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Viral Hemorrhagic Septicemia Virus (Vhsv) Great Lakes Strain Ivb: Viral Detection, Mechanisms of Infection, and Host-Virus Interactions in the Yellow Perch (Perca Flavescens)

Wendy Joy Olson
University of Wisconsin-Milwaukee

VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) GREAT LAKES STRAIN IVB: VIRAL DETECTION, MECHANISMS OF INFECTION, AND HOST-VIRUS INTERACTIONS IN THE YELLOW PERCH *(PERCA FLAVESCENS)*

by

Wendy Joy Olson

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ABSTRACT
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Abstract

Viral hemorrhagic septicemia virus (VHSV) is one of the most devastating and problematic viral fish diseases to plague the European aquaculture industry, and due to its pathogenicity, disease course, mortality rates, and wide host range, remains one of the most pathogenic viral diseases of finfish worldwide. A new freshwater strain of viral hemorrhagic septicemia virus IVb (VHSV-IVb) in the Great Lakes has been found capable of infecting a wide number of naive species, and has been associated with large fish kills in the Midwestern United States since its discovery in 2005. In this study, the yellow perch, *Perca flavescence*, one such species documented in several fish kills affiliated with VHSV, was used as a research model to elucidate host-virus interactions to better understand the mechanisms of viral infection. A direct comparison of viral infection kinetics and net mortality among yellow perch stocks derived from distinct genetic and geographic regions found large variation in susceptibility to the disease, suggesting that genetic variance within a population can play a significant role in survival after infection with VHSV-IVb. Upon investigation of the early acute innate immune

response after exposure to the VHS virus, a significant up-regulation of Mx expression in the liver, as well as the inflammatory response genes IL-1β and SAA, in all three tissues sampled, head kidney, spleen, and liver, was directly correlated to viral load indicating the role of these genes in the initial stages of infection. Viral load increased most rapidly in the head kidney and spleen, suggesting that potential down-regulation of Mx in these tissues may represent a viral strategy to increase replication. Finally, when the pathology and distribution of the virus were monitored in different tissues of adult fish exhibiting a low level of VHSV infection over time, the finding of significant viral load in the gills and blood suggest that the sampling of these tissues may offer a more accurate, non-lethal alternative to viral screening from the currently, more traditional and lethal cell culture analysis of the head kidney/spleen. A significant viral load in the brain, however, particularly in the later stages of infection, also suggests that latent virus may remain in the brain neurons undetected in asymptomatic carriers of the virus, classifying the VHS virus as neurotropic, as well as a hemorrhagic.

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Yellow Perch

Chapter 1 - Introduction & Objectives

Due to its broad host range and acute nature, **viral haemorrhagic septicaemia (VHS)** is one of the most problematic viral fish diseases to plague the aquaculture industry, and remains one of the most pathogenic viral diseases of finfish worldwide (Kim & Faisal, 2011; Wolf, 1988). In areas of Europe, VHS infections in farmed rainbow trout (*Oncorhynchus mykiss*) have resulted in extremely high mortalities, causing extensive economic losses (Einer-Jensen *et al.*, 2004; Olesen, 1998; Skall *et al.*, 2005; Smail, 1999; Wolf, 1988) and, thus, is currently listed as a reportable disease by the World Organization for Animal Health (OIE, 2012). Viral haemorrhagic septicaemia virus (VHSV) isolates of marine origin have been identified in North America, Europe, and Asia where they cause disease in both farmed and wild species (Kim *et al.*, 2003; King *et al.*, 2001; Meyers & Winton, 1995; Mortensen *et al.*, 1999; Nishizawa *et al.*, 2002). In 2005, a new freshwater strain of VHSV was isolated from tissue of an adult muskellunge, (*Esox masquinongy*), collected in 2003 from Lake St. Clair, Michigan, part of the Great Lakes of North America (Elsayed *et al.*, 2006). This novel sublineage, named Great Lakes VHSV-IVb, was subsequently associated with massive fish kills that included

freshwater drum (*Aplodinotus grunniens*) (Lumsden *et al.*, 2007), muskellunge, round goby (*Neogobius melanostomas*) (Groocock *et al.*, 2007), gizzard shad (*Dorosoma cepedianum*), and yellow perch (*Perca flavescens*), in Lake St. Clair, Lake Erie, and Lake Ontario (Figure 1-1.) (Kim & Faisal, 2011). When VHSV was confirmed for the first time in the U.S. in the Great Lakes of North America in 2005, the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) immediately took action to prevent any further spread of the disease by issuing a Federal Order prohibiting the transport of any VHS-susceptible fish species from any VHS-affected or at risk states without specific disease testing and inspection of the facility (Figure 1-2). In an effort to slow the spread of the virus to inland lakes and rivers, the Federal government ordered strict restrictions on the transfer of susceptible species from the Great Lakes watershed into other regions of the U.S. (USDA, 2008). Because of the severe pathogenicity of the disease and the ever-increasing number of host-environments in which the virus is found, the study of VHS remains a top priority worldwide.

Figure 1-1. A VHS-induced fish kill documented in the Great Lakes of North America shortly after the discovery of a new freshwater strain of virus, VHSV-IVb *(U.S. Fish & Wildlife, 2008).*

September 9, 2008

Species regulated by title 9 CFR Parts 83.1 through 83.7, 93.900 and 93.910 through 93.916 (the Viral Hemorrhagic Septicemia (VHS) Interim Rule)

The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS) has identified the following species as having originated in freshwater locations in the United States and/or Canada, and as having been infected by VHS virus under natural (i.e. non-experimental) conditions of exposure; and from which VHS virus has been isolated by cell culture, with confirmation of strain identity through molecular detection. Anadromous fish species that have migrated into freshwater and from which VHS strain type IV(a) is isolated are excluded from this definition.

For regulatory purposes, presence of the viral pathogen and clinical expression of disease caused by the virus are considered synonymous.

Black crappie	*Pomoxis nigromaculatus*
Bluegill	*Lepomis macrochirus*
Bluntnose minnow	*Pimephales notatus*
Brown bullhead	*Amieurus nebulosus*
Brown trout	*Salmo trutta*
Burbot	*Lota lota*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Emerald shiner	*Notropis atherinoides*
Freshwater drum	*Aplodinotus grunniens*
Gizzard shad	*Dorosoma cepedianum*
Lake whitefish	*Coregonus clupeaformis*
Largemouth bass	*Micropterus salmoides*
Muskellunge	*Esox masquinongy*
Shorthead redhorse	*Moxostoma macrolepidotum*
Northern Pike	*Esox lucius*
Pumpkinseed	*Lepomis gibbosus*
Rainbow trout	*Oncorhynchus mykiss*
Rock bass	*Ambloplites rupestris*
Round goby	*Neogobius melanostomus*
Silver redhorse	*Moxostoma anisurum*
Smallmouth bass	*Micropterus dolomieu*
Spottail shiner	*Notropis hudsonius*
Trout-Perch	*Percopsis omiscomaycus*
Walleye	*Sander vitreus*
White bass	*Morone chrysops*
White perch	*Morone americana*
Yellow perch	*Perca flavescens*

Figure 1-2. As of 2008, 28 new species of freshwater fish in the Great Lakes demonstrated susceptibility to VHSV-IVb resulting in the transfer of live fish to uninfected regions of the U.S. to become federally regulated (USDA, 2008).

Genotypic analysis has classified VHSV as a negative-sense RNA virus. Its bullet shaped structure and 11kb genome have classified it as part of the *Rhadboviridae* family of the newly recognized *Novirhabdovirus* genus. While rhabdoviruses are among the most widely distributed viruses in nature, infecting plants, invertebrates and vertebrate hosts, the most well-known, and well-studied include the mammalian rhabdoviruses, rabies and vesicular stomatitis virus (VSV). The 11kb VHSV genome consists of six genes including,-the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV), and the viral RNA polymerase (L) (Figure 1-3A.) (Schutze *et al.*, 1999). Like all negative-sense RNA viruses, once inside a host-cell the viral RNA cannot be directly accessed by host ribosomes to form proteins. Instead, the RNA must first be transcribed by its own viral RNA polymerase into a "readable" complementary positive-sense mRNA transcript. Thus, intact, functional viral polymerase (L) must be safely transported in the host cell by the virus in order for it to replicate and become infectious (Figure 1-3).

The sixth gene, which encodes a non-virion protein (NV) is unique to fish rhabdoviruses including VHSV and has resulted in their sub-classification into the novirhabdovirus genus. While the function of the non-virion protein remains unknown, a large variation in the NV genomic sequence and subsequent protein structure between fish rhabdoviruses suggests that there is a relatively low level of evolutionary constraint on the NV gene (Kurath *et al.*, 1997).

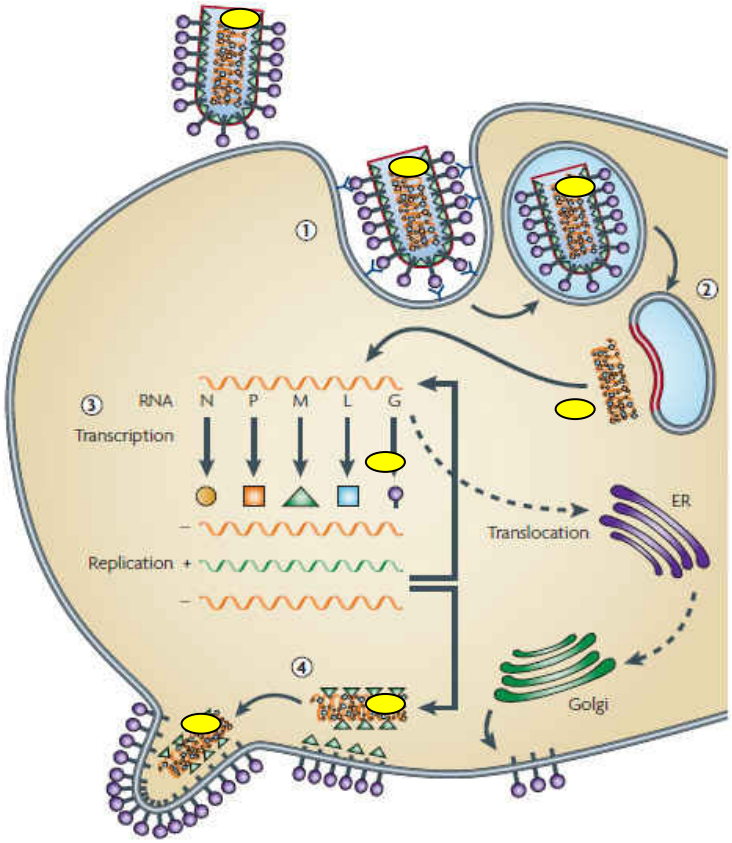

Figure 1-3. The Rhabdovirus virus life cycle. A simplified rhabdovirus life cycle can be divided into four phases. (1) The virus binds to the cell and enters by phagocytosis, (2) followed by fusion of the glycoprotein and matrix membrane to release the viral genome and viral polymerase into the cell cytoplasm (uncoating), (3) resulting in transcription of a positive-sense RNA that translated into viral proteins by the cellular endoplasmic reticulum (ER) and Golgi apparatus. (4) The pos-sense RNA is replicated back into a neg-sense RNA and the life cycle completed by assembly of the viral components and release of the virus through budding (Schnell *et al.*, 2010).

The clinical signs of an acute VHS manifestation of the disease are often non-specific, appearing singly or in a combination of either dermal petichial hemorrhages, hemorrhaging at the base of the fins (Figure 1-4 & 1-5A), muscles, internal organs (Figure 1-5B), and exophthalmia (Figure 1-5C). Ascites is often observed upon necropsy. In acute forms of the disease, mortality is rapid and commonly as high as 100% in juvenile fish, and as much as 30-90% in adults. In chronic forms of the disease, however, fish become infected with the virus without showing any outward clinical signs or pathology of the disease (Batts & Winton, 2007; Skall *et al.*, 2005). These fish experience a prolonged course with lingering low levels of mortality, and possibly shed the virus into the surrounding environment acting as covert carriers of the disease. In some hosts, a chronic infection is characterized by nervous behavior and erratic swimming (Kim & Faisal, 2010a; Wolf, 1988). Recent studies also demonstrate that the main portal of viral entry and replication is at the base of the pelvic and pectoral fins (Harmache *et al.*, 2006; Montero *et al.*, 2011).

Figure 1-4. Juvenile northern pike exhibiting characteristic external clinical signs of VHS consisting primarily of severe hemorrhage throughout the body. *Image by Dr. Mohamed Faisal (MSU,Lansing MI)*

Figure 1-5. Clinical manifestation of VHS most often consists of (**A.**) moderate to severe petichial hemorrhage throughout the body, (B.) hemorrhage of the muscle and internal organs, (C.) swelling behind the eye, or exophthalmia. *Images credit of Dr. Jim Winton (USGS, Seattle, WA), Dr. Mohamed Faisal (MSU, Lansing, MI), and Dr. Paul Bowser (Cornell, Ithaca, NY)*

History of VHSV

The first reported clinical signs of VHS infecting freshwater rainbow trout in Europe date back to 1938. It was not until 1962, however, that the virus was isolated and viral etiology confirmed. Until the late 1980s, the VHS virus was thought to exclusively target rainbow trout farmed in Europe until, in 1988, through routine viral screening of spawning salmonid broodstocks, a marine form of the VHSV was isolated for the first time in the Pacific Northwest in Washington State in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus tshawytscha*) (Winton *et al.*, 1989). At the time, VHSV had never been isolated outside of Europe, therefore, it was speculated that the virus must have originated through imported Atlantic salmon eggs from Europe. In 1990, however, a pacific cod (*Gadus macrocephalus*) caught in Prince William Sound, Alaska, exhibited lesions similar to those described in Europe. Upon examination by the pathology staff of the Alaska Department of Fish and Game, the marine strain of the virus

was again confirmed. This isolation provided the first evidence that a marine reservoir of the virus existed and may have led to the infected salmonids isolated earlier in Washington state (Amos *et al.*, 1998). Following this discovery, isolations of VHSV became widespread and problematic in hatcheries along the marine coastal regions of North America, where it has been particularly associated with substantial mortality in Pacific herring (*Clupea harengus pallas*), Pacific hake (*Merluccius productus*), and walleye pollock (*Theragra chalcomgramma*) (Kocan *et al.*, 1997; Meyers *et al.*, 1999; Meyers & Winton, 1995).

Since its discovery in the North Pacific, VHSV has been found in other marine fish worldwide, including turbot (*Scophthalmus maximus*) farmed in the Baltic Sea, and olive flounder (*Paralichthys olivaceus*) farmed in Japan (Isshik *et al.*, 2001; King *et al.*, 2001; Mortensen *et al.*, 1999; Nishizawa *et al.*, 2002). As VHSV continued to be isolated from an increasing number of marine species around the world, it soon became clear that the virus was more geographically widespread and less species-specific than previously thought. With further genetic analysis, it was concluded that the freshwater virus, originally known to infect rainbow trout in Europe, was actually derived from the marine form of the virus (Skall *et al.*, 2005).

In an effort to prevent the spread of this voracious pathogen, the European Union enforced strict restrictions on trade between zones where the pathogen was endemic, and between zones which were considered VHSV-free. While an aggressive VHS virus eradication program has successfully reduced the number of infected farms, the virus continues to be problematic throughout fish farms in continental Europe. Several studies

have recently indicated that VHS non-susceptible individuals may be acting as active covert carriers of VHS, serving as the primary source of viral spread into fish farms. It is suggested that these carrier fish act as viral reservoirs, continually shedding the virus at low to non-detectable levels while showing no clinical pathology of the disease. In addition, while the practice of pasteurizing fish feeds has significantly contributed to the reduction of VHSV in fish culture facilities, the introduction of the virus can still occur through the import of contaminated fish or fish eggs, or by waterborne virus in the water supply (Kurath, 2012). Once the virus establishes itself in a population, this opportunistic pathogen may remain hidden or latent in apparently healthy populations of fish until a significant stressor, such as spawning, sustained harassment by predators, severe temperature change, nutritional deprivation, or acute exposure to pollutants (hydrocarbons) trigger an active infection and subsequently lethal outbreak of the disease (Amos *et al.*, 1998; King *et al.*, 2001; Mortensen *et al.*, 1999; Skall *et al.*, 2005; Smail, 1999).

VHSV in the Great Lakes

The first isolate of VHSV was discovered in 2005 in the Great Lakes during a routine survey to determine the spread of the intracellular bacterium, *Piscirickettsia sp.,* in adult muskellunge (*Esox masquinongy)* from Lake St. Clair, Michigan (Elsayed *et al.*, 2006). Upon more intense investigation, the same exact strain of VHSV was then isolated from 4 of 42 muskellunge that had been collected in 2003. Since no other form of the virus had been previously reported in Great Lakes fish species prior to this discovery, a study was

immediately initiated to sequence the virus in an effort to identify its possible source. Nucleotide sequence analysis demonstrated that this was an entirely new strain of freshwater VHSV, most closely related to the North American genotype IV of marine VHSV and clearly distinct from the three known European genotypes (Figure 1-6.). This implies that, the Great Lakes strain of freshwater VHS, now referred to as the North American IVb virus, evolved from the North American marine coastal virus, now referred to as IVa, through adaptation to its new freshwater hosts of the Great Lakes (Ammayappan & Vakharia, 2009; Einer-Jensen *et al.*, 2004; Elsayed *et al.*, 2006).

Following this discovery, in 2005 VHSV was associated with a large fish kill consisting of freshwater drum (*Aplodinotus grunniens),* muskellunge, round goby (*Neogobius melanostomas*), striped bass (*Morone saxatilis*), three-spined stickleback (*Gasterosteus aculeatus*), mud minnows (*Fundulus heteroclitus*), and brown trout (*Salmo trutta*) in Lake Ontario, as well as muskellunge in Lake St. Clair. In 2006, another large die-off of round goby and muskellunge in the St. Lawrence River, followed by significant fish kills of muskellunge, northern pike (*Esox lucius*), gizzard shad (*Dorosoma cepedianum*), smallmouth bass (*Micropterus dolomieu*), walleye (*Sander vitreus*), and yellow perch (*Perca flavescens*) throughout Lake St. Clair, Lake Erie and Lake Ontario (USDA, 2006), were all found to be associated with VHSV. In 2008, the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, listed 28 different fish species native to the Great Lakes as VHS-susceptible (Figure 1-2.); however this list is expected to grow as more potentially susceptible species are investigated. Since its discovery, VHS has been isolated in all five of the Laurentian

Great Lakes of North America, Lake Winnebago, WI, and several smaller inland lakes located in Michigan, Ohio, and New York (Figure 1-7.).

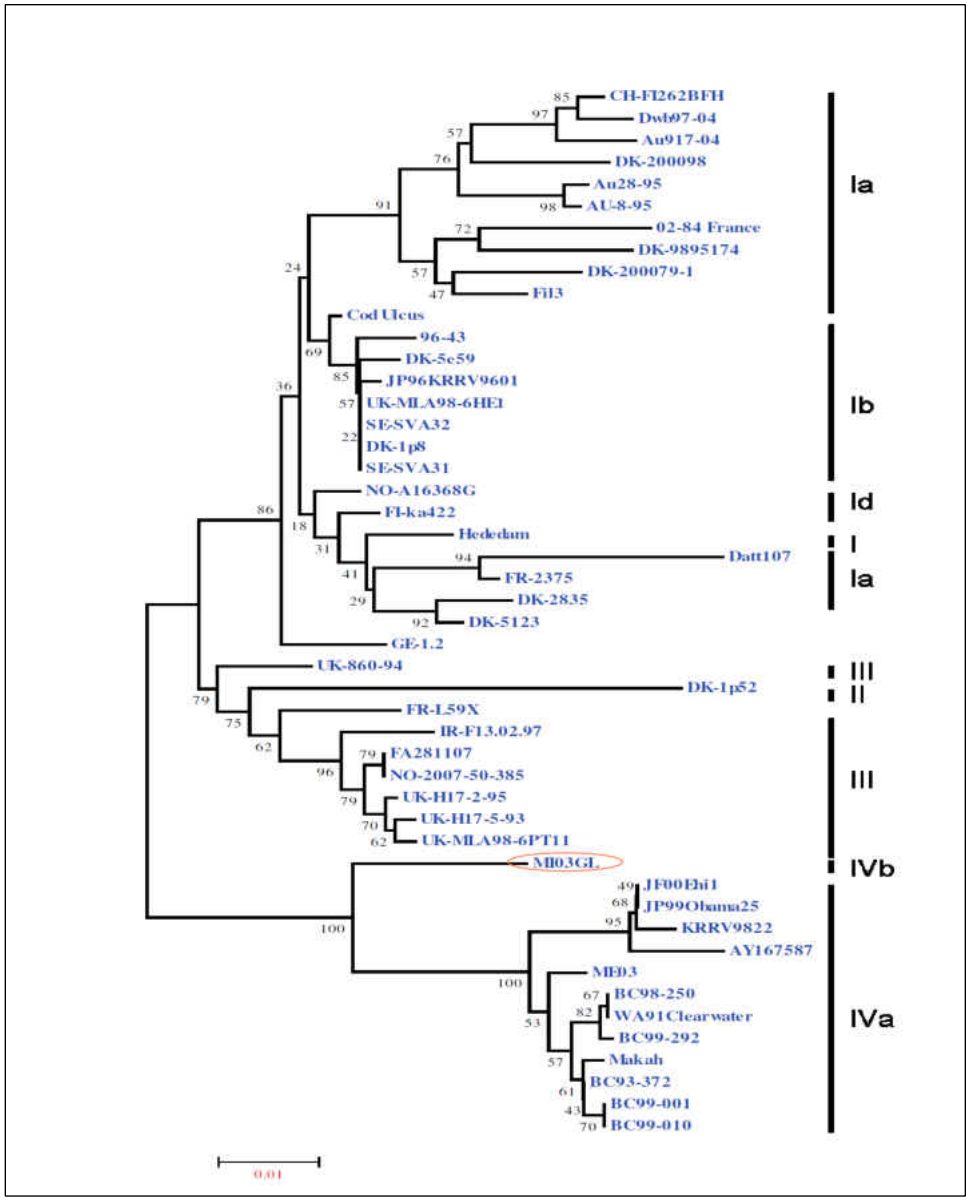

Figure 1-6. Phylogenetic relationship of the glycoprotein sequences across 48 different VHSV isolates. The Great Lakes strain M103GL (circled) forms its own unique sublineage IVb, whereas all other North American isolates collected thus far fall under sublineage IVa (*from Ammayappan and Vakharia, (2009)*).

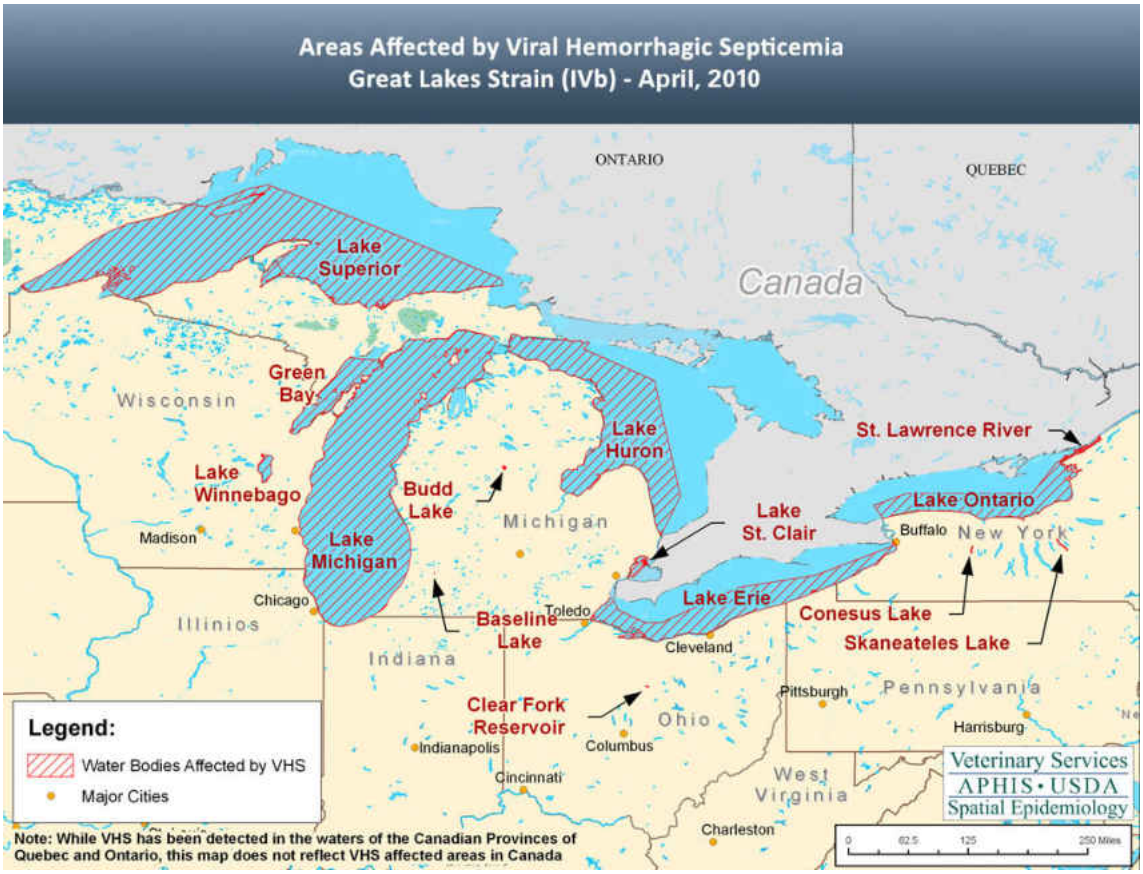

Figure 1-7. VHSV-IVb has been confirmed in each of the Great Lakes as well as several smaller lakes in the states of Michigan, Ohio, and New York (*USDA-APHIS, 2010*).

The VHSV-IVb strain is noteworthy to the field of virology, as well as the wild fisheries and aquaculture industry, in that it represents a geographic invasion of an extensive freshwater ecosystem and it is the first time the virus has been known to cause large-scale epidemics in wild freshwater fish populations. Several distinct features set this virus apart from its European counterparts and its marine ancestors, the most striking being the broad new host range of freshwater fish species this virus has acquired in a very short period of time. Genetic typing of the virus isolated from fish kills throughout the Great Lakes currently indicate very low genetic diversity, implying that this is a very early point in the emergence of the virus. In contrast, the much more familiar and diverse European strain of freshwater VHSV continues to exclusively target only freshwater salmonids. Gradual, species-specific adaptation to a host environment based on successful replication and disbursement is the pathogenic behavior exhibited most commonly by viruses. However, it is very unusual to witness the evolution of this process over such a short period of time (Kurath, 2010). Therefore, studying the mechanisms of how such a virus can infect its host successfully, will not only provide valuable insight into the teleost immune system, but possibly offer insight regarding how viruses may quickly evolve to infect multiple host species with tremendous diversity and speed.

The Yellow Perch as a Research Model

The discovery of a novel strain of viral hemorrhagic septicemia virus (VHSV) in the Laurentian Great Lakes and the associated fish kills have raised much concern over the ability of this virus to infect a large range of freshwater species with devastating

consequences. The yellow perch (*Perca flavescens)* has been a large part of many of the die-offs associated with VHSV in the Great Lakes, and thus, has been listed as a highly susceptible species to the disease (Kane-Sutton *et al.*, 2010). The natural distribution of the yellow perch ranges throughout much of North America from the Northern provinces of Canada, into the eastern and southern United States. In addition, human introductions and dispersal has resulted in the yellow perch being introduced into several western regions of the United States (Figure 1-8.) (Brown *et al.*, 2009).

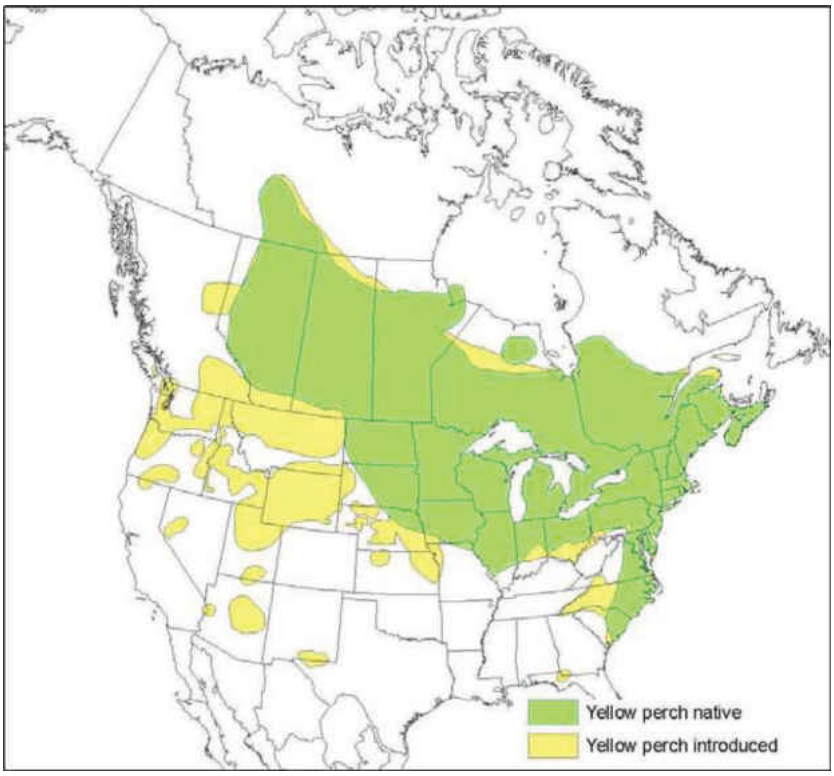

Figure 1-8. Native and introduced distribution of yellow perch in North America from (Bradford *et al.*, 2008). While the virus has been contained primarily to the Great Lakes basin, the virus susceptibility and wide range of the yellow perch, however, may allow for the virus to spread undetected into new regions.

The yellow perch is also known for its extremely high commercial and aquaculture value. Commercial fishing is predominantly successful in the Great Lakes, particularly in Lake Erie and in Green Bay, WI (directly connected to Lake Michigan) (Brown *et al.*, 2009). Yellow perch harvests from the Great Lakes peaked at 14,900 metric tons/year in the 1950s, but have continued to decline to 5,000 – 8,200 metric tons/year in the 1980s and 1990s (Malison, 2003) due to predation, pollution, invasive organisms, and over-fishing. A high consumer demand, combined with an increase on commercial fisheries closures and restrictions, has thus fueled a recent increase in yellow perch aquaculture. The challenges and risks associated with preventing potential VHS outbreaks in fisheries and aquaculture systems outside of the Great Lakes have been high; however they can be expected to rise to an even greater extent if the virus is successful at spreading across the region. In addition, while the virus to date has seemingly been contained to the Great Lakes region, the high susceptibility of the yellow perch to the Great Lakes virus, combined with its wide range of habitats and distribution as a prey species, may allow this fish species to potentially act as a covert carrier, facilitating the spread of VHS into the inland rivers and lakes throughout North America. Thus, the yellow perch will serve as an ideal research model to aid in understanding how freshwater fish are recognizing this pathogen, as well as the mechanisms this virus can utilize to replicate and spread.

Summary/Main Objectives

Using the yellow perch as a research model in a controlled laboratory setting, the basic objectives outlined in this study will aid in the preliminary development of a disease resistant stock, new methods of non-lethal sampling and enhanced viral detection, and contribute towards a greater understanding of the mechanisms of viral infection as well as the fish's primary antiviral immune defenses. The first objective will compare differences in resistance or susceptibility to the Great Lakes strain of VHS within three different broodstock populations of fish with varying geographic and genetic origins. The second objective focuses specifically on elucidating the early immune response necessary for an effective defense against invading viral pathogens. Improving our understanding of the innate mechanism used by the fish to combat invading pathogens will not only result in improved aquaculture practices, potential vaccine development, and treatment strategies leading to more sustainable healthy fish populations, but contribute towards our greater understanding of the evolution of various immune system mechanisms and responses in lower vertebrates. The final objective will examine how and where the virus replicates and spreads within different tissues throughout the fish after exposure, ranging from the fins, gills, blood, liver, kidney, spleen, brain, and blood, and how this distribution changes over time. This knowledge will not only aid in our understanding of the viral mechanisms of infection, but may challenge the current methodologies in viral detection and surveillance as previously unknown potential reservoirs of the virus are explored.

Main objectives are proposed in this study:

I. **Assess the susceptibility of different yellow perch broodstocks of genetic variability and geographic origin to the Great Lakes strain of VHS IVb to evaluate the potential for a disease resistance stock. (Chapter 2)**

II. **Conduct an analysis of immunologically relevant tissue in yellow perch infected with VHSV describing innate immune response pathways characteristic to early, or pre-disease, stages of infection. (Chapter 3)**

III. **Determine how the relative distribution of virus within key internal organs of infected fish develops and changes over the course of infection, as well as identify potential organs where virus may be found in asymptomatic carriers. (Chapter 4)**

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Chapter 2

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Comparative susceptibility among three stocks of yellow perch (*Perca flavescens*) to viral haemorrhagic septicaemia virus (VHSV) strain IVb from the Great Lakes

Olson, W[1]., Emmenegger, E[2]., Glenn, J[2]., Winton,J[2]., & Goetz, F.[1,3]

[1]University of WI-Milwaukee, [2]U.S. Geological Survey (USGS), Seattle, WA, [3]National Oceanic and Atmospheric Administration (NOAA), Port Orchard, WA

"The Great Lakes strain of viral haemorrhagic septicaemia virus IVb (VHSV-IVb) is capable of infecting a wide number of naive species and has been associated with large fish kills in the Midwestern United States since its discovery in 2005. The yellow perch (Perca flavescens),a freshwater species commonly found throughout inland waters of the United States and prized for its high value in sport and commercial fisheries, is a species documented in several fish kills affiliated with VHS. In the present study, differences in survival after infection with VHSV-IVb were observed among juvenile fish from three yellow perch broodstocks that were originally derived from distinct wild populations, suggesting innate differences in susceptibility due to genetic variance. While all three stocks were susceptible upon water-born exposure to VHS virus infection, fish derived from the Midwest (Lake Winnebago, WI) showed significantly lower cumulative percent survival compared with two perch stocks derived from the East Coast (Perquimans River, NC and Choptank River, MD) of the United States. However, despite differences in apparent susceptibility, clinical signs did not vary between stocks and included moderate to severe haemorrhages at the pelvic and pectoral fin bases and exophthalmia. After the 28-day challenge was complete, VHS virus was analyzed in subsets of whole fish that had either survived or succumbed to the infection using both plaque assay and quantitative PCR methodologies. A direct correlation was identified between the two methods, suggesting the potential for both methods to be used to detect virus in a research setting."

Introduction

Due to its broad host range and acute nature, viral hemorrhagic septicemia (VHS) is one of the most problematic viral fish diseases to plague the aquaculture industry, and remains one of the most pathogenic viral diseases of finfish worldwide (Kim & Faisal, 2011; Wolf, 1988). In areas of Europe, VHS infections in farmed rainbow trout, *Oncorhynchus mykiss*, have resulted in extremely high mortalities, causing extensive economic losses (Einer-Jensen *et al.*, 2004; Olesen, 1998; Skall *et al.*, 2005; Smail, 1999; Wolf, 1988). Viral hemorrhagic septicemia virus (VHSV) isolates of marine origin have been identified in North America, Europe, and Asia where they cause disease in both farmed and wild species (Kim *et al.*, 2003; King *et al.*, 2001; Meyers & Winton, 1995; Mortensen *et al.*, 1999; Nishizawa *et al.*, 2002). In 2005, a new freshwater strain of VHSV was isolated from tissue of an adult muskellunge (*Esox masquinongy*), collected in 2003 from Lake St. Clair, Michigan, part of the Great Lakes of North America (Elsayed *et al.*, 2006). This novel sublineage, named Great Lakes VHSV-IVb, was subsequently associated with large fish kills that included freshwater drum (*Aplodinotus grunniens*) (Lumsden *et al.*, 2007), muskellunge, round goby (*Neogobius melanostomas*) (Groocock *et al.*, 2007), gizzard shad (*Dorosoma cepedianum*), and yellow perch (*Perca flavescens*), in Lake St. Clair, Lake Erie, and Lake Ontario (Kim & Faisal, 2011). To date, the Great Lakes strain of VHSV-IVb has been isolated from at least 31 species of fish in the Great Lakes, several smaller inland lakes and the upper portion of the St. Lawrence River (Thompson *et al.*, 2011a). Because of the severe pathogenicity of the

disease and the ever-increasing number of host-environments in which the virus is found, the study of VHS remains a top priority worldwide.

The yellow perch is well known throughout North America for its high value to aquaculture, commercial and sport fisheries. This species has also been included in several of the fish kills that have occurred throughout the region in association with the Great Lakes VHS-IVb virus, and was the primary species affected in a large die-off in central Lake Erie (Kane-Sutton *et al.*, 2010). While yellow perch susceptibility to Great Lakes VHSV-IVb has been tested relative to other fish species (Kim & Faisal, 2010b), variations in susceptibility within different perch populations have not been addressed. Because yellow perch primarily live in lake ecosystems, gene flow between these inland ecosystems is limited and, thus, there are great genetic differences between populations. Therefore, populations of yellow perch that originate from different genetic backgrounds and geographic regions may have developed fundamental differences in their susceptibility or resistance to the virus.

In the present study, three yellow perch broodstocks derived from wild populations having different geographic origins and genetic backgrounds (Grzybowski *et al.*, 2010; Todd & Hatcher, 1993), were challenged with VHSV-IVb to evaluate (measured by % survival) the VHS resistance of each stock. Offspring from the three stocks were propagated under identical conditions with environmental factors such as diet, water temperature, flow rates, and fish densities kept the same between fish stocks. Thus, with all other variables held equal, the genetic origins of the fish would be the principle component involved in disease susceptibility or resistance.

Materials and Methods

Yellow perch stocks

The yellow perch populations used in the present study are part of a broodstock development program for aquaculture (Rosauer *et al.*, 2011) at the University of Wisconsin-Milwaukee (UWM) School of Freshwater Science (SFS). The three broodstocks chosen for the study were originally derived in 2006 from the gametes of adult perch collected from three distinct locations: the Perquimans River of the Albemarle Sound (NC), the Choptank River of the Chesapeake Bay (MD), and Lake Winnebago (WI), a watershed connected to Lake Michigan by the Fox River. Thus, all three stocks originated from three separate drainage basins and genetic analyses confirmed that each represented a separate population (Grzybowski *et al.*, 2010; Todd & Hatcher, 1993). Since their origin, two subsequent generations of these broodstocks have been produced from crosses within the stocks. Juvenile fish for the current study were produced from the second generation of crosses and were reared under identical environmental conditions at the UWM SFS Aquaculture Research Facility according to the guidelines of the Animal Care and Use Committee of the UWM as previously described (Rosauer *et al.*, 2011).

Virus challenge

When the juvenile perch were 1-2g, they were transferred to the USGS Western Fisheries Research Center in Seattle, WA and held for 6-8 weeks prior to virus challenge. Choptank River perch were used in a preliminary experiment to determine the dose of

VHSV for subsequent challenges (data not shown) and optimal dose was determined such that the full effect of the virus could be observed within 28 days post-infection. For the main challenge experiment, all three stocks of fish were exposed to VHSV-IVb by static water-borne immersion with aeration at the predetermined viral titre of 5×10^5 PFU mL^{-1} for 2 hrs at 30 fish per tank (30 L) in triplicate tanks (n=90 fish) per treatment group. VHSV-IVb Great Lakes (isolate Muskie MI03) virus strain used in the challenge was propagated using the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan *et al.*, 1983a) and initial virus titre was determined by plaque assay (Batts & Winton, 2007). Initial water temperature was 10°C and raised 0.5°C daily until it reached 12°C for the duration of the 28 day experiment. Identical triplicate tanks of each fish stock serving as negative controls, were exposed to the same volume of tris-buffered Eagle's minimum essential medium (MEM-10) used to formulate the virus treatments at the same temperature regime. All control and virus challenge treatments were observed for 28 days and fish were fed every other day during the challenge. Dead fish were recorded and removed daily from each of the tanks. All survivors were euthanized after 28 days with an overdose of $NaHCO_3$ buffered tricaine methanesulfonate (MS-222; Western Chemical Inc.). All fish in the study were immediately frozen and stored at -80°C until virus analysis could be performed.

Virus analysis by plaque assay

Virus titre was determined in a representative subset of 7 fish that had succumbed to infection and 15 surviving fish from each yellow perch stock (total n= 66 fish, 22 fish

stock^{-1}). Whole fish were diluted 1:8 (weight:volume) in MEM-0 and homogenized with a Stomacher-80 (Seward Laboratory Systems, Biomaster). Sample homogenates were transferred into 15 mL conical tubes and spun at 4°C for 10 minutes at 1000 x g. One milliliter (mL) of supernatant from each sample was removed to quantify infectious virus by plaque assay, and another 1 mL stored at -80°C to assess virus genome concentrations by qRT-PCR. For plaque assay, serial 10-fold dilutions (1:40 to 1:4 x 10^6) of tissue homogenate supernatants were plated in duplicate onto polyethylene glycol (7% PEG) treated EPC cell monolayers in 24-well plates. Cell-cultures were overlaid with methylcellulose and incubated at 15°C for 7 days before fixation with crystal violet-formalin solution and plaque enumeration as previously described (Batts & Winton, 1989a). Fish tissue viral titres of VHSV are reported as plaque forming units per gram (PFU g^{-1}).

Virus analysis by qRT-PCR

The qRT-PCR assay for the detection of VHS virus nucleic acids was developed and described by (Garver *et al.*, 2011). Thawed 210 µL samples of homogenate supernatant were used for total RNA extraction using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Complimentary DNA was synthesized from 1 µg RNA by RT-PCR using the Applied Biosystems (ABI) High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. VHS virus primers (*Forward primer* 5'-ATG AGG CAG GTG TCG GAG G-3', *Reverse primer* 5'-TGT AGT AGG ACT CTC CCA GCA TCC-3', Invitrogen) and MGB probe (5'-6 FAM-TAC GCC ATC ATG ATG

ATG -MGBNFQ-3', ABI) were utilized for the qPCR assay as described in Garver *et al.* (2011). The RNA standard used for qPCR virus quantification, generated by a plasmid vector containing the N-gene of the virus, was generously provided by Dr. Kyle Garver (Pacific Biological Station, Nanaimo, BC). RNA transcripts were quantified using a NanoDrop ND-1000 and copy number was determined such that one µg of N RNA transcript contained 1.32×10^{12} gene copies. A standard curve based on a dilution series of N-transcripts ranging from 1×10^{8} to 1×10^{1} viral gene copies was included on each qPCR plate and used to calculate virus loads based on the resulting CT values. The VHSV qPCR assay was performed using a LightCycler® 480 (Roche). Each qPCR assay was conducted in a 25 µL reaction volume containing 1 µL of cDNA, 600 nM of each primer, 200 nM probe, and 12.5 µL 2x TaqMan Universal PCR Master mix (Applied Biosystems). The thermal cycle profile was: 2 minutes at 50ºC, 10 minutes at 95ºC, 40 cycles of 15 seconds at 95ºC, and 1 minute at 60°C (Garver *et al.*, 2011).

Statistical analysis

Percent survival was calculated as the cumulative number of surviving fish in the pooled triplicate tanks for each stock divided by the total number of fish for each stock originally exposed to the virus x 100. All statistical analyses of % survival, multivariate comparisons (ANOVA), and linear regression analysis on plaque assays and qRT-PCR values were completed using Sigma Plot® software (Systat Inc.).

Results

Clinical signs of VHSV infection

Clinical signs of VHS infection were similar among all three stocks of yellow perch and included internal and external hemorrhages consistent with signs in other fish species infected with VHSV. In addition to petechial hemorrhages observed throughout the body, signs of hemorrhage were particularly visible on the brain and optical lobes when the fish were viewed dorsally (Fig. 2-1A). Hemorrhage was also evident in the eye socket in association with exophthalmia (Fig. 2-1B), and at the base of the pectoral and pelvic fins (Fig. 2-1B & 2-1C). Internally, abdominal swelling as a result of severe ascites, and petechial hemorrhages on the swim bladder and liver were commonly observed.

Survival among stocks

The cumulative number of survivors for each yellow perch stock was used to calculate % survival (Fig. 2-2). In the VHSV exposed fish, yellow perch stocks from the East Coast (Choptank River and the Perquimans River) were nearly identical in susceptibility, with relatively high survival of 74% (67/90) and 73% (66/90), respectively. Alternatively, the Midwest (Lake Winnebago) perch exhibited relatively low survival of 20% (18/90). An initial Kaplan-Meier Log-Rank Survival Analysis as well as a pair-wise multiple comparison test using the Holm-Sidak method demonstrated a significant ($P = <0.001$) difference in % survival between Lake Winnebago and Choptank River fish, and between Lake Winnebago and Perquimans River fish, but not between Choptank River and Perquimans River fish ($P= 0.735$). This indicates that the yellow perch stocks originating

from Lake Winnebago in Wisconsin were significantly more susceptible to Great Lakes VHSV-IVb than perch originating from the two East Coast populations (Choptank River, MD, Perquimans River, NC). In equivalent tanks of negative control fish, 99% (89/90) of the Lake Winnebago and Choptank River fish and 94% (85/90) of the Perquimans River fish survived the 28 day experiment.

When virus load was determined in subsamples of fish from each stock, the Lake Winnebago fish that survived the challenge with VHSV had significantly higher virus titres and viral N-gene copy numbers (ANOVA, $P<0.05$) then Choptank or Perquimans River fish (Table 2-1). The prevalence of virus in the surviving VHSV exposed fish differed as well. Only 4 of 15 Perquimans River fish and 8 of 15 Choptank River fish tested positive for VHSV at the end of the 28-day challenge period, while 13 of 15 surviving Lake Winnebago fish had detectable virus titres verifiable by both plaque assay and qRT-PCR. All VHSV exposed fish that died during the challenge had detectable virus (100% prevalence) in all three stocks by both plaque assay and qRT-PCR. Statistical analysis using ANOVA indicated no significant differences in detectable virus loads in these fish. None of the negative control fish that died during the 28-day challenge tested positive for VHS virus.

Differences between the three stocks of yellow perch were also apparent in the kinetics of VHSV infection (Fig. 2-3). Fish from Lake Winnebago had an acute high mortality response on day 9, followed by an even greater mortality response that peaked on day 14, resulting in a bimodal disease progression pattern. The Choptank River perch had a relatively delayed bimodal response to infection with the first peak in mortalities

occurring on day 13 and the second mortality peak on day 21. The Perquimans River fish, alternatively, were the first stock of fish to succumb to VHSV infection with a mortality peak on day 4; however, a slow progression of the disease resulted in a wide-spread temporal distribution of mortality.

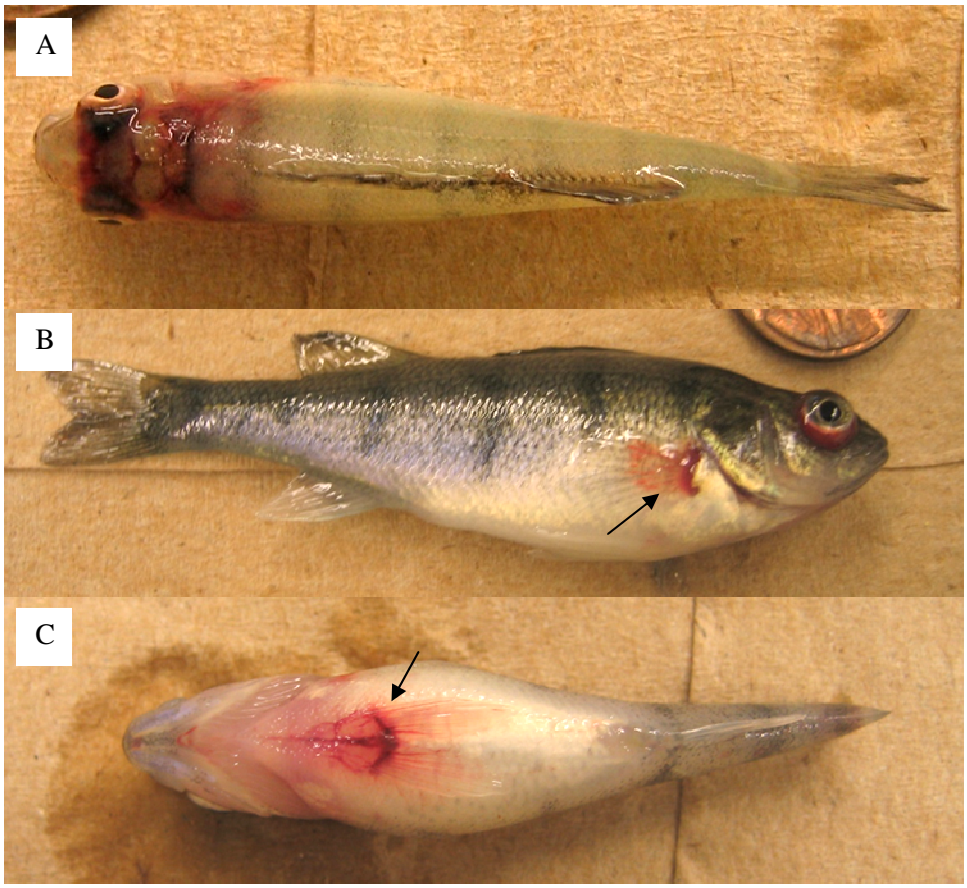

Figure 2-1. Clinical signs affiliated with VHSV-IVb infection in three different juvenile yellow perch after immersion exposure to the Great Lakes VHSV strain at a dose of $1x10^5$ PFU mL^{-1}. Hemorrhages were visible around the brain and optical lobes when the fish were viewed dorsally (A), the eye socket (B), and at the base of the pectoral and pelvic fins (B & C).

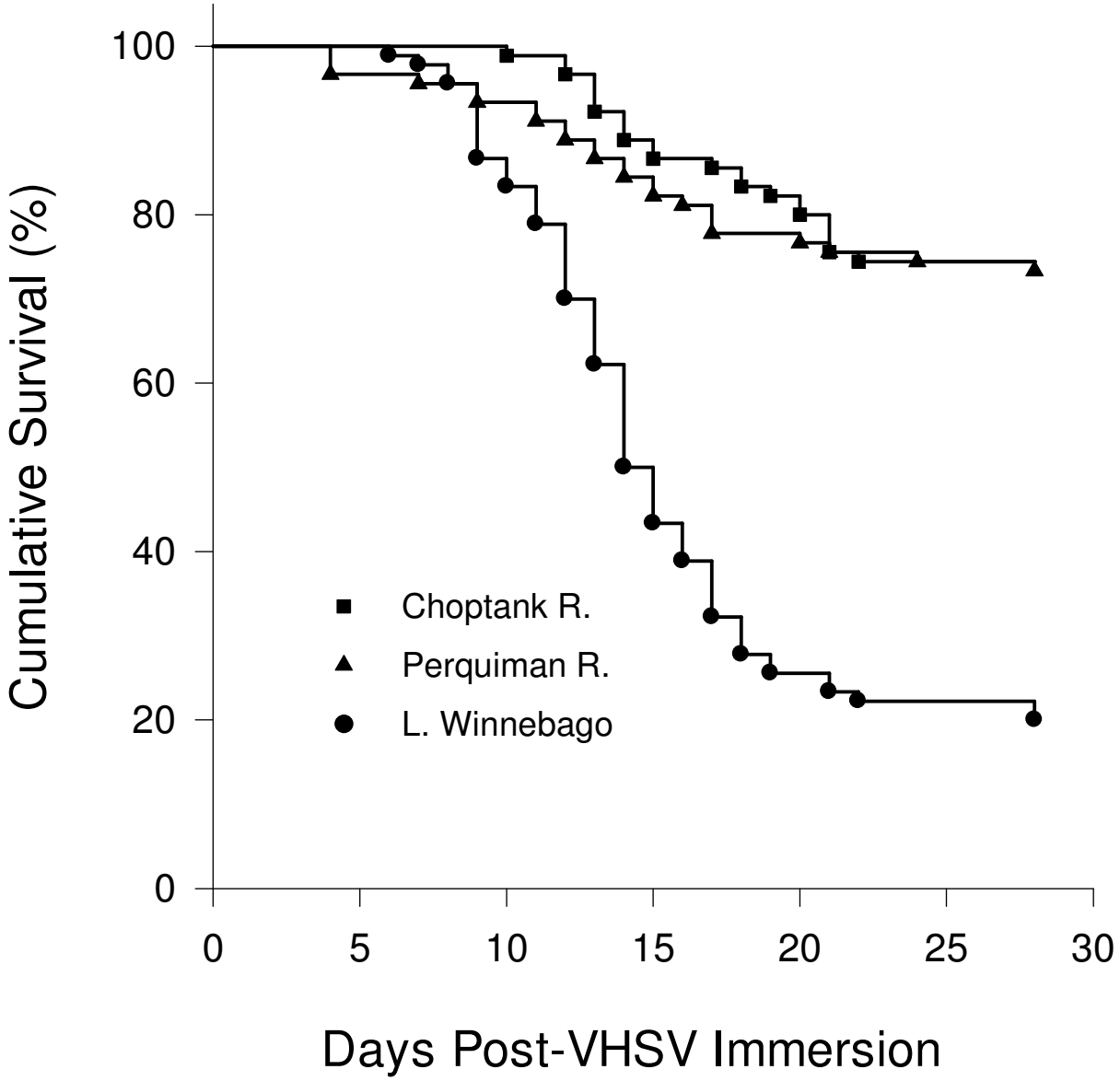

Figure 2-2. The cumulative % survival of Lake Winnebago, Choptank River, and Perquimans River perch after water-born exposure to VHSV-IVb.

	Perquimans R.	Choptank R.	L. Winnebago
Survivors (n = 15 stock^{-1})			
Prevalence of VHSV	(4/15) 27%	(8/15) 53%	(13/15) 87%
PA titer (PFU g^{-1})	64,784	26,483	1,367,489
qRT-PCR (copy #)	1,860	1,473	86,231
Mortalities (n = 7 stock^{-1})			
Prevalence of VHSV	(7/7) 100%	(7/7) 100%	(7/7) 100%
PA titer (PFU g^{-1})	3,040,800	10,977,605	14,380,585
qRT-PCR (copy #)	463,877	1,110,308	1,809,404

Table 2-1: Prevalence of VHSV (#VHSV positives over total # of fish examined) and comparison of the geometric mean virus load by plaque assay (PA) and qRT-PCR.

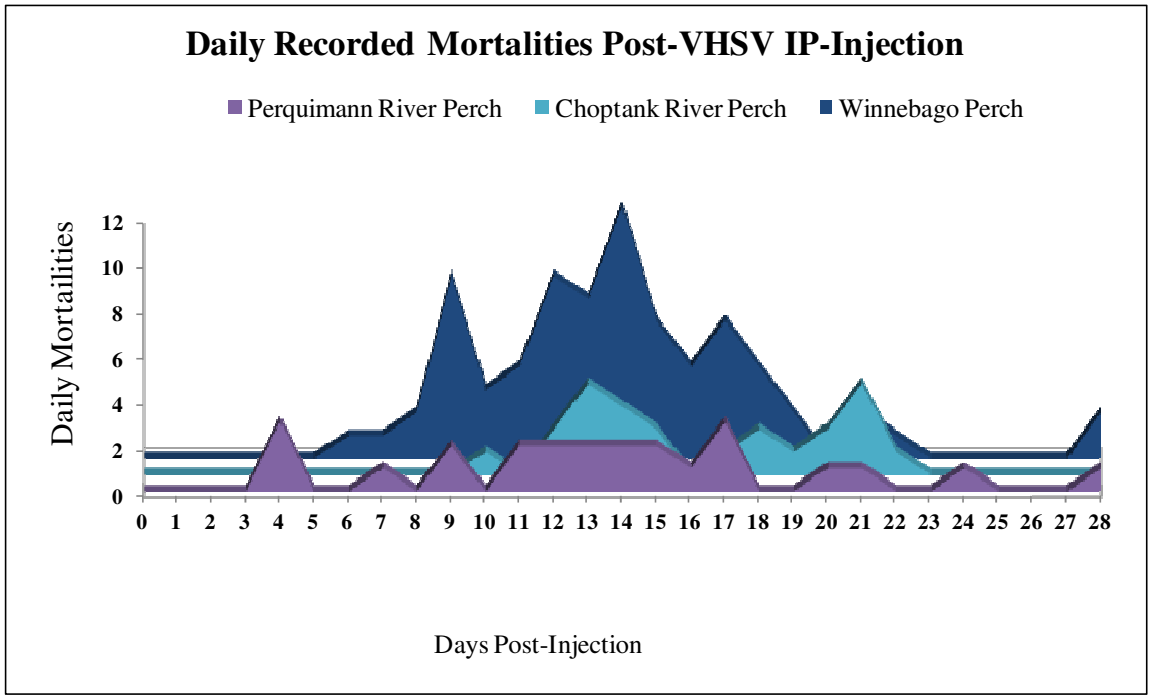

Figure 2-3. Kinetics of VHSV-IVb infection between Lake Winnebago, Choptank River, and Perquimans River perch stocks.

Comparison between plaque assay and qRT-PCR

All whole fish (n=72) homogenates were analyzed for VHSV using both plaque assay (PFU g^{-1}) and qPCR (viral copy # μg^{-1} RNA). When results were directly compared after a logarithmic transformation, a significant positive correlation between virus load as determined by qRT-PCR and the corresponding number of plaques in paired samples was found with a Pearson correlation coefficient of $R^2 = 0.89$ ($P < 0.001$) (Fig. 2-4). The limit of detection for the plaque assay was 500 PFU g^{-1}, while the limit of detection for the qRT-PCR assay was 10 viral copies; therefore, four of the samples contained a low level of virus detectable by qPCR, but were negative by plaque assay.

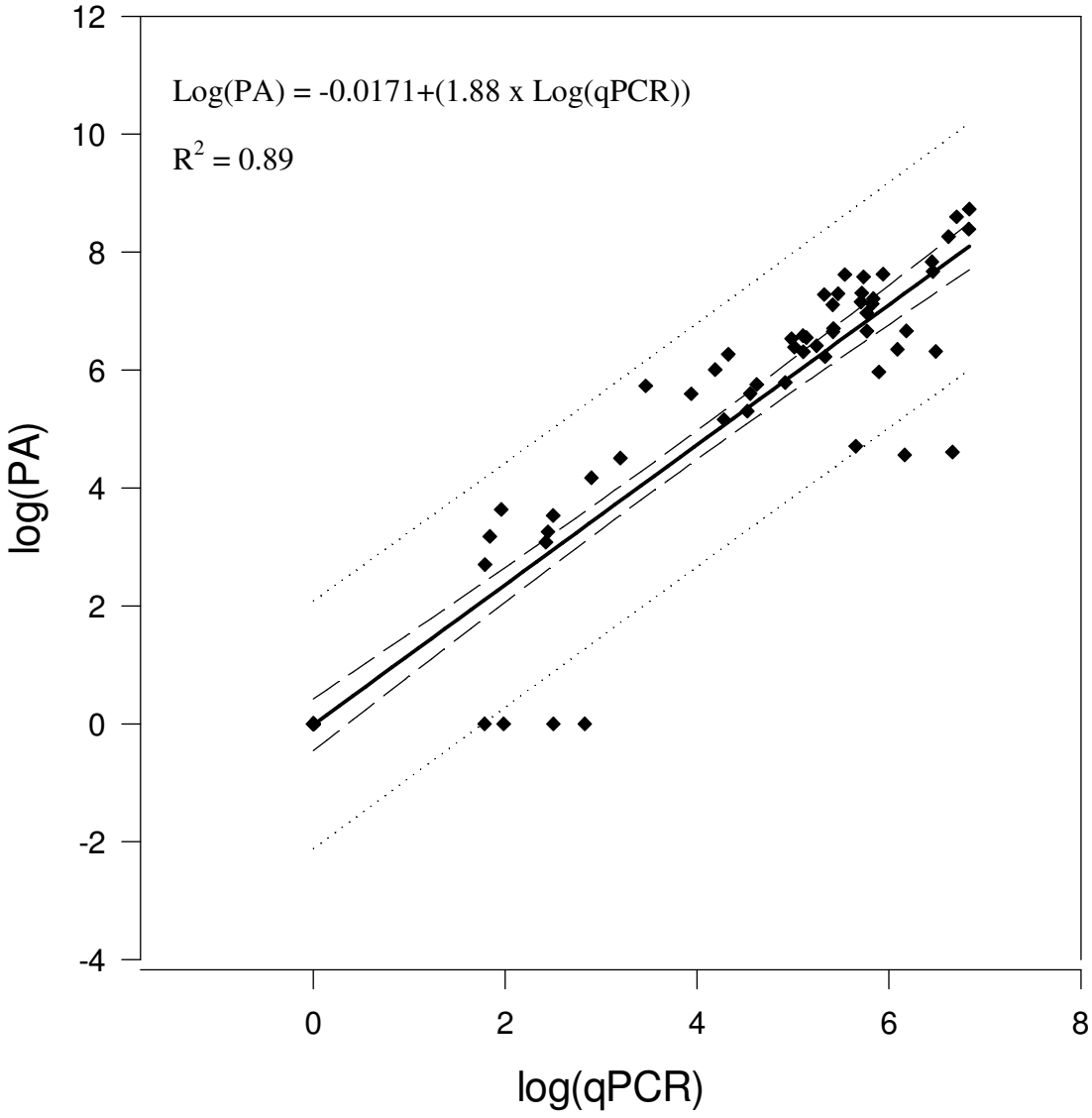

Figure 2-4. Linear regression between plaque assay (PA-PFU g^{-1}) and qRT-PCR (qPCR-viral copy #) on log transformed data ($R^2 = 0.89$). Thin dotted and dashed lines represent the 95% confidence intervals for the population and regression, respectively.

Discussion

This study identified major differences in susceptibility to the Great Lakes VHSV-IVb strain in yellow perch populations originating from different genetic and geographic origins. Because the environmental conditions for rearing and challenging the fish were identical for all three stocks, genetic differences between the populations from which the stocks were derived most likely contributed to the variability in virus susceptibility. In the present study, the major differences in susceptibility were observed between fish from the Lake Winnebago stock versus the two East Coast stocks from the Perquimans and Choptank Rivers. While significant genetic differences were previously observed between all three of the perch populations from which these broodstocks were derived, a greater difference was observed between East Coast and Midwest perch populations than between East Coast populations (Gryzbowski *et al.,* 2010) supporting the results observed here. In aquaculture, the identification of disease resistant stocks of fish is a vital first step towards the development of healthier broodstocks. Because many disease resistance traits have been shown to be heritable, there is large potential to continue this form of genetic improvement within current fish broodstocks throughout the aquaculture industry (Norris *et al.*, 2008; Ødegård *et al.*, 2011; Ødegård *et al.*, 2007; Wetten *et al.*, 2007).

A previous study examining the three yellow perch stocks used in this study also found significant differences in other phenotypes, such as growth (Rosauer *et al.*, 2011). The Lake Winnebago population had a significantly lower growth rate overall than both Choptank River and Perquimans River fish. It has been proposed that, because growth

and an active immune response are both resource-demanding processes, a trade-off between fast growth and an effective immune response would most likely exist (Johansen *et al.*, 2006). In contrast, more recent studies evaluating the correlation between body weight and disease resistance have demonstrated no such adverse relationship (Silverstein *et al.*, 2009). The results of these previous studies, as well as the results reported here, imply that simultaneous genetic improvement for both growth and disease resistance can be accomplished. Rosauer *et al.* (2011) also documented differences in apparent stress level between Lake Winnebago, Choptank and Perquimans River stocks, which may be linked to growth rate and the potential to resist infection. In the study by Rosauer *et al.* (2011), it was reported that Lake Winnebago fish seemed more easily stressed by human interactions (e.g., tank cleaning, adjusting feeders, and peering into or walking by the tank), than Choptank or Perquimans River fish. This increased level of stress may lead to lower growth rates and reduced resistance to infection.

An additional factor that may be affecting susceptibility is that fish from the Great Lakes region may have never been exposed to VHS virus or any similar rhabdovirus in the past, making them particularly vulnerable. Alternatively, the perch stocks from the Choptank River and Perquimans River, both river estuary environments on the east coast of the U.S., may have encountered strains of VHSV (Gagne *et al.*, 2007) or other marine viruses and, thus, natural selection would have allowed for the development of more resistant stocks. Similar pathogen-driven selection mechanisms have been proposed for disease resistance among different populations of Chinook salmon (*Oncorhynchus tshawytscha*), which were comparable in genetic diversity, but separated by a

geographical land barrier (Purcell *et al.*, 2008). Alternatively, marine populations of Pacific herring (*Clupea pallasii*) with distinct genetic differences and similarly high diversity were found equally susceptible to the North American marine strain of VHSV-IVa (Hershberger *et al.*, 2010) possibly due to similar selection pressures of a shared environment.

The clinical signs observed in the infected perch were similar to signs of VHS virus in other species, including septicemic hemorrhages throughout the body and at the base of the fins. The transparency of dead juvenile perch allowed us to observe severe internal haemorrhages around the lobes of the brain, suggesting that infected fish may suffer permanent damage to the central nervous system and that the brain may be a potential viral reservoir in surviving fish.

The plaque assay currently remains the 'gold standard' for the verification of infectious virus and is the best method for the detection of infectious virus particles. The plaque assay, however, is also relatively labor intensive, time consuming (requires a minimum of 5 days to complete), and the resulting titre can be highly susceptible to the presence of proteolytic enzymes, the quality and quantity of the cell monolayer used in the assay, changes in temperature, and differences in incubation time. Alternatively, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has become an increasingly common method for the diagnosis of a wide variety of viruses due to its speed (a large number of samples can be analyzed in <1 day) and sensitivity (detection of less than 10 copies of RNA target genes can be achieved). It has become particularly useful in the medical diagnosis of other RNA viruses, including Ebola and Marburg viruses, Lassa

virus, Crimean-Congo haemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus (Drosten *et al.*, 2002). Unlike the plaque assay, this method can be used to detect all viral RNA, not just infectious particles. This is particularly useful in the detection of a negative-strand RNA rhabdovirus such as VHS virus, where the mutation rate and thus the presence of non-infectious particles and RNA is typically much higher than that of other viruses (Flint *et al.*, 2004). However, in most cases, the detection of viral RNA by qRT-PCR is only an indicator of infection, and provides no information regarding the infectious capabilities of the virions detected. Only a few studies have worked towards identifying the relationship between qRT-PCR and plaque assay analysis of the VHS virus in fish (Chico *et al.*, 2006; Garver *et al.*, 2011; Hope *et al.*, 2010). Here, we directly compared VHS virus concentrations in whole fish by plaque assay using cell culture and qRT-PCR using primer/probe sets designed by Garver *et al.* (2011) to test whether a correlation between the two methodologies could be observed.

The strong positive correlation between the VHSV qRT-PCR and plaque assays results suggests that the viral copy number as determined by the qRT-PCR assay of Garver *et al.* (2011) is directly correlated to levels of replicating virus and can be used together in virus screening practices. Although not true for all viruses, a similar correlation between qRT-PCR (viral copy #) and plaque assay (PFU $g^{-}1$) was also identified for the detection of the RNA virus responsible for yellow fever (Bae *et al.*, 2003), and qRT-PCR is currently used to estimate the titre of infectious measles, mumps, and rubella in live virus vaccines (Schalk *et al.*, 2005; Schalk *et al.*, 2004).

In this present study, four fish had detectable virus by qRT-PCR that was undetected by plaque assay (Figure 2-4). While a negative plaque assay result indicates that any infectious virus present was below the limits of detection, qRT-PCR confirmed the presence of viral RNA in these fish. Because these four samples represented surviving fish from the 28-day viral challenge, this may mean that either the fish have successfully cleared the virus, or that latent virus may still be present but below the detection limit of the plaque assay. In either scenario, the indication of viral RNA may still warrant further evaluation of the fish as a potential viral reservoir of latent infection, or may simply indicate previous exposure to the virus.

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Chapter 3

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Expression Kinetics of Key Genes in the Early Innate Immune Response to Great Lakes Viral Hemorrhagic Septicemia Virus IVb Infection in Yellow Perch *(Perca flavescens)*

Wendy Olson[1], Eveline Emmenegger[2], Jolene Glenn[3], Crystal Simchick[4], Jim Winton[2], & Frederick Goetz[1,4]

[1]University of WI-Milwaukee, [2]U.S. Geological Survey (USGS), Seattle, WA, [3]Seattle Biomedical Research Institute, Seattle, WA, [4]National Oceanic and Atmospheric Administration (NOAA), Port Orchard, WA

" The recently discovered strain of viral hemorrhagic septicemia virus, VHSV-IVb, represents an example of the introduction of an extremely pathogenic rhabdovirus capable of infecting a wide variety of new fish species in a new host-environment. The goal of the present study was to delineate the expression kinetics of key genes in the innate immune response relative to the very early stages of VHSV-IVb infection using the yellow perch (Perca flavescens) as a model. Administration of VHSV-IVb by IP-injection into juvenile yellow perch resulted in 84% cumulative mortality, indicating their high susceptibility to this disease. In fish sampled in the very early stages of infection, a significant up-regulation of Mx gene expression in the liver, as well as IL-1β and SAA activation in the head kidney, spleen, and liver was directly correlated to viral load. The potential down-regulation of Mx in the hematopoietic tissues, head kidney and spleen, may represent a strategy utilized by the virus to increase replication."

Introduction

Due to its extremely has a long history as being one of the most pathogenic viral diseases of finfish worldwide (Skall et al., 2005; Smail, 1999; Wolf, 1988)Outbreaks of the highly pathogenic fish virus, viral hemorrhagic septicemia virus (VHSV) are characterized by systemic hemorrhage throughout the body of the fish and mortality rates that can be as high as 90% in a wide number of host species. The causative agent of VHS is a negative-sense RNA virus (VHSV) belonging to the family *Rhadboviridae*, and classified in the genus *Novirhabdovirus* (ICTV, 2012). Because of its severe impact on trout farms in Western Europe, the virus has been historically considered a European fish pathogen exclusively targeting freshwater salmonids. Thus, the majority of viral-host interaction studies have been conducted on the rainbow trout *(Oncorhynchus mykiss).* More recent phylogenetic analyses of VHSV isolates from a large number of marine fish species in the Atlantic and Pacific Oceans, however, have identified VHSV as essentially a marine virus in origin and categorized VHSV isolates into four genotypes (I, II, III, and IV) (Einer-Jensen et al., 2004; Pierce and Stepien, 2012; Smail, 1999). These findings suggest that the marine to freshwater species host-jump associated with the virus's ability to exclusively infect salmonids in Europe in the 1900s was due to repeated introductions of the virus into salmonid stocks through anadromous migration or, more likely, via the practice of feeding raw, infected marine fish over a long period of time (Kurath and Winton, 2011). Therefore, the emergence of a freshwater VHSV genotype IVb in the Great Lakes basin of North America (Elsayed et al., 2006), emphasizes the ability of this RNA virus to once again adapt from a saltwater to a freshwater environment, however,

the causal mechanisms of this adaptation remain unknown. Unlike the rainbow trout adapted strain of VHSV in Europe (genotype VHSV-Ia), the Great Lakes strain of VHSV-IVb has also demonstrated the ability to infect a very large number of new host species in a relatively short amount of time (Ammayappan & Vakharia, 2009; Kim & Faisal, 2010b; Thompson *et al.*, 2011b). Since its discovery, questions and concerns regarding the ability of the virus to spread rapidly throughout the inland waters of the U.S. and infect naive populations of freshwater fish are being addressed (Kim & Faisal, 2010b; Kim & Faisal, 2011).

The yellow perch (*Perca flavescens*) is a freshwater species of fish that has been a part of large fish kills in the Great Lakes associated with VHSV-IVb, and is listed as a susceptible species to the virus (USDA, 2008). Therefore, the potential impact that VHSV could have on yellow perch commercial and sport fisheries is of great concern to fisheries and aquaculture managers (Kane-Sutton *et al.*, 2010). The yellow perch, however, is also a fish species in which the physiological and immune responses to viral infection have not been well studied and, thus, are poorly understood. Most of the research on the host response to VHSV has been completed on the rainbow trout, an ancestral teleost salmonid species. However, the yellow perch is a more derived teleost, in which differences in the antiviral immune response may have evolved over time. Vaccine development, the enhancement of feed immunostimulants, and the development of hardier, more disease resistant fish stocks, are some of the potential strategies proposed to mitigate the impact that VHSV-IVb will have on fisheries and aquaculture

populations. To accomplish any of these disease prevention strategies, however, requires an understanding of the host immune defenses involved.

Many studies have demonstrated that the most successful vaccines against virus diseases of fish will confer a non-specific anti-viral response at early stages post-vaccination (Lorenzen *et al.*, 2002; McLauchlan *et al.*, 2003). It has also been observed that the successful activation of non-specific innate defense mechanisms induced shortly after infection will often determine the outcome, or survival of the fish, after viral exposure. Thus, to better understand the fish host-virus relationship between the VHS IVb virus and its naive freshwater hosts, the present study focuses exclusively on the early innate immune pathways triggered relative to viral load by VHSV-IVb infection during the very early stages of infection.

While a large repertoire of complex pathways and molecules are involved in the early innate immune response, the expression of Mx, interleukin 1β (IL-1β), and serum amyloid A (SAA), representative cytokines that function in three different components of the fish's innate immune system, were the focus in the current study. The expression of the Mx transcript has become a direct indicator of type I interferon (IFNα and IFNβ) activation, a crucial component of the antiviral innate immune response conserved in many species. Interleukin-1β is a pro-inflammatory cytokine that has been implicated as a key gene in early VHSV infection in rainbow trout (Tafalla *et al.*, 2005). In mammals, SAA is primarily synthesized in the liver and induced by the inflammatory cytokines interleukins 1 and 6 and tumor necrosis factor (Thorn & Whitehead, 2002). In salmonids, SAA induction has been well documented in the liver upon bacterial infections and

stimulation with various pathogen-associated molecular patterns (PAMPs) (Bayne & Gerwick, 2001; Jensen *et al.*, 1997), however, SAA transcription and up-regulation have also been described in rainbow trout macrophages in response to lipopolysaccharide (Goetz *et al.*, 2004), and the skin of parasite-infected carp (Gonzalez *et al.*, 2007). The results of this study increase not only our understanding of the antiviral mechanisms utilized by a more recently evolved fish species to a novel virus, but provides insight into the anti-viral role these cytokines may play in lower vertebrates such as the yellow perch.

Materials & Methods

Yellow Perch, VHSV Propagation, and Intraperitoneal-Injection Challenge

Juvenile yellow perch were obtained from the University of Wisconsin-Milwaukee (UWM) School of Freshwater Science (SFS). All fish used in the study were originally derived from spawnings of yellow perch broodstocks collected as gametes from the Choptank River, MD, and reared under identical environmental conditions at the UWM SFS Aquaculture Research Facility according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the UWM as previously described (Rosauer *et al.*, 2011). The fish used in the current study were offspring from the second generation of fish derived from the Choptank River broodstock. When the juvenile perch were approximately 5-10g, they were transferred to the USGS Western Fisheries Research Center (WFRC), Seattle, WA, where they were reared and used for the subsequent challenge experiment under protocols approved by the IACUC of the WFRC.

All virus exposure experiments were performed in the WFRC aquatic biosafety level-3 containment laboratory.

The Great Lakes VHS virus strain IVb (MI03) was isolated from adult muskellunge, *Esox masquinongy* (Mitchill) from Lake St. Clair in 2003 (Elsayed *et al.*, 2006). The virus was propagated using the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan *et al.*, 1983b) and concentration determined by plaque assay as previously described (Batts & Winton, 2007)

For the challenge experiment, 100 fish (7-10g) were equally divided into four tanks, each containing 25 fish, and exposed to the VHSV by intraperitoneal (IP) -injection at a titer of 1×10^5 PFU/fish. Four additional tanks, each containing 25 fish, served as negative control groups, and were challenged by IP injection with the same volume of tris-buffered Eagle's minimum essential medium (MEM-10-T) used to formulate the virus treatments to simulate the stresses of handling and injection. Water temperature was 9°C at the start of the challenge and then raised 1.0°C daily until it reached 12°C. All fish were fed every other day throughout out the entire challenge experiment. Of the negative control tanks and virus challenged tanks, fish from one tank in each group were monitored daily for a total of 17 days only for mortality events to assess disease progression. From these tanks, dead fish were removed daily and mortality data recorded. Percent survival in each tank was calculated as the cumulative number of surviving fish divided by the total number of fish x 100. In the other three virus and negative control treatment tanks, four fish were lethally sampled from each tank on days 1, 2, 3, 4, 5 and 6 post-virus exposure (n=12 fish/treatment group/day) (Figure 1). Each fish sampled was

euthanized by an overdose (25 mg mL^{-1}) of buffered tricaine methanesulfate (MS-222, Western Chemical Inc.), and the liver, spleen, and head kidney were collected into RNAlater (Qiagen) and kept at -80°C until RNA extraction.

cDNA synthesis

Tissues stored in RNAlater were weighed and transferred into Qiagen Lysis Buffer for homogenization using lysing matrix D beads (MP Biomedicals). Total RNA was isolated from the homogenized tissue using the RNAqueous®-Micro Scale RNA Isolation Kit (Ambion) according to the manufacturer's protocol that included a 20-minute in column DNase treatment. Total RNA quality and concentration were determined using a Bio-Rad Experian Electrophoresis Station. All RNA used in the study had a RNA Quality Indicator (RQI) value >7.0 and a 260/280 index of 2.00 ± 0.20. Complimentary DNA was synthesized using an oligo-dT-primer and ImProm-II Reverse Transcriptase kit (Promega) according to the manufacturer's protocol. The thermal cycle profile was: 25°C for 5 min., 42°C for 1 hr., 70°C for 15 min., and a 4°C hold.

Gene Expression Analysis by qPCR

The expression of Mx, IL-1β, and SAA was quantified by real time quantitative reverse transcription PCR (qRT-PCR) using Power SYBR Green PCR Master Mix (Applied Biosystems), cDNA and gene specific primer sets (Table 1) on either a Stratagene Mx 3000P qPCR system or a LightCycler® 480 (Roche) and analysis was completed with Real-time PCR Miner software (Zhao & Fernald, 2005). Quantification

was performed by calculating the relative mRNA concentration (*Ro*) for each gene per individual sample using the following equation: $Ro = 1/(1+E)^{Ct}$, where *E* is the gene efficiency calculated as the average of all individual sample efficiencies across all reactions for a given gene per qPCR, and *Ct* is the cycle number at threshold (Zhao & Fernald, 2005). Gene expression analysis in all fish (virus-exposed and mocks) was normalized to the control genes, ribosomal S4 (RS4) in the head kidney and liver, and elongation factor-α (EF-1α) in the spleen (Table 1). Elongation factor-α, rather than RS4, was used as a normalizing gene in the spleen since RS4 expression in the spleen was found to change relative to VHSV detection. To determine the level of gene regulation, transcript expression of virus-exposed fish was quantified relative to transcript expression in negative control fish.

Virus load by qRT-PCR

To assess the amount of VHSV in infected fish, we used the qRT-PCR protocol described by Garver et al. (2011). The RNA standard used for qPCR virus quantification generated by a plasmid vector containing the N-gene of the virus was generously provided by Dr. Kyle Garver (Pacific Biological Station, Nanaimo, BC). The VHSV qPCR assay was performed using a Stratagene Mx 3000P qPCR system (Roche). Each qPCR assay was conducted in a 25 µl reaction volume containing 1 µl of cDNA, 600 nM of each primer, 200 nM probe, and 12.5 µl 2x TaqMan Universal PCR Master mix (Applied Biosystems) (Table 3-1). The qPCR cycle profile was: 2 min at 50ºC, 10 min at 95ºC, 40 cycles of 15 sec at 95ºC, and 1 min at 60°C (Garver *et al.*, 2011). A standard

curve based on a dilution series of N-transcripts ranging from 1 x10^8 to 1 x 10^1 viral gene copies was included on each plate and used to calculate virus loads based on the resulting CT value.

Statistics

Statistical relevance was calculated using Sigma Plot® software (Systat Inc.). Statistical analysis of gene expression relative to time of expression and tissue type was completed using Tukey's pairwise multivariate comparisons procedure within a two-way analysis of variance (ANOVA). Correlation results were completed using linear regression analysis on log transformed data.

Gene	5'- Forward-3'	5'- Reverse-3'	Size of PCR product (bp)	Genebank accession #/ Source
IL-1β	ATC TTG AGG TTG TGG AGG CA	GCA CAT TTC CAC TGG CTT GT	176 bp	GO656767.1
Mx	AAG AGG CAG TGG CAT TGT	AAT GAG CGT CAG GTC TGG AA	244 bp	GO654167.1
SAA	ACC ATG CTC GTT TGC CTT CT	TGT GGC GAG CAT ACA GTG AT	209 bp	KC306947
EF-1α	TGA CAA CGT CGG CTT CAA CA	GCA CAT TTC CAC TGG CTT GT	124 bp	GO658156.1
RS4	ACA TCA CCT ACC CTG CTG GAT T	AAT GCC CTT GGT GCC AAT CA	174 bp	FK819484.1
VHSV	ATG AGG CAG GTG TCG GAG G	TGT AGT AGG ACT CTC CCA GCA TCC	82 bp	Garver et al. 2011
MGB probe	5'-6 FAM-TAC GCC ATC ATG ATG - MGBNFQ-3'			

Table 3-1. Yellow perch primers used for immune gene expression analysis and VHSV (N-gene) primer (Garver et al. 2011).

Results

Clinical Signs and Progression of VHS in Yellow Perch

External signs were only noted on the fish that had succumbed to the disease in the mortality monitoring tanks and most typically included petechial hemorrhages throughout the entire body as well as exophthalmia (Figure 3-1.). In the tanks reserved for monitoring mortality events, the first documented death occurred on day 9 post-VHSV injection, three days after the last sampling date in the experiment (Figure 3-2). Total mortalities within this tank peaked on day 11. At the end of the 17-day challenge period, cumulative mortality was 84% (21/25). No deaths occurred in the negative control tank. No fish, VHSV-exposed or mock treated, died in tanks used to assess disease progression during the six-day sampling period of the experiment. Clinical signs of disease as hemorrhage of the internal organs and moderate to severe ascites were first documented within fish on days 5 and 6 post-VHSV injection.

Figure 3-1. The clinical signs observed in yellow perch that succumbed to VHS infection by VHSV-IVb IP-injection included petechial hemorrhage throughout the entire body of the fish as well as exophthalmia within the eye sockets.

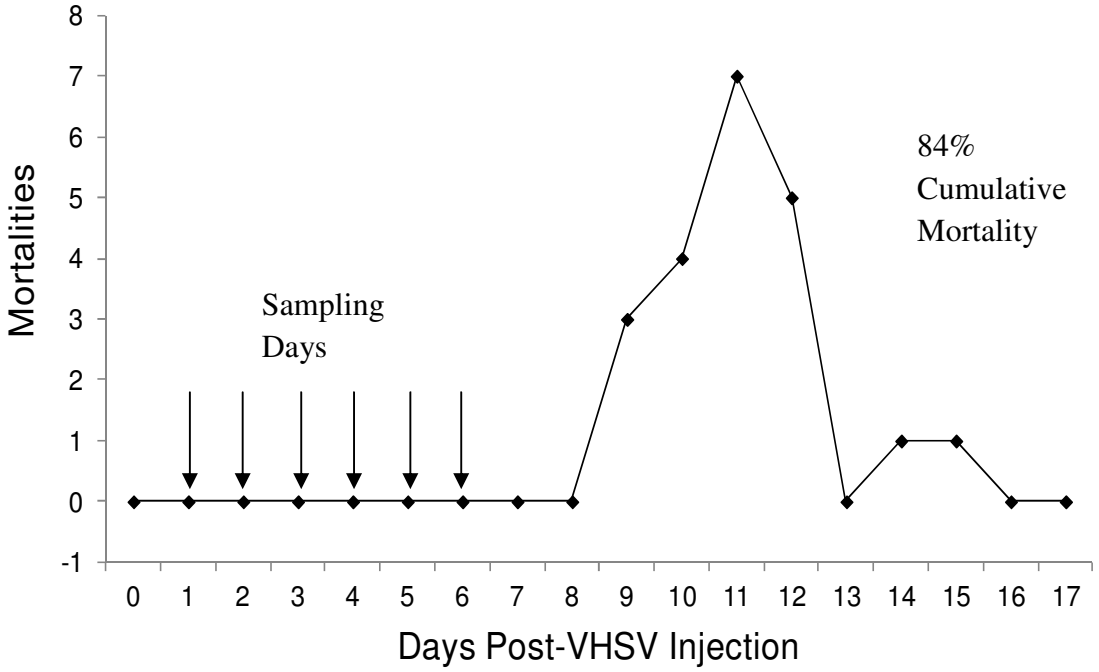

Figure 3-2. Daily mortalities recorded over a 17-day period post-virus challenge. Fish were sampled for gene expression and viral load on days 1-6 (arrows) post-virus injection before any mortality had occurred to capture the early innate immune response to infection.

VHS Viral Load in Tissues

The VHSV N-gene copy number was measured separately in each of the three tissues within each fish sampled (N=54 fish) post-VHSV injection by qRT-PCR, and the geometric mean of detected viral load (copy #) was analyzed over time. Significant increases in viral load were observed in all three tissues on days 3, 5 and 6 relative to day 1. While the accumulation of viral RNA was directly correlated with time post-challenge in all three tissues ($P < 0.001$), the accumulation of virus was the most rapid and significantly correlated with time within the spleen ($F_{(1, 52)} = 66.61$, $R^2 = 0.56$) (Figure 3-3); however, differences in the total viral load measured in the head kidney and spleen were not significant on any of the sampling days. Total viral load remained lowest in the liver. Because of a more rapid accumulation of virus within the head kidney and spleen, virus load detected in these organs became significantly greater than virus load detected in the liver by day 6 of the challenge (Figure 3-4).

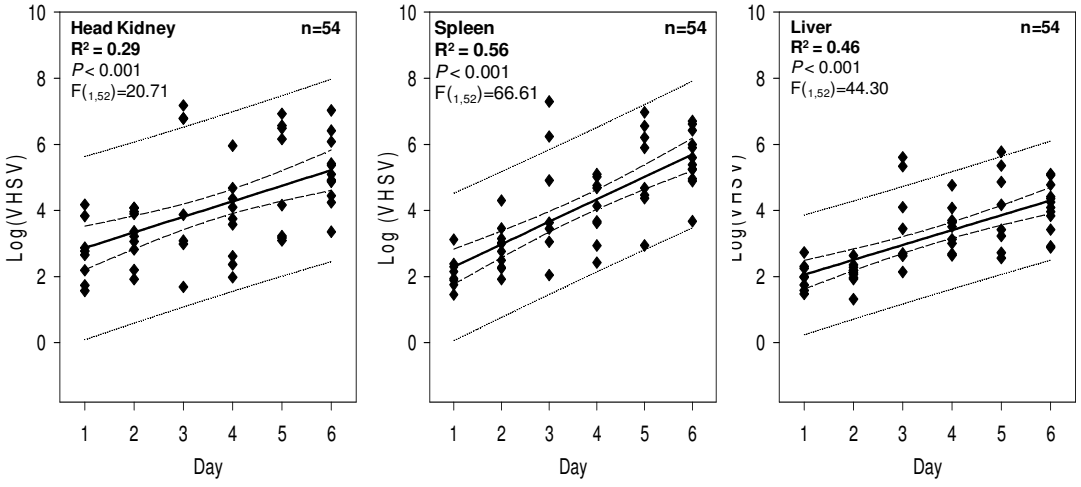

Figure 3-3. Linear regression analysis of log transformed VHS viral load against day post-VHSV injection. Dashed lines show the 95% confidence interval of the regression line and dotted lines indicated to 95% confidence interval for the entire data set.

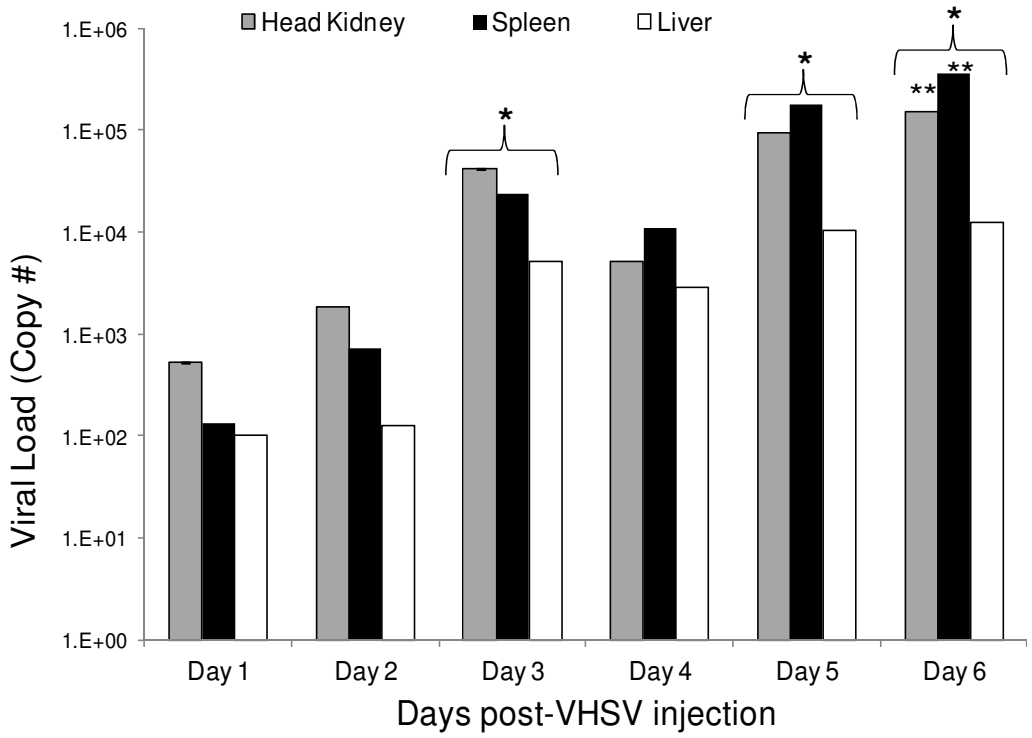

Figure 3-4. The geometric mean of VHS viral load (copy #) in head kidney, spleen, and liver measured on days 1-6 post-VHSV injection using RT-qPCR. Single asterisks indicate significant increases in viral load in all three tissues on days 3, 5 and 6 relative to day 1. Double asterisks indicate significant differences in viral load between spleen and head kidney as compared to the liver on day 6 of the challenge.

Mx Gene Expression

Mx gene expression within the tissues of VHSV infected fish was measured and analyzed relative to expression in the tissues of control fish on each of the six sampling days (Figure 3-5). The expression of Mx in the tissues of the control fish averaged 0.041 (± 0.017) in the head kidney, 0.086 (± 0.053) in the spleen, and 0.0046 (± 0.0045) in the liver. Mx expression in the head kidney of VHSV-exposed fish peaked at a 6.5-fold increase relative to Mx expression in control fish on day 4 post-VHSV injection, and remained relatively unchanged on days 5 and 6, at a 6.4- and 6.2-fold increase, respectively. Mx expression in the spleen of VHSV-injected fish gradually increased and peaked at a 7.0-fold increase relative to the control fish on day 5 post-VHSV injection. On day 6 post-VHSV injection, Mx expression in the spleen declined to a 0.5-fold level relative to Mx expression in the control fish. Mx expression in the liver, alternatively, was significantly elevated relative to controls on day 6 post-VHSV injection ($P < 0.001$), as well as to levels in the head kidney and spleen on days 4-6 post-VHSV injection ($P < 0.05$). A linear regression analysis indicated that no significant correlation between Mx expression and virus load was found in either the head kidney or the spleen (Figure 3-6). Mx expression in the liver, however, was significantly correlated to viral load ($P < 0.001$, $F_{(1,41)} = 56.46$, $R_2 = 0.58$).

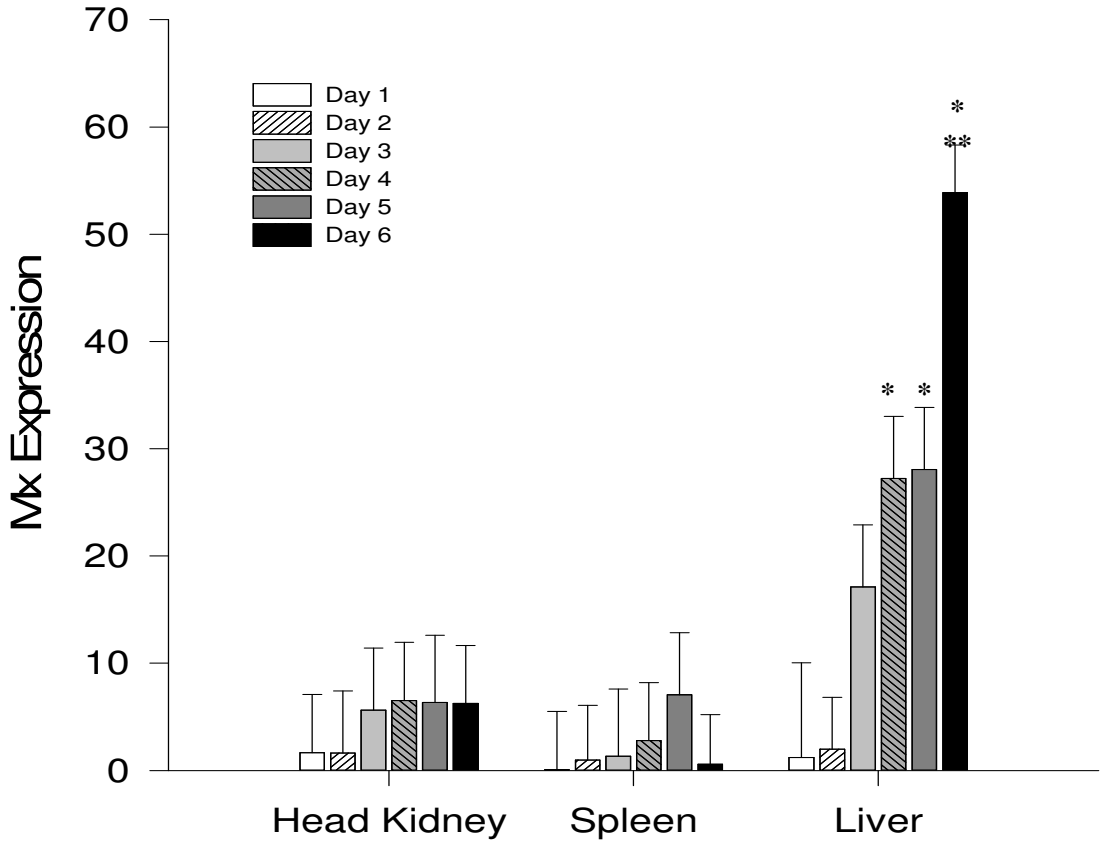

Figure 3-5. Mx gene expression (relative to mock-infected control fish) in head kidney, spleen and liver on days 1-6 post-VHSV injection. Single asterisks indicate significant increases in Mx expression in the liver on days 4, 5 and 6 relative to day 1. Double asterisks indicate significant differences in Mx expression between spleen and head kidney as compared to the liver on day 6 of the challenge.

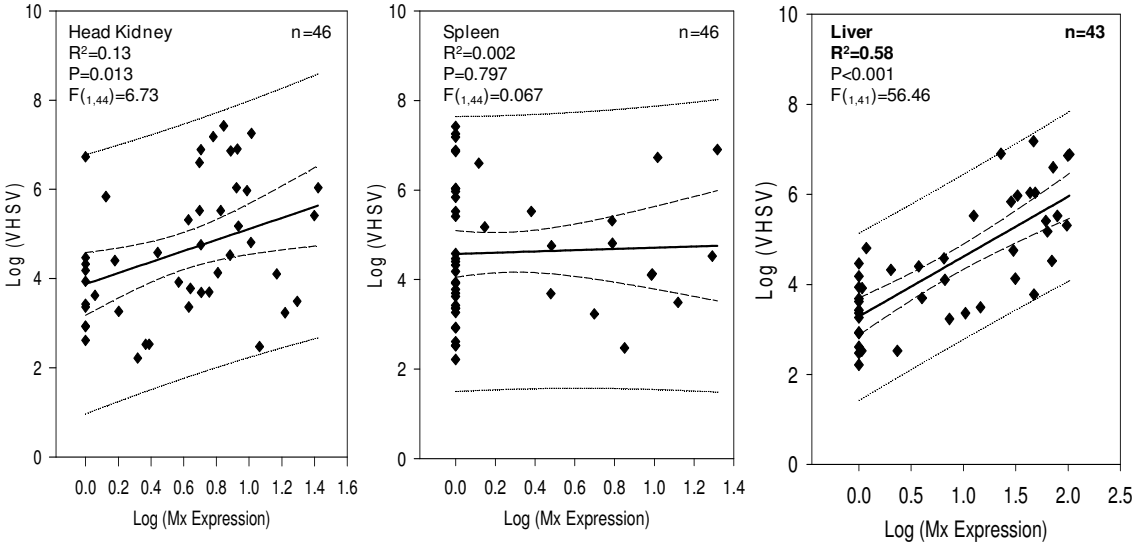

Figure 3-6. Linear regression analysis of log transformed VHS viral load against log transformed Mx expression. Dashed lines show the 95% confidence interval of the regression line and dotted lines indicated to 95% confidence interval for the entire data set.

IL-1β Gene Expression

Il-1β gene expression within the tissues of VHSV infected fish was measured and analyzed relative to expression in the tissues of control fish on each of the six sampling days (Figure 3-7). The expression of IL-1β in the tissues of the control fish averaged 0.001 (± 0.0008) in the head kidney, 0.002 (± 0.0004) in the spleen, and 0.0001 (± 0.0001) in the liver. Within each of the tissues, IL-1β gene expression in the head kidney peaked on day 3 with a 74- fold increase relative to the control before decreasing to 27-fold and 30-fold increases on days 5 and 6 post-VHSV injection, respectively. IL-1β gene expression in the spleen peaked on day 4 with a 122-fold increase relative to the control before decreasing to a 31-fold increase one day later. The expression level on day 4 was significantly higher relative to days 1, 2, 3, 5 & 6 ($P < 0.05$). IL-1β gene expression in the liver peaked on day 5 with a 173-fold increase relative to the control before decreasing to a 36-fold increase on day 6 post-VHSV injection. The expression level on days 4 and 5 were significant relative to days 1, 2, 3 & 6 ($P < 0.05$). Overall, IL-1β gene expression within the tissues of VHSV-injected fish was significantly correlated with viral load in all three tissues ($P<0.001$) (Head Kidney: $F_{(1, 41)} = 41.21$, $R^2 = 0.50$; Spleen: $F_{(1, 43)} = 26.93$, $R^2 = 0.39$; Liver: $F_{(1, 43)} = 35.23$, $R^2 = 0.45$) (Figure 3-8).

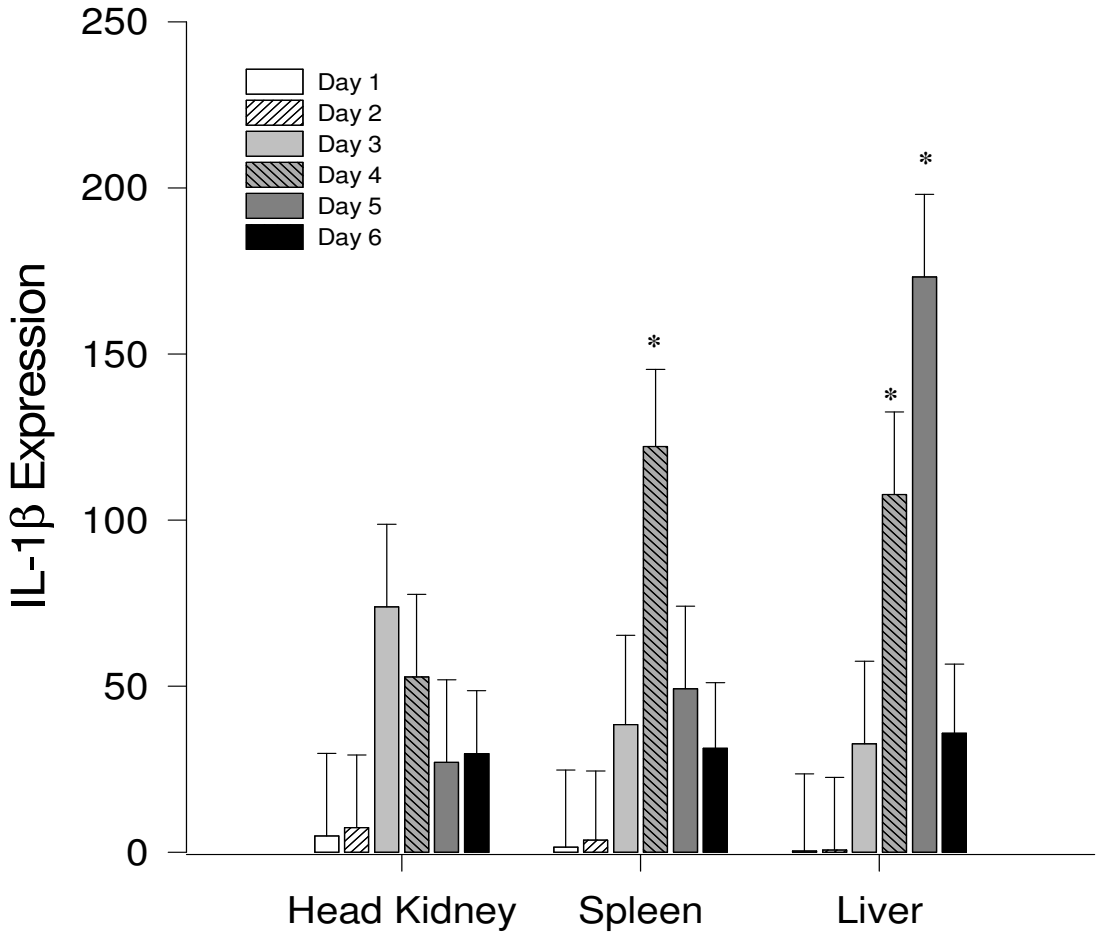

Figure 3-7. IL-1β gene expression (relative to mock-infected control fish) in head kidney, spleen and liver on days 1-6 post-VHSV injection. Single asterisks indicate a significant increase in IL-1β expression in the spleen on day 4 relative to all other days measured, as well as in the liver on days 4 and 5 relative to all other days measured.

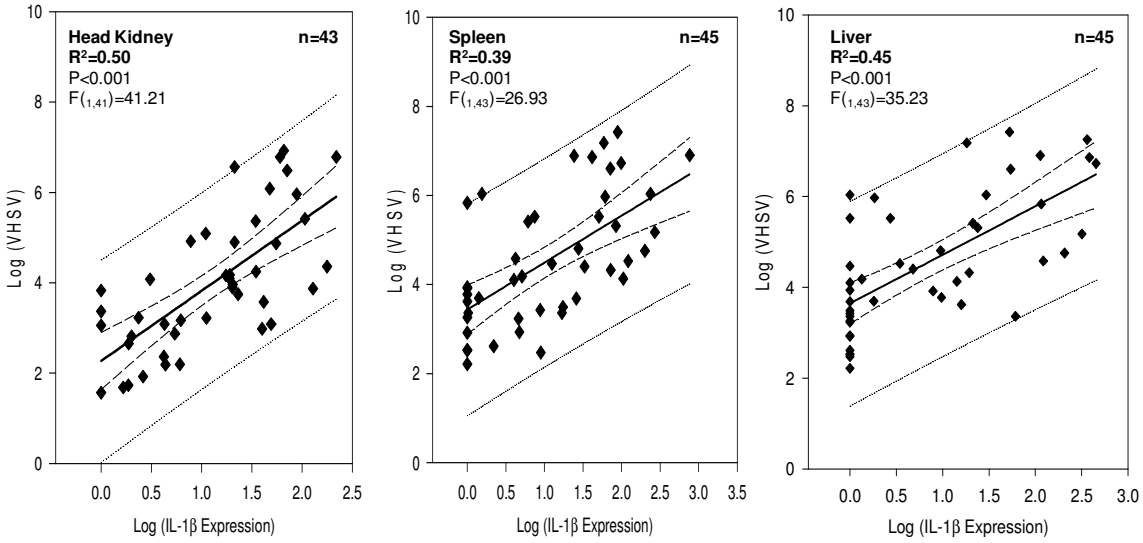

Figure 3-8. Linear regression analysis of log transformed VHS viral load against log transformed IL-1β expression post-VHSV injection. Dashed lines show the 95% confidence interval of the regression line and dotted lines indicated to 95% confidence interval for the entire data set.

SAA Gene Expression

SAA gene expression within the tissues of VHSV infected fish was measured and analyzed relative to constitutive expression maintained in the tissues of control fish on each of the six sampling days (Figure 3-9). The expression of SAA in the tissues of the control fish averaged 0.0007 (± 0.0006) in the head kidney, 0.014 (± 0.014) in the spleen, and 0.087 (± 0.046) in the liver. SAA expression gradually increased over time and peaked on day 6 in all three tissues, with a 12.2-fold increase in the head kidney, a 42.6-fold increase in the spleen, and a 119-fold increase in the liver. Significant increases in SAA expression were identified on days 4, 5, & 6 in the liver and day 6 in the spleen ($P \leq 0.05$) (Figure 3-9). SAA expression was also significantly correlated with viral load in all three tissues ($P<0.001$) (Head Kidney: $F_{(1, 38)} = 135.83$, $R^2 = 0.78$; Spleen: $F_{(1, 38)} = 89.48$, $R^2 = 0.70$; Liver: $F_{(1, 40)} = 81.41$, $R^2 = 0.67$) (Figure 3-10).

Discussion

An increasing number of studies have focused on the early innate immune response to the virus as a key factor controlling the outcome of infection. Past studies on the immune response to VHSV have focused primarily on rainbow trout and the response to virus isolates of genotype VHSV-I, II, and III. In contrast, the current study investigated the viral accumulation and innate immune response towards an emerging invasive virus, VHSV-IVb, in yellow perch, a percid phylogenetically distant from salmonids and relatively unstudied compared to rainbow trout. In this study, the administration of

VHSV-IVb by IP-injection into juvenile yellow perch resulted in 84% mortality, indicating their high susceptibility to this disease. In fish sampled in the very early stages

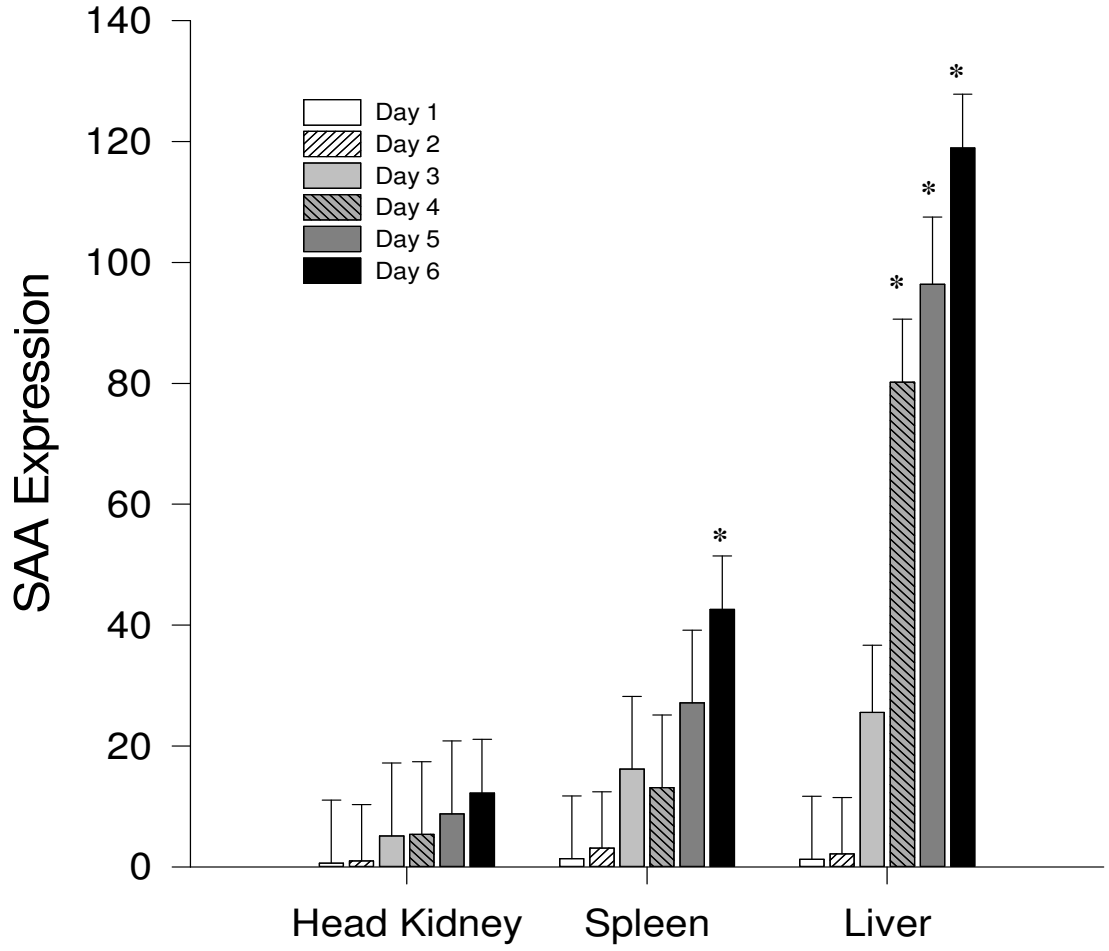

Figure 3-9. SAA gene expression (relative to mock-infected control fish) in head kidney, spleen and liver on days 1-6 post-VHSV injection. Single asterisks indicate significant increases in SAA expression in the liver on days 4, 5 and 6 relative to day 1 and in the spleen on day 6 relative to day 1.

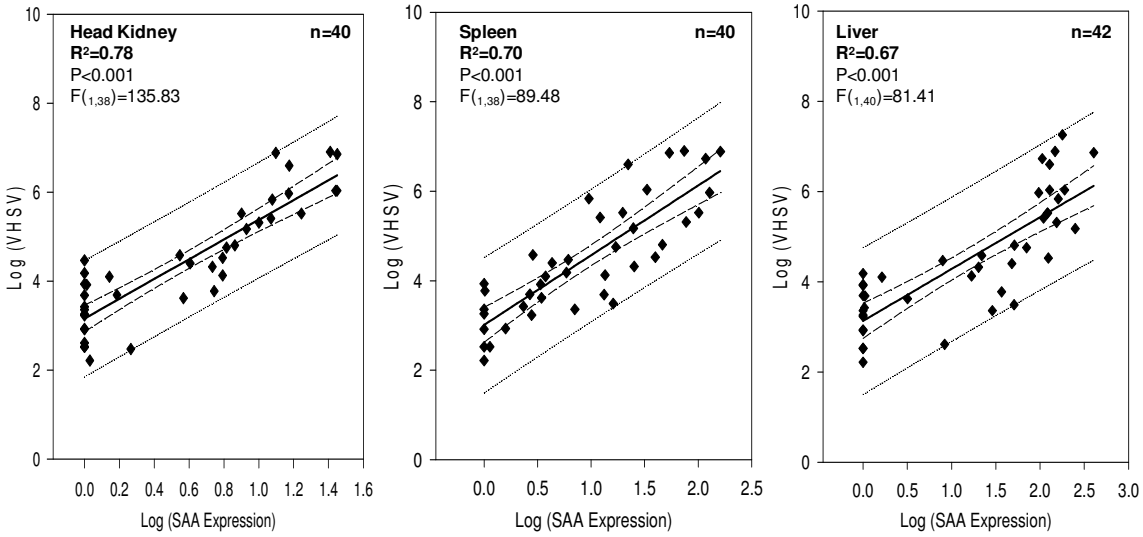

Figure 3-10. Linear regression analysis of log transformed VHS viral load against log transformed SAA expression post-VHSV injection. Dashed lines show the 95% confidence interval of the regression line and dotted lines indicated to 95% confidence interval for the entire data set.

of infection, a significant up-regulation of Mx gene expression in the liver, as well as IL-1β and SAA activation in the head kidney, spleen, and liver was directly correlated to viral load. In addition, the relatively low level of Mx expression observed in the hematopoietic tissues, head kidney and spleen, may represent the host response to a strategy utilized by the virus to down-regulate or block Mx expression in these tissues, thus increasing virus replication.

When the kinetics of viral RNA accumulation was monitored in each of the three tissues, the accumulation of virus was the most rapid and significantly temporally correlated within the spleen and head kidney. Even so, viral load also remained highly correlated with time in the liver, suggesting that the liver might have a secondary role in VHSV-IVb infection. Since the spleen and head kidney are the primary producers of hematopoietic cells, including mature macrophages in fish, these results also suggest that cells in these tissues may be specifically targeted and critical for successful VHSV replication.

Mx proteins comprise a small family of GTPases and have been shown to induce a high degree of resistance against virus infections in a number of vertebrates (Haller *et al.*, 2007), and even in the abalone (*Haliotis discus*), an invertebrate mollusk (De *et al.*, 2007). Mx genes, however, are highly polymorphic and the resulting amino acid differences account for a large number of allelic gene products and a large degree of variation between species, complicating their study. The presence of multiple isoforms of Mx suggests that different protein products, with and without antiviral mechanisms, may be unique to each species, and thus heavily influenced by the environment in which a

species has evolved over time. In fish, for example, three isoforms of Mx have been identified in rainbow trout (Trobridge *et al.*, 1997; Trobridge & Leong, 1995), Atlantic salmon (*Salmo salar*)(Robertsen *et al.*, 1997), rock bream (*Oplegnathus fasciatus*)(Zenke & Kim, 2009), and Gilthead seabream (*Sparus aurata*) (Fernandez-Trujillo *et al.*, 2011), seven in zebrafish (*Danio rerio*)(Altmann *et al.*, 2004), five in the channel catfish (*Ictalurus punctatus*)(Plant & Thune, 2008), two in the Atlantic halibut (*Hippoglossus hippoglossus*)(Jensen & Robertsen, 2000), turbot *(Scophthalmus maximus)* (Abollo *et al.*, 2005), and goldfish (*Carassius auratus*)(Zhang *et al.*, 2004), and one in the pufferfish (*Takifugu rubripes*)(Yap *et al.*, 2003), Japanese flounder (*Paralichthys olivaceus*)(Lee *et al.*, 2000), Senegalese sole (*Solea senegalensis*)(Fernandez-Trujillo *et al.*, 2006), orange-spotted grouper (*Epinephelus coioides*)(Chen *et al.*, 2006), and barramundi (*Lates calcarifer*) (Wu *et al.*, 2010).

Contrary to similar studies examining Mx activation in rainbow trout (McLauchlan *et al.*, 2003), the present study identified a relatively high level of Mx expression in the perch liver that while directly correlated to viral load, appeared to be associated with relatively poor protection against VHSV disease progression (84% mortality). Unlike the rainbow trout study, however, which examined the antiviral response of three Mx genes, only one Mx gene was analyzed in this study, thus it may be possible that alternate forms exist in perch with different expression kinetics. The sequences used to obtain qPCR primers for this Mx form were obtained from a yellow perch expression sequence tag (EST; Accession #GO654167.1), derived from sequencing the ovary. This EST contains only a partial open reading frame so it is not possible to determine the precise Mx form

that it represents. However, the amino acid sequence of this open reading frame aligns most closely with an Mx protein from *Perca fluviatilis*, the European perch (Staeheli et al., 1989). Only one Mx form was reported for *P. fluviatilis* but there was no attempt to look for multiple forms so it may be possible that alternate forms exist in perch. Alternatively, lower levels of Mx transcription and no correlation to viral load were observed in the head kidney. Further, no correlation and even potential down-regulation of Mx was observed in the spleen where virus accumulation was highest. These observations may be due to interferon down-regulation as part of a strategy utilized by the virus for enhanced replication within these tissues.

The pro-inflammatory cytokine IL-1β has also been well-characterized in a wide variety of fish species and directly correlated to early viral infection in rainbow trout (Purcell *et al.*, 2010; Tafalla *et al.*, 2005), although the role of IL-1β function in antiviral defenses remains largely unknown. Allelic variations of IL-1β in fish suggest alternative unknown functions may also exist, the activation of specific immune responses including the induced expression of COX2 and MHC II in rainbow trout macrophages (Iliev *et al.*, 2005), as well as the *in vivo* enhancement of phagocytosis and increased resistance against bacterial infections have been observed (Secombes *et al.*, 2011).

IL-1β was significantly correlated to viral load in all three tissues though the duration of the experiment, peaking at different stages of the infection within different tissues. The kinetics of IL-1β activation were very different than those observed in a similar study examining IL-1β expression in tissues of the rainbow trout after exposure to a very low level of VHSV-Ia infection induced by IP-injection (Tafalla *et al.*, 2005). In the rainbow

trout, IL-1β activation was immediately induced (days 1 & 2 post-VHSV infection) before returning to control levels by day 3 in the spleen. In addition, IL-1β was only slightly activated in the head kidney and liver. In contrast, in the yellow perch, IL-1β expression peaked at much higher levels later (days 3, 4, & 5 post-VHSV exposure) in each of the tissues, particularly the liver, before a rapid down-regulation. In addition, IL-1β was significantly correlated to viral load throughout the duration of the experiment. The differences observed here may again be due to the heightened susceptibility of yellow perch to the VHS virus, and suggest this inflammatory cytokine plays a significant role in the disease progression.

Of the three genes analyzed, SAA activation was the most strongly correlated with viral RNA accumulation, particularly in the head kidney, although SAA expression reached its highest levels in the liver. This appears to be the first published report of SAA activation directly tied to anti-VHSV immune response in fish. Human SAA is an acute-phase protein that circulates in the blood and is known to be primarily synthesized in the liver. It has been implicated in antiviral immune defense against hepatitis C virus by inhibiting viral entry into cells (Cai et al., 2007; Lavie et al., 2006), as well as numerous other immune defense functions ranging from cytokine activation (Patel et al., 1998), to leukocyte recruitment (Mullan et al., 2006), and antimicrobial activity (Badolato et al., 2000). The extrahepatic production of anti-viral SAA proteins in the plasma has been observed in a variety of mammalian species (Urieli-Shoval et al., 1998). However, a recent study examining SAA in rainbow trout challenged with bacterial proteins, found significant up-regulation of SAA expression in immune relevant tissues, but no SAA

proteins were detected in the blood or plasma, suggesting that in fish, a more localized alternate function may exist (Villarroel *et al.*, 2008). The results of the present study support this hypothesis, that SAA may have a more localized, anti-viral role in all three immune tissues of the yellow perch.

In summary, we present evidence that yellow perch mount a strong innate immune response to VHSV-IVb as demonstrated by significant changes in the expression of IL-1β, Mx and SAA in various immune related tissues during the early stages of VHSV infection. Further, VHSV accumulation was highest and most directly correlated to SAA expression in the spleen and head kidney, suggesting that SAA activation in these tissues may be a direct indicator of VHSV-IVb infection. The continued study of emerging viruses in non-traditional fish species, such VHSV-IVb in yellow perch, can provide valuable insight into alternative immunological host defenses, as well as novel strategies utilized by a rapidly evolving virus. Such knowledge is beneficial, not only for the mitigation of the impact VHSV-IVb and similar viruses in aquaculture and fisheries, but for our own understanding of the evolution of host-virus immune defenses.

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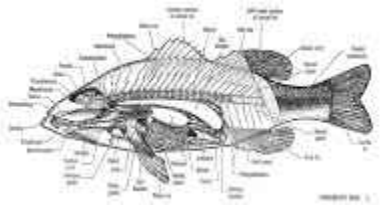
Chapter 4

Viral distribution and pathology of the Great Lakes viral hemorrhagic septicemia virus (VHSV-IVb) in adult yellow perch



"Since the discovery of VHSV-IVb in the Great Lakes of North America, over 31 species of freshwater fish commonly found throughout the inland waters of the U.S. have been classified as being susceptible to the disease. While studies have begun to examine viral tissue distribution and pathology using highly susceptible juvenile fish, the goal of this study was to better understand the viral pathogenesis as an infection progresses over time in asymptomatic adult fish. Adult yellow perch (Perca flavescens) were exposed to VHSV-IVb by IP-injection at two different titers, 1 x 10^2 PFU/fish and 1x10^4 PFU/fish and sampled for viral tissue distribution. These titers produced infections that resulted in 67% and 60% cumulative survivals, respectively, with the onset of mild to no clinical signs of the disease. Plaque assays on the tissues from VHSV-injected fish sampled over the course of 28 days, revealed that virus was detected mainly in the gills as well as the pooled head kidney and spleen. VHSV titers determined by qPCR detected viral load in the blood that was highly correlated to viral detection in the pooled head kidney and spleen over all sampling days, while viral load in the brain gradually increased over time. Fluorescent microscopy imaging of brain tissues of VHSV-exposed fish at 28 days indicated mild hemorrhage with VHSV-associated erythrocytes, as well as leukocytes, and heavy infection in cell body layers within the optic tectum and in axonal tracts throughout the brain. These results suggest that blood is an active component of VHSV transport, and that the analysis of gills by plaque assay, and blood by qPCR, may offer reliable and accurate methods of non-lethal sampling for VHSV in adult fish. These results also indicate, however, that the brain of the fish may be a target organ of the virus and heavy staining of VHSV in neurons suggest that the VHSV-IVb virus may be neurotropic as well as hemorrhagic."

Introduction

Viral hemorrhagic septicemia virus (VHSV) is a fish virus best known for its severe pathology and high mortality rates in rainbow trout (*Oncorhynchus mykiss*) hatcheries throughout Europe, where outbreak of disease has resulted in extensive economic losses since its discovery in 1962 (Einer-Jensen *et al.*, 2004; Olesen, 1998; Skall *et al.*, 2005; Smail, 1999; Wolf, 1988). Because potential outbreaks of this highly transmissible virus have had such profound socio-economic consequences, the Office of International Epizooties (OIE), also known as the World Organization of Animal Health, has categorized VHSV as a reportable disease of great international concern. Thus, the increasing number of aquatic environments in which the virus has been found is of great concern to fisheries and aquaculture managers throughout the world. With the exception of the VHSV-Ia strain, VHSV was typically known as a marine virus until a new freshwater strain, now classified as VHSV-IVb, was reported in 2005 in the Great Lakes of North America from adult muskellunge sampled in 2003 (Elsayed *et al.*, 2006). Since then, the VHS virus has been found capable of infecting over 31 species of freshwater fish commonly found throughout the inland waters of the U.S. (Kim & Faisal, 2010b; Thompson *et al.*, 2011b). Never before had VHSV, or any other rhabdovirus been isolated in any of the Great Lakes, reflecting the ability of VHSV to rapidly evolve into a new-host environment (Pierce & Stepien, 2012), and opening the door for a large number of new freshwater host-species to become infected with the virus.

Genotypic analysis has classified VHSV as part of the *Rhabdoviridae* family, which includes some of the most well-known and most well-studied mammalian viruses, rabies

and vesicular stomatitis virus (VSV). Like all rhabdoviruses, VHSV is a negative-strand RNA virus consisting of an 11.2 kb nucleotide genome which includes five proteins including, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral RNA polymerase (L). Unique to VHSV and other fish rhabdoviruses, however, is a unique sixth gene that encodes a non-virion (NV) protein of unknown function. This non-virion protein is expressed in infected cells but is not present in purified virions (Kurath *et al.*, 1997), and has resulted in the placement of fish rhabdoviruses into of the newly recognized *Novirhabdovirus* genus within the *Rhabdoviridae* family.

Clinical signs of an acute VHS manifestation of the disease in fish are often non-specific, appearing as petichial hemorrhages throughout the body, hemorrhaging at the base of the fins, muscles, internal organs and eyes, exophthalmia, darkening of the body and pale gills. Ascites and hemorrhaging of the liver and other internal organs are often observed upon necropsy (Kim & Faisal, 2010a; Olson *et al.*, 2013; Wolf, 1988). In acute forms of the disease, mortality is rapid and commonly as high as 100% in juvenile fish, and as much as 30-90% in adults. In chronic forms of the disease, however, fish become infected with the virus without showing any outward clinical signs or pathology of the disease. These fish experience a prolonged course with lingering low levels of mortality, and possibly shed the virus into the surrounding environment acting as covert carriers of the disease. In some hosts, a chronic infection is characterized by nervous manifestations, often associated with erratic swimming behavior (Batts & Winton, 2007; Lovy *et al.*, 2012; Skall *et al.*, 2005).

Currently, sampling of the head kidney and spleen for virus isolation using cell culture or plaque assay, followed by confirmation by reverse-transcriptase polymerase chain reaction (RT-PCR), is the gold standard diagnostic test for VHSV in fish (Batts & Winton, 2007; OIE, 2009). Unfortunately, sampling of these tissues is lethal and, in addition, the nature of this assay requires that virus be present in sufficient quantities in these organs to produce a positive titer in a susceptible cell line, a condition that does not always exist in fish infected with low levels of virus (Lovy *et al.*, 2012). Because of the wide-species host range and the active screening for virus in fish, the impact of euthanizing a statistically sufficient number of fish can have a negative impact on a natural population in a lake, such as the case for muskellunge (*Esox masquinongy*) or northern pike *(Esox lucius)*, or for valuable broodstocks in aquaculture. Thus, if virus can be more accurately detected in a non-lethal manner, such as blood sampling or gill and fin biopsy, larger numbers of fish may be screened more effectively and efficiently at a lower cost and valuable fish stocks could be spared. To do this, however, requires a more detailed understanding of how the pathogenicity and distribution of the VHS virus changes in a susceptible host-fish species over time.

Using the adult yellow perch as a research model and multifaceted methods such as quantitative PCR and fluorescence microscopy in conjunction with plaque assay, this study examines the relative distribution of VHSV-IVb throughout the head kidney, spleen, liver, brain, fin, gills, and blood over the course of infection in an effort to track how the virus distribution between these tissues changes over time. This knowledge is useful for identifying similarities and differences between VHSV and other rhabdoviruses

in host-virus interactions and pathogenesis of disease, and will aid in the development of a more efficient, accurate, and potentially non-lethal, identification of chronically infected asymptomatic adult fish.

Materials & Methods

Adult yellow perch were obtained from the University of Wisconsin-Milwaukee (UWM) School of Freshwater Science (SFS). All fish used in the study were spawned and reared at the UWM SFS Aquaculture Research Facility according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the UWM as previously described (Rosauer *et al.*, 2011). Approximately 3-4 months prior to the challenge, all fish used in the study were transferred to the USGS Western Fisheries Research Center (WFRC), Seattle, WA, where they were used for the subsequent challenge experiment under protocols approved by the IACUC of the WFRC. All virus exposure experiments were performed in the WFRC aquatic biosafety level-3 containment laboratory.

The Great Lakes VHS virus strain IVb (MI03) was isolated from adult muskellunge, *Esox masquinongy* (Mitchill) from Lake St. Clair in 2003 (Elsayed *et al.*, 2006). The virus was propagated using the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan *et al.*, 1983b) and concentration determined by plaque assay as previously described (Batts & Winton, 2007).

Virus Quantification by Plaque Assay Analysis

For the first challenge experiment, 180 fish (100-150g) were equally divided between 3 treatments, each treatment conducted in 4 replicates of 15 fish/replicate. The treatments consisted of one group (n=60 fish) exposed to VHSV by intraperitoneal (IP) -injection at a titer of 1 x 10^4 PFU/fish, another group (n=60 fish) exposed to VHSV by intraperitoneal (IP) -injection at a titer of 1 x 10^2 PFU/fish, and a third group (n=60 fish) served as a control, and was challenged by IP injection with the same volume of Tris-buffered Eagle's minimum essential medium (MEM-10-T) used to formulate the virus treatments. This group served to simulate the stresses of handing and injection. Water temperature was 9°C at the start of the challenge and then raised 1.0°C daily until it reached 12°C. All fish were fed every other day throughout the entire challenge experiment. Of the negative control tanks and virus challenged tanks, fish from one replicate tank in each group were monitored daily for a total of 28 days only for mortality events to assess disease progression. From these tanks, dead fish were removed daily and mortality data recorded. Percent survival in each tank was calculated as the cumulative number of surviving fish divided by the total number of fish x 100. In the other three virus treated and negative control tanks, fish were lethally sampled on days 2, 4, 7, 10, 14, 21 and 28 post-virus exposure. Each fish sampled was euthanized by an overdose (25 mg mL^{-1}) of buffered tricaine methanesulfate (MS-222, Western Chemical Inc.), and samples of the spleen, head kidney, gill, and fin were collected and immediately frozen (-80°C) until plaque assay analyses could be conducted. The head kidney and spleen samples were pooled for each individual prior to freezing.

Plaque assay analysis on each specimen collected was conducted according to protocol establish by the AFS Fish Health Section for the detection of VHSV (Batts and Winton, 2007). Each sample was diluted 1:8 (weight:volume) in MEM-0 and homogenized with a Stomacher-80 (Seward Laboratory Systems, Biomaster). Sample homogenates were transferred into 15 mL conical tubes and spun at 4°C for 10 minutes at 1000 x g. One milliliter (mL) of supernatant from each sample was removed to quantify infectious virus by plaque assay, and another 1 mL stored at -80°C to assess virus genome concentrations by qRT-PCR. For plaque assay, serial 10-fold dilutions (1:40 to 1:40k) of tissue homogenate supernatants were plated in duplicate onto polyethylene glycol (7% PEG) treated EPC cell monolayers in 24-well plates. Cell-cultures were overlaid with methylcellulose and incubated at 15°C for 7 days before fixation with crystal violet-formalin solution and plaque enumeration as previously described (Batts & Winton, 1989b). Fish tissue viral titers of VHSV are reported as plaque forming units per gram (PFU g^{-1}).

Virus Quantification by qPCR analysis

For the second challenge experiment, only one group of triplicate tanks, containing 16 yellow perch/tank, was exposed to the VHSV by IP-injection at a titer of 1 x 10^4 PFU/fish as described above. Identical triplicate tanks of each fish stock serving as negative controls were exposed to the same volume of MEM-10 used to formulate the virus treatments at the same temperature regime. On days 5, 7, 9, 12, 14 & 21 post-virus exposure one fish was sampled from each tank. Each fish was euthanized by an overdose

(25 mg mL^{-1}) of buffered tricaine methanesulfate (MS-222, Western Chemical Inc.) and samples of the spleen, head kidney, blood, brain, gill, and fin were collected. Tissue samples were immediately frozen (-80°C) until qPCR analyses could be completed. The head kidney and spleen samples were pooled for each individual prior to freezing.

Blood samples of approximately 1mL were taken from each fish in a non-heperinized syringe and kept overnight at 4°C for clotting to occur. After 12-24 hrs, samples were spun at 13,000 rpm for 20 minutes at 4°C and serum was carefully removed. The resulting pellet (~0.4-0.5 mL) was transferred to a new 2ml tube containing 1mL of TRI Reagent® RT-Blood with 75µl 4-bromoanisle (MRC, Inc, cat.# RB211), vortexed for 5 minutes and frozen at -80°C until further processing could be completed. RNA extraction into 50µl H_2O was completed according to the manufactures protocol. RNA was processed a second time to remove any interfering DNA and/or inhibiting enzymes using the RNAqueous®-Micro Scale RNA Isolation Kit (Ambion) according to the manufacturer's protocol. The pooled head kidney and spleen, brain, gill, and fin samples were collected and immediately froze at -80°C and total RNA extraction using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Complimentary DNA was synthesized from 1 µg RNA by RT-PCR using the Applied Biosystems (ABI) High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. VHS virus primers (*Forward primer* 5'-ATG AGG CAG GTG TCG GAG G-3', *Reverse primer* 5'-TGT AGT AGG ACT CTC CCA GCA TCC-3', Invitrogen) and MGB probe (5'-6 FAM-TAC GCC ATC ATG ATG ATG -MGBNFQ-3', ABI) were utilized for the qPCR assay as described in Garver *et al.* (2011). The RNA standard used for qPCR virus

quantification, generated by a plasmid vector containing the N-gene of the virus, was generously provided by Dr. Kyle Garver (Pacific Biological Station, Nanaimo, BC). A standard curve based on a dilution series of N-transcripts ranging from 1 $x10^8$ to 1 x 10^1 viral gene copies was included on each qPCR plate and used to calculate virus loads based on the resulting CT values. The VHSV qPCR assay was performed using a LightCycler® 480 (Roche). Each qPCR assay was conducted in a 25 µL reaction volume containing 1 µL of cDNA, 600 nM of each primer, 200 nM probe, and 12.5 µL 2x TaqMan Universal PCR Master mix (Applied Biosystems). The thermal cycle profile was: 2 minutes at 50ºC, 10 minutes at 95ºC, 40 cycles of 15 seconds at 95ºC, and 1 minute at 60°C (Garver *et al.*, 2011).

Statistics

Statistical relevance was calculated using Sigma Plot® software (Systat Inc.). Statistical analysis of gene expression relative to time of expression and tissue type was completed using Tukey's pairwise multivariate comparisons procedure within a two-way analysis of variance (ANOVA). Correlation results were completed using linear regression analysis on log transformed data.

Fluorescence Microscopy

In a third challenge experiment, yellow perch were exposed to virus as described above and on day 28 post-virus exposure, all surviving fish were euthanized and the heads removed and immediately fixed (30% ethanol, 10% formalin, 2% glacial acetic

acid, and 58% distilled water). Samples were shipped to and processed for microscopy at UWM-SFS in Milwaukee, WI. The brains were carefully removed from each of the fish heads and placed in a 20% sucrose / phosphate-buffered saline (PBS) solution overnight at 4°C. After sucrose saturation, brain tissues were embedded in a 1:2 OTC sucrose medium. Frozen blocks were cut into 7µm sagittal or horizontal sections onto silicone coated slides and fixed in cold acetone for 5 min, followed by a 30 min drying period at -20°C. Tissues were then rehydrated with a series of 5 min incubations with PBS followed by a 30 min block with 10% normal goat serum (NGS) in PBS with 0.1% Tween. Sections were blocked a second time with a commercially available Image-iT® FX Signal Enhancer Reagent (Molecular Probes, cat# R37107) to further reduce non-specific staining or background fluorescence. Tissues were washed two more times using 10% NGS/PBS with 0.1% Tween before incubation with anti-VHSV monoclonal antibody (Aquatic Diagnostics Ltd., Scotland) diluted 1:10 in PBS in a humid chamber for 1 hour at room temperature. Tissue sections were washed an addition 3 times, 30 min each, using 10% NGS/PBS before incubation with Alexa Fluor 594 (AF594) goat anti-mouse IgG fluorescent conjugate (Molecular Probes, cat# A31624) diluted 1:1000 in 10% NGS/PBS for 15 min at 4°C. This was followed by 5 consecutive 5-10 min washes with 10% NGS/PBS at room temperature and mounted with Vectashield® with DAPI for nuclear staining (Vector Laboratories, cat# H1200).

Results

In the tanks of fish reserved only for mortality monitoring, fish exposed to VHSV at a dose of 1 x 10^2 PFU/fish had 67% cumulative survival (10/15), while fish exposed to VHSV at a dose of 1 x 10^4 PFU/fish had 60% cumulative survival (9/15) (Figure 4-1). No mortalities were observed in any of the negative control tanks. Statistical analysis verified that no significant difference in cumulative survival existed between fish exposed to the two VHSV titers (P = 0.532).

Despite a relatively high mortality in each of the tanks (33% low dose and 40% high dose), external clinical signs of the disease remained very mild and were often not visible in fish that had succumbed to the disease in either treatment group. When external clinical signs were visible, they most frequently consisted of mild exophthalmia, or swelling of the eye, as well as slight hemorrhage on the fins and the base of the anus. Fish with external signs did not always die from the disease, and most appeared to recover from clinical signs of VHS by day 21 post-VHSV injection. Hemorrhages around the brain, eye sockets, and liver, as well as moderate ascites in the abdominal cavity, however, were the most often apparent internal signs of the sampled fish. Since many of the fish that had died during the 28-day challenge did not exhibit external signs of the disease, plaque assay analysis was conducted on the pooled head kidney and spleen on all individual fish to confirm the presence of VHSV. Because mild exophthalmia was the most common clinical sign exhibited during the challenge, the brain was also extracted and included in the analysis. This analysis of the fish that had succumbed to the disease confirmed relatively high titers of VHSV in the pooled head kidney/spleen and brain. The

geometric mean viral titer in the brain was 5.57 x 10^8 PFU g^{-1}, while the geometric mean viral titer in the pooled head kidney/spleen was 6.72 x 10^8 PFU g^{-1}. No significant difference in viral titer between the brain and pooled kidney/ spleen was detected (n=8 fish, data not shown).

VHSV titer was analyzed by plaque assay in the tissues, head kidney/spleen, gill, and fin over the first 28 days post-VHSV injection. In fish exposed to the virus at a dose of 1 x 10^2 PFU/fish, three fish were sampled (1 fish/tank) on days 2, 4, 7, 10, 14, 21 and 28 days post-VHSV exposure (Figure 4-2, Table 4-1). On day 2 post-VHSV exposure, virus was only detected in the fin in 1/3 fish analyzed. While viral detection in the pooled head kidney and spleen became evident on day 4. On day 10, VHSV was only detected in 2/3 fish, but in the gill and fin it was detected in all 3/3 fish sampled. By day 28 post-VHSV exposure, VHSV was detected in the pooled head kidney and spleen in 1/3 fish (62% of fish overall), the gill in 2/3 fish (67% of fish overall), and the fin in 0/3 fish (57% of fish

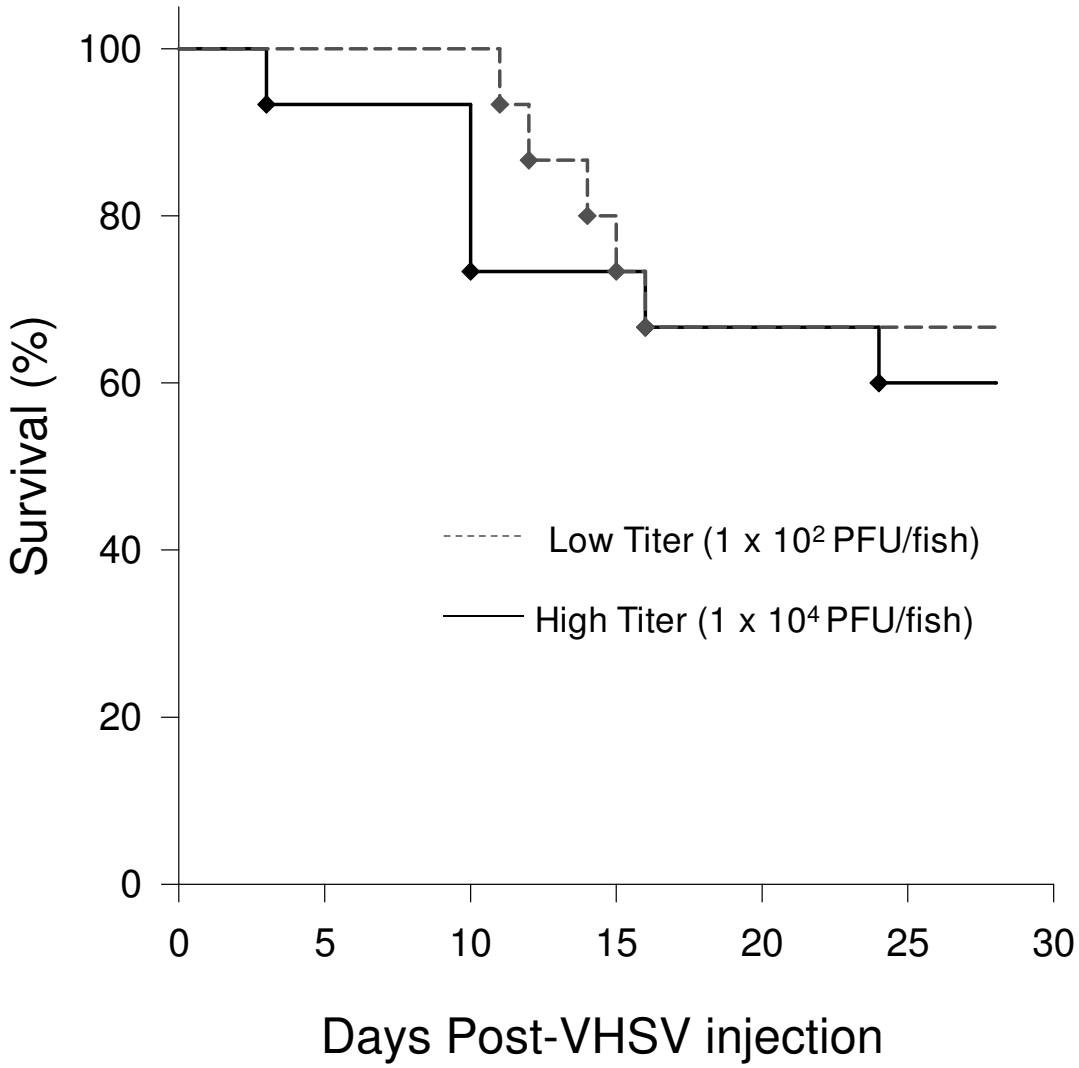


Figure 4-1. Adult yellow perch were exposed to VHSV-IVb at two viral titers, 1 x 10^2 and 1 x 10^4 PFU/fish, and survival monitored daily in each group over 28 days. Overall, cumulative percent survival (67% vs 60%) was not significantly different between the two groups.

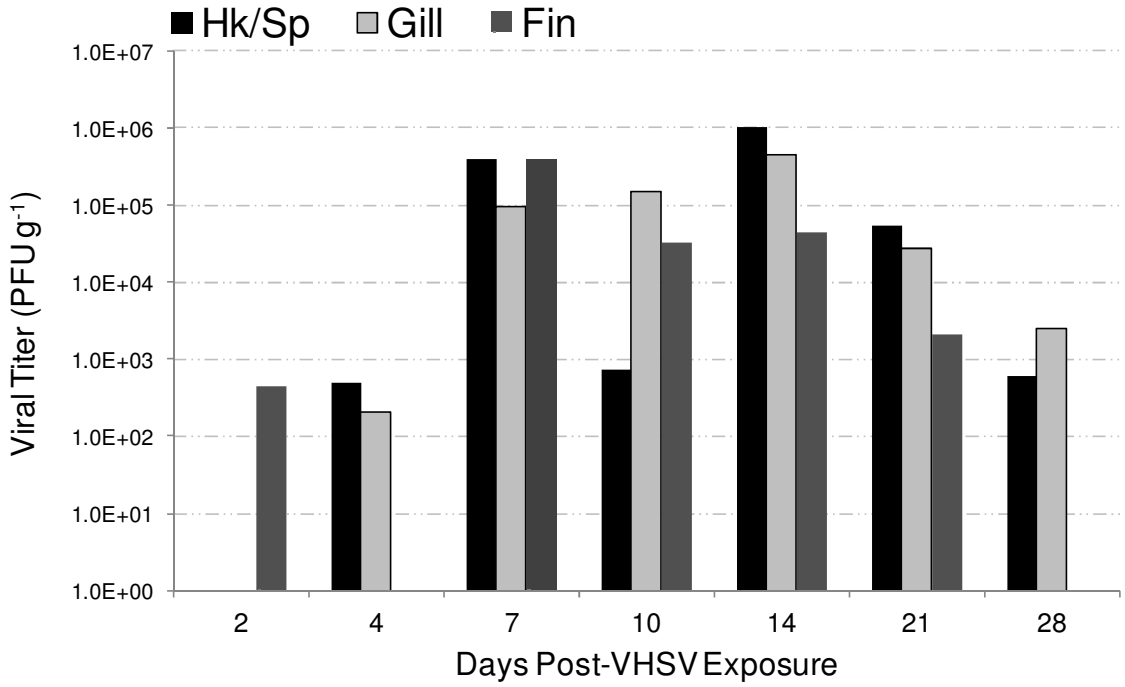


Figure 4-2. The geometric mean of VHS viral titer (PFU g^{-1}) determined by plaque assay in all three tissues (pooled head kidney/spleen (Hk/Sp), fin and gill) in fish exposed to a viral titer of 1 $x10^2$ PFU/fish over time.

Low Titer - Day	2	4	7	10	14	21	28	Total	% Pos.
Kd/sp	0/3	2/3	3/3	2/3	3/3	2/3	1/3	13/21	62%
Gill	0/3	1/3	3/3	3/3	3/3	2/3	2/3	14/21	67%
Fin	1/3	0/3	3/3	3/3	3/3	2/3	0/3	12/21	57%

Table 4-1. VHSV prevalence determined by PA in fish exposed to a viral titer of 1 $x10^2$ PFU/fish.

overall) (Table 4-1). When viral titer in time point-sampled fish was analyzed, no significant difference was found between viral titer in the three tissues. All mock-injected fish sampled during the were negative for virus, and fish that had died in the VHSV-exposed sampling tanks showed significant levels of virus in the pooled head kidney and brain as described above.

In fish exposed to the higher viral titer of 1 x 10^4 PFU/fish, three fish were sampled (1 fish/tank) on days 2, 4, 7, 10, 14, 21 and 28 days post-VHSV exposure (Figure 4-3, Table 4-2). Unlike fish in the low titer challenge, a relatively high viral titer was immediately detected in only the pooled head kidney/spleen on day 2 post-VHSV exposure (Figure 4-3, Table 4-2). However, as with the fin in the lower titer challenge, this was only detected in 1/3 fish tested, although the gill and fin from the same fish tested negative. While virus was detected in the pooled head kidney/spleen in all 3/3 fish sampled on days 4 and 21 post-VHSV exposure, it was only detected in 2/3 fish on days 7, 10 and 14, despite a positive detection of VHSV in the gill and fin in all 3/3 fish on each sampling day. By day 28 post-VHSV exposure, VHSV was not detected in the pooled head kidney and spleen in any of the fish tested (62% of fish overall), the gill in 1/3 fish (71% of fish overall), and the fin in 0/3 fish (62% of fish overall) (Table 4-2.). When viral titer in positive fish was analyzed, no significant difference was found between the three tissues, however, overall viral titer was statistically significantly higher in each tissue in fish exposed to the high titer (1 x 10^4 PFU/fish) on days 10 and 21 post-VHSV exposure relative to fish exposed to the lower titer (1 x 10^2 PFU/fish) ($P<0.05$).

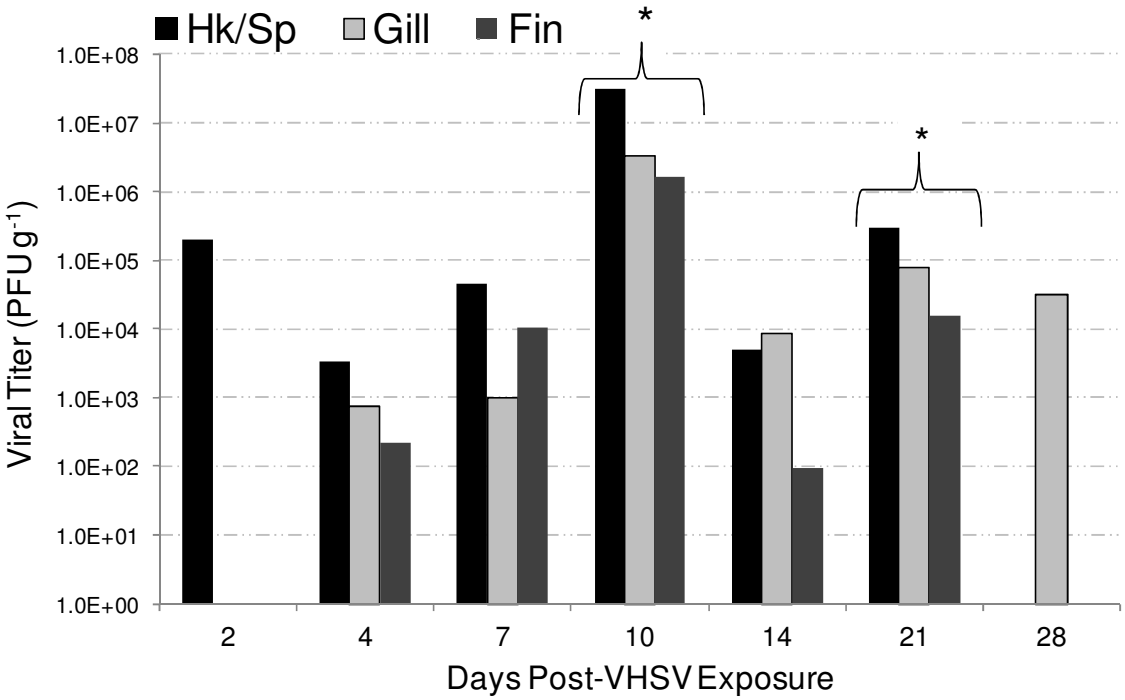


Figure 4-3. The geometric mean of VHS viral titer (PFU g^{-1}) determined by plaque assay (PA) in all three tissues (pooled head kidney/spleen (Hk/Sp), fin and gill) in fish exposed to a viral titer of 1 x10^4 PFU/fish over time. Astericks indicate where viral titer was statistically significantly higher in each tissue in fish exposed to the high titer (1 x 10^4 PFU/fish) on days 10 and 21 post-VHSV exposure relative to fish exposed to the lower titer (1 x 10^2 PFU/fish) ($P<0.05$).

High Titer - Day	2	4	7	10	14	21	28	Total	% Pos.
Kd/sp	1/3	3/3	2/3	2/3	2/3	3/3	0/3	13/21	62%
Gill	0/3	2/3	3/3	3/3	3/3	3/3	1/3	15/21	71%
Fin	0/3	1/3	3/3	3/3	3/3	3/3	0/3	13/21	62%

Table 4-2. VHSV prevalence determined by plaque assay (PA) in fish exposed to a viral titer of 1 x10^4 PFU/fish.

In the second challenge experiment, fish were exposed to a viral titer of 1 x 10^4 PFU/fish as in the previous challenge, however, VHSV load was analyzed by qPCR in the pooled head kidney/spleen, blood, brain gill, and fin in 3 fish sampled (1 from each tank) at each time point, days 5, 7, 9, 12, 14 and 21 days post-VHSV exposure (Figure 4-4, Table 4-3). While virus was detected in the pooled head kidney and blood on every sampling day (88% and 94% of all samples respectively), virus was not detected in the fin until day 12, 14 & 21 (31% of all fish sampled), and in the gill on days 5, 12, 14, and 21 (45% of all fish sampled). Only on day 9 was VHSV not detected in the pooled head kidney and spleen, but positive in the blood of one of the fish sampled (Table 4-3). Alternatively, in no case was there a positive detection of VHSV in the pooled head kidney and spleen, but not in the blood. VHSV detection in the brain also began on day 9 (44% of all fish sampled), however peaked to the highest viral load detected in all tissues on day 21. Viral titer in the pooled head kidney/spleen, blood, and brain was statistically significant relative to the fin and gill on day 14 post-VHSV exposure ($P<0.05$). Linear regression analysis confirmed a significant correlation between viral load detected in the pooled head kidney/spleen and blood over the course of the challenge experiment ($F_{(1, 14)} =42.783$, $R^2=0.75$) (Figure 4-5).

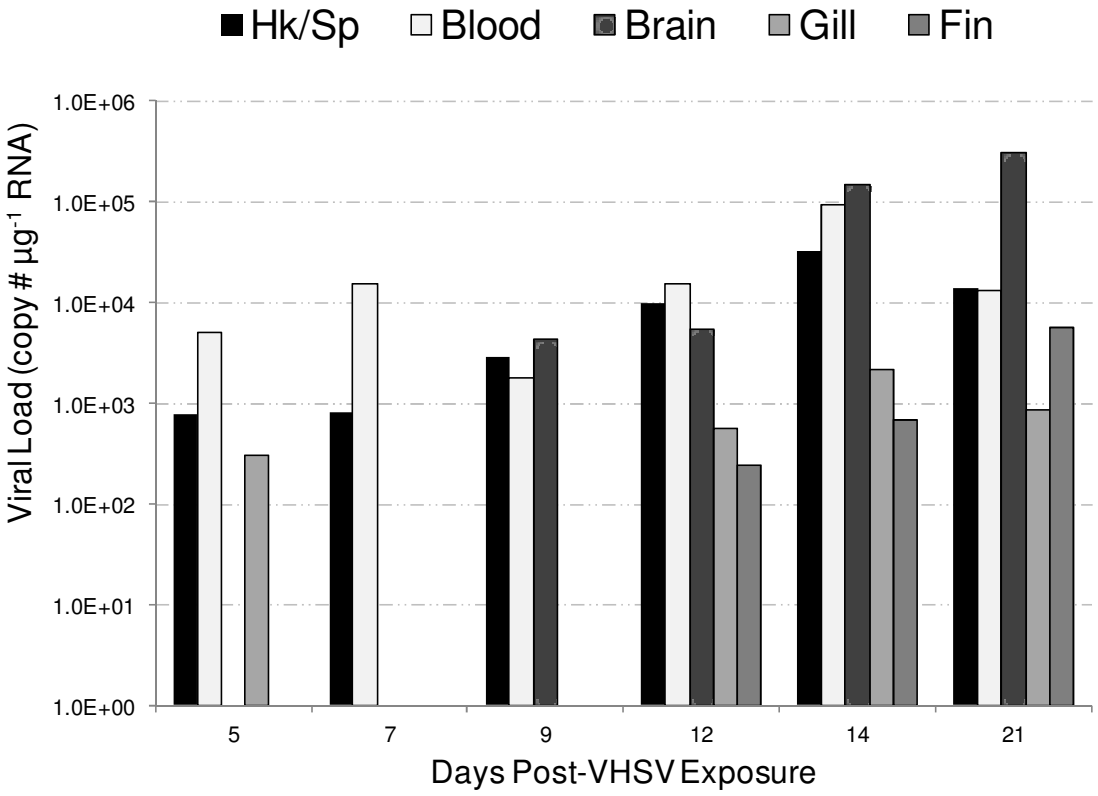


Figure 4-4. The geometric mean of VHS viral load (copy # μg^{-1} RNA) determined by qPCR assay in the pooled head kidney/spleen (Hk/Sp), fin, gill, blood and brain in fish exposed to a viral titer of 1 $x10^4$ PFU/fish over time.

qPCR High titer - Day	5	7	9	12	14	21	Total	% Pos.
Hk/Sp	3/3	2/3	2/3	3/3	2/2	2/2	14/16	88%
Blood	3/3	2/3	3/3	3/3	2/2	2/2	15/16	94%
Brain	0/3	0/3	1/3	2/3	2/2	2/2	7/16	44%
Gill	1/2	0/2	0/2	1/2	1/1	2/2	5/11	45%
Fin	0/2	0/2	0/3	1/3	1/2	2/2	4/14	29%

Table 4-3. VHSV prevalence determined by qPCR in fish exposed to a viral titer of 1 $x10^4$ PFU/fish.

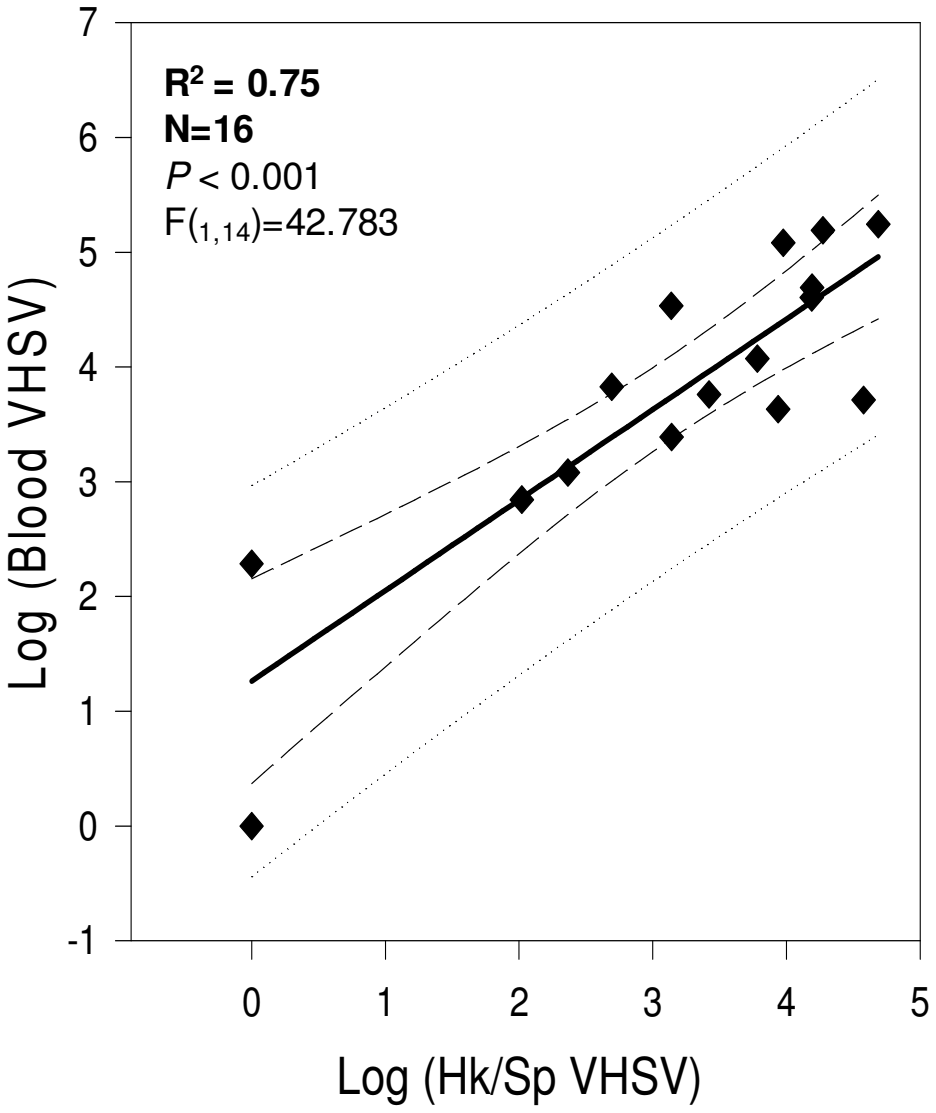


Figure 4-5. Linear regression analysis indicating a significant correlation between VHS viral load (copy # μg^{-1} RNA) detected in the blood versus the pooled head kidney and spleen (Hk/Sp) by qPCR.

Fluorescence microscopy imaging using a fluorescently conjugated monoclonal anti-VHSV antibody was conducted on sectioned brain tissues of fish 28 days-post-VHSV exposure. Despite being asymptomatic for VHS infection, the presence of VHSV positive erythrocytes was identified within the optic tectum of the brain (Figure 4-6 A & B). VHS virus was also identified with leukocytes in the same region (Figure 4-7). Additional tissue analysis also indicated heavy staining for VHSV positive neural cell bodies of the optic tectum (Figure 4-8 A), and axonal tracts within the telencephalon (Figure 4-9 A). The presence of VHSV in peripheral nerve axons was also visible in a longitudinal section of a cranial nerve (Figure 4-10 A), a cross-section of an axonal tract running through the thalamus (Figure 4-11 A), and hindbrain (Figure 4-11 B). Nuclear staining of the tissue revealed that virus remained in the cytoplasm of the neurons and was not associated with the nucleus in any stage of infection (Figure 4-8 B, Figure 4-9 B, Figure 4-10 B).

Discussion

This study was unique in that it examined the viral distribution and pathology in a population of largely asymptomatic adult yellow perch infected with the Great Lakes strain VHSV-IVb. This is in contrast to other studies that have only examined the susceptibility of juvenile fish to the VHSV-IVb, where young fish exhibited severe to moderate external signs of the disease including hemorrhage throughout the body, fin bases and exophthalmia at similar viral titer exposures, and viral distribution not yet

explored (Kim & Faisal, 2010b; Olson *et al.*, 2013). Alternatively, in the current study, clinical manifestations of VHS as external signs, including mild hemorrhage of the fins

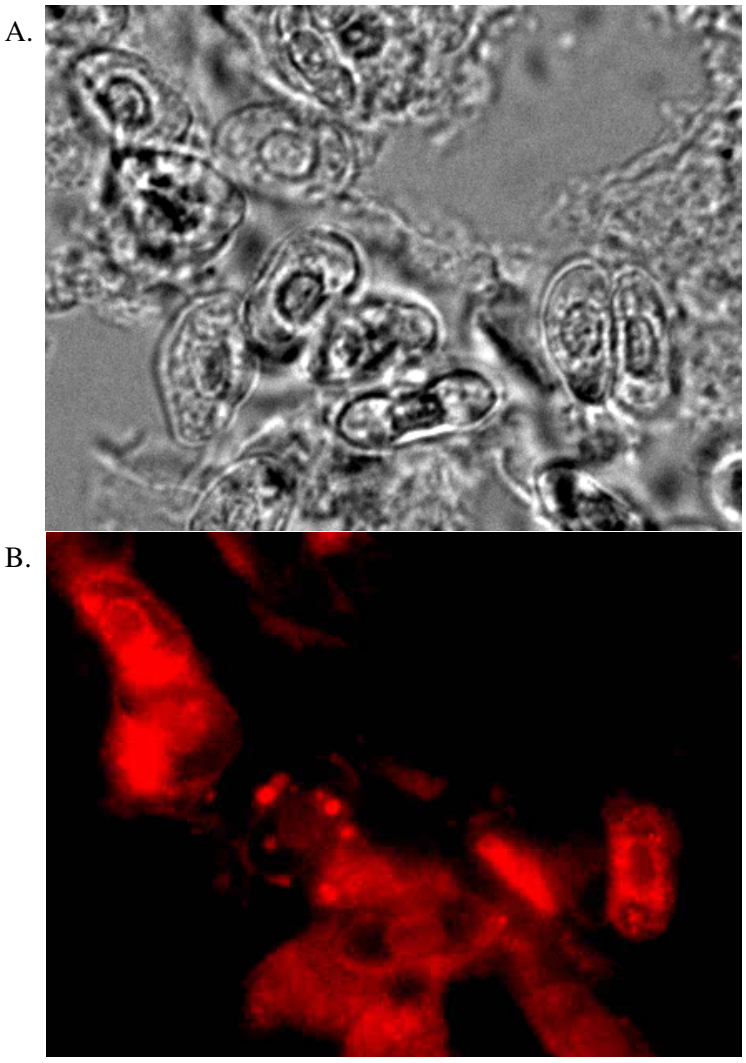


Figure 4-6. VHS virus positive erythrocytes within the brain tissue of VHSV-exposed fish. (A) Light microscopy DIC image of erythrocytes identified within the optic tectum of an adult yellow perch brain. (B) Fluorescent microscopy image using a monoclonal antibody to identify VHSV (*red*).

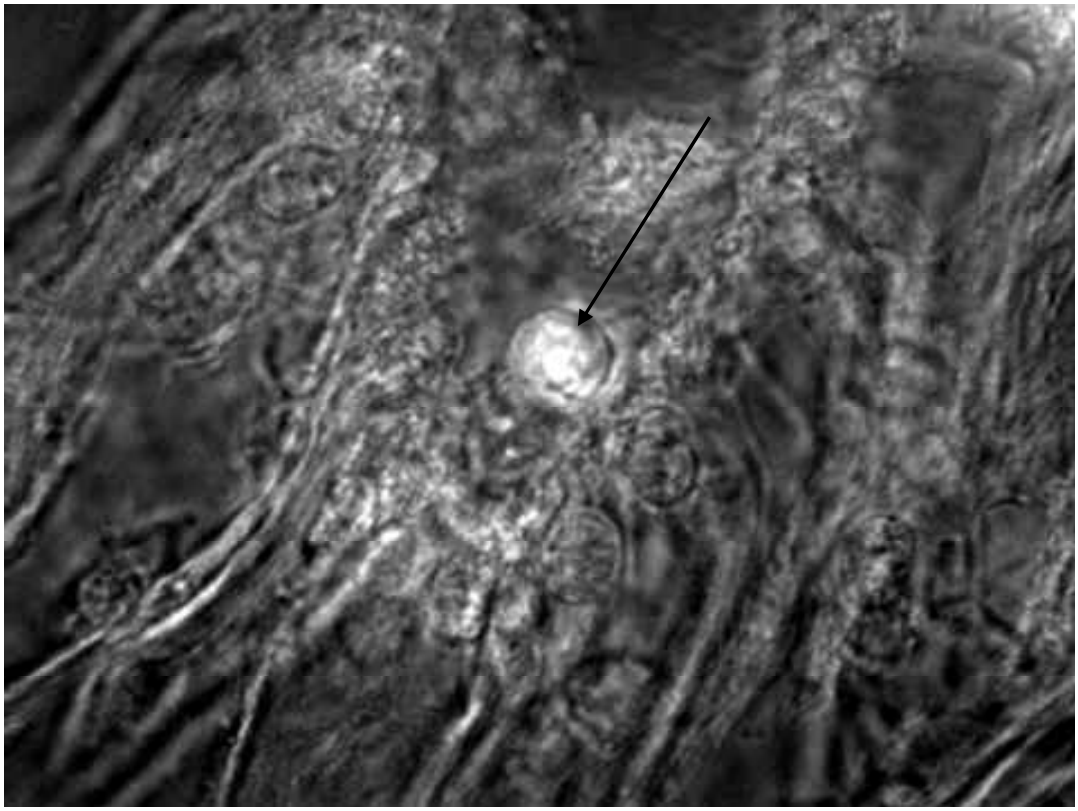
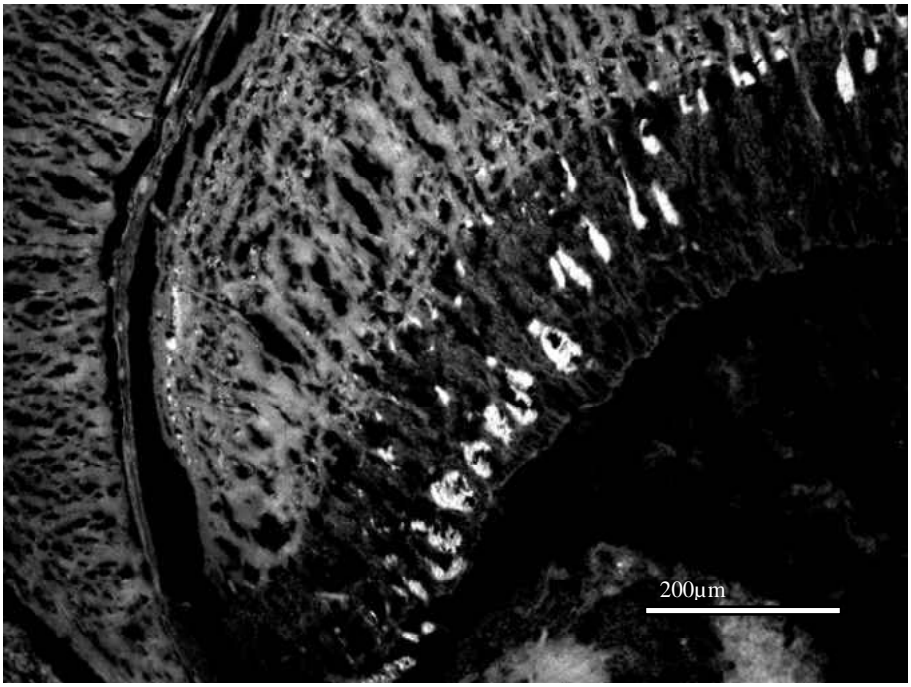


Figure 4-7. Fluorescence microscopy image indicating a VHS virus (white) positive leukocyte in a brain section of a VHSV-exposed adult yellow perch.

A.



B.

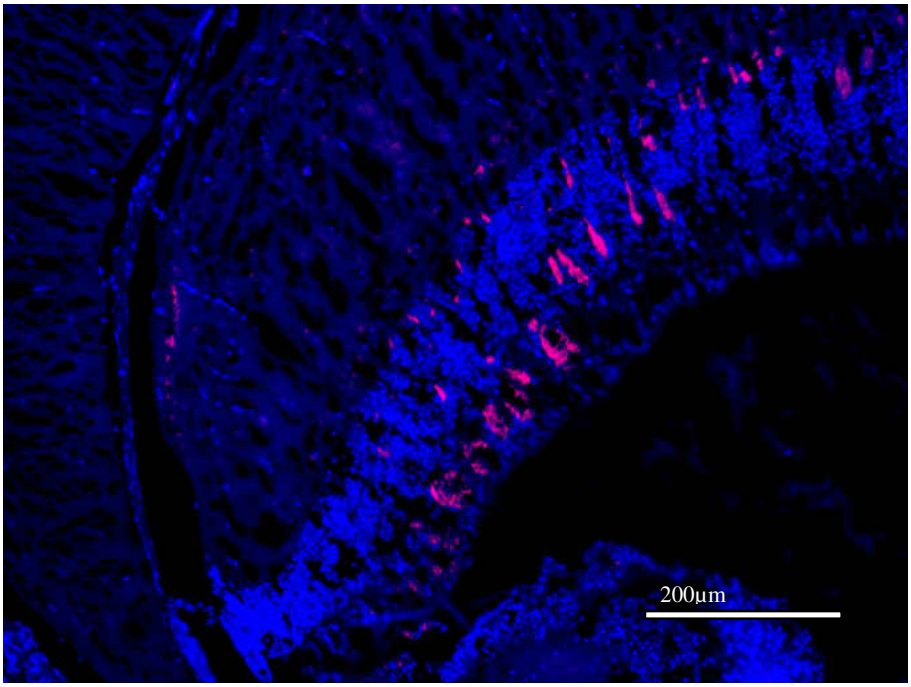
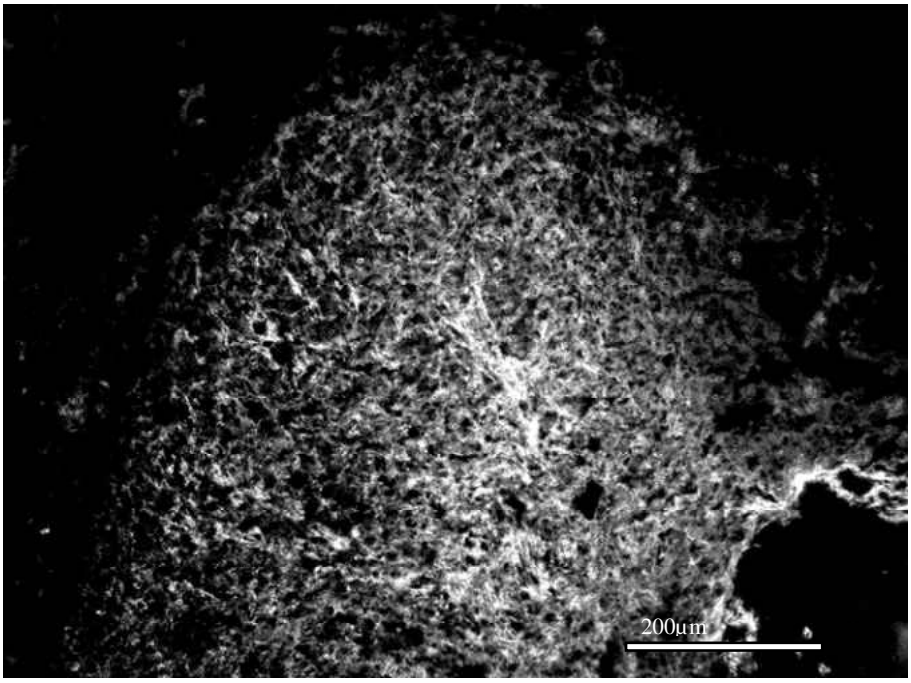


Figure 4-8. Fluorescence microscopy indicating VHS virus (A) (*white*) within neural cell bodies of the optic tectum. (B) Staining of the cellular nuclei within the tissues (*blue*) revealed that VHS virus (*pink*) remained in the cytoplasm of the neurons.

A.



B.

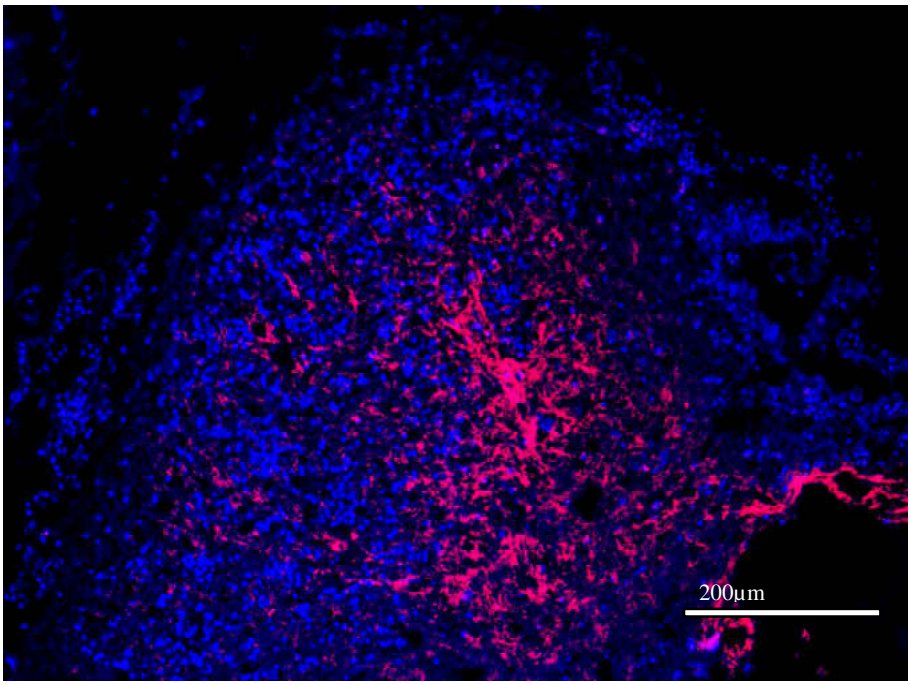
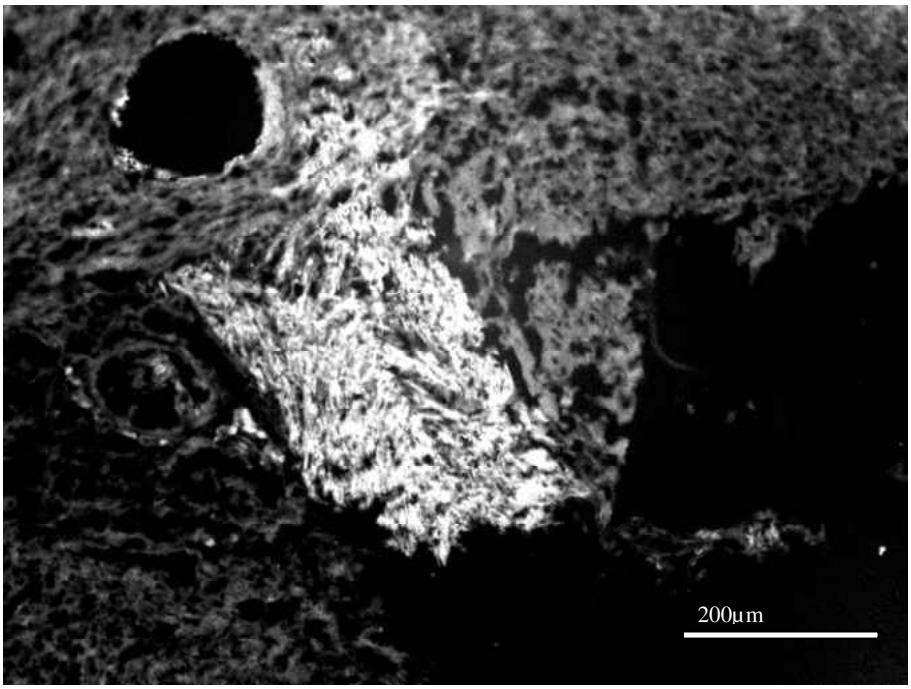


Figure 4-9. Fluorescence microscopy image in a brain section of a VHSV-exposed adult yellow perch indicating VHS virus (A) (*white*) within the axonal tracts of the telencephalon, and (B), VHS virus *(pink)* does not translocate with the cellular nuclei (*blue*).

A.



B.

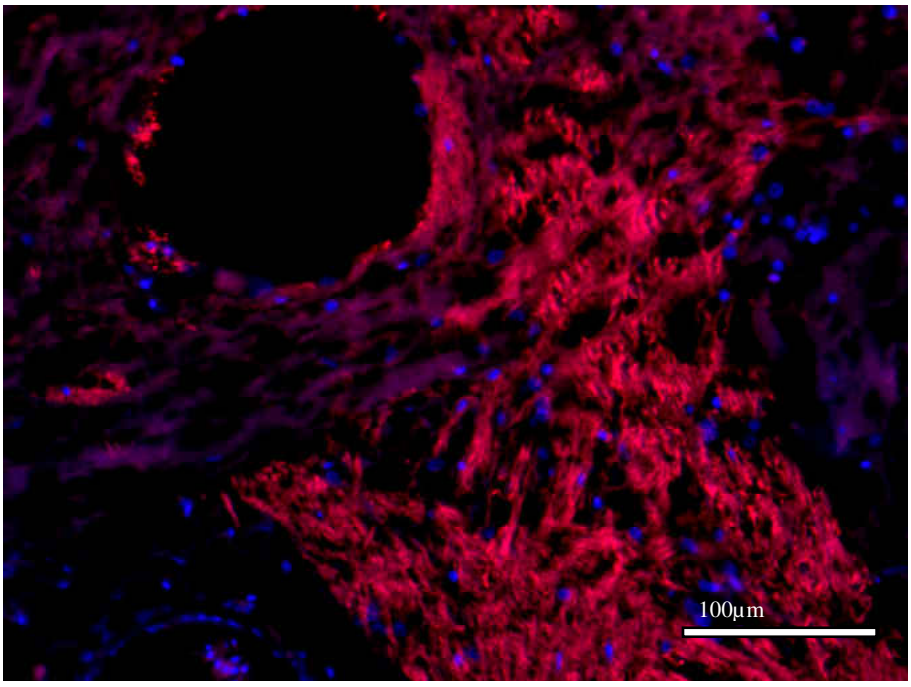
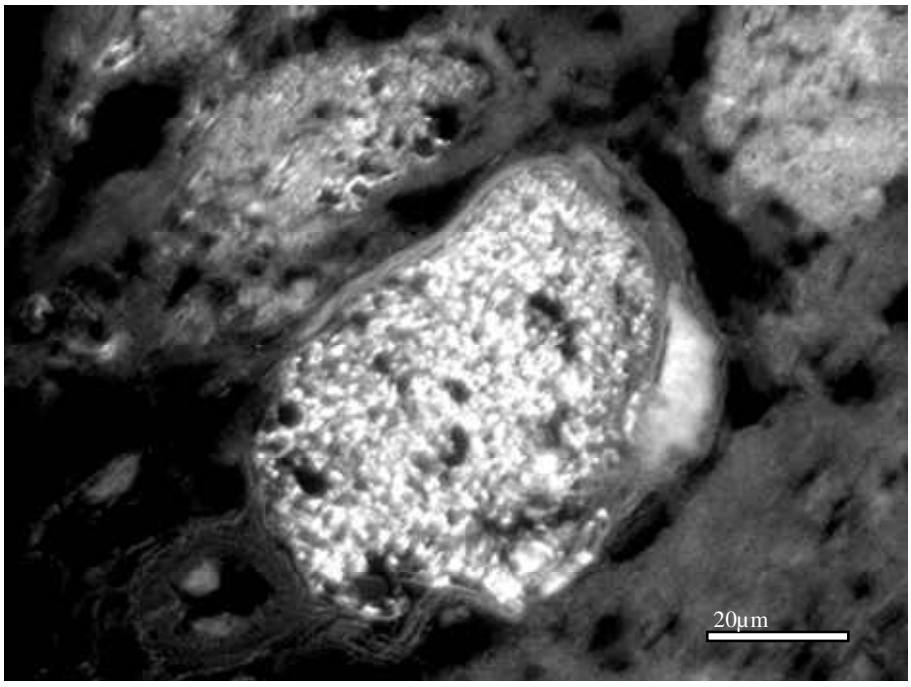


Figure 4-10. Fluorescence microscopy image in a brain section of a VHSV-exposed adult yellow perch indicating VHS virus (A.) (*white*) within a longitudinal section of a cranial nerve, and (B), VHS virus (*pink*) does not translocate with the cellular nuclei (*blue*).

A.



B.

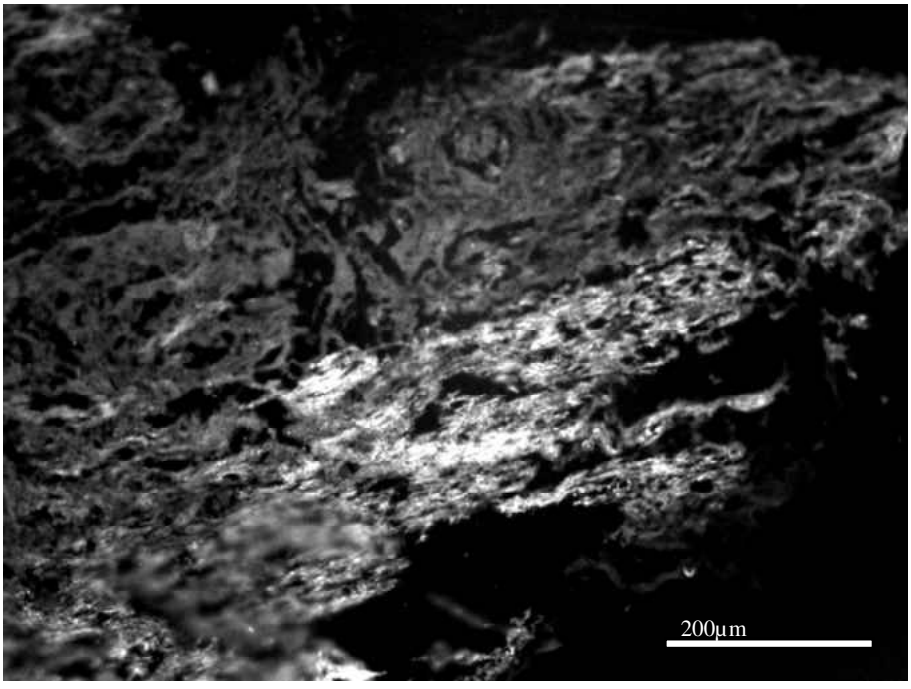


Figure 4-11. Fluorescence microscopy image in a brain section of a VHSV-exposed adult yellow perch indicating VHS virus (*white*) within (A) a cross-section of an axonal tract running through the thalamus, and (B) hindbrain.

and exophthalmia, were rarely observed in fish that had died during the adult yellow perch virus challenge; only internal signs were observed. In addition, particularly in fish injected with the lower titer of VHSV, the fish died very gradually instead of as a group. The occasional death of only a few fish at a time, combined with the lack of external signs, suggests that die-offs resulting from the manifestation VHSV in adult fish may easily go unnoticed in a natural lake environment. Fish that had demonstrated clinical signs during the early stages of infection also appeared to recover as survivors of the 28-day challenge.

In the present study, the identification of virus in the gill by plaque assay in fish which tested negative for infection in the head kidney/spleen suggests that this tissue may harbor virus in cases of low level infection. The detection of live, replication capable VHSV in the gill may be useful for the monitoring for VHSV in aquatic organisms since VHSV in the gill may also be an indicator of viral shedding in a chronically infected carrier fish. Similar studies with VHSV-Ia have identified the gill epithelium as a target for viral replication (Yamamoto *et al.*, 1992), and a recent study confirmed that a rainbow trout gill epithelial cell line (RTgill-W1), but not spleen macrophage cell line (RTS11), will support replication of VHSV-IVb (Pham *et al.*, 2013). This correlates with our findings of VHS virus in the gill, but not the pooled head/spleen samples, suggesting that the macrophage rich organs of the head kidney and spleen maybe capable of clearing the virus from these tissues, while the gill epithelium may not. Thus, the relatively high prevalence of VHS virus detected by plaque assay suggest that the gill in particular may

serve as a useful tool for non-lethal detection measures, particularly in fish that may not have actively replicating virus in the head kidney or spleen.

When VHS virus was examined by qPCR, virus was consistently detected in the blood in nearly all (94%) of the fish that were sampled with a significant correlation to viral load detected in the pooled head kidney/spleen ($F_{(1, 14)} = 42.783$, $R^2=0.75$). In one of the fish analyzed, a positive detection of virus in the blood correlated with a negative detection in the pooled head kidney and spleen, suggesting that analysis of the blood may be even more representative of viral infection. Excluding this fish from the analysis (as a possible outlier), however, increases the correlation between the pooled head kidney and spleen even greater ($F_{(1, 14)} = 91.152$, $R^2=0.87$). The finding of a significant correlation between viral load detected in the blood and the pooled head kidney/spleen over the course of the 21-day challenge suggests that blood could be used as an accurate non-lethal sampling tool in place of the lethal analysis of the head kidney and spleen.

As in this study, VHSV-IVb antigen-associated leukocytes have been detected in several other Great Lakes fish species (Al-Hussinee *et al.*, 2010; Al-Hussinee *et al.*, 2011). Blood has also been used for the detection of VHSV-Ia relative to viral load in head kidney and spleen in infected rainbow trout (*Oncorhynchus mykiss*) (Cuesta & Tafalla, 2009), however, with less robust results, possibly due to inhibitory enzymes present in the extracted RNA due to the presence of serum. Alternatively, VHSV-IVb may have evolved a slightly higher affinity for cells in the blood, particularly erythrocytes, resulting in a more pathogenic virus capable of infecting a wide array of new host-species. In addition, as research in anti-VHSV antibody production and detection continues to

improve (McLauchlan *et al.*, 2003; Millard & Faisal, 2012; Pereiro *et al.*, 2012; Purcell *et al.*, 2012), the potential use of blood and serum as a non-lethal means for both the detection of VHSV and anti-VHSV antibody holds more promise than ever before.

The discovery of VHSV antigen associated erythrocytes in the brain tissue of infected fish also provides valuable insight into how the virus is potentially spread throughout various tissues within the fish. Unlike mammalian erythrocytes, nucleated red blood cells of fish have been shown to be fully functional immune cells capable of eliciting pathogen associated molecular pattern-specific responses (Montero *et al.*, 2011). Because fish lack a lymphoid system, and teleost erythrocytes maintain a nuclei and functional cellular machinery, it would be highly beneficial for the virus to have adapted the use of these cells for quick transport and dispersal to tissues throughout the body. The infection of red blood cells in fish is not novel in that is has long been the characteristic signature of the iridovirus, erythocytic necrosis virus (ENV) (Glenn *et al.*, 2012; Smail, 1982). Unlike ENV, however, which is easily visualized by the identification of intracytoplasmic inclusion bodies in infected cells, VHSV, like all rhabdoviruses, sheds its glycoprotein upon cellular entry and replicates in the cytoplasm, thus the viral capsid cannot be easily visualized. While in this study, fluorescence microscopy confirmed the presence of VHS virus in red blood cells using an N-gene specific monoclonal anti-VHSV antibody, studies to determine whether or not the virus is still infectious, or can replicate within these cells remain.

Viral detection by plaque assay, qPCR, as well as fluorescence microscopy, also confirmed VHS virus accumulation in the brain, particularly in the later stages of

infection in association with hemorrhage and the likely introduction through VHSV infected blood and virally infected neural axons. This finding is consistent with other reports of VHSV in the brain tissues of chronically infected juvenile rainbow trout (*Oncorhynchus mykiss*) (Brudeseth *et al.*, 2002) and Pacific herring (*Clupea pallasii*) (Lovy *et al.*, 2012), providing further evidence that the same is true of VHSV-IVb in adult yellow perch. Targeting of the brain is a characteristic similar to another rhabdovirus capable of infecting multiple host-species, rabies. This small, negative strand RNA virus is exclusively known as a prototypical neurotrophic virus, but the mechanisms of neural transport via peripheral axons to the brain may be similar. Like rabies, our results suggests that the VHS virus spreads in the blood, and it can enter CNS neurons at a peripheral sites and travel through the spinal cord to the brain of the infected host (Kelly & Strick, 2000). Unlike the rabies virus, however, it is unknown if the VHS virus is capable of replicating within the neuron and inducing apoptosis, or if the virus can sustain in the cranial neurons long-term.

In summary, the results of this study suggest a low level of VHSV-IVb infection in adult yellow perch may easily go undetected since no clinical signs were apparent in most of the fish in this study. When blood and brain tissues were included in the second challenge, analysis by qPCR demonstrated that blood, in combination with plaque assay of the gill, might provide a viable and accurate way of screening for VHSV in larger, more valuable fish in a non-lethal manner. The finding of significant virus in the brain, possibly through the transport of VHSV associated erythrocytes and neural axons,

however, also suggests that this tissue may be the target organ and source of chronic infection in long-term asymptomatic carriers of infection in adult yellow perch.

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Chapter 5

Final Discussion

Overall, for a successful viral infection and the onset of disease to occur, proper conditions must be met within three primary areas, the external environment, the species population, and the individual fish immune system (Figure 5-1). Previous studies have verified that the VHSV-IVb virus is only capable of replicating in water temperatures below 18°C, with optimal replication occurring between 9-12°C (Goodwin & Merry, 2011). External stressors in a lake such as low oxygen as in hypoxia or anoxia, combined with rapid temperature changes characteristic to large lake environments in the winter and spring, can also compromise the fish immune system, increasing chances of susceptibility. Farm raised fish held at similar temperatures may also become immune compromised due to stresses such as shipping and crowding during transport, making them more susceptible to disease outbreak as well. Another significant factor that will determine disease susceptibility within a population is the species of fish, since large differences appear to exist between fish species in susceptibility to VHSV-IVb.

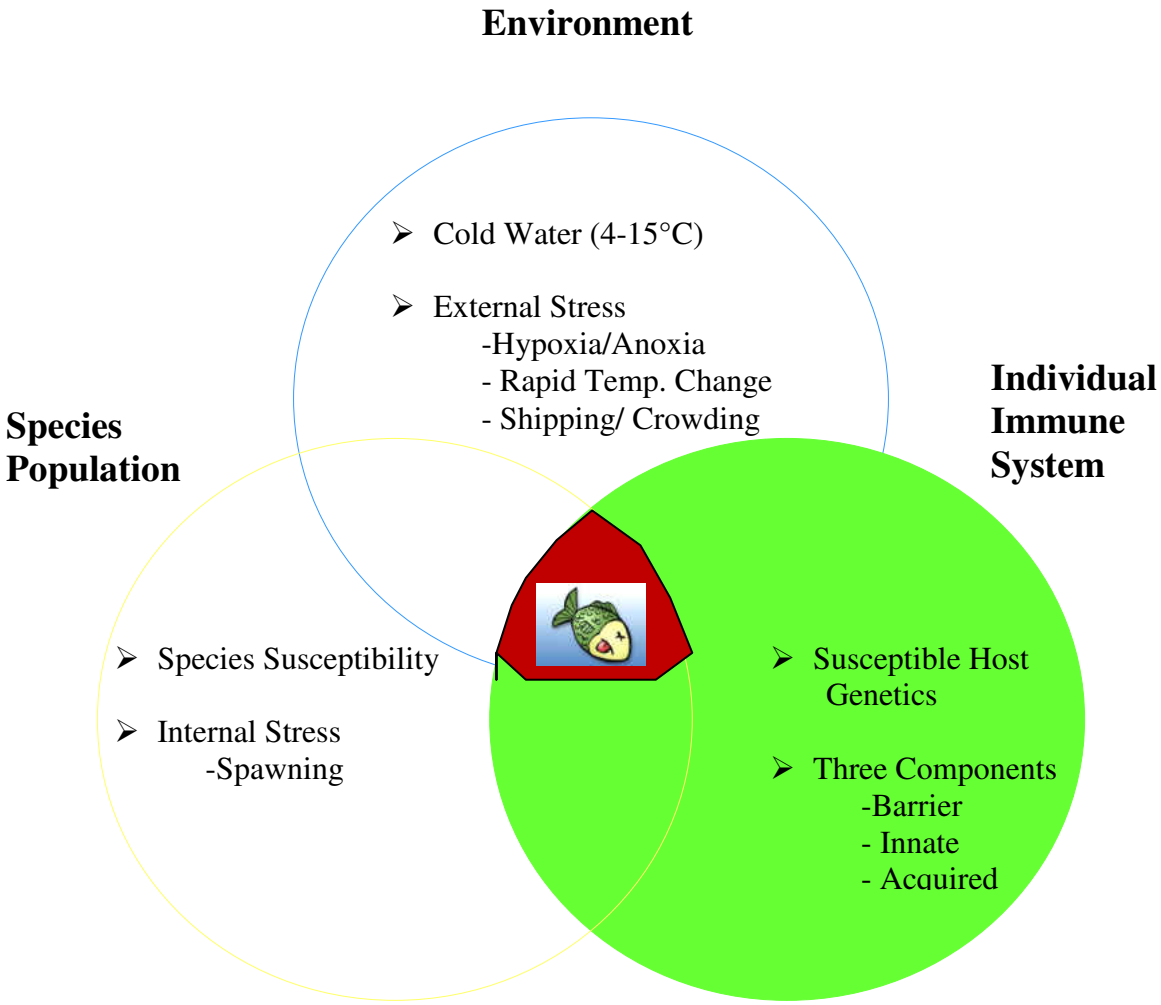

Figure 5-1. Conditions must be met in three generalized components for successful VHSV infection and the onset of disease to occur. A common variable influenced and shared in all three components to VHS susceptibility is stress.

Populations within a species, however, will react differently to VHSV exposure due to genetic differences and stresses brought on by external changes in the environment, and/or internal stress such as spawning. Within an individual fish, activation of a proper immune response will ultimately dictate whether or not the disease will progress. The results presented here indicate that a large component of a healthy immune response will depend on genetic factors and stress, both internal and external. Thus, provided general conditions for the virus to replicate are met (i.e., water temperature and species susceptibility), stress and genetics are ultimately the two factors which will influence disease susceptibility and outbreak the most.

The first chapter of this study identified major differences in susceptibility to the Great Lakes VHSV-IVb strain within a single species of yellow perch in three populations derived from different genetic and geographic origins. With all environmental conditions held equal, the most predominant differences between individuals, and thus the factors that would determine the outcome of VHSV infection and disease, were host genetics. Because genetic diversity was not significantly different between populations, a factor often attributed to differences in susceptibility to disease, it may be hypothesized that the existence of certain disease resistance genetic traits shaped by the host population's previous environment are responsible. As mentioned earlier, studies have confirmed that fish from the Great Lakes region most likely have never been exposed to VHS virus or any similar rhabdovirus before the identification of VHSV-IVb (Elsayed *et al.*, 2006; Kim & Faisal, 2010a; Kim & Faisal, 2011; Olson *et al.*, 2013; Pierce & Stepien, 2012).

Therefore, previous generations have not been influenced by the same natural selection pressures as the fish from the East coast. Alternatively, the perch stocks from the Choptank River and Perquimans River, both river estuary environments on the east coast of the U.S., may have encountered marine strains of VHSV (Gagne *et al.*, 2007) and other viruses. Thus, natural selection pressures would have eliminated fish that did not survive viral exposure, allowing for the development of more virus resistant offspring to occur. In addition, however, the individual stress level also appeared to be highest in the susceptible population originating from Lake Winnebago, WI. As mentioned earlier, it was reported that Lake Winnebago fish seemed more easily stressed by human interactions (e.g., tank cleaning, adjusting feeders, and peering into or walking by the tank), than Choptank or Perquimans River fish. In all vertebrates, as well as fish, elevated levels of stress can result in elevated cortisol and a chronic overactive pro-inflammatory state, which can contribute to an increase susceptibility to disease (Miller *et al.*, 2008).

The second chapter of this study focuses exclusively on the early innate immune pathways triggered relative to viral load by VHSV-IVb infection in a highly susceptible population of fish during the very early stages of infection. The administration of VHSV-IVb by IP-injection into juvenile yellow perch resulted in 84% mortality, indicating their high susceptibility to this disease. However, it was also found that yellow perch mount a strong innate immune response to VHSV-IVb as demonstrated by significant increase in expression by pro-inflammatory genes IL-1β and SAA. In addition, a down-regulation of an anti-viral immune response indicated by a decrease in Mx gene expression in the hematopoietic tissues, head kidney and spleen, may represent a strategy utilized by the

virus to increase replication. It is therefore also possible that the fish from Lake Winnebago, WI may have experienced significantly high down-regulation of the Mx gene, resulting in an insufficient antiviral innate immune response and a high pro-inflammatory response, possibly as a result of increased chronic stress, resulting in an increase in overall susceptibility to the disease relative to the other stocks.

In the final chapter of the study, the viral distribution was tracked over time in infected fish. Interestingly, in the juvenile yellow perch infected with a similar dose of VHSV in the first two chapters of the study, the clinical signs observed in the infected perch were similar to signs of VHS virus in other species, including septicaemic hemorrhages throughout the body and at the base of the fins. The transparency of dead juvenile perch allowed us to observe severe internal hemorrhages around the brain, suggesting that infected fish may suffer permanent damage to the central nervous system and that the brain may be a potential viral reservoir in surviving fish. In the adult fish, however, rarely were external clinical signs of the disease observed. Mild exophthalmia, or swelling of the eyes was the most common sign exhibited, however, it also seemed to disappear in the fish over time, again suggesting internal hemorrhage around the brain of the fish (Figure 5-2). The final study confirmed this hypothesis with the discovery of high virus loads in the brain of VHSV infected fish, particularly in the later stages of infection. In addition, this study demonstrated that analysis for VHSV in the blood by qPCR, in combination with plaque assay of the gill, may provide a viable and accurate way of screening for VHSV in larger, more valuable fish in a non-lethal manner.

Figure 5-2. Exophthalmia, or swelling of the eye was the most common clinical sign associated with VHSV infection in adult yellow perch.

From the culmination of this and previous studies, we can begin to understand how VHSV-IVb infects yellow perch in different stages of infection. In the waterborne transmission of virus, recent studies suggest that the main portal of viral entry and replication may be at the base of the fins (Harmache *et al.*, 2006; Montero *et al.*, 2011), as well as oral transmission of VHSV through the predation of infected fish (Schonherz *et al.*, 2012). Once a fish is infected with the VHSV, the suppression of the interferon response, combined with activation of a pro-inflammatory response, may result in successful replication of virus in the target tissues, head kidney and spleen. The spleen and head kidney of the fish represent hematopoietic tissues that are rich in leukocytes and erythrocytes from the continual circulation of blood. Therefore, the detection of VHSV in the blood and VHSV-associated erythrocytes and leukocytes, suggest a mechanism for

the virus to be quickly transported and dispersed to all tissues of the fish in conjunction with internal hemorrhaging. Since VHSV-IVb is capable of replication primarily within epithelial cells (Batts & Winton, 2007), translocation to the skin and gills (*shown in chapter 4*) can rapidly occur and represent key tissues where virus shedding may take place. Targeting of the brain by VHSV-IVb was also demonstrated in this study, possibly through the transport of VHSV-associated erythrocytes and retrograde travel through peripheral axons. This and other studies also suggest (Brudeseth *et al.*, 2002; Lovy *et al.*, 2012) that the brain may be an additional target organ and source of chronic infection in long-term asymptomatic carriers of infection in adult yellow perch. Once the virus establishes itself, this opportunistic pathogen may remain hidden or latent in apparently healthy populations of fish until a significant stressor again triggers an active infection, repeating the cycle of infection and disease once more.

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Wendy J. Olson

EDUCATION

Doctorate of Philosophy in Biology *Spring 2013*
University of Wisconsin-Milwaukee
Pathogenicity of the VHS Virus in the Yellow Perch (Perca flavescens)

Masters of Science in Aquatic Biology *December 2009*
University of Wisconsin-Milwaukee
Great Lakes Water Research Institute
Coastal Biogeochemistry in Lake Michigan

Bachelors of Science in Biochemistry
Bachelors of Science in Conservation & Environmental Science *June 2006*
University of Wisconsin-Milwaukee
Mercury Toxicology

RESEARCH EXPERIENCE

04/12-10/12 **Research Biologist**
International Pacific Halibut Commission (IPHC), Seattle, WA
United States Geological Survey (USGS) - Marrowstone Marine Field Station, Nordland, WA

- Conducted a disease survey for the protist parasite, Ichthyophonus, in the Pacific Halibut (*Hippoglossus stenolepis*) throughout the Northeast Pacific Ocean.
- Responsible for the processing and analysis of tissue samples by microscopy and culturing techniques under the direction and collaboration with scientists at the USGS Marrowstone Marine Field Station.

9/09-06/12 **Research Assistant / PhD candidate**

University of Wisconsin-Milwaukee Department of Biological Sciences
United States Geological Survey (USGS) - Western Fisheries Research Center, Seattle, WA

- Designed and implemented laboratory experiments to answer basic and applied research questions pertaining to the study of the VHS virus in the yellow perch .
- Utilized molecular techniques including RT-PCR & qPCR to directly measure virus and immune gene expression.
- Incorporated cell culture techniques, flow cytometry, and immunofluorescent microscopy to study the activation of various types of immune cells in response to different pathogen signals.
- Completed Aquavet I, a four-week training program in Aquatic Veterinary Medicine taught by Cornell University College of Veterinary Medicine on full scholarship.

06/07-9/09 **Research Assistant / MS Graduate Student**

University of Wisconsin-Milwaukee Great Lakes Water Research Institute

- MS thesis research project examining how biogeochemical processes in coastal Lake Michigan influence CO_2 and nutrient dynamics.
- Extensive lab and field experience collecting and culturing predominant phytoplankton species.

01/08-05/08 **General Chemistry Teaching Assistant**

Concordia University Department of Natural Sciences, Milwaukee, WI

- Taught second semester General Chemistry lab and led a weekly class discussion.

12/05- 06/07 **Research Technologist**

Medical College of Wisconsin, Milwaukee, WI

- Independently managed research projects studying the mechanisms of HIV-1 genetic integration and replication within dendritic cells.
- Conducted multiple cell based assays examining protein function and interaction using fluorescence microscopy, confocal imaging, ELISA and flow cytometry.
- Used molecular techniques involving siRNA to silence specific genes of interest.

- Regularly organized, presented, and discussed experiment results with a team of other scientists.
- Assisted/trained a steady rotation of medical and new graduate students on lab research procedures and specific projects.
- Maintained budget for new laboratory set-up and maintenance operations.

9/05-12/05 **Independent Senior Research**

University of Wisconsin-Milwaukee Department of Chemistry and Biochemistry

- Utilized protein separation, spectroscopic analysis, AA and ICP-MS techniques to locate and identify mercury binding proteins in the Chinook salmon *(Oncorhynchus tshawytscha*).
- *Formally presented the results of this research at the Eighth International Conference on Mercury as a Global Pollutant in Madison, WI. (Aug 6-11, 2006).*

6/00- 6/05 **Quality Control Chemist**

Sigma-Aldrich Corporation Fine Chemicals Inorganics Division, Milwaukee, WI

- Designed and implemented methods of analysis involving new technologies resulting in greater speed and accuracy of testing quality and quantity of inorganic compounds.
- Regularly trained new employees as well as provided troubleshooting support to fellow coworkers regarding inorganic analysis instrumentation and methods.
- Actively involved in the validation/testing of various chemical reagents to meet specific pharmaceutical and/or research grade quality guidelines.
- Continually involved in compliance issues regarding documentation and process control to meet ISO 9000 regulations.

PUBLICATIONS

Olson W, Emmenegger E, Glenn J, Winton J, Goetz F. Viral distribution and pathology of the Great Lakes viral hemorrhagic septicemia virus (VHSV-IVb) in adult yellow perch *(Perca flavescens)*
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