphipods may have different effects on the disease ecology of VHSV IVb depending on taxonomy as well as age class of resident fishes.

It would not be surprising that if crustaceans as small as amphipods are harboring the virus, leeches from Budd Lake would be similar in this aspect. However, no virus was isolated from this category of invertebrates, despite their similar littoral habitat as the amphipods. A probable explanation for this is that the leeches in this study were not of the family Piscicolidae, as they were in Faisal and Schulz (2009), thus they may not have had the same opportunities to acquire the virus as piscicolids would from latching directly onto fish.

Oral infection by ingestion of invertebrates containing the VHS virus may have an important effect on pathogen control and management, but the context in which these factors operate determines the outcome. Schönherz et al. (2012) makes the distinction between when two modes of transmission, oral infection and water immersion, have the most impact on disease dynamics; VHSV transmitted orally progresses slower and is thought to be more important during initiation of infection in juvenile fish, while virus transmitted through the water progresses faster and may be more significant as an epizootic becomes established.

Since only 1 of 84 total pools was positive for VHSV IVb in the enclosure study, ample information was lacking regarding any patterns between likelihood of infection and density of fish per enclosure. Yet, the positive pool was from an enclosure comprising 15 fish (the highest density examined in the study) that was removed from the south end of the lake in late June, when the water temperature was approximately 20°C. This portion of the study was originally designed to detect VHSV IVb in a sensitive sentinel species in the case that it could not be detected in resident fish, based on the idea that if VHSV IVb was still circulating in the lake, it

would most likely be expressed in naïve fish. As was the case with the amphipods, this pool was

only detected using real-time RT-PCR, as viral infection was not evident in cell culture and

clinical signs of VHS were not visible in these fish. Exponential amplification began around the

30th of 40 cycles, thus the copy number was apparently low. Interestingly however, viral titer

will be below the infection threshold during the naïve stage (Schönherz et al. 2012), and Eckerlin

et al. (2011) reported that although VHSV IVb was clearly still present in their study site in the

summer, detection of the viral RNA was significantly reduced; these examples are consistent

with our findings. In addition to the VHSV IVb-positive pool, another pool of fish from the

enclosure study was positive for antibodies detected by the cELISA. As with the Bluegills and

Pumpkinseeds, the inhibition level for the cELISA that is considered to be positive with 95%

confidence is based on experimental studies with Muskellunge, so this must be taken into

account with considering the enclosure study results. The presence of VHSV IVb-specific

antibodies from fish in the enclosure study corroborates that the naïve fish were indeed exposed

to the virus at some point before collection, and their immune systems are responding

appropriately.

This is the first study of its kind to assess such a wide range of potential reservoirs of

VHSV IVb. Clearly, the full collection of factors that direct VHSV IVb replication, transmission,

virulence, and persistence in the environment is difficult to ascertain. Future research should be

targeted towards deciphering whether VHSV IVb is able to achieve a true non-replicating state in

entities other than fish, such as invertebrates or abiotic matter as discussed in the present study; if

this state is possible, it would be useful to delineate the amount of time it is able to stay in this

state before it is no longer able to replicate or cause disease upon encountering a fish host.

Additionally, based on the various evidence of bivalves inhibiting or enhancing replication of

pathogens that infect other organisms, and the potential impacts of invasive bivalves such as the zebra mussel (*Dreissena polymorpha*), it would be interesting to know if VHSV IVb is manipulated by bivalves in the Great Lakes region, and to pinpoint the underlying mechanisms of these manipulations.

APPENDIX

Table 2-1. VHSV IVb-positive samples from 2011 and 2012.

Sample Type	Year	Virus	Viremia	PNT	cELISA
Prey Fish					
Bluegills	2011	0/22	0/22	0/40*	1/62* [a]
Pumpkinseeds	2011	0/21	0/21	0/71*	1/77* [b]
Notropis	2011	0/70	n/a	n/a	n/a
Invertebrates					
Mussels	2011	0/40	n/a	n/a	n/a
Amphipods	2011	2/39 [c]	n/a	n/a	n/a
	2012	3/46 [d]	n/a	n/a	n/a
Leeches	2011	0/29	n/a	n/a	n/a
Abiotic Matter					
Water	2011	0/6	n/a	n/a	n/a
	2012	0/20	n/a	n/a	n/a
Sediment	2011	0/6	n/a	n/a	n/a
	2012	0/20	n/a	n/a	n/a
Naïve Fish					
5/enclosure	2011	0/18	0/18	0/13	0/17
10/enclosure	2011	0/28	0/28	0/20	0/28
15/enclosure	2011	1/39 [e]	1/39 [f]	0/32	0/39

* = Individually tested

[a] Fall, North

[b] Summer, North

[c] (1) Summer, North; (1) Fall, North

[d] (2) Spring, North; (1) Spring, South

[e] June 28 Removal, South

[f] July 26 Removal, South

Figure 2-1. Budd Lake, Clare County, Michigan. Solid triangles represent amphipods from which VHSV IVb was isolated. Open circles represent resident Bluegills and Pumpkinseeds from which antibodies were detected. The solid square represents sentinel Largemouth Bass from which VHSV IVb was isolated. The gray square represents sentinel Largemouth Bass from which VHSV IVb was detected in sera (viremia).
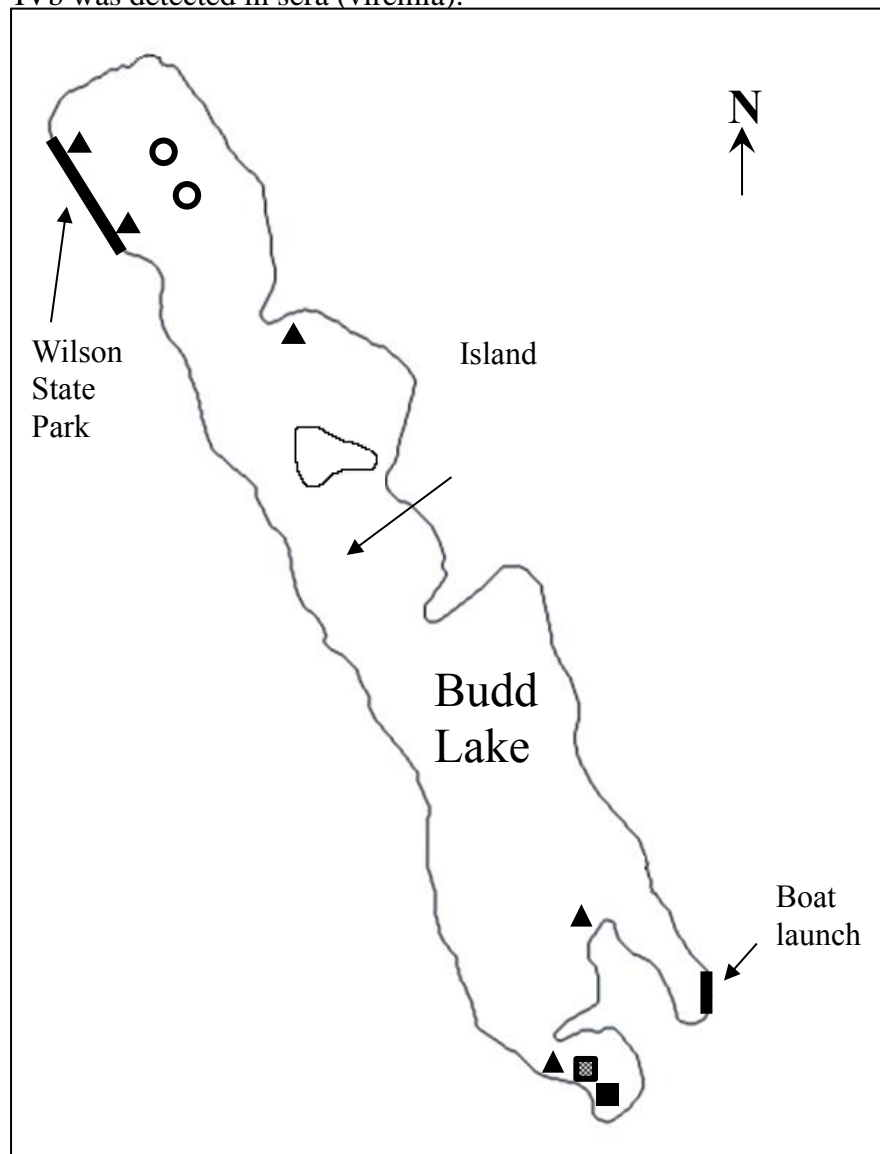
CONCLUSIONS

When introduced into an aquatic system, VHSV IVb possesses the ability to cause widespread damage to many species of fish. When it has existed in an area for an extended period of time, many fish species will recover from the initial damage and successfully form a protective immunity to avoid succumbing to future infections. This is evidenced by the exceptionally high titers of antibodies detected in fishes of Budd Lake, Michigan, four years following the initial mortality event.

Non-lethal sampling was effective at isolating the virus from mucus, fin, and gill tissues of esocids, promoting the likelihood that these fish are asymptomatic carriers that may be shedding the virus to other individuals or into the environment. In addition, one third of sampled Largemouth Bass collected in the spring of 2011 yielded positive virus results from internal tissues, yet few clinical signs were observed indicating that these fish may also be continuing the disease cycle without actually suffering from the disease itself. Largemouth Bass have an evident capability of developing immunity against VHSV IVb as well. Esocids ranging in length from 47.2-106 cm and Largemouth Bass ranging in length from 16.3-42.2 cm displayed antibodies. Out of all seasons examined, spring was the peak time to detect antibodies in esocids and virus in Largemouth Bass. There was no apparent relationship between the probability of virus detection and the sex of Largemouth Bass or between the probability of antibody detection and the sex of esocids.

In addition to analyzing virus load and antibody production in esocids and Largemouth Bass, several potential incidental viral reservoirs were examined for presence of VHSV IVb. Prey fish from the *Lepomis* and *Notropis* genera were tested for virus and the *Lepomis* genera

was also tested for antibodies. Virus was not isolated from any of these prey fish, signifying that they may be a minor source of infection for larger game species. However, antibodies were discovered in two *Lepomis* individuals, so these fish may be getting infected but are building immunity; the clear development of antibodies in several species of fish may likely contribute to herd immunity, protecting the fish populations in Budd Lake from another VHSV IVb-induced outbreak.

One organism in particular, the amphipod, presented positive virus results in 2011. Because of this, amphipods were extensively sampled 2012, and virus was again isolated. These findings, along with previous literature reporting similar results, suggest that non-fish organisms may play a greater role in persistence of VHSV IVb in the environment than previously anticipated. Leeches, mussels, water, and sediment did not appear to be significant actors in environmental persistence of the virus in this study, but these results may vary with temperature, location, and season.

Additionally, an experiment was conducted in 2011 to consider the impact that stocking and density will have on a VHSV IVb-endemic fishery such as Budd Lake. If stocking were to disrupt the balance of fish populations in a lake, which could therefore trigger excess stress, the probability of an epizootic event would likely increase. Naïve (disease-free), juvenile Largemouth Bass were placed into enclosures at different densities in Budd Lake, and one quarter of the total amount of enclosures were removed every four weeks. Fish were tested for VHSV IVb and antibodies. One pool of five fish tested positive for VHSV IVb in tissues, and a different pool tested positive for VHSV IVb in blood sera. No clear trends indicated if fish at higher densities were more susceptible to infection, but the virus was detected in tissues and sera from the 15- and 10-density enclosures, respectively. Clearly, these fish received the virus from

either water or food floating through the enclosures, so there is a potential risk for introduced fish to contract VHSV IVb. However, considering that 85 pools of tissues and 46 pools of sera were tested, and only one pool of each tested positive for virus, the risk of a VHSV IVb-related epizootic event may be minimal. This point is further supported by the fact that VHSV IVb was detected in tissues of resident fishes in the concurrent year, an ideal situation for the virus to infect naïve fish.

In conclusion, the objectives of this study were to (1) evaluate the presence of VHSV IVb in Budd Lake fishes, invertebrates, and abiotic matter, (2) assess the presence of antibodies to VHSV IVb in Budd Lake fishes, and (3) consider the risk of future epizootic events caused by VHSV IVb in Budd Lake by exposing susceptible, naïve fish (sentinels) to Budd Lake by immersion and all of the objectives were met. Since both virus and antibodies were detected in the lake, it is clear that the virus is still persisting in this environment and that fish are mounting an effective immune response. This response, produced by the most susceptible fishes, may be contributing to a herd immunity that has protected the fish from a VHSV IVb-induced mortality event since the initial die-off in 2007. Furthermore, other organisms may be involved in ecological persistence of this virus, apart from the host and pathogen. Whether these organisms play a significant role is unclear at this time. Lastly, the results of the enclosure study indicate that while naïve fish may be at risk for succumbing to VHS after being stocked into an endemic water body, the risk may be low as the virus appears to only be detectable with the employment of extremely sensitive, real-time PCR assays.

FUTURE RESEARCH

Budd Lake is an ideal site to study VHSV IVb in wild fish populations not only because of its small and closed geological characteristics, but because there are several years of VHSV IVb records from the resident fish in the lake. In Chapter 1, it was referenced that disease at the individual level may not always lead to drastic effects at the population level (Riley et al. 2008); so, it would be extremely useful and interesting to have an estimate of the population sizes over time of fishes in a lake such as Budd Lake. By having this data, the population-level effects of VHSV IVb on important game fishes over a long period would be much more apparent. However, it would be even more advantageous to have this information beginning with the first year of an outbreak, and to track the populations from that point on, but this could not be accomplished until another lake experiences its initial mortality event.

Since incidental reservoirs of VHSV IVb may play a larger role in persistence and transmission of the virus than previously thought, and the Aquatic Animal Health Laboratory at Michigan State University has identified two non-fish reservoirs of VHSV IVb, there are possibly many other animals (or even plants) that are contributing to the success of the virus that have yet to be discovered and these are worth investigating. Even if these organisms are inactivating the virus, managers would certainly benefit from knowing that information as those organisms could be utilized as biocontrol agents. If VHSV IVb continues to be a serious issue in the Great Lakes Region, exploration into the molecular functions in non-fish reservoirs that allow the virus to be stabilized, multiplied, or inactivated should be a priority if managers can use that information to control this devastating disease.

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