IDENTIFICATION OF HOST RANGE, SUSCEPTIBILITY, AND DISEASE COURSE OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IN GREAT LAKES FISH SPECIES

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ABSTRACT

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Viral hemorrhagic septicemia virus (VHSV) is an emergent pathogen throughout the Laurentian Great Lakes basin that has been associated with mass mortality events in a number of freshwater fish species and poses one of the greatest challenges to fisheries managers. As a result, a number of studies were undertaken to compare interspecies susceptibility to VHSV and investigate the virus-host interactions in order to gain insights into the potential ecologic impacts of VHSV. The first of these experiments included, equal groups of representative Great Lakes fish species that were intraperitoneally (i.p.) injected with a single dose of VHSV strain MI03. Findings from the study revealed that ten of the eleven species could succumb to VHSV infection, albeit at differing cumulative mortalities. The muskellunge (*Esox masquinongy*) and largemouth bass (*Micropterus salmoides*) experienced 100% cumulative mortality, while salmonid species experienced little to no mortalities. However, the study could not be used to compare susceptibility among Great Lakes fish, therefore another study was designed, using a broader range of doses and increased numbers of fish to compare the susceptibility of juvenile fish species based on the calculated median lethal dose of infection by i.p. injection (IP-LD_{50}). The study identified fish species of high, medium, and low susceptibility based on the LD_{50} values. The

largemouth bass were the most susceptible, with an IP-LD_{50} value of 1.5×10^2 plaque forming units (PFU), while salmonids were the least susceptible, with IP-LD_{50} values no less than 1.4×10^6 PFU. Despite the variability noticed in susceptibility, dead and moribund fish from all species exhibited the classical signs of VHSV infection. In a similar study, the IP-LD_{50} muskellunge resulted in an IP-LD_{50} of 2.2 PFU and an immersion median lethal dose (IM-LD_{50}) of 1.7×10^4 PFU / mL. As the most susceptible host, muskellunge were then exposed to doses of high, medium and low virus concentration by immersion which led to an acute, subacute, and chronic development of disease. Given the variability noted in the progression of disease, a fourth study was conducted to evaluate VHSV distribution, load, and histopathologic changes in muskellunge tissues collected in parallel at predetermined timepoints. The highest levels of VHSV appeared in the heart and liver at 6 days post infection (d p.i.) which was approximately 5.93×10^8 and 5.20×10^9 PFU / g tissue, respectively. The last study entailed assessment of viral shedding from survivors of VHSV IVb infection. When juvenile muskellunge were exposed to VHSV IVb at a relatively low concentration, viral shedding continued for up to 105 d p.i. and at rates that were highly variable, yet as high as 1.36×10^5 PFU $fish^{-1}$ $hour^{-1}$. Survivors of infection were re-challenged, at which time shedding of VHSV IVb was reinitiated. Viral shedding was noted in one group of fish not re-exposed to VHSV IVb, and was thus attributed to handling stress.

DEDICATION

To Sunnie, Mom, Richard, Shelly, Elmer, and Greyson.

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INTRODUCTION

Viral hemorrhagic septicemia (VHS) is a serious viral infection of teleosts in the northern hemisphere and is a reportable disease to the World Organization for Animal Health (OIE). The disease course of infected fish may vary, but the most feared aspect of VHS infection has been its ability to cause widespread mortality events within a relatively short period of time. The causative virus, Viral hemorrhagic septicemia virus (VHSV) has caused fish kills that have been widely reported from as early as the 1930s in farmed rainbow trout (*Oncorhynchus mykiss*) of Europe (Wolf 1988) to wild marine and freshwater fish populations in North America (Wolf 1988; Meyers and Winton 1995; Smail 1999; Gagné et al. 2007; Groocock et al. 2007; Lumsden et al. 2007).

The virus is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Tordo et al. 2005), which is enveloped, bullet-shaped, and contains a negative single strand of ribonucleic acid (RNA; Wagner and Rose 1996). The non-segmented genome is approximately 11 kb in length and encodes for five structural proteins and a non-structural protein: nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), non-structural protein (NV), and the RNA-polymerase (L) (Wagner and Rose 1996; Schütze et al. 1999; Ammayappan and Vakharia 2009).

Presently, VHSV isolates can be differentiated into one of four major genotypes (designated I-IV) based upon sequence analysis of the G and N genes (Snow et al. 1999, 2004; Einer-Jensen et al. 2004, 2005). Each of these

genotypes has its geographic and host ranges. Genotype IV pertains to isolates from North America, Japan and Korea.

In 2003, VHSV invaded the Great Lakes basin, and was isolated from kidney and spleen tissues collected the same year from muskellunge (*Esox masquinongy*) from Lake St. Clair, MI. Phylogenetic analysis of the N and G genes later revealed that the VHSV muskellunge isolate was distinct from all four previously known genotypes, yet closely related to VHSV IVa, and therefore described as VHSV IVb (Elsayed et al. 2006). In subsequent years, a number of mass mortality episodes associated with VHSV infection have been reported throughout the Great Lakes in a number of fish species (Gagne´ et al. 2007; Groocock et al. 2007; Lumsden et al. 2007). The virus has since been isolated from over 28 Great Lakes fish species, many of which are game fish. This ever increasing list of species, which is the most of any isolate of VHSV to date, demonstrates the ability of this particular strain of VHSV (MI03) to infect a wide host-range of freshwater fish species. Isolations of VHSV in field surveys have yielded important data into the virus' origins and epizootiology, but little information about host susceptibility and disease course has been gained from spontaneously infected fish, as they are often dead for an unspecified period of time, which can lead to tissue autolysis, or were sampled as apparently healthy adults. Given that the virus has been identified in subclinical fish and amidst mass mortality episodes of fish, there is no clear indication of what species are likely to succumb to death, clear the viral infection, or become reservoir hosts.

Hence, there is a dire need to better define the interactions of this emerging VHSV sublineage with it wide range of susceptible hosts. For this reason, the studies outlined in this dissertation have been designed with the ultimate goal to better define VHSV IVb infection and thereby fill existing knowledge gaps.

Chapter 1 provides a detailed summary of viral hemorrhagic septicemia virus in the context of history, etiology, diagnosis, and research completed to date. In chapter 2, eleven fish species were screened by intraperitoneal (i.p.) injection using VHSV IVb strain (MI03) to evaluate whether fish would succumb to VHSV infection. Soon thereafter, a more extensive experimental infection via i.p. could be completed using stratified dose levels of virus in determination of a median lethal dose of infection (LD_{50}), as described in chapter 3. The calculated LD_{50} values were compared to provide an overview of susceptibility to VHSV IVb, and identify species that appear highly susceptible or resistant to infection.

Investigations into the diathesis of infection were conducted thereafter using muskellunge, as model host for disease course analysis. In chapter 4, the LD_{50} by intraperitoneal injection was compared to immersion challenge. Results of the waterborne exposure yielded information into the relationship of mortality event and exposure dose, and provided baselines for which a high, medium, and low dose to VHSV could be considered.

In chapter 5, muskellunge were experimentally infected with VHSV IVb by waterborne exposure at a relatively medium dose and sampled at predetermined

timepoints. The sequential pathology in a multitude of organs were assessed in parallel by the viral plaque assay, cell culture reisolation, and histology and evaluated for VHSV distribution, viral load, and microscopic damage.

Since our previous studies demonstrated the juvenile muskellunge could survive a low dose exposure of VHSV IVb, the studies described in chapter 6 reveal that muskellunge could shed VHSV in the surrounding environment at variable rates over an extended period of time. Once shedding ceased, the survivors were re-challenged to VHSV IVb at varying doses to determine whether shedding of virus could be reinitiated, correlated to exposure dose, and if the LD_{50} in previously exposed fish differed from those that were naïve.

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CHAPTER 1

Literature Review

CHAPTER 1

Literature Review

Abstract

Viral hemorrhagic septicemia virus (VHSV) is one of the most serious pathogens of finfish worldwide in terms of its wide host range, pathogenicity, disease course, and mortality rates. The disease was first documented in the 1930s in Europe in association with heavy losses in rainbow trout. Data collected over 50 years show that VHSV is a virus of marine origin: its ability to alternate between marine and freshwater environments remains an enigma which requires further investigation. In 2003, VHSV invaded the Laurentian Great Lakes basin causing devastating losses. VHSV is believed to have negative impacts on a number of important Atlantic, Pacific, and Great Lakes fish species. Phylogenetic analysis has offered clues into the geographic and host range of the virus, but sporadic outbreaks of the disease in uncommon locations have imparted unforeseen challenges in delineating the virus' regional distribution. The virus' ability to gain access to aquaculture farms has also positioned it as a disease of utmost priority once detected in these settings. Current diagnostic methods, while greatly improved, are hampered by the variability of disease course among susceptible species. In general, VHSV causes severe degeneration in the hematopoietic tissues of affected fish. Based on historical and current data, it is feared that VHSV will continue to mutate, expand to other geographic areas, and infect new host species. As a result, immediate international attention and coordination of

efforts are needed. The objective of this review article is to provide an updated synopsis on the current status of VHSV epizootiology and pathogenicity.

Historical background

Viral hemorrhagic septicemia virus (VHSV) is one of the most pathogenic viral diseases of finfish worldwide (Wolf 1988). The disease was first recognized as early as 1938 when Danish rainbow trout farms became stricken with what Schäperclaus described as “infectious kidney swelling and liver degeneration” (Schäperclaus 1938). A multitude of outbreaks were soon documented that were associated with heavy economic losses (Hill 1992). A viral etiology was strongly suspected by Schäperclaus (Schäperclaus 1954) because the disease was transmitted using bacteria-free filtrates of tissue homogenates from affected fish. Within three decades since the initial report, the viral etiology was confirmed by Jensen in 1963 when he isolated the virus on a rainbow trout gonad cell line (RTG-2) (Wolf & Quimby 1962), and promptly described it as “Egtved” virus, but the disease was renamed as Viral Hemorrhagic Septicemia by the Office of International Epizooties currently known as the World Animal Health Organization (Altara 1963). This terminology remained until 2000 when the International Committee for Taxonomy of Viruses changed the name of the virus into VHSV (Tordo et al. 2004). The virus was then isolated from a number of other European countries such as France, Italy and Poland (Bellet 1965, Besse 1965, Ghittino 1965, Miaczynski 1965).

By the 1970s, researchers embarked on a series of studies attempting to characterize and elucidate the biological and biophysical aspects of the virus (De Kinkelin & Scherrer 1970, De Kinkelin 1972, Ahne 1982a,b). Serologic investigations revealed the presence of two distinct strains of VHSV (Jørgensen 1971, Jørgensen & Meyling 1972), but isolates obtained from brown trout later revealed the presence of a third strain (De Kinkelin 1977). The discovery that a virus was responsible for mass mortalities in cultured rainbow trout prompted many investigations in Europe to characterize the newly isolated deadly virus (Lenoir & De Kinkelin 1975) and to develop sensitive diagnostic assays (Jørgensen 1972). Moreover, Koch's postulates were fulfilled through a series of experimental infection studies (Ord et al. 1976, Yasutake & Rasmussen 1968). By the late 1970s, it was believed that the "Egtved" virus was strictly a disease of rainbow trout farmed in freshwater.

Until the late 1980s, the geographic and host range of VHSV was believed to be confined to the waters of continental Europe (Wolf 1988), but Hopper (1989) and Brunson's (1989) discovery of VHSV in spawning chinook salmon (*Oncorhynchus tshawytscha*) at the Glenwood Springs Hatchery on Orcas Island, Washington State (WA), USA and from spawning coho salmon (*Oncorhynchus kisutch*) at the Makah National Fish Hatchery, WA confirmed the expansion of VHSV range to involve North America. The following year, two more isolations occurred in wild adult coho salmon from Lummi Bay Hatchery (Stewart et al. 1990) and Soleduck Hatchery in WA (Eaton & Hulett 1991). This surprise prompted North American regulatory agencies to implement health inspections

and surveillance of salmonids and other fish species. By 1990-1993, VHSV was isolated from sport caught Pacific cod (*Gadus macrocephalus*) in Prince William Sound (PWS), Alaska (Meyers et al. 1991, 1992, 1994). During this same time frame, VHSV continued to devastate freshwater and marine waters in Europe. Simultaneous reports of VHSV outbreaks were described in another marine fish; the turbot (*Scophthalmus maximus*) farmed in the Baltic Sea (Schlotfeldt et al. 1991). These findings raised the suspicion that VHSV was of marine origin.

In 1993, adult Pacific herring (*Clupea harengus pallasi*) returning to spawn in PWS were found positive for VHSV (Meyers et al. 1994). Meyers and Winton (1995) raised the suspicion that VHSV may negatively impact Pacific herring at the population level as it affected all age classes. Additional VHSV positive Pacific herring were sampled from British Columbia, Canada and Puget Sound, WA, which suggested that this particular strain of VHSV was spreading regionally and that it may have become enzootic in Pacific herring populations from Alaska to Washington (Meyers & Winton 1995). Then in 1999, VHSV was isolated from a mass mortality episode that involved multiple species in Alaska such as the Pacific herring, Pacific hake (*Merluccius productus*) and walleye pollock (*Theragra chalcomgramma*) (Meyers et al. 1999), thereby providing evidence that VHSV was more widespread in the marine environment than previously thought. Such discoveries in the coastal waters of Alaska and the Pacific Northwest led to a study conducted by Hedrick et al. (2003) which revealed the presence of VHSV in sardine (*Sardinops sagax*), mackerel (*Scomber japonicus*), Pacific herring, shiner perch (*Cymatogaster aggregata*), eulachon (*Thaleichthys*

pacificus), sablefish (*Anoplopoma fimbria*) and surf smelt (*Hypomesus pretiosus pretiosus*) sampled from British Columbia, Canada to the southern California coast.

The North American VHSV isolations ignited further testing of marine fish populations in European waters. Such marine surveys revealed the presence of VHSV in new geographic areas and infecting new hosts such as farmed turbot off the west coast of Scotland (Ross et al. 1994), Atlantic cod in the North Sea (Smail 1995), Atlantic herring (*Clupea harengus*) from the English Channel (Dixon et al. 1997), haddock (*Melanogrammus aeglefinus*) and sprat (*Sprattus sprattus*), blue whiting (*Micromesistus poutassou*), whiting (*Merlangius merlangus*), and lesser argentine (*Argentina sphyraena*) throughout the Baltic Sea, Kattegat, Skagerrak, and North Sea (Mortensen et al. 1999). In addition to Europe and North America, VHSV has also been isolated in Japan from wild Japanese flounder (*Paralichthys olivaceus*) in Wakasa Bay of the western Pacific Ocean (Takano et al. 2000, 2001) in 1999 and in Korea (Kim et al. 2003). Based on the information gathered from all over the world, by the late 1990s, a consensus started building that VHSV primarily affects marine fish and that VHSV may have a marine origin (Stone et al. 1997).

In 2003, a virus was isolated from samples of kidney and spleen tissues of adult muskellunge from Grosse Pointe Yacht Club, Lake St. Clair, Michigan by the Michigan State University Aquatic Animal Health Laboratory (Elsayed et al. 2006). Electron microscopy observations revealed the virus shared the characteristics bullet shape morphology of rhabdoviruses. However, this virus

was not fully until 2005. The muskellunge isolate characterization included genetic sequencing which revealed the Michigan isolate was VHSV and was closely related to the Pacific coast VHSV genotype IV isolates (Elsayed et al. 2006).

The devastative effects of VHSV IVb was first realized during the spring of 2005 when a mass mortality of freshwater drum (*Aplodinotus grunniens*), round goby (*Neogobius melanostomus*), and some muskellunge occurred within the Bay of Quinte, Lake Ontario, Canada (Lumsden et al. 2007d). Modeling estimates conducted by the authors approximated upwards of 30,000 freshwater drum to have died over a 40-day period. While this mortality event was the only one reported for 2005, subsequent VHSV isolations from mortality events and field surveys followed soon thereafter.

In 2006, isolations of VHSV dramatically increased during the spring and early summer months. Mass mortality events of a number of fish species were reported from different parts of Lake Erie, Lake Ontario, Lake St. Clair, and the St. Lawrence River (*Micropterus dolomieu*), white bass (*Morone chrysops*), and yellow perch (*Perca flavescens*) (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan; Records of the Aquatic Animal Health Program, College of Veterinary Medicine, Cornell University, New York; (Groocock et al. 2007). Species collected from these fish kills included the bluntnose minnow (*Pimephales notatus*), burbot (*Lota lota*), freshwater drum, gizzard shad (*Dorosoma cepedianum*), muskellunge, northern pike (*Esox lucius*), rock bass (*Ambloplites rupestris*), round goby, shorthead redhorse sucker

(*Moxostoma macrolepidotum*), silver redhorse sucker (*Moxostoma anisurum*), and smallmouth bass.

Mortality events in the Great Lakes ceased until August 2006, when large numbers of walleye (*Sander vitreus*) were reported dead in Conesus Lake, an inland lake of New York (Records of the Aquatic Animal Health Program, College of Veterinary Medicine, Cornell University, New York)**.** This surprising emergence of VHSV in a an inland lake approximately 35 miles south of Lake Ontario was the first isolation of VHSV in waters that were completely isolated from the Great Lakes. Field surveys of healthy fish conducted by the State University of New York, College of Environmental Science and Forestry isolated VHSV in over ten species, six of which were previously unreported and included black crappie (*Pomoxis nigromaculatus*), bluegill (*Lepomis macrochirus*), brown bullhead (*Ameiurus nebulosus*), channel catfish (*Ictalarus punctatus*), largemouth bass (*Micropterus salmoides*), pumpkinseed (*Lepomis gibbosus*), and rock bass (Records of the Aquatic Animal Health Program, College of Veterinary Medicine, Cornell University, New York). In the same year, VHSV was isolated from chinook salmon, lake whitefish (*Coregonus clupeaformis*) and walleye sampled during the fall of 2006 from areas of northern Lake Huron. An isolation of VHSV would also occur from archived lake whitefish samples collected in waters near Cheboygan, Michigan, in the fall of 2005 by the Chippewa Ottawa Resource Agency. Surveillance conducted in Lake St. Clair would also reveal the presence of VHSV in spottail shiners (*Notropis hudsonius*) and emerald shiners (*N.*

atherinoides) (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan).

By the early spring of 2007, reports of VHSV isolation were reported from Wisconsin, Michigan, and New York. The Wisconsin Department of Natural Resources (WI DNR) reported large numbers of dead freshwater drum in Lake Winnebago and Little Lake Butte des Morts during the spring (WI DNR News Release May 18, 2007) confirming the presence of VHSV in waters close to Lake Michigan. Soon thereafter, isolations were made in smallmouth bass, lake whitefish, and brown trout from nearby Wisconsin waters of Green Bay and Lake Michigan (WI DNR News Release May 24, 2007). At that same year, VHSV IVb was isolated from dead and dying black crappie, bluegill, largemouth bass, and pumpkinseed from Budd Lake, Michigan (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan). This particular isolation was surprising given that it was from a lake that is highly isolated from either Lakes Michigan or Huron.

During the same time several fish kills were reported from New York from which VHSV was isolated. In a press release by the New York Department of Environmental Conservation (NY DEC) dated June 19, 2008, VHSV was isolated from scores of dead smallmouth bass, rock bass and from one sampled lake trout (*Salvelinus namaycush*) in Skaneateles Lake. Subsequent isolations were reported from rainbow trout within the Little Salmon River, sunfish from the Seneca-Cayuga Canal, as well as sunfish collected from a privately owned pond in Ransomville, New York (NY DEC press release July 23, 2007).

In 2008, reports of VHSV isolation in Lake Michigan were common in the early summer months. Reports of a mass mortality of round goby were reported by the WI DNR in an area of Lake Michigan near Milwaukee (WI DNR press release June 5, 2008), which was soon followed by VHSV isolation in yellow perch collected during a survey in neighboring waters (WI DNR press release June 13, 2008). In a press release dated July 2, 2008 by the Illinois Department of Natural Resources, VHSV was isolated from subclinically infected rock bass and round goby from Lake Michigan. Other notable isolations of VHSV were reported by U.S. Fish and Wildlife personnel at the La Crosse Fish Health Center in the same year which were obtained from sea lamprey (*Petromyzon marinus*) collected at multiple sites within the Cheboygan River, Greek Creek, and Ocqueoc River (La Crosse Fish Health Center, Fish Health Newsletter Winter 2008) and from ovarian fluids of muskellunge collected from Clear Fork Reservoir, Ohio which is an inland lake located in north central Ohio (U.S. Fish and Wildlife Service press release June 17, 2008).

Around mid-May of 2009, VHSV was isolated by the MSU-AAHL from brown bullhead and muskellunge collected during a routine fish survey from Baseline Lake (Livingston County, Michigan) and Lake St. Clair, Michigan. The isolation of VHSV from Baseline Lake was the second inland lake of Michigan where VHSV was found. Within a matter of weeks, a mass mortality event of smallmouth bass was reported in Lake St. Clair, Michigan from which VHSV was once again isolated (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan). Positive isolations of VHSV were once again

obtained from smallmouth bass in Sturgeon Bay, which is a part of Green Bay, Wisconsin (WI DNR press release June 12, 2009). Three fish from Sturgeon Bay were submitted to the WI DNR after anglers had noticed skin lesions.

In January of 2010, VHSV IVb was positively identified in Lake Superior for the first time following field surveys in which over 874 fish samples were tested (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan; Records of the Aquatic Animal Health Program, College of Veterinary Medicine, Cornell University, New York). Mortality was not associated with these positive detections in areas near Paradise, Skanee, and the Apostle Islands of Michigan and St. Louis and Superior Bay of Wisconsin.

Prior to the original report of the invasion of the Laurentian Great Lakes by VHSV genotype IVb in 2006 by Elsayed et al., Gagné et al. (2007) isolated VHSV IVb from the tissues of mummichog (*Fundulus heteroclitus*) and three-spine stickleback (*Gasterosteus aculeatus aculeatus*) collected as early as 2000 in waters near New Brunswick (Canada). Subsequent isolations of VHSV IVb also occurred in 2002 and 2004 from striped bass (*Morone saxatilis*) and brown trout (*Salmo trutta trutta*) collected in waters near New Brunswick and Nova Scotia (Gagné et al. 2007). The studies of Gagné et al. (2007), suggested that VHSV IVb may have existed off the Atlantic coast of North America prior to its emergence in the St. Lawrence River and the Great Lakes basin.

Locations of VHSV IVb detection between 2005-2010 are shown in Figure 1-1 and a comprehensive list of Great Lakes fish species in which VHSV has been detected from are shown in Table 1-1.

More recently VHSV was isolated from rainbow trout in the United Kingdom (Stone et al. 2008). While this resurgence of VHSV in Europe is not surprising, the isolation reported by Stone et al. (2008) demonstrated the first VHSV isolate in the United Kingdom (UK) obtained from a freshwater species in a freshwater environment. The investigators attributed the outbreak to a nearby stream that may have been harboring the virus for a long period of time. The results of this report emphasized the fact that while legislation, biosecurity, and sound management practices can greatly reduce the spread of VHSV, the virus' covert nature enables it to continue expanding into new geographic locales. The re-emergence of VHSV was also experienced in Norway in seawater-farmed rainbow trout (Dale et al. 2009). The authors concluded that the outbreak was most likely a result of viral transmission from the marine environment. Both isolations have thus confirmed VHSV's capacity to emerge and resurge in the northern hemisphere.

Virus characterization

VHSV is a member of the order, *Mononegavirales*, family *Rhabdoviridae* and genus *Novirhabdovirus* (Tordo et al. 2007), which belongs to a group of bullet shaped viruses that measure approximately 70 nm in width by 180 nm in length. An envelope, which contains the major surface antigen, glycoprotein, encases the virus and is believed to be important for virus adsorption and attachment to susceptible cells (Smail 1999). Four structural proteins are present, along with a RNA dependent RNA polymerase (Lenoir & De Kinkelin

1975, Smail 1999, De Kinkelin 1980, Benmansour 1994). The VHSV genome is a single, negative-stranded ribonucleic acid (RNA) and is approximately 11-12 kb containing six open reading frames (Schu□tze et al. 1999) in the following order; nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonstructural protein (NV) and lastly the viral RNA polymerase (L) (Figure 1-2). Translated proteins have been measured with molecular masses as follows; 38-41 kDa (N), 21.5-25 kDa (P), 19 kDa (M), 72-80 kDa (G), and 157-190 kDa (L) (Lenoir & De Kinkelin 1975, McAllister & Wagner 1975).

Information regarding the gene functions of VHSV are largely provided by studies conducted on mammalian or other fish rhabdoviruses. The glycoprotein gene is particularly important in that it is the primary neutralizing surface antigen of VHSV and other rhabdoviruses (Jørgensen et al. 1995, Wagner & Rose 1996). The nucleoprotein is responsible for tightly enclosing the RNA genome thereby creating the ribonucleoprotein core (Wagner & Rose 1996). Both the glycoprotein and nucleoprotein genes have enabled investigators to conduct phylogenetic studies. The phosphoprotein, also known as the internal matrix protein is considered the “viral transcriptase” when associated with the L and P proteins (Emerson et al. 1975). The M protein acts as a bridge between the viral envelope and nucleocapsid (Wagner & Rose 1996), but is also said to play a regulatory role in viral transcription, replication, production, and budding (Finke et al. 2003a,b, Mebatsion et al. 1996).

Disease

Pathogenesis

The initial emergence of VHSV in rainbow trout farms led to a wealth of information about VHSV-associated clinical signs and histopathologic alterations in spontaneously and experimentally infected rainbow trout. Clinical signs, although non-specific can appear singly or in a combination of either dermal petechial hemorrhages, hemorrhages at the base of fins, severely pale gills, periocular hemorrhage, lethargy, and abnormal to erratic swimming (Yasutake 1975). These findings have been reproduced through experimental infection (Kim & Faisal 2010, Figure 1-3).

In the rainbow trout, endothelial cells of the kidney and spleen are the first targets of the virus within the first 1-4 days of infection (Yasutake & Rasmussen 1968, Kim & Faisal 2010, Hoffman et al. 1979, Evensen et al. 1994, Brudeseth et al. 2002, Estepa et al. 1992). Soon thereafter the hematopoietic elements of the kidney and spleen tissue undergo necrosis and degeneration, most notably at melanomacrophage centers (Brudeseth et al. 2002). Immunohistochemistry has previously revealed that VHSV can be detected in macrophages and melanomacrophages, supporting the claim that replication occurs in these cells during both acute and chronic infection (Estepa et al. 1992, Evensen & Olesen 1997, Chilmonczyk et al. 1995). By days 4-7 after experimental infection, multifocal degeneration of the liver can be observed in histopathologic specimens (Yasutake 1975, Kim & Faisal 2010, Evensen et al. 1994, Brudeseth et al. 2004). While conflicting data exists whether the liver is considered a primary target of

the virus, disease course studies have collectively supported the idea that endothelial cells of the liver do not appear to be affected owing to the idea that liver endothelial cells may lack the appropriate receptors for viral attachment (Evensen et al. 1994, Brudeseth et al. 2002).

Forms of Disease

Spontaneous and experimental infection studies have demonstrated acute, subacute, and chronic forms of the disease. In some hosts, the chronic form is characterized by nervous manifestations (Wolf 1988, Smail 1999). The clinical and associated pathologies vary with disease form, with the acute disease being the most rapid and resulting in greater mortality. Chronically infected fish experience a prolonged course with lingering low level mortality and often resulting in fish that are virus carriers, which shed VHSV virus particles into the surrounding environment (Neukirch 1985). The nervous form is often associated with erratic swimming behavior characterized as spiraling and/or flashing (Yasutake 1975).

Transmission

Lateral transmission is the primary method by which VHSV infects new hosts. Fish that are chronically infected often become carriers and shedders of the virus. Once the virus has been shed into the water through urine, the virus attaches to the gill epithelium and/or skin of nearby hosts (Chilmonczyk 1995). Whether the virus enters the host immediately or replicates at the attachment for an extended period of time before entry is unclear, however viral replication has been shown to occur in both the skin and gills (Yamamoto et al. 1992). More

specifically, viral replication has been observed in gill pilar cells (Chilmonczyk 1980, Neukirch 1984), which suggest the gills may not serve merely as a portal of viral entry (Hoffman et al. 1979). When gill epithelia of rainbow trout were cultured and infected with VHSV isolates from the marine and freshwater environment, investigators discovered that the predilection of certain VHSV isolates for gill tissues depends on the origin of virus and fish species, respectively (Brudeseth et al. 2008). While several studies have indicated the gills as a major site of entry, a recent study conducted by Harmache et al. (2006) also showed the fin base as a site of viral entry through a noninvasive bioluminescence assay. The skin has also been proposed as an entry site for virus but experimental data has revealed that VHSV can be detected after fish have become viremic (Brudeseth et al. 2002, Yamamoto et al. 1992).

The transmission of VHSV from fish to fish through urine as the only means has also been challenged. Traditional insights into viral pathogenesis strongly suggest this pathway as the only means of transmitting virus. Oral transmission has been demonstrated (Ahne 1980) by the feeding of infected fish. Investigators have also proposed that leeches (Faisal & Schulz 2009) could serve as mechanical vectors, yet additional studies are required to determine if VHSV replication takes place in the leech internal organs. Furthermore, fish-eating birds have been proposed as possible vectors, but only if the fish has been regurgitated to a different body of water. Fecal transmission in birds has not been strongly suspected given the acidity of digestive secretions and the bird's body temperature which are not ideal for VHSV. While these assumptions have

yet to be verified, the source of outbreaks is all too often attributed to covertly infected fish or transfer from the surrounding environment. Therefore the need to clarify the relationships between the virus and environment requires further study.

Experimental studies have also provided insights into how the virus can be transmitted. One of the most common methods utilized by investigators has been through waterborne challenge, which mimics a natural course of infection (Castric & De Kinkelin 1980, Jørgensen 1980, Meier et al. 1980, De Kinkelin & Castric 1982, Castric & De Kinkelin 1984, Meier et al. 1986, Dorson et al. 1991, Meier et al. 1994, Follett et al. 1997, Kocan et al. 1997, Snow & Smail 1999, Snow et al. 2000, King et al. 2001, Bowden 2003, Muroga et al. 2004, Skall et al. 2004, Snow et al. 2005, Hershberger et al. 2007, López Vázquez et al. 2007). The method involves immersion of a group of fish into a known concentration of virus infected water, usually expressed as plaque forming units (pfu/mL) or median tissue culture infectious dose ($TCID_{50}$). Alternatively, infection with VHSV has also been demonstrated by intraperitoneal injection, which allows for approximately equal amounts of virus delivered to each fish (Castric & De Kinkelin 1980, Jørgensen 1980, De Kinkelin & Castric 1982, Castric & De Kinkelin 1984, Meier et al. 1986, Dorson et al. 1991, Meier et al. 1994, Follett et al. 1997, Kocan et al. 1997, Snow & Smail 1999, Snow et al. 2000, Bowden 2003, Muroga et al. 2004, Skall et al. 2004, Snow et al. 2005, Hershberger et al. 2007, López Vázquez et al. 2007). A third method of transmission known as cohabitation requires that VHSV carrier fish are placed inside a tank with naïve

fish (López Vázquez et al. 2007). Although each of the transmission modes are different mechanically and approach, all methods are capable of producing the clinical diathesis of VHSV infection.

Factors influencing disease course

A number of factors predispose fish to VHSV infection including stress, age, and water temperature. Stressors such as dramatic changes in the environment have been noted to be responsible for the development and onset of infection. Hoerlyck et al. (1984) reported an instance of isolating VHSV from maricultured rainbow trout that were VHSV negative prior to transport from the freshwater environment. VHSV is considered to be a coldwater disease with an optimal temperature of 9-12 $^{\circ}$C (Smail 1999). This is interesting considering that VHSV replicates best on cell lines at 14-15 $^{\circ}$C (De Kinkelin & Scherrer 1970); however, this phenomena is not unique among aquatic pathogens. These differences may be the result of differences in the origins of VHSV isolates which may require different environmental parameters *in vitro* versus *in vivo*. The pathogenicity of VHSV is greatly diminished at or above 20 $^{\circ}$C (De Kinkelin & Scherrer 1970). More recently, it was determined that VHSV can survive up to 14 days in freshwater and only 4 days in seawater (Hawley & Garver 2008). Factors that also influence survivability of the virus include whether the water was filtered prior to the addition of virus and the temperature at which the virus and water mix was held. These results were partially corroborated by Kocan et al. (2001), who

also found that virus survival was greatly enhanced if the virus was maintained in serum enriched cell culture medium or water that contained ovarian fluid.

The age at which fish become infected also determines the course and outcome of disease. Infected fry-juvenile age fish may experience up to 100% mortality while fish that are older when infected may exhibit 25-75% mortality rates (Meyers & Winton 1995, Skall et al. 2005). Experimental infections have demonstrated marked mortality rates especially in the case of juvenile herring at 5 months of age compared to 9 and 13 months of age (Kocan et al. 1997). However, Ghittino (1965) determined that fish from any age group could become infected, with younger fish being more susceptible and fish greater than 6 months appearing resistant. The findings were further supported by studies conducted on rainbow trout by Roberts (1978) and Bellett (1965), who determined rainbow trout greater than 2-6 months to have milder cases of disease, while fish older than 2 years were almost completely resistant.

Tissue alterations caused by VHSV

Histopathologic examination of certain tissues from diseased fish has offered invaluable insights into the pathogenesis of VHSV. The most pronounced changes have been reported in the muscle, liver, spleen, kidney, and at times in the swimbladder (Figure 1-4a) (Yasutake 1975). Despite no actual damage to muscular tissues, bleeding in organized discrete foci can often be observed between muscle bundles and fibers (Figure 1-4b). Liver sinusoids are often distended along with hepatocellular vacuolation and degenerative changes. Kidney tissues can exhibit variable changes depending on the stage of infection.

Yasutake reported lymphopenia to be the predominant alteration during acute infection, while hyperplastic changes are observed in chronic stages of disease (Yasutake 1975). When Yasutake and Rasmussen investigated histopathogenesis in experimentally infected rainbow trout, they found that the kidneys were the initial target site of VHSV, which was followed by changes in the spleen, skeletal muscle, liver, pancreas, and adrenal cortex (Kim & Faisal 2010). Given the clinical signs of most infected fish, massive hemorrhages were not as commonly noted as compared to petechia in scattered foci. Changes observed in the kidney and liver were consistent with those from epizootic specimens.

The histopathologic alterations predominantly consist of necrosis and degeneration (Wolf 1988). Although various tissues are involved, Wolf (1988) describes the kidney and liver as the primary targeted organs. Hyperplasia within renal tissue present as foci of macrophages while destruction of melanomacrophage centers can be observed in more advanced stages of disease. Alterations of liver tissue are most notably appreciated in hepatocytes, which often become vacuolated, pyknotic and karyolitic. Aggregations of erythrocytes are also noted within skeletal muscle with minimal damage to fibers or bundles. These findings were initially described in rainbow trout, but were eventually noted in other species including those from the marine environment.

Widespread hemorrhagic changes are the direct result of VHSV's predilection for endothelial cells (Smail 1999, Brudeseth 2002, Liversidge & Munro 1978, De Kinkelin et al. 1979). As a result, extravasations of the blood

supply into the interstitium of muscle and organs are believed to be responsible for the dramatic decrease in circulating blood volume. However, VHSV has also exhibited leukotropic tendencies, especially for macrophages (Evensen et al. 1994, Brudeseth et al. 2002, Neukirch 1085). Immunohistochemistry demonstrated the presence of VHSV within macrophages and melanomacrophages (Brudeseth et al. 2002, Estepa et al. 1992), but would be expected as it has a preference for leucopoietic tissues.

Experimental infections have allowed investigators to observe histopathologic changes under controlled conditions. In experimentally infected sea bass (*Dicentrarchus labrax*) and turbot, tissue alterations were observed in kidney tissues which displayed severe interstitial necrosis, while severe necrosis was noted throughout the spleen and liver (Castric & De Kinkelin 1984). When VHSV was initially isolated from whitefish in 1984 (Meier et al. 1986), the same virus was used for a bath infection of whitefish (*Coregonus sp.*) and rainbow trout. Noted histopathologic changes included focal necrosis of the liver, spleen and pancreas. Additionally, intestinal mucosa was necrotic and the anterior kidney exhibited focal necrosis and degeneration of individual cells with a loss of detail in capillaries. Tubular epithelia were often detached with pyknotic nuclei within a granular cytoplasm. Hyaline casts and necrotic debris was noted in the lumen of tubules while the mesangium of glomeruli were mildly swollen. These findings were also confirmed in histopathologic data from grayling (*Thymallus thymallus* L.) (Meier & Wahli 1988). Similar findings were described in important

Great Lakes fish species experimentally infected with VHSV (Kim & Faisal 2010) (Figure 1-4c and d).

More recently, *in situ* hybridization was performed on histologic sections of yellow perch tissues collected from moribund individuals during an experimental infection using VHSV. The virus was detected in the glomeruli and tubular epithelia of kidney tissues and endothelial cells of muscle (Kim and Faisal, unpublished; Figure 1-5). The identification of virus in these particular cells suggests they are both targets for viral attachment and viral replication. The stage of disease however could not be determined as the tissues were from a moribund fish. As a result, further investigation is warranted whereby a number of tissues collected at varying timepoints following infection could be monitored.

Diagnosis

The diagnosis of VHSV can be accomplished through a number of diagnostic methods. Detailed recommendations by which VHSV can be detected and confirmed are provided by the World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals 2009 and American Fisheries Society (AFS) Fish Health Section – Bluebook 2007 (Batts & Winton 2007). A presumptive diagnosis can be completed on the basis of clinical signs, histopathologic changes and through the use of established immortal fish cells lines for initial isolation. Recommended cell lines for freshwater European VHSV isolates have included bluegill fry (BF-2) or rainbow trout gonad (RTG-2) cells in Europe (OIE Manual of Diagnostic Tests for Aquatic Animals 2009, Batts &

Winton 2007, Olesen & Jørgensen 1992). Whereas VHSV isolates from North America can be cultured on *Epitheliosum papulosum cyprini* (EPC), fathead minnow (FHM), BF-2 cells, and chinook salmon embryo (CHSE-214) cell lines (OIE Manual of Diagnostic Tests for Aquatic Animals 2009, Batts & Winton 2007, Olesen & Jørgensen 1992). Early reports recommend a pH of 7.4 to 7.8 as ideal for culture medium (Campbell & Wolf 1969, Wolf & Quimby 1976), while a pH of 7.1 and temperature of 15 $^{\circ}$C in cell culture medium is recommended (Batts et al. 1991). Enhancement of viral detection has also been suggested through pretreatment of polyethylene glycol or DEAE dextran (Campbell & Wolf 1969, Batts & Winton 1989). Other methods include electron microscopy or gross, clinical, and microscopic pathology. While all of the aforementioned methods are useful in guiding the decisions of the diagnostician, they do not provide a definitive diagnosis for VHSV or any other fish virus given the similarities in clinical signs.

According to the OIE and AFS Bluebook, confirmation of VHSV can be completed through a variety of serologic techniques. These include the plaque neutralization test (PNT), immunoblot (McAllister & Owens 1987, McAllister & Schill 1986), fluorescent antibody test (Lorenzen et al. 1988), enzyme linked immunosorbent assay (ELISA) (McAllister & Schill 1986, Olesen & Jørgensen 1991, Meier & Jørgensen 1975, Ahne 1981, De Kinkelin 1983), and sequence analysis (Snow et al. 2004). The PNT technique is dependent on heat-sensitive serum factors known as complement which aid the cross-linking between antigens and antibodies. Serology is also useful to detect viral antigen through

the use of indirect fluorescent antibody and immunoperoxidase staining (Ahne 1981, Faisal & Ahne 1980).

An immunofluorescent technique developed by Jørgensen and Meyling (1972) was utilized to identify viral antigens in infected tissues. This technique permits investigators to detect VHSV in tissue during the climax of protein synthesis and assembly, which occurs 18-26 h following infection. The idea is to allow a primary monoclonal antibody to attach to the virus within the tissue, followed by the addition of a secondary antibody that is labeled with a fluorescein isothiocyanate. The same technique was later refined by the same authors in 1974 (Jørgensen 1974).

As mentioned, ELISA is considered to be the mainstay method by which virus can be detected in cell culture supernatants (McAllister & Schill 1986, Way & Dixon 1988). The method is performed by placing a primary layer, also known as the catching layer, of a polyclonal or monoclonal antibody. Secondly, a blocking reagent, generally bovine serum albumin is then applied to eliminate any extraneous sites that may interfere with the assay. Next the virus is added and then followed by a MAb that will attach to the G protein. The final step is to then add a tagged anti-species antibody to attach to the last monoclonal antibody added. These methods have been described by Way and Dixon (1988) using polyclonal antibodies, Mourton et al. (1990) in comparison of indirect, direct and antigen-capture ELISAs.

Virus gene probes have also been developed by Batts et al. (1993), which are used to detect complementary deoxyribonucleic acid (cDNA) or viral

ribonucleic acids. After thorough investigation, the authors were able to utilize the developed probes in confirmation and differentiation of the two main groups of VHSV. One of the most specific techniques, that also has the ability to differentiate serologically similar strains is the reverse transcriptase polymerase chain reaction (RT-PCR). The goal in this case is to obtain the viral RNA and produce a cDNA through the use of a reverse transcriptase and nucleotides within appropriate buffers. The cDNA is then amplified by polymerase chain reaction (PCR) using a TaqPolymerase in a temperature cycler. The amplification of cDNA results in an adequate sample to be electrophoresed in an agarose gel and visualized. This highly specific technique has allowed investigators to identify VHSV more rapidly than other techniques (Einer Jensen et al. 1995, Bruchhof et al. 1995, Winton & Einer Jensen 2002). Since most of the recommended primer sets by the OIE and AFS Bluebook can detect all genotypes, it is also recommended that new isolations be sent to a reference laboratory for serologic and molecular analysis.

Prevention and control

At present, the best approach to avoid threats of VHSV infection is by prevention as there are no current chemotherapeutic options available. The primary measure by which fisheries and hatchery managers can prevent the spread of disease is by properly disinfecting contaminated equipment and holding areas and ultraviolet (280-200 nm wavelength) irradiation of incoming water (Øye et al. 2001). Other effective control measures include increasing the

temperature to above 20 °C (Parry & Dixon 1997) or dilution in seawater (Kurita et al. 2002). However, a recent study investigating the stability of virus in fresh- and saltwater revealed that VHSV, regardless of its environmental origin, could remain stable in either water type (Hawley & Garver 2008). Interestingly the virus survived an average of 13 days in freshwater at 15 °C, yet was inactivated on average of 4 days in seawater. If the temperature of the water was increased in either water type, the stability of the virus was greatly reduced owing to the sensitivity of the virus to higher temperatures. Some disinfectants have also been effective, such as chlorine, hypochlorite, formalin, sodium hydroxide and iodophors (Wolf 1988, Smail 1999). Application of any of the aforementioned preventative measures could limit the exposure and recurrence of future outbreaks. Currently, viral hemorrhagic septicemia remains a reportable disease to the World Organization for Animal Health (OIE) which is a critical step in preventing and controlling the spread of the disease before the virus is unintentionally imported and exported through fish and/or fish products. The OIE has also implemented for identifying and confirming the disease which has allowed various countries to initiate management and regulatory guidelines rapidly once the virus was detected.

Vaccination

Vaccine development has shown some promising data in the literature for rainbow trout culture. In 1995, De Kinkelin et al. (1995) evaluated vaccination protocols which revealed varying degrees of efficacy and protection between inactivated, live attenuated and recombinant vaccines. The investigation revealed

that the virus strain 07/71 inactivated by 2-propiolactone and intraperitoneally injected was effective and immunogenic in trout of all sizes but not all life stages (De Kinkelin 1988). Cross protection between the three serotypes was observed as well as neutralizing antibodies in the sera of vaccinated fish. The production of live vaccines against VHSV was initiated by Vestergård-Jørgensen (1982) by attenuating the virus through several subcultures in rainbow trout gonad cells (RTG-2) at 14 °C. Rainbow trout fry were shown to be protected by immersion up to 150 days following infection at 10 °C. An EPC cell attenuated virus (De Kinkelin et al. 1981) increased the survival rate of 30% in rainbow trout fry when compared to unvaccinated fry. Interestingly, the virus provided immunity against the parent (i.e. pre-attenuated) virus strain but not the wild-type virus.

Subunit vaccines or recombinant virus have also been investigated to determine whether glycoprotein like particles could provide immunity. Given that the glycoprotein gene plays a critical role in antigenicity and virus attachment, studies have revealed that injection of synthetically produced glycoprotein stimulates production of VHSV specific antibodies in rainbow trout (Lorenzen et al. 1993). Even more interesting was the production of a subunit vaccine in insect cells through the use of a baculovirus vector (Lecocq et al. 1994). By intraperitoneal injection alone, investigators were able to induce virus neutralizing antibodies and protection against infection in rainbow trout using the baculovirus. These findings were encouraging with respect to vaccination, but the inability to induce protection through immersion made the technique unfeasible for large scale production purposes.

Within the last decade, vaccination against VHSV using DNA plasmid constructs has gained more attention. The earliest studies were those of Lorenzen et al. (1998), which demonstrated that intramuscular vaccination using DNA encoding the glycoprotein gene could induce protective immunity and thus produce neutralizing antibodies in rainbow trout. Alternatively, the nucleoprotein gene was protective but did not induce production of neutralizing antibodies. In 2001, Fernandez-Alonso et al. utilized short pulses of low intensity ultrasound to deliver a VHSV DNA vaccine to fingerling rainbow trout. When ultrasound vaccination was compared to immersion or injection, ultrasound delivery of the vaccine was the only method by which humoral immunity and survival post reinfection occurred. Perhaps one of the most promising studies has been through dual DNA vaccination using the glycoprotein gene of infectious hematopoietic necrosis virus (IHNV) and VHSV (Einer Jensen 2009). After a single intramuscular injection, fish were protected against both viruses for an extended period of time. When DNA vaccination with the glycoprotein gene was conducted on fish acclimated to temperatures of 5, 10, and 15 $^{\circ}$C (Lorenzen et al. 2009), protection against reinfection was conferred at all temperatures. However, non-specific protection, seroconversion and the inflammatory response at the site of vaccination were most rapid at 15 $^{\circ}$C. While these studies do not comprise all of the available information on DNA vaccination against VHSV, it does provide compelling and encouraging data.

These early studies eventually sparked numerous trials and investigations into creating an economically feasible, efficacious and practical vaccine. An oral

vaccine was recently developed by using a lyophilized virus encased in polyethylene glycol and extruded to create a feed sized pellet (Adelmann et al. 2008). Results indicated that VHSV antibodies could be detected following oral vaccination. After vaccination, the fish were immersion challenged using VHSV strain Fi13 and protection was found to occur, thus presenting a method that could be both effective and feasible in aquaculture settings. Orally vaccinated fish showed significantly lower mortalities compared to fish given a placebo.

In spite of the growing interest in vaccination against VHSV, De Kinkelin (De Kinkelin 1988) indicates that a vaccine must meet several criteria. The vaccine must allow producers to administer the vaccine once without a booster, deliver it easily, purchase it at a reasonable cost and provide universal protection and resistance to infection. Furthermore, the vaccine must not impose the risk of reverting to a virulent form, remain stable in storage and allow investigators to continue sero-surveillance operations. Fulfilling these criteria would be essential in marketing a vaccine that would prevent future economic losses from mass mortalities.

VHSV typing by serological methods

Since the early discovery and isolation of VHSV, scientists realized that VHSV is very heterogenous in nature. For example, based on plaque neutralization assay (PNT) results, three VHSV serotypes have been recognized: Type 1 is primarily represented by the F1 strain (originally isolated from Denmark); Type 2 is represented by the Hededam isolate from Danish rainbow

trout (Jørgensen & Meyling 172, Jørgensen 1980); and Type 3 is represented by the French strain 23/75, isolated from brown trout by De Kinkelin and Le Berre (1977). The experiments designed by Olesen et al. (1993) were critical in providing a more detailed characterization of VHSV serogroups. The author was able to demonstrate that the three serogroups while different, were in actuality descendents of one another, explaining the subtle differences and overlap between serogroups. Such discrepancies encouraged investigators to pursue alternative methods of differentiating isolates through sequence comparisons of the glycoprotein gene for phylogenetic analysis.

Phylogeny

Once believed to be a disease unique to cultured rainbow trout, subsequent reports demonstrate that VHSV has both a wide host and geographic range. The compiled lists of isolations from various fish species and their locations throughout the last two decades have been instrumental in unraveling the phylogenetic relationships that exist between isolates. Among the earliest phylogenetic studies on VHSV were those performed by Benmansour et al. (1997) and Stone et al. (2008), who sequenced the G-gene from a number of VHSV isolates and reported the presence of three distinct genotypes, which correlates with geographic ranges. Genotype I included isolates from Europe, Genotype II included isolates from in and around the British Isles, and Genotype III included North American isolates. Prior to that, the distinction between North American and European isolates was suggested by Oshima et al. (1993) Bernard

et al. (1992), Batts et al. (1993). The studies of Benmansour et al. (1997) and Stone et al. (2008) ignited more interest in phylogenetic studies on VHSV as opposed to continuing the serologic testing for strain differentiation. This approach was so sensitive that it differentiated among various isolates within the same genotype. For example, Thiéry et al. (2002), who studied the sequence of the V2 region of the G gene of 63 French VHSV isolates, concluded the presence of four sublineages (designated a-d) within Genotype I.

In 2004, Snow et al. (2004) published the most comprehensive phylogenetic tree that has lead to current understanding of VHSV genotypes and their geographic ranges. Using 128 VHSV isolates from numerous geographic and host origins, the authors were able to validate previously reported molecular epidemiological data and gain insights into the subtle differences within each genotype. Analysis of the isolates revealed that genotype I included freshwater farmed rainbow trout in continental Europe, denoted Ia (Ston et al. 2008, Batts & Winton 2007, Benmansour et al. 1997), and marine isolates from the Baltic Sea, Skagerrak and Kattegat, denoted Ib (Batts & Winton 2007). Interestingly genotype Ia included one marine isolation from farmed turbot in Germany, which was surprising given that all of the other isolates of the sublineage were from freshwater. Some studies have also included additional sublineages of genotype I to include Ic, Id, and Ie (Einer Jensen et al. 2004), all of which are rainbow trout. The close designation of this marine isolate to outbreaks in freshwater, was previously suggested by Schlodfeldlt et al. (1991) and attributed to virus contaminated effluent from farms containing infected rainbow trout. Isolates of

genotype Ib also contained one isolate from Atlantic herring from the English Channel (Dixon et al. 1997), which suggests the migratory behavior of herring may have been responsible for this unlikely inclusion. While such differences were critical in understanding VHSV's ability to expand in new locales, the most significant was the close genetic relationship between isolates of genotype Ia (freshwater) and Ib (marine), which, combined with the findings of Einer Jensen et al. (2004), strongly suggested that the origin of VHSV in outbreaks of rainbow trout was from the marine environment. Genotype II included 7 isolates of which were from the Baltic Sea, none of which were linked to rainbow trout aquaculture. This second marine lineage thus provided even more evidence that VHSV originated in the marine environment. Genotype III consisted of over 30 isolates from the British Isles and 2 isolates from Irish and Scottish turbot marine farms. Lastly, genotype IV included marine isolates from North America and Japan.

The discovery and classification of genotype IV isolates from coho and chinook salmon in Washington State (Stone et al. 1997, Snow et al. 1999, Nishizawa et al. 2002, Einer Jensen et al. 2005) reinforced the idea that VHSV was from the marine environment. Surprisingly, in 2003, VHSV was isolated (MI03) from muskellunge caught from the freshwater Lake St. Clair in Michigan (Elsayed et al. 2006). Sequence analysis of G and N genes of MI03 revealed that the Great Lakes isolate was closely related to the North American strain, yet slightly distinct. The authors suggested the establishment of a new sublineage within Genotype IV, designated as IVb with MI03 as the index strain while the Pacific Northwest strains constituted the IVa sublineage within Genotype IV with

the Makah strain as the index strain. Subsequent isolations from a number of fish species within the Great Lakes basin and the east Atlantic coast revealed the widespread distribution of VHSV-IVb and its wide host range (Lumsden et al. 2007, Groocock et al. 2007, Gagné et al. 2007). Sequence data from the virus recovered from these hosts revealed a nearly identical match to the original MI03 muskellunge isolate, which suggested an even more extensive host range than any other isolate of VHSV. Table 1-2 displays the current status of phylogenetic analysis of VHSV isolates retrieved from all over the world.

The conservative nature of the N-gene has proven useful in prolonged evolutionary comparisons of other *Lyssavirus* spp. (Bourhy et al. 1992, 1993) and speculating the origins of new isolates. Partial sequences of the G and P genes revealed that the Japanese isolates belong to two genotypes; seven were closely related to North American isolates, while only one isolate was more closely related to European isolates. In the same context, an overwhelming influx of newly described isolates suggested that VHSV originated from the marine environment. Einer Jensen et al. (2004), who compared the entire G-gene sequences from 74 isolates retrieved from freshwater and marine fish worldwide, demonstrated that the North American and European isolates' division occurred approximately 500 years ago, while the ancestor of the European freshwater isolates emerged relatively recently (~50 years prior). The authors noticed that freshwater isolates have a 2-5 times faster nucleotide substitution rate, which they interpreted as either a result of inherent physiologic differences from fish hosts (marine vs. freshwater), or a consequence of intense aquaculture

practices. Through restriction fragment length polymorphism assays, Einer Jensen et al. (2005) determined that Japanese isolates were closely related to genotype IV with the exception of one, which was from genotype I. Although the discovery of genotype IV isolates in Japan are not surprising, the identification of a genotype I isolate suggests the possibility of accidental importation of infected fish from a foreign country. Consequently, the use of phylogenetic data remains useful in understanding the patterns of distribution between different genotypes and will continue to be a critical tool in understanding the epizootiology of VHSV.

More recently VHSV was isolated from rainbow trout in the United Kingdom (Stone et al. 2008), which demonstrated the first VHSV isolate from the United Kingdom (UK) obtained from a freshwater species in a freshwater environment. Additionally, previous isolations in the region were more closely related to Genotypes III and Ib (Einer Jensen et al. 2004), whereas the newest UK isolate was considered to be of Genotype Ia based on G-gene sequences. The re-emergence of VHSV was also experienced in Norway in seawater-farmed rainbow trout (Dale et al. 2009). Investigators determined the isolate was from Genotype III, which has not been isolated from salmonids. The authors concluded that the outbreak was most likely a result of viral transmission from the marine environment. Both isolations have thus confirmed VHSV's capacity to engage new hosts and challenge the notion that genotype and geography were strongly related. The number of species found within a particular genotype is described in Table 1-2.

Knowledge gaps

In general, our current understanding of VHSV has been largely provided by published data regarding European and Pacific VHSV isolates. Despite the obvious similarities in disease manifestations, there is clear evidence that not all VHSV isolates carry the same host range, function at equivalent environmental parameters, or contain identical genomes. Such differences have compelled investigators to characterize the virus and assess the risk to neighboring species, while also providing regulatory agencies the ability to make informed decisions.

At present, little data has been made available regarding the pathogenicity and host susceptibilities of recent VHSV isolates. Furthermore, little is known about the degree of pathogenicity of VHSV for listed and unlisted freshwater and marine species from which VHSV has been isolated from, nor is there a clear distinction between species that are resistant, become reservoirs, or develop fulminant VHSV infection. While such data would be critical in guiding policy and management, the occurrence of VHSV infections in previously unidentified species each year has owed to VHSV's unpredictable host range. This in combination with the devastative nature of VHSV has sparked the dilemma for fisheries and hatchery managers from a recreational and economic standpoint.

Table 1-1 VHSV isolations in Great Lakes fish species that were either collected dead or during field surveys

Family	Common name	Scientific Name	VHSV Detection(s)	
			Mortality	Surveillance
Catostomidae	Shorthead redhorse	*Moxostoma macrolepidotum*	x	
	Silver redhorse	*Moxostoma anisurum*	x	
Centrarchidae	Black crappie	*Pomoxis nigromaculatus*	x	x
	Bluegill	*Lepomis macrochirus*	x	x
	Largemouth bass	*Micropterus salmoides*	x	x
	Pumpkinseed	*Lepomis gibbosus*	x	x
	Rock bass	*Ambloplites rupestris*		x
	Smallmouth bass	*Micropterus dolomieu*	x	x
Clupeidae	Gizzard shad	*Dorosoma cepedianum*	x	
Cyprinidae	Emerald shiner	*Notropis atherinoides*		x
	Spottail shiner	*Notropis hudsonius*		x
	Bluntnose minnow	*Pimephales notatus*	x	x
Esocidae	Muskellunge	*Esox masquinongy*	x	x
	Northern Pike	*Esox lucius*	x	x
Gobiidae	Round goby	*Neogobius melanostomus*	x	x
Ictaluridae	Channel catfish	*Ictalurus punctatus*		x
	Brown bullhead	*Icatlurus nebulosus*		x
Lotidae	Burbot	*Lota lota*	x	
Moronidae	White bass	*Morone chrysops*	x	x
	White perch	*Morone Americana*		x

Table 1-1 (cont'd) VHSV isolations in Great Lakes fish species that were either collected dead or during field surveys

Family	Common name	Scientific Name	VHSV Detection(s)	
			Mortality	Surveillance
Percidae	Walleye	*Sander vitreus*	x	
	Yellow perch	*Perca flavescens*	x	x
Percopsidae	Trout-Perch	*Percopsis omiscomaycus*		x
Petromyzontidae	Sea lamprey	*Petromyzon marinus*		x
Salmonidae	Brown trout	*Salmo trutta*	x	
	Chinook salmon	*Oncorhynchus tshawytscha*		x
	Lake trout	*Salvelinus namaycush*		x
	Lake whitefish	*Coregonus clupeaformis*	x	x
	Rainbow trout	*Oncorhynchus mykiss*		x
Sciaenidae	Freshwater drum	*Aplodinotus grunniens*	x	x

Table1-2 Phylogenetically classified viral hemorrhagic septicemia virus isolates by species and location.

Genotype	Sub-group	Species	Location	Source
I	Ia	Rainbow trout, *Oncorhynchus mykiss*	France, United Kingdom	Stone et al. 2004, 2008
	Ic		Denmark	Einer Jensen et al. 2004
	Id		Vestrefjord, Norway	Einer Jensen et al. 2004
	Ie		Georgia	Einer Jensen et al. 2004
	Ia	Turbot, *Scophthalmus maximus*	Germany	Schlotfeldt et al. 1991
	Ie		Black Sea	Nishizawa et al. 2006
	Ib	Atlantic herring, *Clupea harengus*	Baltic Sea	Mortensen et al. 1999
		Sprat, *Sprattus sprattus*	Baltic Sea	Skall et al. 2004, King et al. 2001
		Dab, *Limanda limanda*	Kattegat	Einer Jensen et al. 2004
		Sand goby, *Pomatoschistus minutus*	Baltic Sea	Snow et al. 2004
		Fourbeard rockling, *Enchelyopus cimbrius*	Baltic Sea	Mortensen et al. 1999
		Blue whiting, *Micromesistius poutassou*	North Sea	Skall et al. 2004, Snow et al. 1999, Nishzawa et al. 2002
		Japanese flounder, *Paralichthys olivaceus*	Japan	Nishzawa et al. 2002
		Sprat, *Sprattus sprattus*	Baltic Sea	Mortensen et al. 1999
II	II	Atlantic Cod, *G.morhua*	Baltic Sea	Mortensen et al. 1999
		Atlantic herring, *Clupea harengus*	Baltic Sea	Mortensen et al. 1999
		Whiting, *Merlangius merlangus*	North Sea	Mortensen et al. 1999

Table 1-2 (cont'd 1) Phylogenetically classified viral hemorrhagic septicemia virus isolates by species and location.

Genotype	Sub-group	Species	Location	Source
III	III	Turbot, *Scophthalmus maximus*	Ireland	Snow et al. 1999.
		Norway pout, *Trisopterus esmarkii*	North Sea	King et al. 2001
		Haddock, *Melanogrammus aeglefinus*	North Sea	Smail 2000
		Atlantic Cod, *G.morhua*	North Sea	Smail 2000
		Atlantic herring, *Clupea harengus*	Skagerrak	Mortensen et al. 1999
		Argentine, *Argentina sphyraena*	North Sea	Mortensen et al. 1999
		Eel, *Anguilla anguilla*	France	De Kinkelin 1972, Castric et al. 1992, Jorgensen et al. 1994
		Greenland halibut, *Reinhardtius hippoglossoides*	Flemish Cap	Dopazo et al. 2002
		Rainbow trout, *Oncorhynchus mykiss*	Norway	Dale et al. 2009
		Japanese flounder, *Paralichthys olivaceus*	Japan	Kim et al. 2003, isshiki et al. 2001, Byon et al. 2006
		Atlantic salmon, *Salmo salar*	Canada, Pacific	Jimenez et al. 1988, traxler et al. 1995
		Pacific herring, *C. pallasii*	British Columbia (BC) /Alaska/Washington (WA), USA	Benmansour et al. 1997

Table 1-2 (cont'd 2) Phylogenetically classified viral hemorrhagic septicemia virus isolates by species and location.

Genotype	Sub-group	Species	Location	Source
IV	IVa	Pacific sardine, *Sardinops sagax*	BC, Oregon, California	Hedrick et al. 2003.
		Pacific cod, *G. macrocephalus*	Prince William Sound, Alaska, USA	Benmansour et al. 1997
		Chinook salmon, *O. tshawytscha*	Orcas Island, WA, USA	Hopper 1989
		Coho salmon, *O. kisutch*	Makah, WA, USA	Benmansour et al. 1997
		Pacific sardine, *Sardinops sagax*	BC, Oregon, California	Hedrick et al. 2003
		Pacific mackerel, *Scomber japonicus*	Santa Catalina, California, USA	Hedrick et al. 2003
		Eulachon, *Thaleichthys pacificus*	Sandy River, Oregon, USA	Hedrick et al. 2003
		Shiner perch, *Cymatogaster aggregata*	NW Vancouver Island, British Columbia (BC), Canada	Hedrick et al. 2003
		Surf smelt, *Hypomesus pretiosus*	Winchesterbay, Oregon, USA	Hedrick et al. 2003
		Sablefish, *Anoplopoma fimbria*	Queen Charlotte Straits, BC, Canada	Hedrick et al. 2003

Table 1-2 (cont'd 3) Phylogenetically classified viral hemorrhagic septicemia virus isolates by species and location.

Genotype	Sub-group	Species	Location	Source
IV	IVb	Muskellunge, *Esox masquinongy*	Lake St. Clair, MI, USA	Elsayed et al. 2006
		Brown trout, *Salmo trutta*	French River, Nova Scotia	Gagné et al. 2007
		Striped bass, *Morone saxatilis*	Miramichi Bay and River, Baie du Vin, New Brunswick	Gagné et al. 2007
		Mummichog, *Fundulus heteroclitus*	Ruisseau George Collette, near Bouctouche, New Brunswick	Gagné et al. 2007
		Three-spined stickleback, *Gasterosteus aculeatus aculeatus*	Ruisseau George Collette, near Bouctouche, New Brunswick	Gagné et al. 2007
		Freshwater drum, *Aplodinotus grunniens*	Lake Ontario, Canada	Lumsden et al. 2007
		Round gobies, *Neogobius melanostomus*	Lake Ontario and St. Lawrence River, NY, USA	Groocock et al. 2007

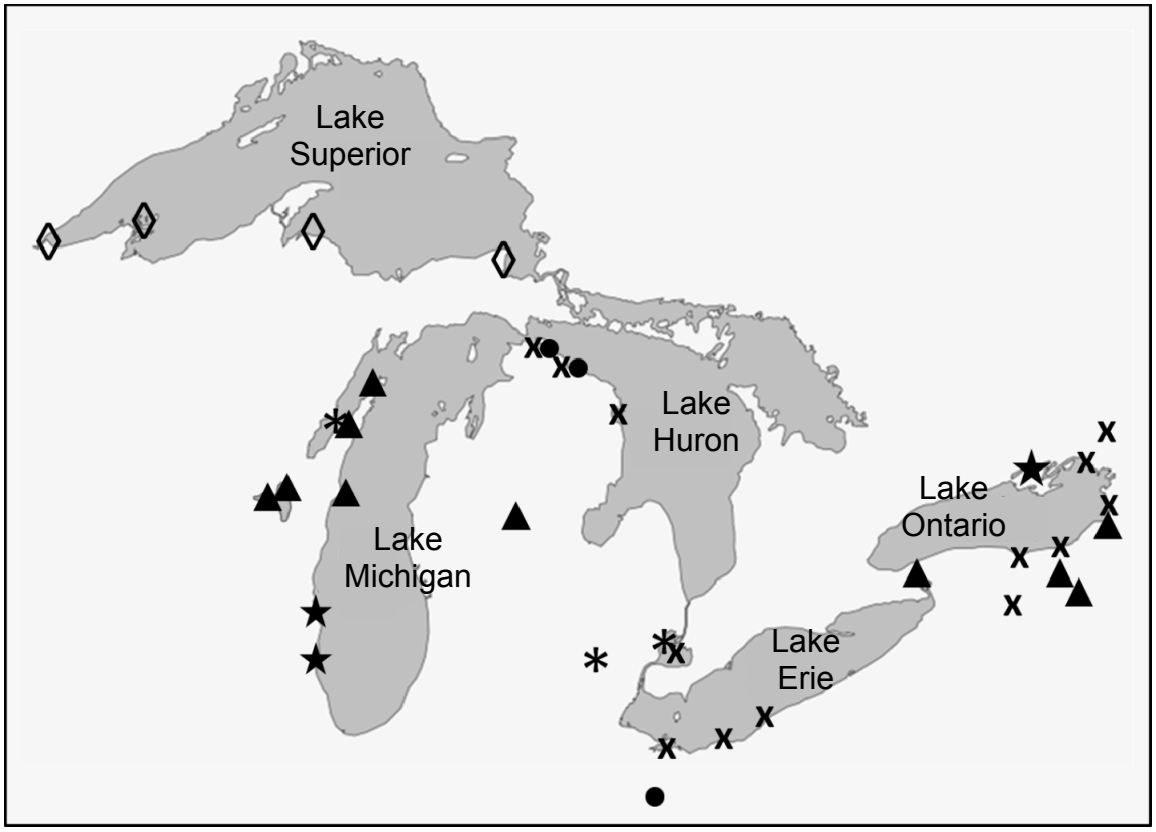

Figure 1-1 Approximate locations of VHSV isolation between 2005-2010 (Star = 2005, Cross = 2006, Triangles = 2007, Circle = 2008, Asterisk = 2009, Diamond = 2010) The information is based on published (43,45,44) and unpublished reports (Records of the Aquatic Animal Health Laboratory, Michigan State University, Michigan; Records of the Aquatic Animal Health Program, College of Veterinary Medicine, Cornell University, New York; Records of the La Crosse Fish Health Center, Onalaska, Wisconsin).

Figure 1-2 Viral hemorrhagic septicemia virus genome comprised of 6 open reading frames and a total length of 11,158 bases.

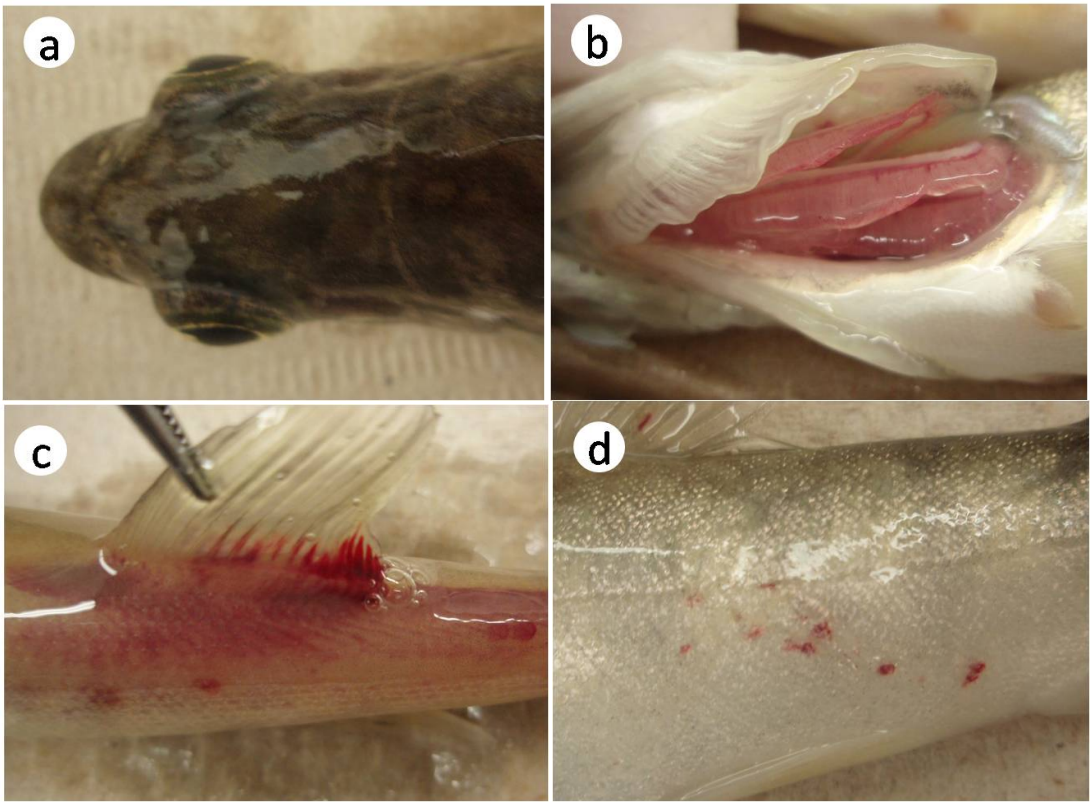

Figure 1-3 Clinical signs of viral hemorrhagic septicemia virus IVb infection. (a) Lake trout (*Salvelinus namaycush*) showing moderate exophthalmia, (b) severe gill palor (c), hemorrhaging at the base of the dorsal fin of a juvenile muskellunge (d), and petechial hemorrhages in the epidermis of a splake (*Salvelinus namaycush* x *S. fontinalis*).

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

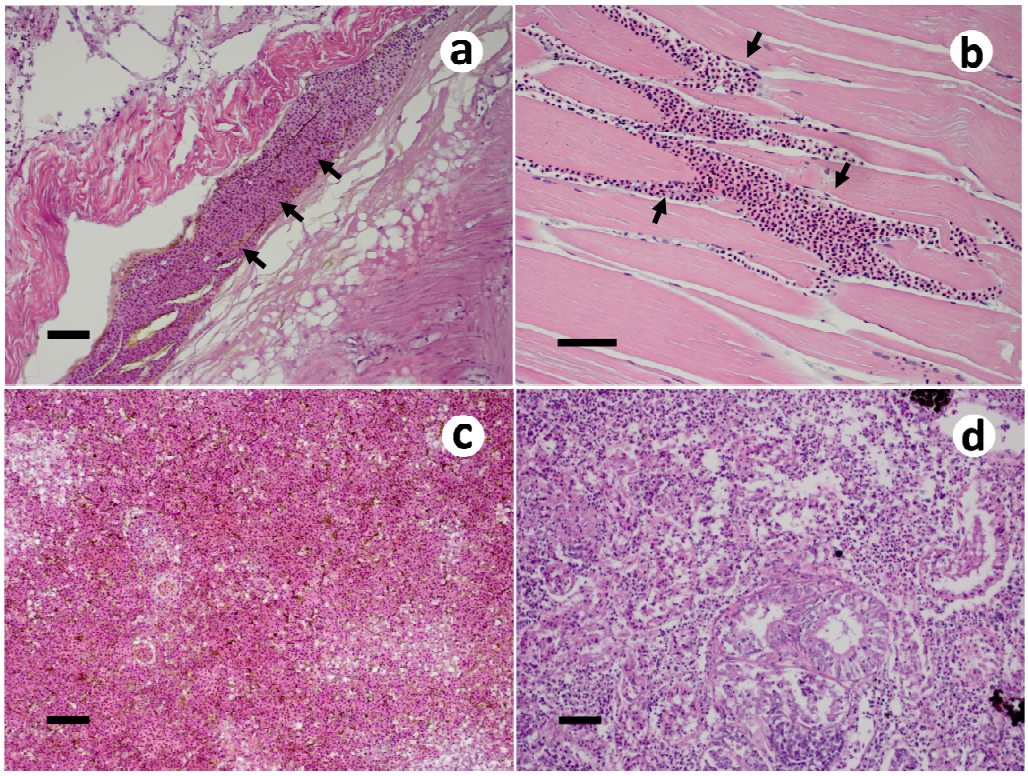

Figure 1-4 Tissue alterations caused by experimental infection of viral hemorrhagic septicemia virus IVb infection.
(a) muskellunge showing widespread hemorrhage in the swimbladder wall. (scale bar = 100μm, black arrows) (b) Infiltration of erythrocytes between muscle bundles and fibers of a lake trout. (scale bar = 100μm, black arrows). (c) Spleen section of a largemouth bass showing severe congestion. (scale bar = 200μm). (d) Tissue section of a muskellunge kidney exhibiting widespread haematopoietic depletion (black arrows) and necrosis of tubular epithelia. (scale bar = 100μm)

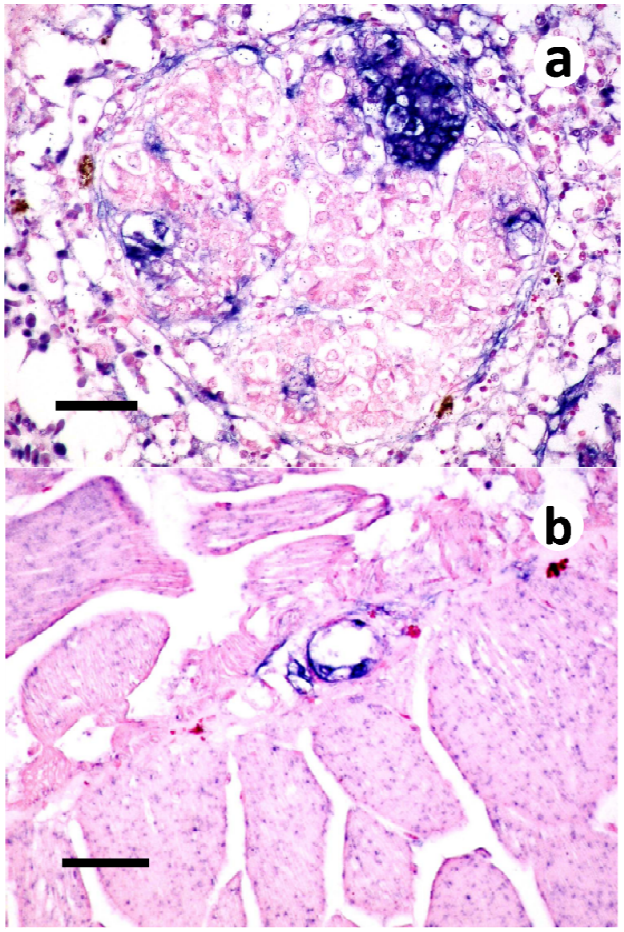

Figure 1-5 *In situ* hybridization of tissue sections from yellow perch experimentally infected with viral hemorrhagic septicemia virus IVb. (a) kidney section showing positive staining within the glomeruli (scale bar = 50μm) (b) muscle exhibiting positive stain in endothelial cells (scale bar = 50μm).

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CHAPTER 2

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CHAPTER 2

Experimental studies confirm the wide host range of the Great Lakes Viral Haemorrhagic Septicaemia Virus Genotype IVb

Viral haemorrhagic septicaemia (VHS) is a serious viral infection of teleosts worldwide (Wolf 1988). The causative agent is the Viral Haemorrhagic Septicaemia Virus (VHSV), a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Tordo, Benmansour, Calisher, Dietzgen, Fang, Jackson, Kurath, Nadin-Davis, Tesh & Walker 2005). Based upon partial sequences of the G and N genes and phylogenetic analyses of VHSV isolates worldwide, four distinct genotypes of VHSV (designated I-IV) have been identified (Einer-Jensen, Ahrens, Forsberg & Lorenzen 2004; Snow, Bain, Black, Taupin, Cunningham, King, Skall & Raynard 2004). While VHSV genotypes I-III are found in continental Europe, genotype IV emerged in the Pacific Northwest region of North America then later isolated in Japan and Korea (Skall, Olesen & Mellergaard 2005). In 2003, a novel sublineage of VHSV invaded the Laurentian Great Lakes basin causing massive kills in a number of freshwater species (Elsayed, Faisal, Thomas, Whelan, Batts & Winton 2006; Gagné, Mackinnon, Boston, Souter, Cook-Versloot, Griffiths & Olivier 2007; Groocock, Getchell, Wooster, Britt, Batts, Winton, Casey, Casey & Bowser 2007; Lumsden, Morrison, Yason, Russell, Young, Yazdanpanah, Huber, Al-Hussinee, Stone & Way 2007). Partial genotyping of the recent VHSV isolates from the Great Lakes has shown that it is distinct from all four previously known genotypes, but most closely related to the marine VHSV-IVa and therefore designated as VHSV-IVb (Elsayed *et al*. 2006). In all VHSV-IVb associated fish kill episodes, mature, large fish were the only

age class reported. This has raised the question of whether or not other age classes of susceptible fish species also succumb to VHSV-IVb. To this end, this study was designed to assess the ability of MI03 to infect juveniles of representative fish species that are residents of the Laurentian Great Lakes basin.

The MI03 virus was maintained by continuous subculture in *epithelioma papulosum cyprini* (EPC) (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & de Kinkelin 1983) and fathead minnow (FHM) (*Pimephales promelas*) (Gravell & Marshberger 1965) cell lines. In order to determine the virus concentration, plaque assay was performed on EPC or FHM cell lines by pretreating the cells with polyethylene glycol and using a methylcellulose overlay as described in Batts, Traxler, Winton (1991) and Batts & Winton (1989). Cell lines were maintained and subcultured in 150 cm^2 tissue culture flasks (Corning) at 25 $^{\circ}C$ using a growth formulation of Earle's salt-based minimal essential medium (MEM) (Invitrogen) supplemented with L-glutamine (29.2 mg mL^{-1}) (Invitrogen), penicillin (100 IU mL^{-1}) and streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% foetal bovine serum (Hyclone), and sodium bicarbonate (7.5% w/v) (Sigma-Aldrich).

Juvenile fish from 11 species were obtained from several sources. Gametes of salmonid species were fertilized, incubated, and raised at our laboratory at the Michigan State University Research Containment Facility (East Lansing, MI). Gametes of coho salmon, *Oncorhynchus kisutch* (Walbaum), were

obtained from the Platte River Weir (Beulah, MI), chinook salmon, *O. tshawytscha* (Walbaum), from the Little Manistee Weir (Stronach, MI), Brown trout, *Salmo trutta* L., and Rainbow trout, *O. mykiss* (Walbaum), from the Oden State Fish Hatchery (Alanson, MI), Brook trout, *Salvelinus fontinalis* (Mitchill), Lake trout, *Salvelinus namaycush* (Walbaum), and their hybrid Splake, *Salvelinus namaycush X Salvelinus fontinalis*, from the Marquette State Fish Hatchery (Marquette, MI) and Atlantic salmon, *Salmo salar* L., from Lake Superior State University (Sault Ste. Marie, MI). Gametes and fingerlings were tested for freedom from VHSV and other pathogens according to the guidelines of the American Fisheries Society Bluebook (2007). In addition, certified VHSV-free juvenile largemouth bass, *Micropterus salmoides* (Lacepéde), were purchased from Stoney Creek Fisheries and Equipment (Grant, MI), yellow perch, *Perca flavescens* (Mitchill), were purchased and transported from Cedar Lane Farms Inc. (Wooster, OH) and juvenile muskellunge were obtained from the Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, IA). Information on weights and lengths of experimental fish are given in Table 2-1. The juvenile muskellunge were fed *ad libitum* with 2.0 mm sinking feed pellets (Silver Cup, Nelson and Sons). Other fish species were fed daily at 1.2-1.5% of body weight using 2.0-4.0 mm feed pellet (Silver Cup).

Prior to infection, five fish from each species were killed using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories) (25 mg mL^{-1}) and tested for the presence of VHSV. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf

Laboratories Ltd) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (Invitrogen) supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}; Invitrogen), and amphotericin B (250 µg mL^{-1}; Invitrogen) to produce a 1:4 dilution (w/v) of original tissues. Samples were centrifuged at 2000 ***g*** and the supernatant was inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% foetal bovine serum). Plates were incubated at 15 $^{\circ}C$ for 7 days, and observed for the formation of cytopathic effects. A second blind passage was performed and assessed for the presence of VHSV.

Fish were challenged with a single dose of MI03 using the intraperitoneal (i.p.) route. The fish were allowed to acclimate to the laboratory conditions for at least 3 weeks prior to infection. All species, except muskellunge and largemouth bass, received 7 x 10^5 pfu in 100 µL MEM supplemented with 2% foetal bovine serum. Muskellunge and largemouth bass were inoculated with 7 x 10^4 pfu in the same manner. Experimental fish were kept in 74 L polyethylene tanks (one tank per fish species) that were continuously supplied with water chilled to 13 $^{\circ}C$. Another group of fish from each species received 100 µL of MEM i.p. and constituted negative controls. Fish were observed daily for up to 28 days post-infection. External and internal lesions were recorded and samples of lesions, spleens, and kidneys were collected and divided into two groups. The first group was subjected to homogenization and virus isolations on EPC and FHM cell lines

and the second group was fixed in 10% formalin and processed for histopathology and haematoxylin and eosin (H & E) staining as detailed in Prophet, Mills & Arrington (1992). Total RNA was extracted from infected cells using Trizol® LS reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription- polymerase chain reaction (RT-PCR) was performed as described in our earlier study (Elsayed *et al.* 2006).

As early as 5 days post-infection, differences among fish species were noted in terms of the severity of clinical signs and onset of mortalities. While 10 species suffered varying degrees of mortalities, infected Chinook salmon juveniles survived the infection and exhibited no disease signs during the observation period. Disease signs included external haemorrhages, diffuse and/or petechial in nature, and whitish, anemic discolouration of the gills (Fig. 2-1a). Haemorrhages were observed in the muscles (Fig.2-1b), liver, abdominal fat (Fig. 2-1c) and swimbladder wall (Fig. 2-1d). Onset of mortalities, daily mortalities, cumulative mortalities, and median days to death are shown in Table 2-1. First mortalities were noticed as early as two days post-infection in the lake trout, while in Atlantic salmon first mortality did not occur until day 12 post-infection. VHSV was isolated from moribund and dead fish on EPC and identity confirmed by RT-PCR. No clinical signs or mortality were observed in the negative controls.

Histopathological alterations involved multifocal haemorrhagic areas in the skin with the underlying musculature exhibiting degenerative changes, oedema, and multifocal to profound haemorrhaging between the myofibres (Fig. 2-2a).

Moreover, swollen and degenerated hepatocytes (Fig. 2-2b) and congested spleens (Fig. 2-2c) were common to all infected fish. In the kidneys, most glomeruli were oedemic with degenerated epithelial lining of the renal tubules (Fig. 2-2d). The connective tissue surrounding the swimbladder exhibited marked haemorrhage with degeneration and necrosis of the mucosal lining (Fig. 2-2e).

Our findings demonstrate that VHSV-IVb is capable of establishing infection in a wide range of cool and coldwater fish species. After i.p. exposure, 10 of the 11 species infected with MI03 exhibited clinical signs consistent with those described for VHS (Wolf 1988) and suffered varying degrees of mortality. VHSV was recovered from internal organs of affected fish and its identity confirmed by PCR. These findings fulfill River's postulates (1937) for the mortalities observed in a number of fish species in the Laurentian Great Lakes basin. The findings of this study also confirm that VHSV-IVb has a relatively wide host range. Such a wide host range has never been reported for any of the VHSV strains.

A limited number of experimental infection studies using the other North American strain (IVa) have been performed. VHSV-IVa was found highly pathogenic to the Pacific herring, *Clupea pallasii* (Valenciennes) (Kocan, Bradley, Elder, Meyers, Batts & Winton 1997; Hershberger, Gregg, Pacheco, Winton, Richard & Traxler 2007), moderately pathogenic to the Pacific sardine (Arkush, Mendonca, McBride, Yun, McDowell & Hedrick 2006) and of minimal pathogenicity to rainbow trout (Winton, Batts, Deering, Brunson, Hopper, Nishizawa & Stehr 1991). Results obtained in the present study tend to display a

similar trend. From the time to death, percent cumulative mortality, and the severity of exhibited clinical signs, it was obvious that muskellunge and largemouth bass were more affected by VHSV, even at a lower dose, than were other fish species. It was also obvious from the mortality and morbidity that coho and chinook salmon were the least affected.

The experimentally infected fish in the present study displayed histopathological lesions that were very similar to those VHSV-induced histopathological changes reported from the rainbow trout (Smail & Munro 1989), pike, *Esox lucius* L. (Meier & Jørgensen 1980), and sea bass, *Dicentrarchus labrax* (L.) (Castric & de Kinkelin 1984), indicating that VHSV-infected fish die from blood extravasation that leads to tissue deprivation of oxygen. In conclusion, this study provides solid evidence that the fish mortality episodes noticed in the Great Lakes basin (2002-2008) were indeed caused by the VHSV-IVb. The study also provides evidence that juvenile fish are susceptible to the infection.

Table 2-1 Mortality in selected Great Lakes fish species following experimental infection with the MI03 index strain of viral haemorrhagic septicaemia virus genotype IVb. Muskellunge and largemouth bass were inoculated intraperitoneally with 7 x 10^4 pfu per fish in 100 µL. Other species, received 7 x 10^5 pfu per fish in the same manner. All fish were monitored daily for an observation period of 28 days post-infection at a water temperature of 13 $^{\circ}$C.

Species	**Length (Cm)**	**Weight (g)**	**Number of fish infected**	**Number of mortalities**	**Median day to death**
Muskellunge	15.8 ± 1.0	17.5 ± 2.3	10	10	5
Largemouth bass	17.5 ± 1.0	82.2 ± 12.3	10	8	8
Splake	15.9 ± 2.7	35.1 ± 17.3	30	13	9
Yellow perch	18.3 ± 0.2	75.5 ± 6.6	10	4	14
Brook trout	16.7 ± 1.1	45.2 ± 10.1	10	4	13
Brown trout	16.9 ± 0.7	47.8 ± 6.0	10	4	9.5
Rainbow trout	13.8 ± 0.9	24.8 ± 3.2	10	3	11
Lake trout	16.3 ± 10.3	29.0 ± 10.8	30	6	2
Atlantic salmon	17.8 ± 1.1	46.1 ± 8.8	30	4	17
Coho salmon	16.5 ± 1.6	38.5 ± 14.5	10	1	7
Chinook salmon	8.9 ± 0.4	6.6 ± 1.0	10	0	-

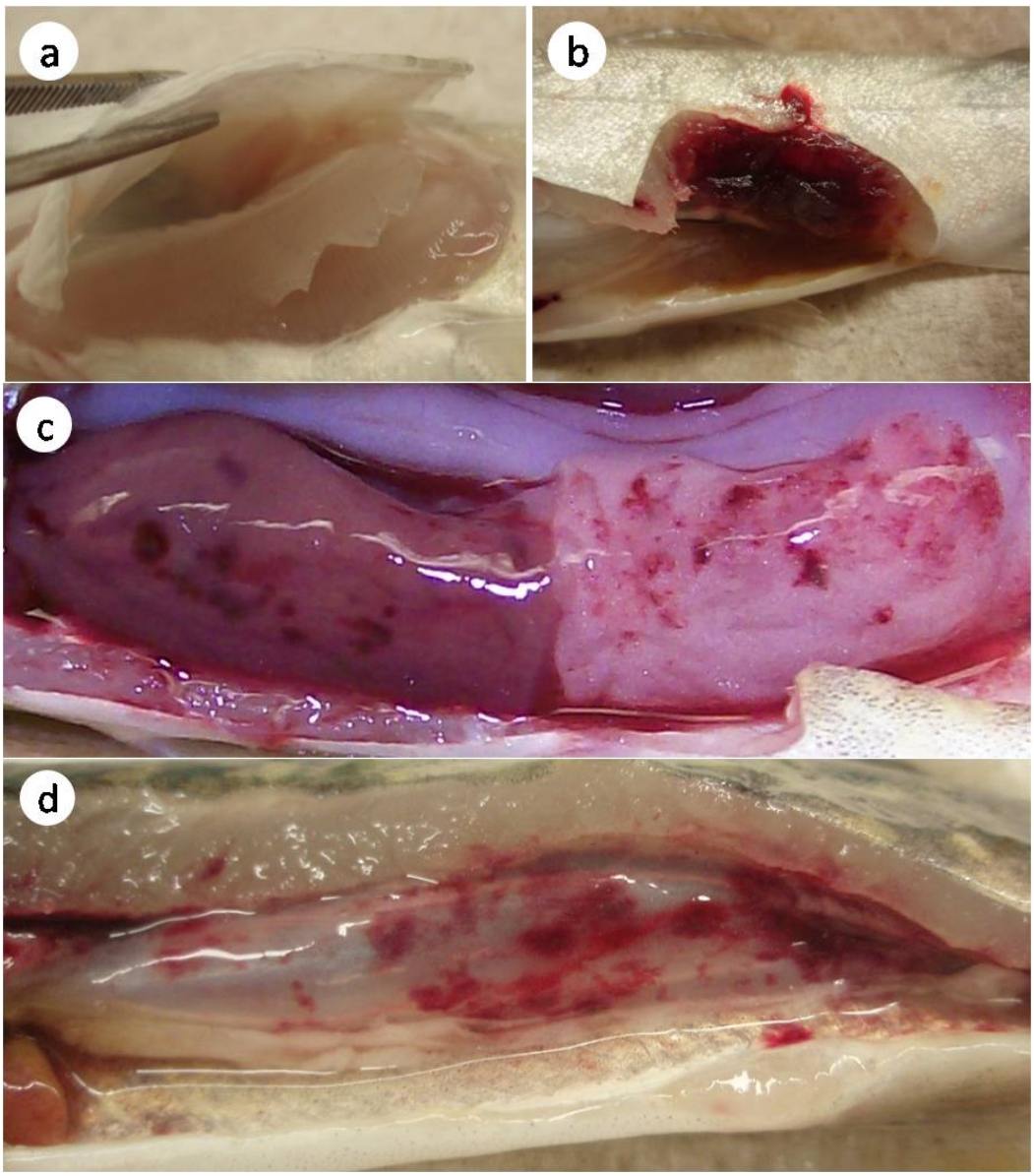

Figure 2-1 Viral haemorrhagic septicaemia virus-infected splake.
(a) pale gills and (b) severe intramuscular hemorrhage. Notice the focal area of darkening centrally and the expansion of the haemorrhage outward. (c) Infected lake trout exhibiting severe hepatomegaly with subserosal petechial and ecchymotic haemorrhages. (d) Infected lake trout exhibiting petechial and ecchymotic haemorrhages on the swimbladder wall.

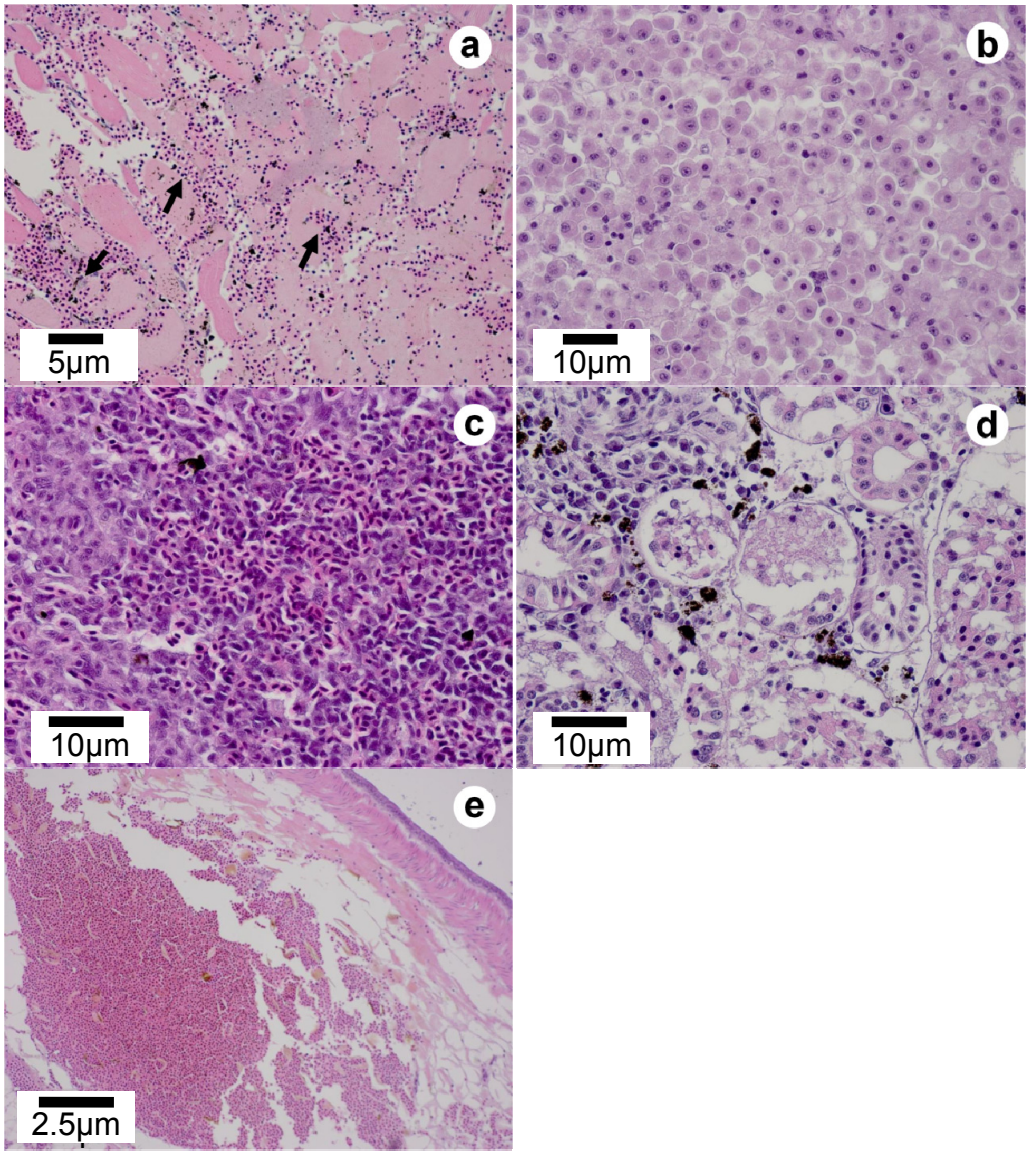

Figure 2-2 Histopathologic descriptions of tissues from fish experimentally infected with viral hemorrhagic septicemia virus IVb.
(a) Tissue section of a viral haemorrhagic septicaemia virus-infected lake trout showing widespread haemorrhage and infiltration of erythrocytes (black arrows) in the lateral trunk muscles (H&E, X200). (b) Liver section of an infected lake trout showing severely swollen and vacuolated hepatocytes (H&E, X400).
(c) Spleen section of an infected Atlantic salmon showing congestion and scattered erythrocytes throughout splenic tissue (H&E, X400). (d) Kidney section of an infected lake trout showing severe tubular necrosis and glomerular degeneration (H&E, X400). (e) Experimentally infected splake exhibiting massive infiltration of erythrocytes into connective tissue surrounding the swim bladder (H&E, X100).

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CHAPTER 3

Kim R. & Faisal M. (2010) Comparative susceptibility of representative Great Lakes fish species to the North American Viral Haemorrhagic Septicaemia Virus (VHSV) Sublineage IVb. *Diseases of Aquatic Organisms*, 91:23-34.

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CHAPTER 3

Comparative susceptibility of representative Great Lakes fish species to the North American viral hemorrhagic septicemia virus (VHSV) sublineage IVb

Abstract

This study was performed in order to compare the susceptibility of representative Laurentian Great Lakes fish species to the emerging viral hemorrhagic septicemia virus (VHSV) genotype IVb. The median lethal dose of infection by intraperitoneal injection (IP-LD_{50}) was obtained from fish that were experimentally infected with the MI03 index strain of VHSV-IVb. Fish were injected at doses ranging from 7×10^{7} plaque forming units (pfu) to 7×10^{-2} pfu and maintained at $12 \pm 1^{\circ}C$. The infection trials identified species of high, medium and low susceptibility based on the IP-LD_{50} values. Pathogenicity of VHSV-IVb was highest in largemouth bass *Micropterus salmoides,* which resulted in an IP-LD_{50} of 1.5×10^{2} pfu, while also demonstrating the clinical diathesis of VHSV infected fish. The virus was moderately pathogenic in yellow perch *Perca flavescens* (IP-LD_{50} of 2.5×10^{5} pfu), but also showed the classical signs of VHSV infection. Salmonids were the least susceptible to VHSV-IVb with IP-LD_{50} values no less than 1.4×10^{6} pfu, however, in fish that succumbed to infection, characteristic VHSV lesions were observed. Histopathologic alterations were most profound in gill, skin, muscle, gonads, and liver of largemouth bass and yellow perch, while in salmonids hemorrhages in the swimbladder and/or

degenerative changes in the liver were the most common lesions noticed. VHSV was isolated from infected fish and its identity confirmed by the reverse transcriptase polymerase chain reaction (RT-PCR). These results highlight the variations among fish species susceptibility to this emerging strain of VHSV and offer insights into the potential impact of VHSV-IVb on the Laurentian Great Lakes fish community.

Introduction

Viral hemorrhagic septicemia (VHS) is a serious viral infection of teleosts in the northern hemisphere (Wolf 1988). The causative agent is the viral hemorrhagic septicemia virus (VHSV), a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Tordo et al. 2005). Since the initial description of VHS by Schäperclaus in 1938 and virus isolation by Jensen (1963) on cell culture, the disease has spread into many European countries devastating rainbow trout *Oncorhynchus mykiss* farmed in freshwater.

Nearly three decades later, VHSV was identified in North America for the first time in coho salmon *Oncorhynchus kisutch* (Hopper 1989) and chinook salmon *Oncorhynchus tshawytscha* (Brunson et al. 1989). Subsequent isolations in other marine fish were soon reported in Europe (Dixon et al. 1997, Mortensen et al. 1999, Smail 2000) and North America (Meyers et al. 1992, 1999, Traxler et al. 1995, Benmansour et al. 1997, Hedrick et al. 2003). VHSV was later isolated in water around Japan (Byon et al. 2006) and Korea (Kim et al. 2003). Such isolations strongly suggest that VHSV may be endemic to the marine environment.

Based upon partial sequences of the G and N genes and phylogenetic analyses of VHSV isolates worldwide, four distinct genotypes of VHSV (designated I-IV) have been identified (Snow et al. 1999, 2004, Einer-Jensen et al. 2004, 2005). Each of these genotypes has its geographic and host ranges. Genotype I consists of five subgroups and is the major cause of VHS epidemics in European rainbow trout farms, but also includes marine species from the Baltic

Sea. Genotype II also includes a number of fish species from the Baltic Sea, while genotype III includes marine fish species around the British Isles. Genotype IV pertains to isolates from North America, Japan and Korea.

Most recently, a novel sublineage of the North American VHSV was found in the Laurentian Great Lakes basin and has been linked to numerous massive kills of freshwater fish species such as the muskellunge *Esox masquinongy* (Elsayed et al. 2006), freshwater drum *Aplodinotus grunniens* (Lumsden et al. 2007), round gobies *Neogobius melanostomus* (Groocock et al. 2007), striped bass *Morone saxatilis*, brown trout *Salmo trutta trutta*, mummichog *Fundulus heteroclitus*, and three-spined stickleback *Gasterosteus aculeatus aculeatus* (Gagné et al. 2007). Genotyping of this recent VHSV isolate from the Great Lakes has shown that it is distinct from all four previously known genotypes, but most closely related to the marine VHSV-IVa (Elsayed et al. 2006). The Great Lakes VHSV isolates have therefore been designated as genotype IVb. This new VHSV differs from VHSV-IVa not only in terms of gene sequences but also in its affinity to freshwater systems, infecting 28 fish species thus far (http://www.aphis.usda.gov). This presumably wide host range within a confined location has never been reported with other VHSV strains or other fish pathogenic rhabdoviruses.

In all VHSV-IVb associated fish kill episodes, mature, large fish were the only age class noticed. This has raised the question of whether or not other age classes of susceptible fish species also succumb to VHSV-IVb. While fish kill episodes demonstrate the destructive and pathogenic nature of VHSV-IVb

(Gagné et al. 2007, Groocock et al. 2007, Lumsden et al. 2007), little data exists on VHSV-IVb pathogenicity to Great Lakes fish, the lesions they induce, and whether this VHSV strain is the only cause of fish kills observed in the Great Lakes basin.

In a preliminary study, 11 juvenile Great Lakes fish species were injected by the intraperitoneal (i.p.) route with VHSV-IVb at equivalent doses which were approximately 7×10^5 pfu or 7×10^4 pfu (Kim & Faisal 2010). Interestingly, the results indicated higher mortalities in muskellunge and largemouth bass, while yellow perch and a number of salmonids experienced moderate to low mortality rates at the same dose. Despite the fact that Rivers' postulates were fulfilled in the study, it was not clear whether variable susceptibility existed among Great Lakes fish. Comparing susceptibilities to VHSV is critical to fisheries managers as it will help guide decisions in conservation and restoration efforts. To this end, a more comprehensive study was designed, using a wider range of doses and greater numbers of fish to compare the susceptibility of juvenile fish species based on the calculated median lethal dose of infection in resident game fish of the Laurentian Great Lakes basin.

Materials and Methods

Cell culture and virus propagation

We used the Great Lakes VHSV-IVb MI03 strain, originally isolated in our laboratory from a naturally infected muskellunge caught from Lake St. Clair (Elsayed et al. 2006). Virus stocks were produced in *Epithelioma papulosum cyprini* (EPC; Fijan 1983), aliquoted, and kept at -80 $^{\circ}$C until used. In order to determine the virus concentration, plaque assay was performed on EPC cell lines by pre-treating the cells with polyethylene glycol and using a methylcellulose overlay as described in Batts et al. (1989, 1991). Titrated virus stocks were aliquoted in cryogenic vials (Corning, Lowell, MA) and kept at -80 $^{\circ}$C until used.

Cell lines were maintained and sub-cultured in 150 cm^2 tissue culture flasks (Corning) at 25 $^{\circ}$C using a growth formulation of Earle's salt-based Minimal Essential Medium (MEM) (Invitrogen) supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich, St. Louis, MO).

Fish and Maintenance

Juvenile fish from seven species were obtained from several sources. Fingerlings of salmonid species were obtained as gametes and subsequently fertilized,

incubated, and raised at the Michigan State University Research Containment Facility (East Lansing, MI). Gametes of coho salmon were obtained from the Platte River Weir (Beulah, MI), chinook salmon from the Little Manistee Weir (Stronach, MI), brown trout and rainbow trout from the Oden State Fish Hatchery (Alanson, MI), and brook trout *Salvelinus fontinalis* from the Marquette State Fish Hatchery (Marquette, MI). Gamete samples and tissues from five fingerlings were tested for the presence of VHSV and other pathogens according to the guidelines of the American Fisheries Society Bluebook (2007). In addition, certified VHSV-free juvenile largemouth bass were purchased from Stoney Creek Fisheries and Equipment (Grant, MI) and yellow perch were purchased and transported from Cedar Lane Farms, Inc. (Wooster, OH). Information on weights and lengths of experimental fish are given in Table 1.

Prior to infection, five fish from each species were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) (25 mg ml^{-1}) and tested for the presence of VHSV. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd. Pocklington, York, UK) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen) supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}) (Invitrogen), and amphotericin B (250 µg ml^{-1}, Invitrogen) to produce 1:4 dilution (w/v) of original

tissues. Samples were centrifuged at 2000 *g* and the supernatant was inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Cultures were incubated at 15 °C for seven days, and observed for the formation of cytopathic effect (CPE). Infected cells were then placed in a -80 °C freezer and removed after 24 hours. The medium was removed, thawed and centrifuged. The supernatant was inoculated for a second time onto EPC cell lines and after seven days was assessed for the presence of VHSV by RT-PCR.

Experimental fish were allowed to acclimate to the laboratory conditions for three weeks, during which the temperature was progressively lowered to 12 ± 1 °C before the experiment was initiated. All fish were initially held in 600 liter circular fiberglass tanks in a continuous flow-through system supplied with oxygenated and facility chilled well water (Michigan State University-Research Containment Facility, East Lansing, MI). All fish species were fed daily at 1.2-1.5% of body weight using 2.0-4.0 mm feed (Silver Cup, Murray, UT). Prior to their use in the experimental infection studies, all fish were acclimated to the laboratory conditions and observed for normal feeding behavior.

Fish were designated as coolwater (largemouth bass and yellow perch) or coldwater species (rainbow trout, brook trout, brown trout, chinook salmon, coho salmon). Morbidity, mortality, and IP-LD_{50} calculation demonstrated the presence

of clear variability among the seven species tested for their susceptibility to VHSV-IVb.

Experimental infection of selected juvenile fish species from the Great Lakes by the i.p. route and calculation of the median lethal intraperitoneal dose (IP-LD_{50})

In this experiment, juveniles of seven fish species were challenged with doses ranging from as high as 7×10^{7} pfu to as low as 7×10^{-2} pfu in 100 µl MEM supplemented with 2% fetal bovine serum by i.p. injection. Each dose level was administered to ten fish shortly after being anesthetized with MS-222 (100-150 ppm). Fish were then recovered from anesthesia in a 72 liter polyethylene tank (Aquatic Eco-Systems, Apopka, FL) in a continuous flow through system. A negative control tank for each species contained ten fish that were injected i.p. with 100 µl MEM only. All fish were monitored daily for an observation period of 28 days post-infection (p.i.). The inflowing water was supplied by an underground well and chilled to 12 ± 1 $^{\circ}$C throughout the duration of the experiment. The outflow was united into a common tract entering an ultraviolet sterilization unit (Aquafine, Valencia, CA). Tanks were monitored every 8-12 hours daily for moribund or dead fish.

Virus recovery

Kidney and spleen tissues were collected from each dead or moribund fish, processed, diluted, and then tested for the presence of VHSV by the methods described above.

Histopathology

Lesions that were grossly observed from dead and severely moribund (euthanized with an overdose of MS-222; 25 mg ml^{-1}) fish were sampled and submitted for histopathology. External and internal lesions were fixed in 10% formalin prior to sectioning. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) as detailed in Prophet et al. (1992).

RNA extraction and RT-PCR

Total RNA was extracted from infected cells using Trizol® LS Reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was accomplished by a two step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR™ (Stratagene, La Jolla, CA) following the manufacturer's instructions. The primer set used in this assay is recommended by the World Organization for Animal Health (OIE 2006) for detection of a 811 base pair sequence of the VHSV nucleocapsid gene: 5'-GGG-GAC-CCC-AGA-CTG-T-3' (forward primer) and 5'-TCT-CTG-TCA-CCT-TGA-TCC-3' (reverse primer). Polymerase chain reaction was achieved by adding into each reaction tube 5 µl of viral RNA, 50 pmol of each primer, 25 µl of Choice™

Taq Mastermix DNA Polymerase (Denville Scientific, Inc. South Plainfield, NJ), and DNA-ase free water to create a final volume of 50 µl. The reverse transcriptase was inactivated by subjecting the mixture to a 94 °C for 2 min, and 30 cycles of PCR (denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and polymerization at 68 °C for 1 min) in a Mastercycler Personal Thermal Cycler (Eppendorf, Hamburg, Germany). The polymerization was finalized by maintaining the mixture for a period of 7 min at 68 °C. The product was visualized by gel electrophoresis in 1.5% agarose gels. A subsample of each species was tested by cell culture and RT-PCR assay using VHSV-specific primers.

Results

Prior to experimental infection, subsamples of experimental fish were found to be free of VHSV or any other virus that can replicate in EPC cell lines.

Largemouth bass

The first incidence of mortality was noted as early as 6 days p.i. at doses of 7 x 10^4 pfu, 7 x 10^3 pfu, and 7 x 10^2 pfu (Figure 3-1). However, fish infected with 7 x 10^3 pfu reached a maximum cumulative mortality of 90% by day 27 p.i., whereas the group infected at a higher dose of 7 x 10^4 pfu reached 80% mortality by day 16 p.i. Rapid rates of mortality were exhibited between days 5 and 10 p.i. in fish infected with 7 x 10^4 pfu to 7 x 10^1 pfu. Doses equal to or less than 7 pfu did not result in any mortality of the fish monitored for 28 days p.i. The Reed and Muench (1938) equation resulted in an IP-LD_{50} dose of 1.5 x 10^2 pfu (Table 1).

Infected fish exhibited gross lesions externally and internally that were identical in moribund and dead fish regardless of the VHSV dose. Pronounced petechiation on most fins was noted (Figure 3-2a). Diffuse dermal erythema (Figure 3-2b) was also appreciable on the ventral abdomen with peri-anal swelling and partial anal prolapse (Figure 3-2b). Focal areas of hemorrhage were noted on gill arches surrounded by pale and whitened lamellae (Figure 3-2c). In some fish, subdermal petechiation was noted caudal to the gills (Figure 3-2d). Internal organs were surrounded by serosanguinous ascitic fluid (Figure 3-2e) and subserosal

petechia was noted on all internal organs and visceral fat (Figure 3-2e), while testes and ovarian tissues were swollen and reddened.

Yellow perch

Initial mortalities occurred later than in the case of largemouth bass with the first mortality noted from days 10 to 12 p.i. at doses ranging from 7×10^7 pfu to 7×10^3 pfu (Figure 3-3). With the exception of fish infected with 7×10^5 pfu, which reached a cumulative mortality of 40%, fish infected with 7×10^7 pfu to 7×10^3 pfu displayed a maximum cumulative mortality of 60-70%. A decrease in the mortalities (10%) was noted beginning with experimental group infected with 7×10^2 pfu which did not experience its first and only mortality until day 21 p.i. The lowest dose of 70 pfu caused no mortality. No mortality occurred in the negative control tanks. Calculation of an IP-LD_{50} using the Reed and Muench (1938) equation resulted in a value of 2.5×10^5 pfu (Table 1).

Infected yellow perch exhibited pale gills, erythematous protruded vent (Figure 3-4a), intramuscular hemorrhages (Figure 3-4b), and an accumulation of ascitic fluid. Severe hepatomegaly with irregular margination of the tissue was noted along with subcapsular petechial hemorrhages (*not shown*). The kidneys of yellow perch were normal in appearance on gross examination.

Rainbow trout

Fish inoculated at a dose of 7×10^7 pfu experienced the highest mortalities (78%) with the majority of deaths occurring between 8 and 11 days p.i. (Figure 3-5). The second highest mortality rate (60%) occurred in fish infected with 7×10^4 pfu with most of the deaths occurring between 11 and 20 days p.i. The remaining groups experienced a maximum mortality between 20-30%. Interestingly, the most rapid rates of mortality were observed between days 8 and 14 p.i. The group of fish infected with 70 pfu was apparently healthy at the end of the study and did not record any mortality throughout the 28 day observation period. There were no mortalities noted in the control tank. The IP-LD_{50} calculation in accordance with the Reed and Muench (1938) equation was 1.4×10^6 pfu (Table 1).

Despite mortality at higher doses, clinical signs were seldom seen. External lesions included mild to moderate subdermal petechial hemorrhages around the base of fins. Pronounced changes were also noted internally with livers that were noticeably pale and friable with petechiation on the sub-serosal surface (Figure 3-6a), splenomegaly, moderate to severe ecchymotic and petechial hemorrhages on the swimbladder wall, and intramuscular hemorrhaging (Figure 3-6b).

Brook trout

The highest mortality rate in brook trout did not occur at the highest level of inoculum delivered (Figure 3-7). Fish injected with 7×10^3 pfu reached 50% mortality between the days of 6 and 9 p.i. Unlike other groups which reached a maximum mortality of 30 to 40%, the lower dosage appeared to be more lethal. The first mortality was noted at 3 days p.i. in fish infected at the highest dose of 7×10^7 pfu but only reached a maximum mortality of 30%. Furthermore, the majority of deaths occurred between days 5 and 14 p.i. which was similar to that observed in rainbow trout. The control tank did not experience any mortality throughout the duration of the experiment. Following the equation generated by Reed and Muench (1938) the IP-LD_{50} was 5×10^6 pfu (Table 1).

External signs of disease were rarely noted. Conversely, internal lesions were in the form of pale, friable and enlarged livers (Figure 3-8). Severe petechiation and ecchymotic hemorrhages were diffusely scattered throughout adipose tissue surrounding the viscera (Figure 3-8). Abdominal organs and viscera were remarkably wet on appearance as a result of severe ascitic fluid accumulation.

Brown trout

Fish inoculated at the highest dose of 7×10^7 pfu experienced the highest mortalities (80%) and the earliest mortality at 3 days p.i. (Figure 3-9). However, a constant rate of mortality was observed throughout the observed 28 days.

Unexpectedly, the second highest dose administered (7×10^6 pfu) achieved a maximum mortality rate of only 10% which occurred on the 17th day p.i. When comparing the doses following thereafter, a correlation between the days p.i. to first mortality and dose administered is evident. In the end, cumulative mortality rates did not clearly exhibit a correlation to dosage administered. No mortalities were noted from the control tank throughout the 28 day period. In accordance with the Reed and Muench (1938) equation, the IP-LD_{50} was 5×10^6 pfu (Table 1).

Clinical signs observed in brown trout were also mild when compared to other species. One fish that was inoculated with 7×10^2 pfu had peri-anal swelling and severely pale gills (Figure 3-10). No internal lesions were noted.

Chinook salmon

Fish inoculated at a dose of 7×10^7 pfu had the highest cumulative mortality (70%) with the majority of mortalities occurring between days 5 and 12 p.i. (Figure 3-11) When assessing the mortality data for the lower doses, cumulative mortality was approximately 20-30% with the exception of groups infected with 7×10^5 pfu and 7×10^4 pfu, which contained fish that were apparently healthy by the end of the 28 day observance period. No mortalities were noted in the control tank. Calculation of the Reed and Muench (1938) equation resulted in a LD_{50} of 1.01×10^7 pfu (Table 1).

Profound external lesions were not noted upon gross examination. Few mortalities were, however, observed to have distended abdomens with palpable fluid contents (Figure 3-12a). The suspicion of ascites was confirmed when approximately 500-1000 µl of fluid were obtained from some individuals (Figure 3-12b).

Coho salmon

Overall, only 10% cumulative mortality was noted for each dose of 7×10^7 pfu and 7×10^5 pfu on days 22 and 7 p.i., respectively (Figure 3-13). All other doses used in this experiment did not result in mortality. No mortalities were noted in the control tank. As a result of being unable to obtain mortalities above and below 50%, the IP-LD_{50} value was estimated to be greater than the highest administered dose of $> 7 \times 10^7$ pfu. One mortality was noticeably emaciated with caudal fin erosion and necrosis.

Kidney and spleen samples from mortalities of all species were confirmed for the presence of VHSV by cell culture and identity of isolated virus confirmed by RT-PCR.

Histopathologic Analysis

Microscopic lesions were identical in VHSV-moribund fish of the seven species. The severity, however, varied from one fish to another. In the case of largemouth bass, histological sections of the fins revealed diffuse congestion and multifocal hemorrhage of all fins that were sampled (Figure 3-14a). Profound changes were noted in submitted gill tissues which often displayed marked congestion in the primary lamellar spaces (Figure 3-14b). Splenic tissue was markedly congested with focal areas of erythrocyte accumulation (Figure 3-14c). The ovarian tissue exhibited diffuse congestion and severe hemorrhaging throughout the interstitium (Figure 3-14d). The anterior kidneys exhibited marked lymphoid depletion in most fish.

Histopathology was demonstrated in the yellow perch in the form of subcutaneous hemorrhage, edema, and congestion between the dermis and subcutaneous tissue (Figure 3-15a) with interstitial hemorrhage between myofibers in deeper skeletal muscle-fibers (Figure 3-15b). The liver exhibited marked multifocal necrosis, vacuolation of hepatocytes, and localized areas of congestion (Figure 3-15c).

All salmonids showed significantly less pathological changes in comparison to those observed in the largemouth bass or the yellow perch. The swimbladders of the rainbow trout displayed multifocal hemorrhages in the outer wall (Figure 3-16a), while the swimbladders of brook trout and brown trout exhibited massive

hemorrhages primarily in the surrounding connective tissue. Rainbow trout livers often demonstrated multifocal areas of necrosis and degeneration with vacuolation of hepatocytes containing pyknotic nuclei (Figure 3-16b), which was similarly noted in the brook trout. Kidneys were not examined microscopically.

Discussion

The generated data from this study confirmed a wide range of susceptible fish hosts to VHSV-IVb and suggests that coolwater fish species are probably more susceptible to VHSV-IVb than salmonid species. The largemouth bass were by far the most susceptible, followed by the yellow perch which required a higher viral dose to reach the IP-LD_{50}. Salmonids required a dose several orders of magnitude higher to reach the median lethal dose, which also resulted in fewer mortalities overall. The coolwater species exhibited clinical signs consistent with VHSV (as reviewed in Wolf 1988), suffered varying degrees of mortality, and succumbed to death by VHSV. Virus was recovered from internal organs of affected fish by cell culture and identity confirmed by RT-PCR. These findings, together with our previous study on muskellunge (Kim & Faisal 2010), fulfill Rivers' postulates (1937) for the mortalities observed in a number of fish species in the Laurentian Great Lakes basin (Elsayed et al. 2006, Gagné et al. 2007, Groocock et al. 2007, Lumsden et al. 2007). The findings of this study also confirm the original field observations that VHSV-IVb has a relatively wide host range.

Most of the data on the host range and fish species susceptibility of the North American VHSV-IVa was obtained from moribund fish during mortality episodes or from apparently healthy fish during routine health inspections. A limited number of experimental infection studies using VHSV-IVa isolates have been performed. Results indicated that VHSV-IVa is highly pathogenic to the Pacific herring (Kocan et al. 1997, Hershberger et al. 2007), moderately

pathogenic to the Pacific sardine (Arkush et al. 2006), and of minimal pathogenicity to rainbow trout (Winton et al. 1991, Follett et al. 1997). Our results with VHSV-IVb indicate a similar trend, with clear differences noted between pathogenicity in coolwater species and coldwater species. From the time to death, percent cumulative mortality, and the severity of exhibited clinical signs data, the largemouth bass and muskellunge (Kim & Faisal 2010) were more affected by VHSV-IVb as confirmed by the low IP-LD_{50} values. Most interesting was the fact that the muskellunge IP-LD_{50} was 2.2 pfu (Kim & Faisal, unpublished), which was lower than that affecting largemouth bass. It was evident from the mortality and morbidity data that coho and chinook salmon were the least affected. Indeed, field observations indicated that these two species do not exhibit clinical signs, even though VHSV-IVb was isolated from them (Faisal, unpublished data). The lack of clinical signs and low susceptibility of brown trout in our study were corroborated with the field studies of Gagné et al (2007) by the VHSV-IVb isolate and experimental infections of Jørgensen (1980), who demonstrated that brown trout were susceptible to VHSV-I isolates only at high concentrations by immersion challenge.

In all of the seven fish species trials, some fish survived VHSV-IVb exposure throughout the observation period and beyond. These survivors were suspected to be carriers of VHSV, but this was not confirmed in our study. The potential for shedding p.i. has been suggested in previous experimental infections with VHSV (Jørgensen 1980, Meier & Jørgensen 1980), demonstrated in Pacific herring (Kocan et al. 1997) and is the basis for which cohabitation

infection trials have been conducted with turbot *Psetta maxima* (López-Vázquez et al. 2007), Japanese flounder *Paralichthys olivaceus* (Muroga et al. 2004) and Atlantic halibut *Hippoglossus hippoglossus* (Bowden 2003). The ability of fish to become carriers and active shedders was recently shown in experimentally infected salmonids that were held in tanks for nine months following infection (Faisal and Shavalier, personal communication). Others have also commented on the ability of VHSV infected fish to shed virus and become sources of infections leading to epizootic mortality (reviewed in Wolf 1988). These data are alarming given the prevalence of salmonid species that are resident to the Great Lakes and are commonly raised in hatchery settings for stocking. If VHSV-IVb should gain access to hatchery facilities, there may be a risk of producing "natural" reservoirs for VHSV throughout the Great Lakes, thereby jeopardizing conservation efforts. Future studies will require that the shedding potential and carrier status of salmonids be evaluated.

Clinical signs and internal lesions observed in this study were predominantly similar in coolwater fish species that experienced morbidity, with hemorrhages being the most prominent sign. The hemorrhage was often so intense that portions of the gills became white in color, a finding consistent with localized ischemia (Figure 3-2). Damage to capillary and sinusoid endothelia leading to hemorrhages has been previously demonstrated (de Kinkelin et al. 1979, Evensen et al. 1994). In the present study, the intensity of hemorrhages varied from one species to another, with largemouth bass showing the most external and internal hemorrhages. Surprisingly, the largemouth bass was the

only species to show congestion of the kidneys, but histopathology was not as severe compared to that of the muskellunge. It seems that severity of hemorrhaging depends on the susceptibility of capillary endothelium, which varies from one fish species to another. Studies performed on other VHSV strains have shown that while hemorrhages are less obvious in the Pacific herring with VHSV-IVa (Kocan et al. 1997), they were most obvious in rainbow trout (Neukrich 1984), pike *Esox lucius* (Meier & Jørgensen 1980), sea bass *Dicentrarchus labrax*, and turbot (Castric & de Kinkelin 1984). Also in the present study, no skin lesions such as those observed with VHSV-infected Pacific herring (Meyers et al. 1994), Pacific cod and hake (Smail 2000) were observed in VHSV-IVb infected fish.

Gross and histological lesions predominated in coolwater species, in comparison to the coldwater species examined. While largemouth bass and yellow perch demonstrated intramuscular hemorrhages, fin hemorrhages, severely pale gills, hemorrhages in gonadal tissue, and ascites, the salmonids rarely exhibited such signs. These changes were probably due to a number of reasons which include increased permeability of blood vessels, decreased clotting mechanisms, virus production in endothelium, and/or increased red cell fragility or intravascular hemolysis. The integrity of endothelial cells in the swimbladder of salmonids may also have been more vulnerable to VHSV, since hemorrhage and congestion in the swimbladder wall and connective tissue were a common finding amongst salmonids. While gross lesions and histopathology varied between cool and coldwater species in our study, some have suggested

experimentally infected non-salmonids and salmonids with VHSV-I isolates demonstrate similar lesions (Meier et al. 1980). The relationship between species type, virus genotype used, and lesions observed is unclear at this time and will require further study to determine whether a correlation exists.

In conclusion, this study provides solid evidence that the fish mortality episodes that occurred in the Great Lakes basin (2002-2008) are most probably caused by VHSV-IVb for a number of reasons since upon experimental infection, disease signs and tissue alterations in fish were similar to those observed in moribund wild fish from which VHSV IVb was re-isolated and confirmed by RT-PCR, thereby fulfilling Rivers' postulates (1937). The study also provides evidence that juvenile fish are susceptible to the infection. This is surprising since in the midst of mortality episodes, adult, sexually mature fish were the only class found. Further studies are being pursued to determine the interactions of each of the fish species with VHSV-IVb. These differences in susceptibility between cool and coldwater species, although determined in a laboratory setting, may offer clues to why VHSV-IVb is not commonly isolated from clinically affected salmonid species.

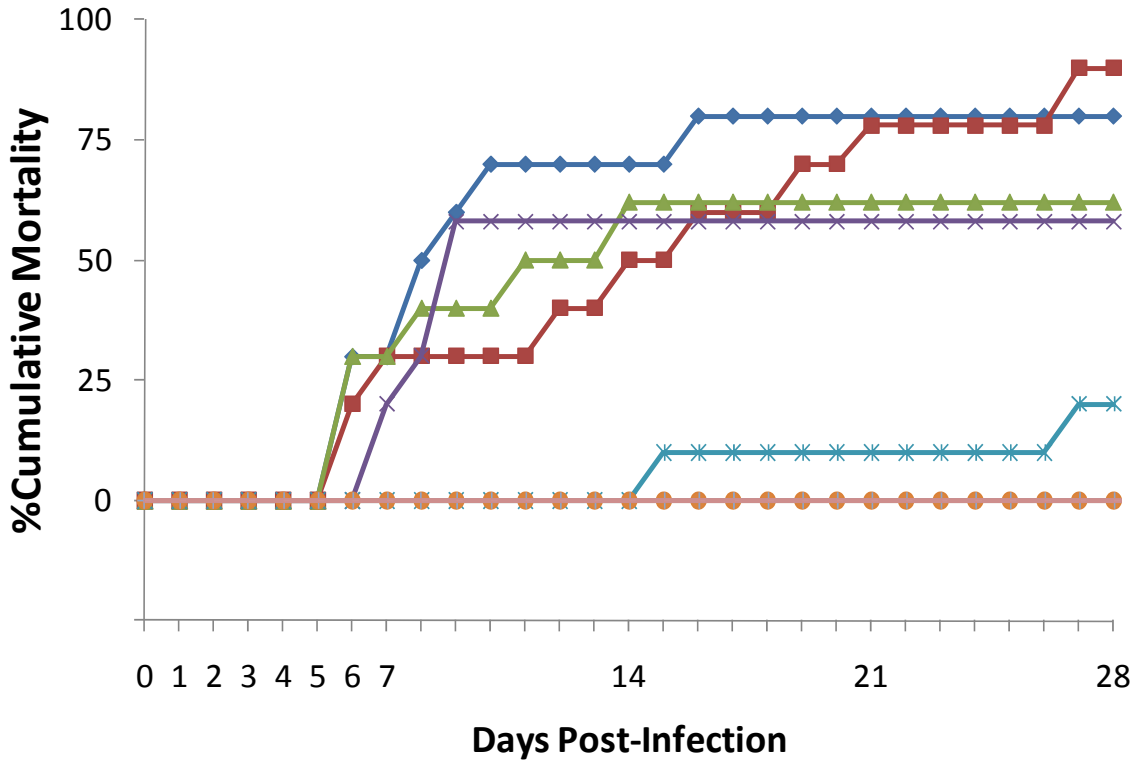

Figure 3-1 Cumulative mortalities of largemouth bass experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^4 pfu; -■-, 7 x10^3 pfu; -▲-, 7 x 10^2 pfu; -X-, 7 x 10^1 pfu; -Ж-, 7 pfu; -●-, 0.7 pfu; -+-, 0.07 pfu; ---, Negative control)

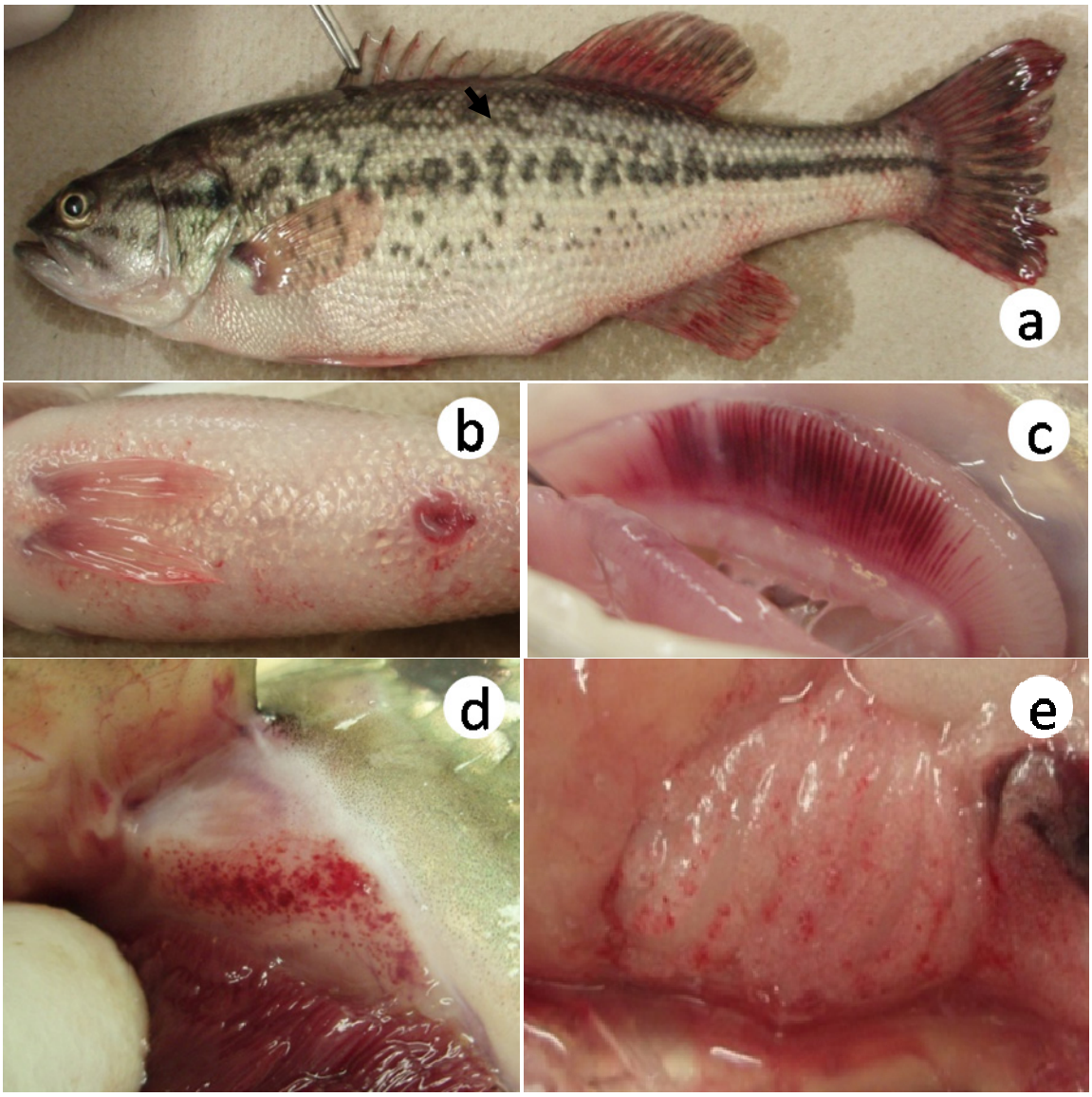

Figure 3-2 Clinical signs of largemouth bass experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection.
(a) pronounced petechiation on all fins, (b) diffuse dermal erythema of the ventral abdomen with a reddened, thickened and partially prolapsed vent (*black arrow*), (c) focal areas of hemorrhage on gill arches surrounded by pale and whitened lamellae, (d) subdermal petechiation on the thymic regions at the dorsal insertion of the gill arch, (e) Subserosal petechia on all internal organs, especially on the pyloric caeca.

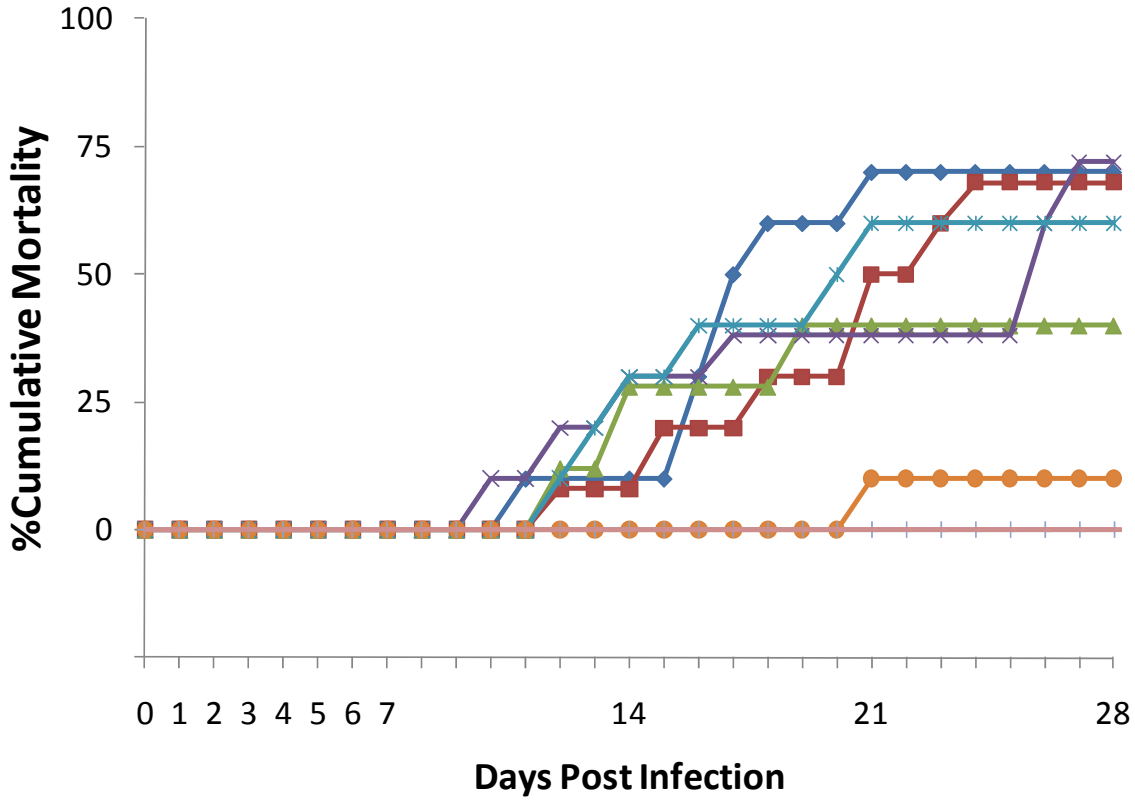

Figure 3-3 Cumulative mortalities of yellow perch experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, 7 x 10^2 pfu; -+-, 70 pfu; ---, Negative control)

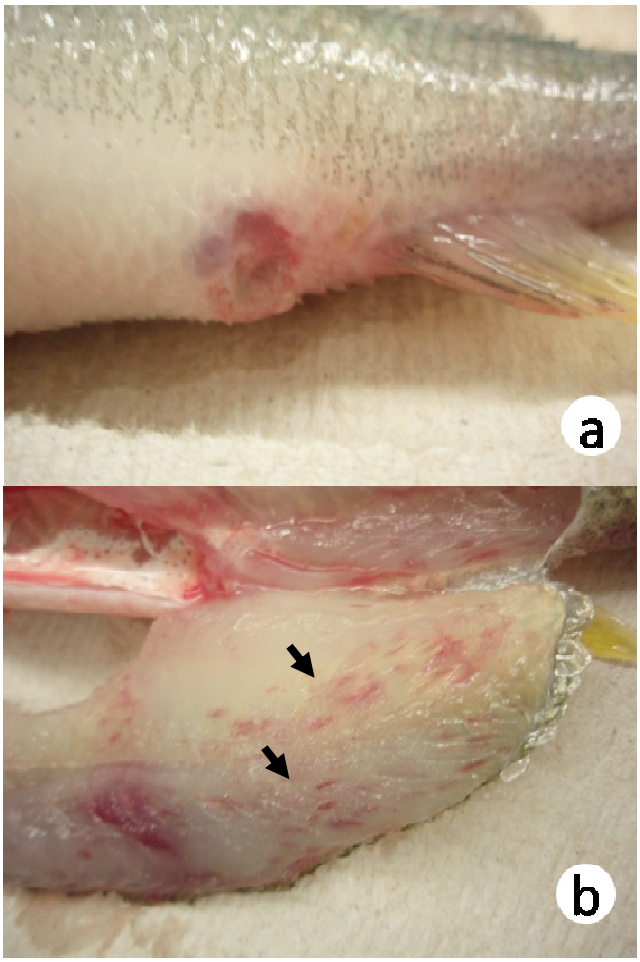

Figure 3-4 Clinical signs of yellow perch experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection.
(a) erythematous vent surrounded by intramuscular hemorrhages, (b) transected lateral muscle tissue exhibited diffuse multifocal intramuscular hemorrhages (*black arrows*).

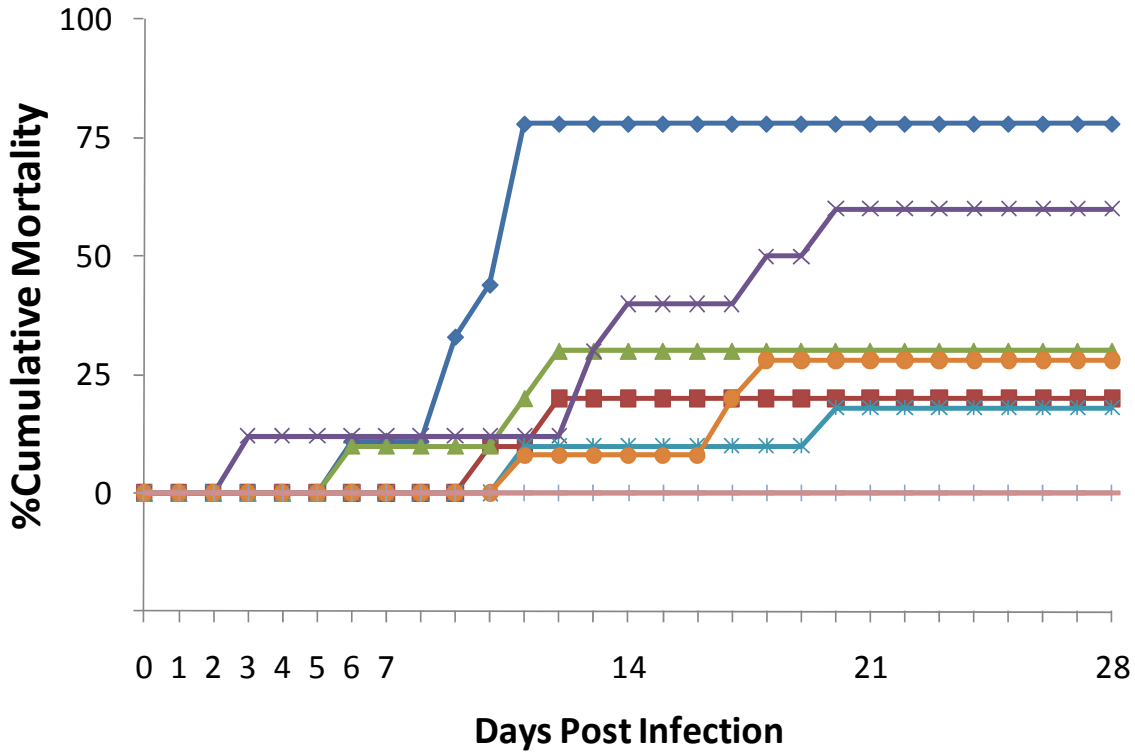

Figure 3-5 Cumulative mortalities of rainbow trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, 7 x 10^2 pfu; -+-, 70 pfu; ---, Negative control)

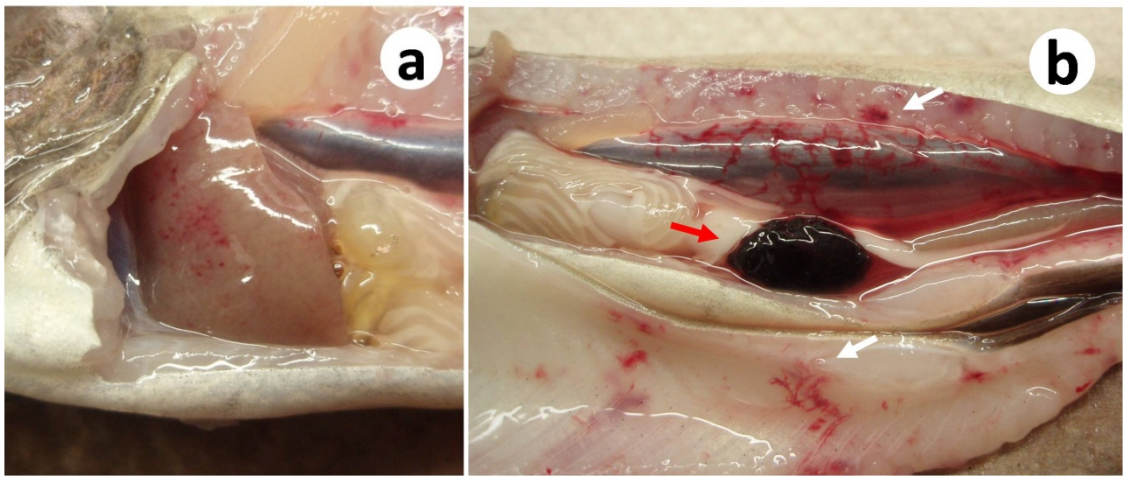

Figure 3-6 Clinical signs of rainbow trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection.
(a) pale, friable liver with subserosal petechiation (b) severe splenomegaly (*red arrow*) with focal areas of intramuscular hemorrhaging in the abdominal wall and lateral musculature (*white arrows*) and petechia on the swimbladder.

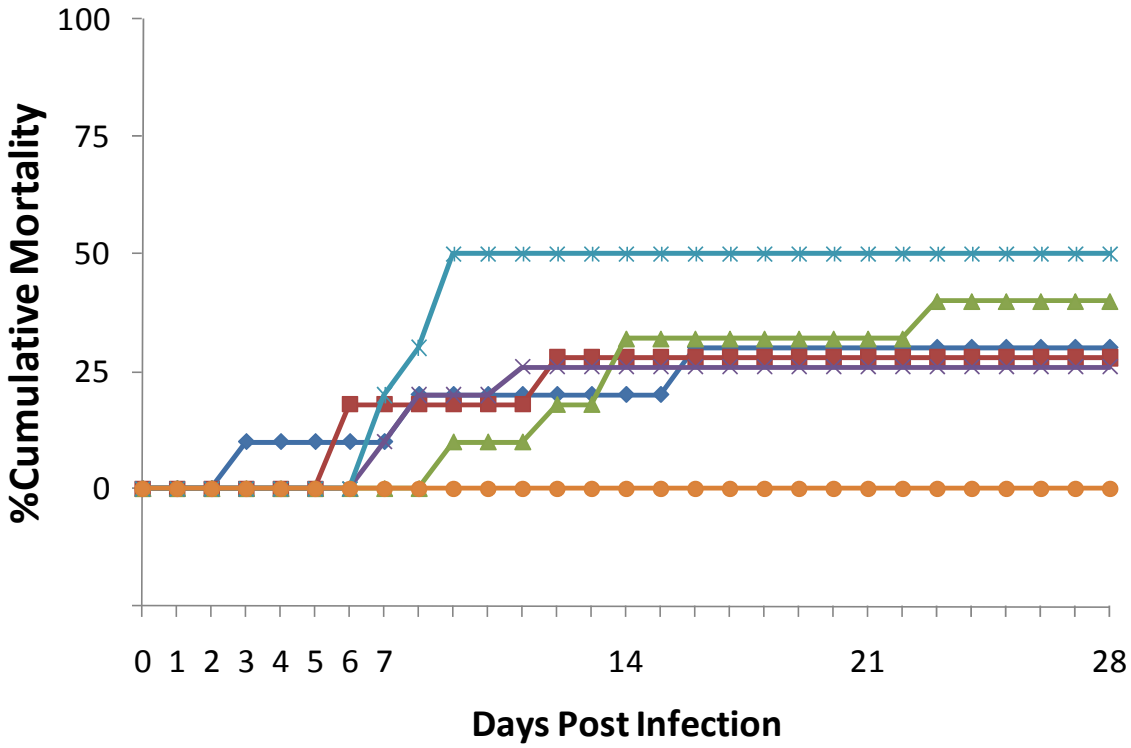

Figure 3-7 Cumulative mortalities of brook trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, Negative control)

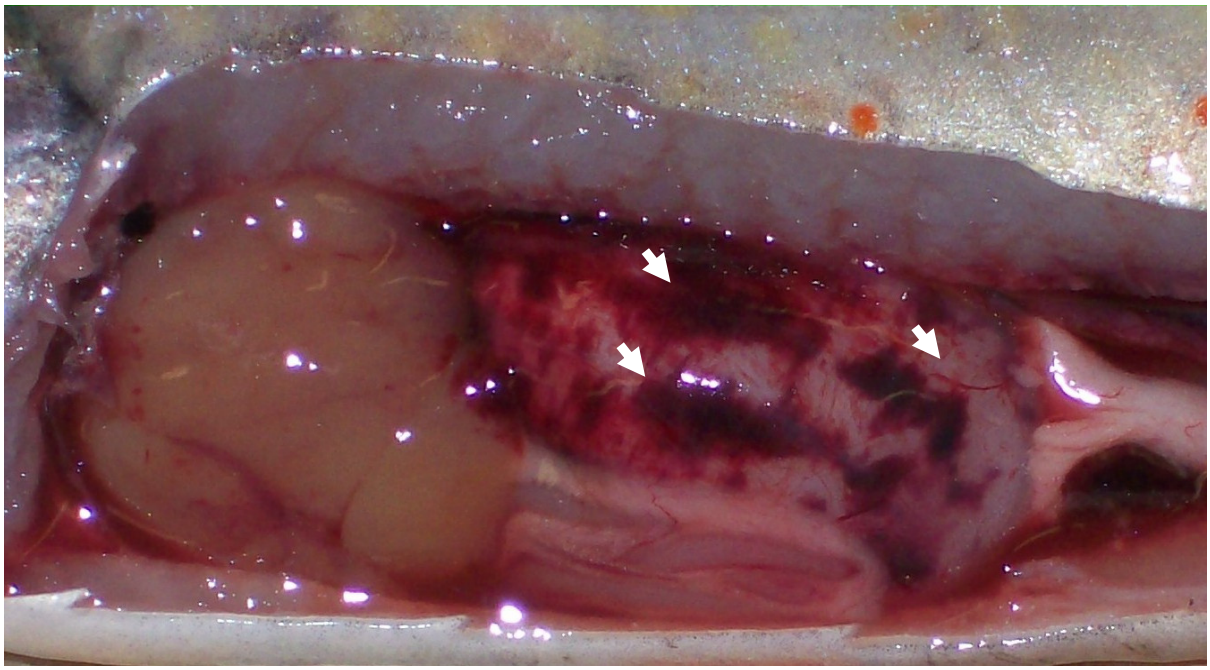

Figure 3-8 Clinical signs of brook trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection.
Livers were markedly pale, friable and enlarged. Severe petechiation and ecchymotic hemorrhages were diffusely scattered throughout adipose tissue surrounding the viscera (*white arrows*).

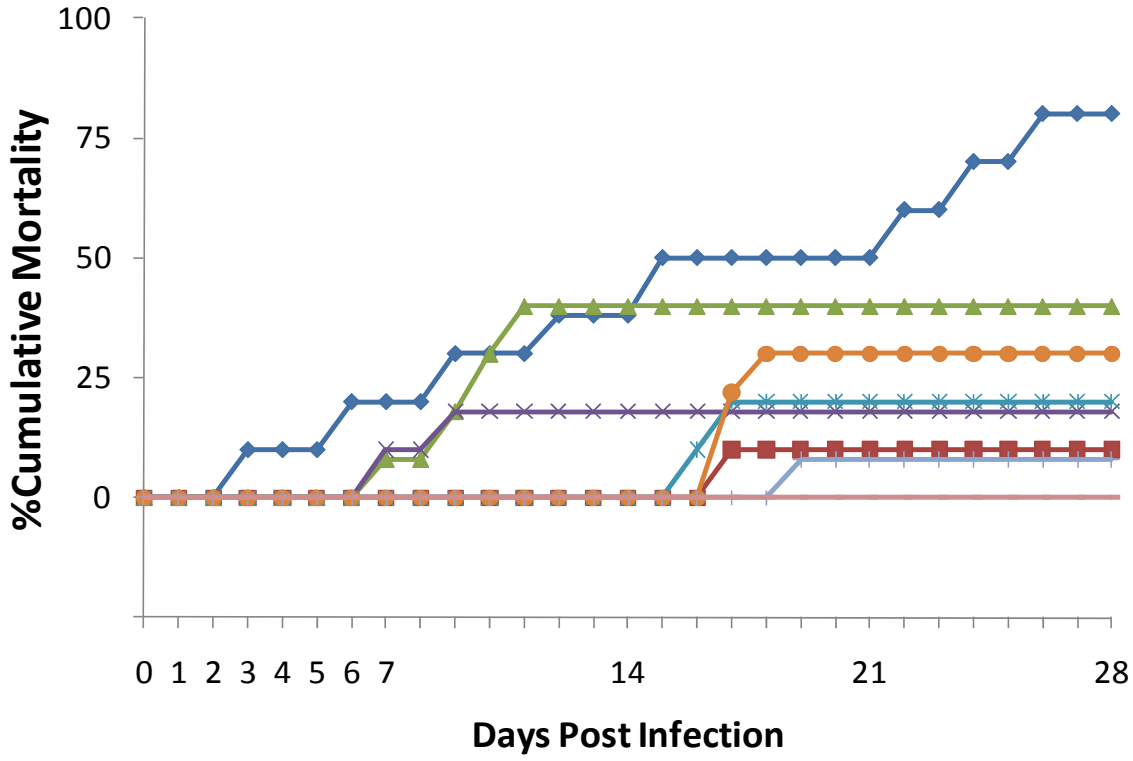

Figure 3-9 Cumulative mortalities of brown trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, 7 x 10^2 pfu; -+-, 70 pfu; ---, Negative control)

Figure 3-10 Clinical signs of brown trout experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal.
External signs of disease were mild. This particular fish was inoculated with 7 x 10^2 pfu and had peri-anal swelling (*white arrow*) and severely pale gills (*black arrows*).

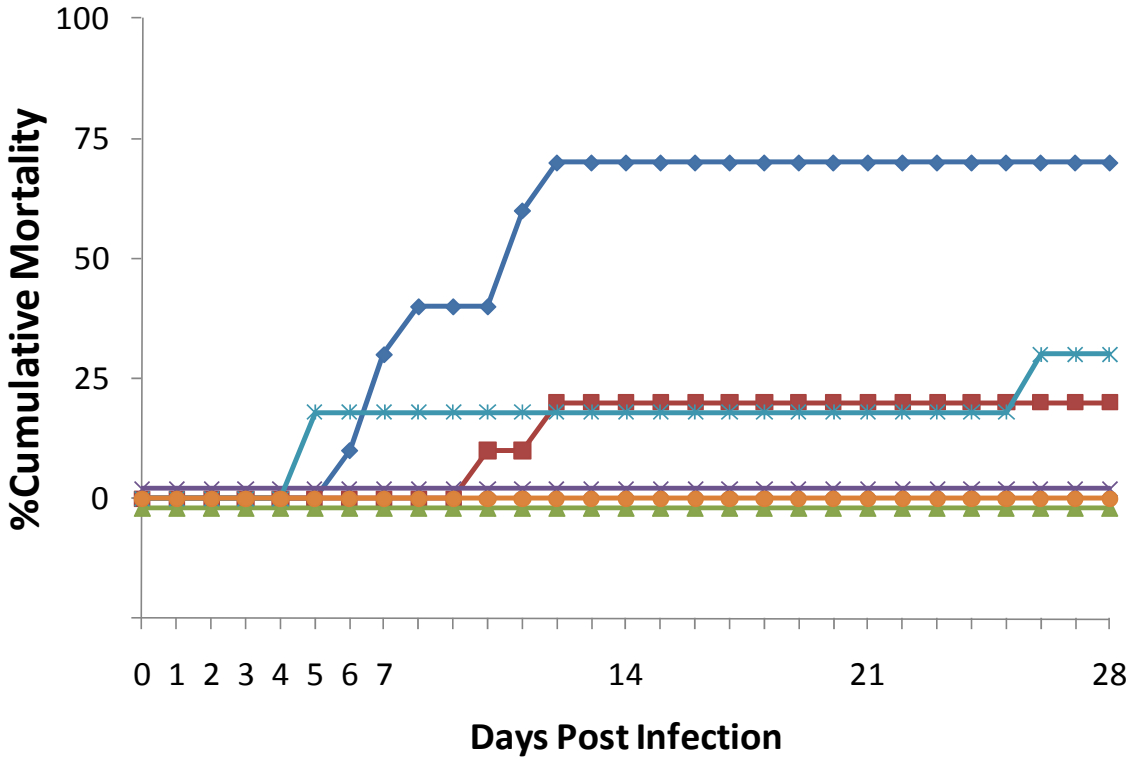

Figure 3-11 Cumulative mortalities of chinook salmon experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, Negative control)

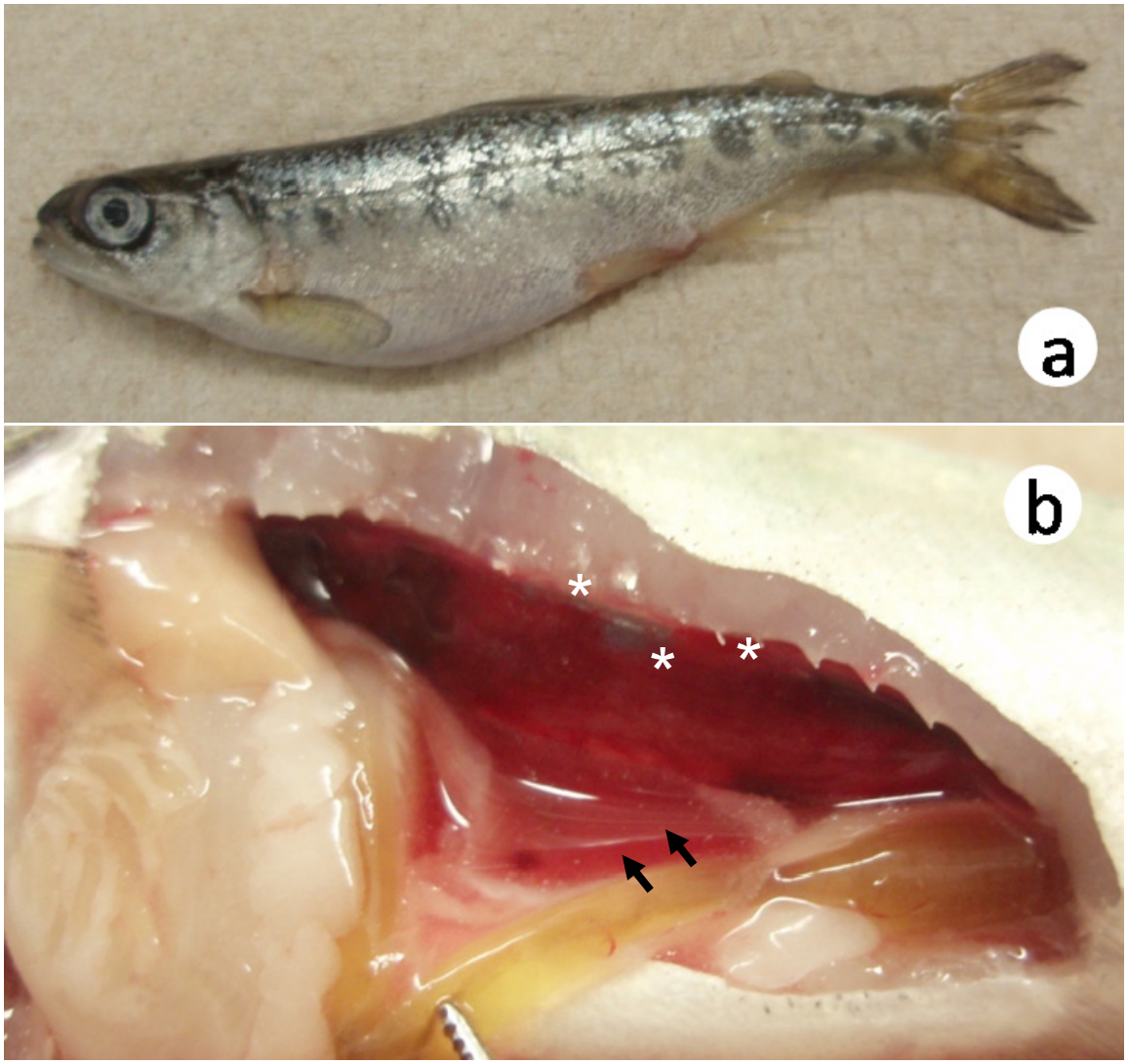

Figure 3-12 Clinical signs of chinook salmon experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection.
(a) distention of the abdomen with palpable, suggestive of ascites. (b) serosanguinous fluid (*white asterisks*) in the peritoneal space. Note the empty fluid filled intestines (*red arrows*).

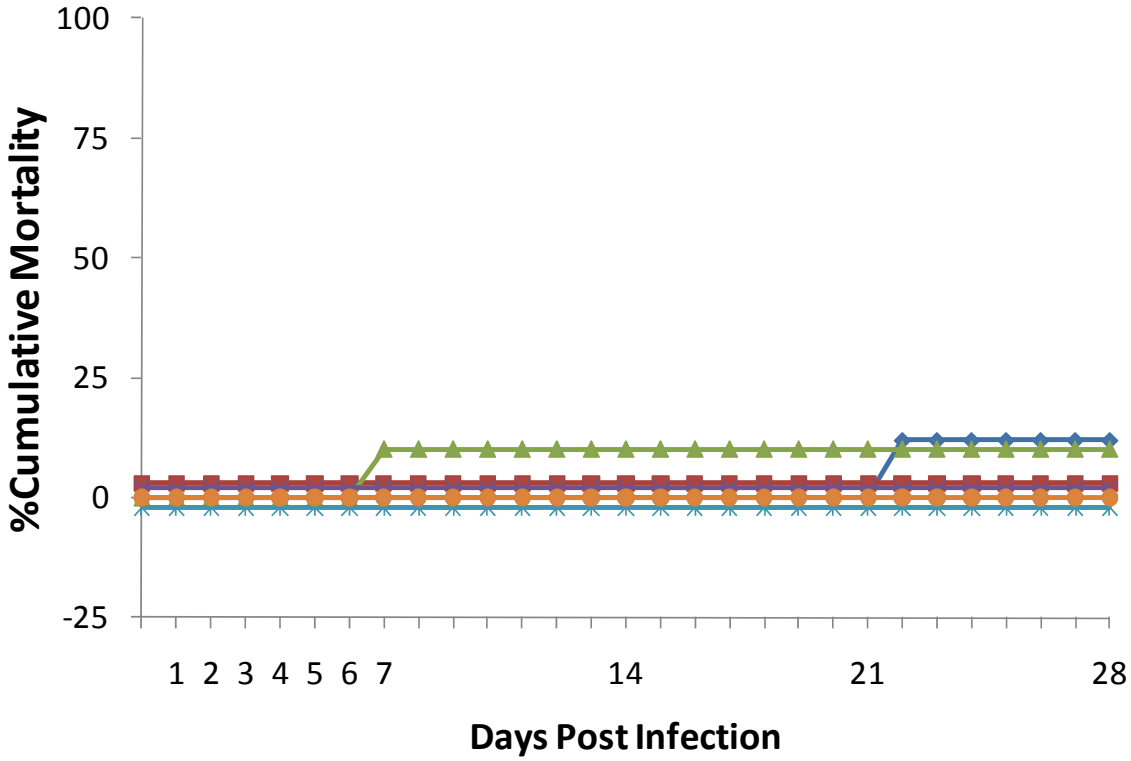

Figure 3-13 Cumulative mortalities of coho salmon experimentally infected with viral hemorrhagic septicemia virus IVb by intraperitoneal injection in calculation of the median lethal dose. (-♦-, 7 x 10^7 pfu; -■-, 7 x10^6 pfu; -▲-, 7 x 10^5 pfu; -X-, 7 x 10^4 pfu; - Ж -, 7 x 10^3 pfu; -●-, Negative control)

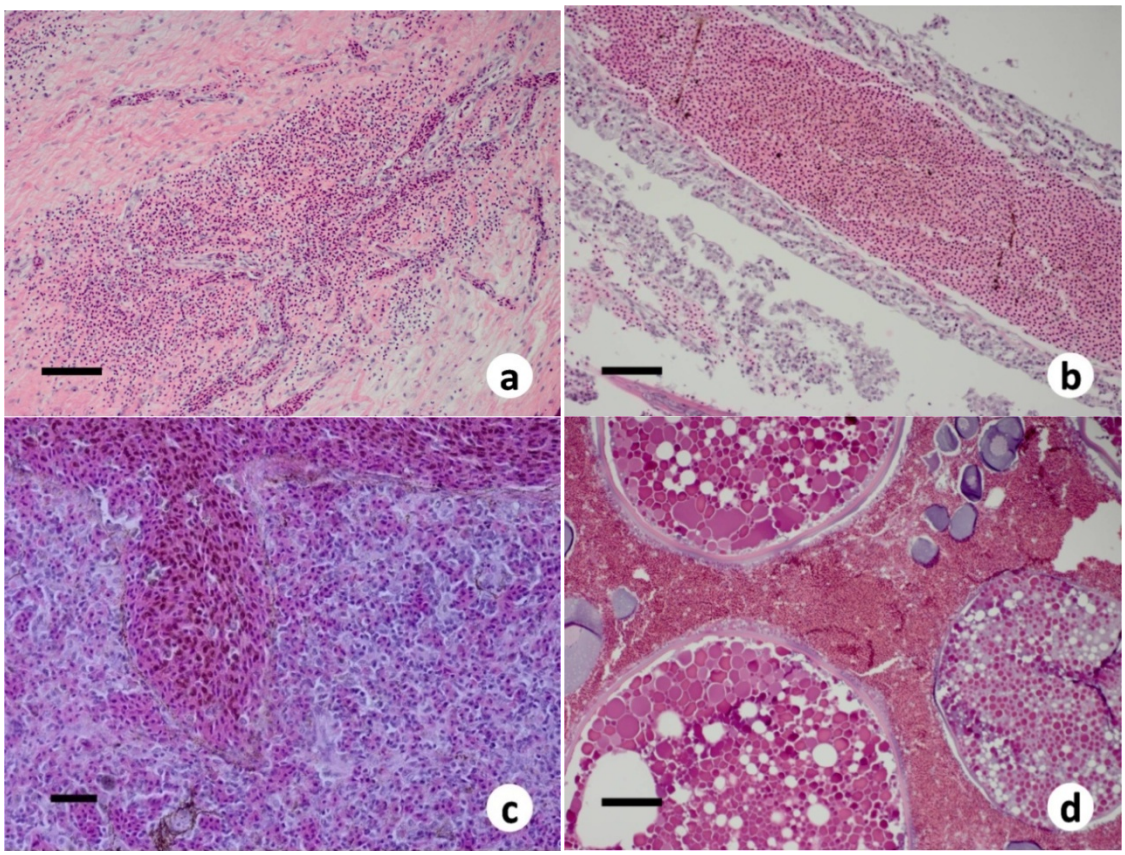

Figure 3-14 Histopathologic lesions in largemouth bass caused by viral hemorrhagic septicemia virus IVb experimental infection by intraperitoneal injection.
(a) diffuse congestion and multifocal hemorrhaging in all fin sections (scale bar = 200 µm), (b) severe congestion in the primary lamellar vessel (scale bar = 200 µm), (c) severe congestion and lymphoid depletion of the spleen (scale bar = 100 µm), and (d) ovarian tissues displayed marked congestion between ova and severe hemorrhaging throughout the interstitium (scale bar = 400 µm). (H&E)

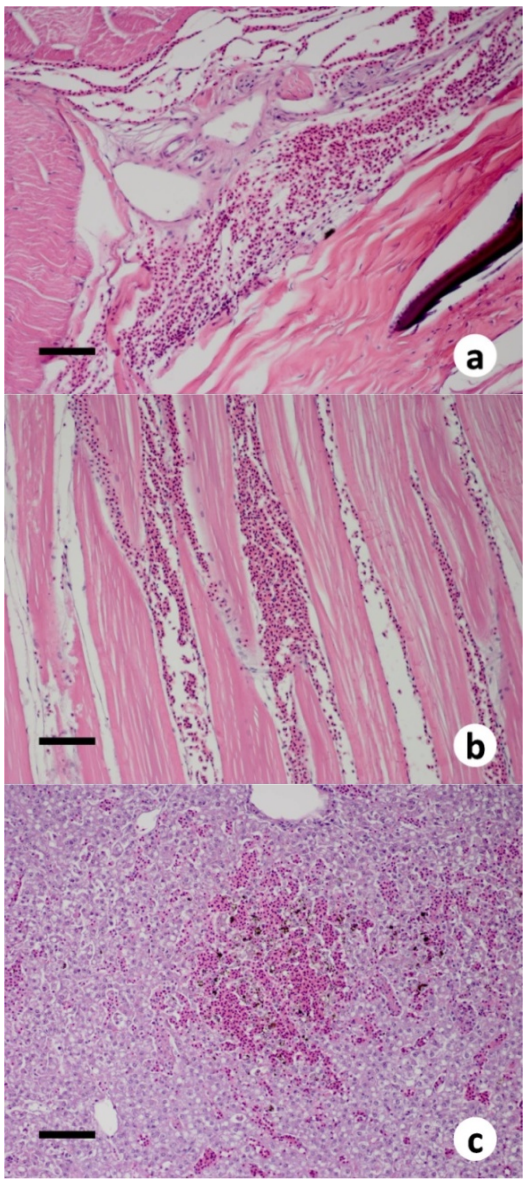

Figure 3-15 Histopathologic lesions in yellow perch caused by viral hemorrhagic septicemia virus IVb experimental infection by intraperitoneal injection.
(a) subcutaneous hemorrhage, edema and congestion in the hypodermis (scale bar = 200 μm) with additional (b) interstitial hemorrhage between myofibers (scale bar = 200 μm), (c) the liver tissue displayed marked multifocal degeneration, necrosis, vacuolation of hepatocytes, and localized areas of congestion and hemorrhage (scale bar = 200 μm). (H&E)

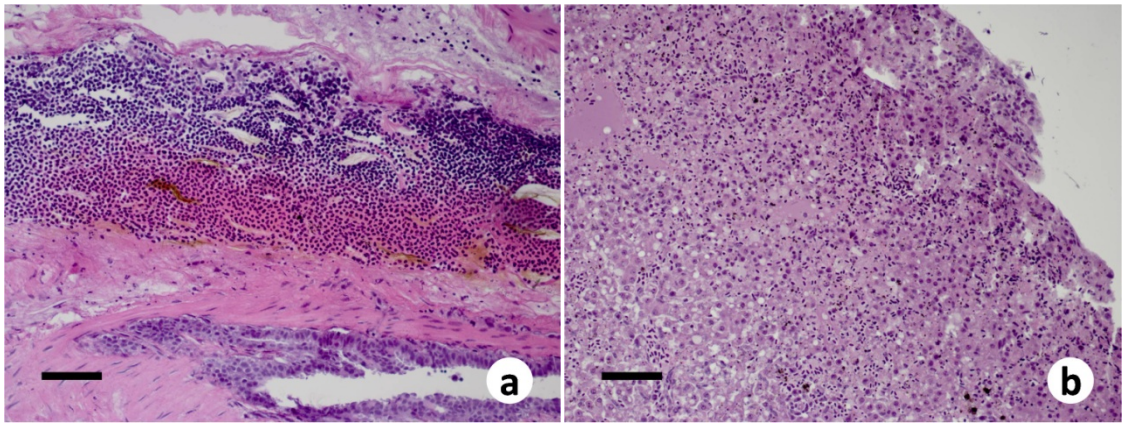

Figure 3-16 Histopathologic lesions in rainbow trout caused by viral hemorrhagic septicemia virus IVb experimental infection by intraperitoneal injection.
(a) massive hemorrhages in the swimbladder wall (scale bar = 200 μm) and (b) moderate congestion and mild vacuolation of some hepatocytes in the liver (scale bar = 200 μm). (H&E)

Table 3-1 The species type, median lethal dose of infection, weights and lengths of each species is presented. Species susceptibility is listed in highest to lowest in descending order based on median lethal dose of infection by intraperitoneal injection (IP-LD_{50}).

Species Type	Species	IP-LD_{50}	Length	Weight
COOL WATER	Muskellunge[a]	2.21 pfu	15.8 ± 1.0 cm	17.5 ± 2.3 g
	Largemouth bass	1.5×10^2 pfu	17.5 ± 1.0 cm	82.2 ± 12.3 g
	Yellow perch	2.5×10^5 pfu	18.3 ± 0.2 cm	75.5 ± 6.6 g
COLD WATER	Rainbow trout	1.4×10^6 pfu	13.8 ± 0.9 cm	24.8 ± 3.2 g
	Brook trout	5×10^6 pfu	16.7 ± 1.1 cm	45.2 ± 10.1 g
	Brown trout	5×10^6 pfu	16.9 ± 0.7 cm	47.8 ± 6.0 g
	Chinook salmon	1.0×10^7 pfu	8.9 ± 0.4 cm	6.6 ± 1.0 g
	Coho salmon	$> 7 \times 10^7$	16.5 ± 1.6 cm	38.5 ± 14.5 g

[a] Muskellunge data was obtained from a previous study (Kim & Faisal 2010)

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CHAPTER 4

Kim R. & Faisal M. (2010) The Laurentian Great Lakes strain (MI03) of the viral haemorrhagic septicaemia virus is highly pathogenic for juvenile muskellunge *Esox masquinongy* (Mitchill). *Journal of Fish Disease*. 33:513-527.

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CHAPTER 4

The Laurentian Great Lakes strain (MI03) of the viral haemorrhagic septicaemia virus is highly pathogenic for juvenile muskellunge *Esox masquinongy* (Mitchill)

Abstract

The Great Lakes strain of viral haemorrhagic septicaemia virus (VHSV) isolated from adult sub-clinical muskellunge, *Esox masquinongy* (Mitchill) in Lake St. Clair, MI, USA was shown to be highly pathogenic in juvenile muskellunge through intraperitoneal (i.p.) injection and waterborne challenge. Mortality began as early as 3 days (d) after exposure in waterborne challenged fish, whereas fish infected by the i.p. route experienced the first mortality by 5 d post infection (p.i.). The median lethal intraperitoneal injection dose (IP-LD_{50}) was approximately 2.21 plaque forming units (pfu) as opposed to the median lethal immersion challenge dose (IM-LD_{50}) of 1.7×10^4 pfu mL^{-1}. A high, medium and low dose of infection caused acute, subacute, and chronic progression of the disease as was evident by the cumulative mortality data, respectively. Clinical signs of disease observed in dead and moribund fish consisted of severely pale gills, dermal petechial haemorrhages along the flanks, severe nuchal haemorrhages, intramuscular haemorrhages at the fin-muscle junction, and focal haemorrhaging on the caudal peduncle. Internal lesions were also appreciated in livers that were pale, discolored, and friable, kidneys that were either congested or degenerative in appearance, and petechial to ecchymotic haemorrhages on the swim bladder wall. Histopathologic examination demonstrated massive haemorrhages in the

swimbladder wall and muscle, severe vacuolation and multifocal necrosis of the liver, multifocal necrosis of the gills, and depletion of lymphoid tissues within the spleen. Kidney tissues also exhibited a mixed pattern of degeneration which included tubular necrosis, interstitial edema and congestion. Virus was recovered from kidney and spleen tissues through tissue culture and reverse transcriptase polymerase chain reaction (RT-PCR). These findings are of great concern when considering the current impact VHSV may have for recruitment of muskellunge in the Great Lakes. Therefore, management and conservation are critical in sustaining future generations of muskellunge and preserve the economic and recreational aspects of these fisheries.

Introduction

Viral haemorrhagic septicaemia virus (VHSV) has been associated with mass mortality episodes in a number of fish species throughout the Laurentian Great Lakes basin (Elsayed, Faisal, Thomas, Whelan, Batts & Winton 2006; Gagné, Mackinnon, Boston, Souter, Cook-Versloot, Griffiths & Olivier 2007; Groocock, Getchell, Wooster, Britt, Batts, Winton, Casey, Casey & Bowser 2007; Lumsden, Morrison, Yason, Russell, Young, Yazdanpanah, Huber, Al-Hussinee, Stone & Way 2007). This World Organization for Animal Health (OIE) notifiable disease has been associated with infections in 28 freshwater fish species and its range extends to include the watersheds of four of the Great Lakes (OIE 2009). Since none of the other VHSV genotypes and strains have shown such tendency to a wide range of freshwater fish species (Winton, Batts, Deering, Brunson, Hopper, Nishizawa & Stehr 1991; Meyers, Short, Lipson, Batts, Winton, Wilcock & Brown 1994; Dixon, Feist, Kehoe, Parry, Stone & Way 1997; Follett, Meyers, Burton & Geesin 1997), there are mounting concerns that VHSV may continue its spread outside the Great Lakes into neighboring water systems. As a result, there is an urgent need to better understand the biological characteristics of this emerging VHSV strain, host-virus interactions in particular.

The original isolation of VHSV in the Great Lakes was from adult muskellunge *Esox masquinongy* (Mitchill) collected from Lake St. Clair in 2003. Partial sequence analyses of the G and N genes of the Lake St. Clair VHSV proved that it is closely related to the North American VHSV-genotype IV, yet distinct from the VHSV isolates originally retrieved from marine fish in Japan,

British Columbia, and Washington State. As a result, the Great Lakes VHSV strain formed a novel sublineage within VHSV genotype IV, designated IVb (Elsayed *et al.* 2006). By 2006, a number of mortality episodes caused by VHSV-IVb in Lake Erie, Lake Ontario, and the St. Lawrence River watersheds involved muskellunge among other fish species. Among affected fish species, muskellunge, in particular, exhibited severe clinical signs typical of VHSV infection. This observation has prompted a preliminary study in which 10 freshwater fish species received the same dose of VHSV-IVb intraperitoneally. Unlike other fish species tested, muskellunge mortality reached 100% within five days by the i.p. route (Kim & Faisal 2010), indicating the potential high susceptibility of this species to VHSV-IVb.

The muskellunge is an important recreational fishery in the Great Lakes and represents one of the few self-sustaining populations in North America (Bryant & Smith 1988). Therefore, there are mounting concerns on the impact(s) of VHSV-IVb on muskellunge stocks. Most of the current knowledge on the susceptibility and disease course of VHSV-IVb in muskellunge has been provided from reported fish kills and field surveys. Some of the fish used for these purposes were either dead for an unspecified period of time or were clinically healthy and therefore, many aspects of VHSV-IVb pathogenicity are currently lacking. To date, experimental infection continues to be an efficient method to unravel the virus-host interactions, to determine disease course, and to ascertain tissue alterations caused by the virus in the absence of other pathogens or toxic chemicals (Ord, Le Berre & de Kinkelin 1976; Jørgensen

1980; Neukrich 1984; Kocan, Bradley, Elder, Meyers, Batts & Winton 1997). In this context, experimental infection studies conducted on European and, to a lesser degree, North American VHSV marine isolates have revealed that the pathogenicity of each virus strain can vary depending on the species and geographic origin (Skall, Olesen & Mellergaard 2005). For instance, experimental infections of the rainbow trout, *Oncorhynchus mykiss* (Walbaum) using VHSV-IV strain, originally isolated from salmonids in the Pacific Northwest (now designated VHSV-IVa) demonstrated that this VHSV strain was of non- to low pathogenicity (Winton *et al.* 1991; Meyers *et al.* 1994; Dixon *et al.* 1997; Follett *et al.* 1997), while VHSV-I caused significantly high levels of mortality in experimentally infected rainbow trout (Skall, Slierendrecht, King & Olesen 2004).

Therefore, this study was designed to assess the extent of muskellunge susceptibility to VHSV IVb and to determine the disease course following i.p. and bath exposure to VHSV-IVb. This information is essential to foster the current understanding about this emerging VHSV strain.

Materials and Methods

Cell culture and virus propagation & titration

Throughout this study, the Great Lakes VHSV IVb index strain MI03, originally isolated in our laboratory in 2003 from Muskellunge (Elsayed et al. 2006), was used. The virus was maintained by continuous subcultures in the cell lines *Epithelioma papulosum cyprini* (EPC) (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & de Kinkelin 1983) and fathead minnow (*Pimephales promelas*) (Gravell and Marshberger 1965). In order to determine the virus concentration, plaque assay was performed on EPC cells or FHM cell lines with polyethylene glycol and using a methycellulose overlay as described in Batts & Winton 1989; Batts, Traxler & Winton 1991). Virus stocks were aliquoted in cryogenic vials (Corning, Lowell, MA) and kept at -80 $^{\circ}$C until used.

Virus was titrated by inoculation onto 24-well cell culture plates. Ten fold serial dilutions were applied to the virus stock beginning with 1:100 to $1:10^{12}$. After seven days of observation, the median tissue culture infectious dose ($TCID_{50}$) was determined to be $1:10^{9.5}$ mL^{-1}.

Cell lines were maintained and subcultured in 150 cm^2 tissue culture flasks (Corning, Lowell, MA) at 25 $^{\circ}$C using a growth formulation of Earle's salt-based Minimal Essential Media (MEM) (Invitrogen, Carlsbad, CA) supplemented with

29.2 mg mL^{-1} L-glutamine (Invitrogen), Penicillin (100 IU mL^{-1}) and Streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich, St. Louis, MO).

Fish and Maintenance

Certified VHSV-free juvenile muskellunge were obtained from the Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, IA) in August of 2007 and May of 2008. It should be noted that the fish obtained in 2007 were much larger than those obtained in 2008; however, each age group was used separately for a particular experiment. Prior to infection, five fish were euthanatized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) (25 mg mL^{-1}) and tested for the presence of VHSV. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd, Pocklington, York, UK) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen) supplemented with 12 mM tris buffer (Sigma), Penicillin (100 IU mL^{-1}), Streptomycin (100 µg mL^{-1}) (Invitrogen), and Amphotericin B (250 µg mL^{-1}) (Invitrogen) to produce 1:4 dilution (w/v) of original tissues. Samples were centrifuged at 2000 *g* and the supernatant was inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Plates were incubated at 15

°C for 7 d, and observed for the formation of cytopathic effects (CPE). A second blind passage was performed and assessed for the presence of VHSV.

Fish were acclimated to temperatures from 16 °C to 11 °C over a three week period before the experiment was initiated. All fish were initially held in 1,900 L circular fiberglass tanks in a continuous flow-through system supplied by oxygenated and facility chilled well water at Michigan State University - Research Containment Facility (East Lansing, MI). The juvenile muskellunge were fed *ad lib* with 2.0 mm sinking feed (Silver Cup, Nelson and Sons, Murray, UT).

Exposure to VHSV IVb by intraperitoneal injection

***Experiment 1* – Experimental Infection of juvenile muskellunge by the intraperitoneal route and calculation of the median lethal dose (IP-LD_{50}):** Juvenile muskellunge (weight 17.5 ± 2.3 g; total length 15.8 ± 1.0 cm) were infected in groups of 10 fish at doses that range from 7×10^4 pfu (3.2×10^4 $TCID_{50}$) to <1 pfu (3.2×10^{-2} $TCID_{50}$) per fish suspended in 100 µL of maintenance culture medium. Injection was performed by the i.p. route. Fish were then recovered in a 74 L polyethylene tank (Aquatic Eco-Systems, Apopka, FL) in continuous flow through system. An additional 10 fish for negative control were each injected by the i.p. route using 100 µL of a maintenance formulation of MEM supplemented with 2% fetal bovine serum and monitored throughout the

experiment. All fish were monitored daily for an observation period of 28 d p.i. Mortalities were recorded daily and a LD_{50} was calculated by the method of Reed and Muench (1938). The in-flowing water was supplied by an underground well and chilled to 12 ± 1 $^{\circ}$C throughout the duration of all experiments. The out-flow was united into a common tract entering an ultraviolet sterilization unit (Aquafine, Valencia, CA). Virus reisolation and tissue sample collection for histology were performed as outlined below and completed likewise for each subsequent experiment.

Exposure to VHSV-IVb by immersion

Originally, we wished to determine if muskellunge could contract VHSV-IVb by immersion exposure. Therefore, we exposed them to VHSV concentration of 1 x 10^5 pfu mL^{-1} (Experiment 2). However, the rapid onset and disease mortality of this group prompted us to first determine the LD_{50} by immersion. Therefore, Experiment 3 was performed to determine the immersion LD_{50}, followed by Experiment 4 in which fish were exposed to LD_{50} and LD_{25} in order to be able to keep infected fish for a longer observation period.

***Experiment 2* – Disease following immersion exposure to a high VHSV-IVb dose:** A group of 60 fish (weight 20.0 ± 7.6 g; total length 18.9 ± 5.2 cm) were experimentally infected via immersion challenge at the high dose (1 x 10^5 pfu

mL^{-1}, 4.5 x 10^4 $TCID_{50}$) in order to produce an acute to peracute course of infection. An additional group of 25 fish was immersed in water mixed with 1 mL of sterile maintenance-MEM and was considered the negative control group. The two experimental groups were held in static, oxygenated water at their respective doses for 90 min as described above. All fish were monitored for up to 60 d p.i. in order to assess morbidity, time to death, cumulative mortality, and to determine tissue alterations in stained tissue sections.

***Experiment 3* – Experimental Infection of juvenile muskellunge by immersion in VHSV containing water and calculation of the median lethal dose (IM-LD_{50}):** Juvenile muskellunge (weight 0.7 ± 0.2 g; total length 5.6 ± 0.7 cm) were infected by immersion. Fish were divided in groups of 10 fish at doses ranging from 1 x 10^5 pfu mL^{-1} (4.5 x 10^4 $TCID_{50}$) to 10 pfu mL^{-1} (4.5 $TCID_{50}$). Virus stocks were diluted and added accordingly to achieve the aforementioned doses in 2 L of water within a Nalgene polystyrene beaker (Rochester, NY) for 1.5 h. A negative control immersion challenge was also conducted using 10 fish in water mixed with 1 mL of maintenance-MEM. Infected fish were monitored daily for 28 d p.i.

***Experiment 4* – Disease following immersion exposure to low-medium VHSV-IVb doses:** A group of 125 fish (weight 1.8 ± 0.6 g; total length 8.5 ± 0.7 cm) were randomly assigned into three groups (Group 1-3). Fish in Group 1 (50 fish) were challenged by immersion in water containing MI03 at a concentration

that caused 50% mortality in Experiment 3 (1.7×10^4 pfu mL^{-1}). Group 2 (50 fish) was infected at a low dose (4×10^3 pfu mL^{-1}) so as to follow a more prolonged course of VHSV infection. Group 3 of 25 fish was immersed in water mixed with 1 mL of sterile maintenance-MEM and was considered the negative control group. The three experimental groups were held in static, oxygenated water at their respective doses for 90 min as described above. All fish were monitored for up to 60 d p.i. to assess morbidity, time to death, cumulative mortality, and histopathology in muskellunge suffering from an extended course of infection.

Histopathology

Dead (<8 hours) and clearly moribund fish were euthanized with an overdose of MS-222 (25 mg mL^{-1}), examined for external and internal lesions and then sampled for histopathology. Samples were taken from lesions in skin, gill, muscle, and internal organs (kidney, spleen, liver, swim bladder, intestine) were fixed in 10% formalin, paraffin embedded, sectioned (5 µm), and then stained with hematoxylin and eosin as detailed in Prophet et al. (1992).

Virus re-isolation and confirmation

Kidney and spleen tissues were collected from each dead or moribund fish, processed, diluted, and then tested for the presence of VHSV using EPC cell line. For PCR confirmation, total RNA was extracted using Trizol® LS Reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription

was accomplished by a two step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR™ (Stratagene, Ja Jolla, CA) following the manufacturer's instruction. The primer set used in this assay is recommended by the Office of International Epizootics (OIE) for detection of a 811 base pair sequence of the VHSV nucleocapsid gene: 5'-GGG-GAC-CCC-AGA-CTG-T-3' (forward primer) and 5'-TCT-CTG-TCA-CCT-TGA-TCC-3' (reverse primer). Polymerase chain reaction was achieved by adding into each reaction tube 5 µL of viral RNA, 50 pmol of each primer, 25 µL of Choice™ *Taq* Mastermix DNA Polymerase (Denville Scientific Inc, South Plainfield, NJ), and the DNA-ase free water to create a final volume of 50 µL. The reverse transcriptase was inactivated by subjecting the mixture to a 94 °C for 2 min, and 30 cycles of PCR (denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and polymerization at 68 °C for 1 min) in a Mastercycler Personal Thermal Cycler (Eppendorf, Hamburg, Germany). The polymerization was finalized by maintaining the mixture for a period of 7 min at 68 °C. The product was visualized by gel electrophoresis in 1.5% agarose gels.

Statistical analyses

Mortality data from the muskellunge obtained from fish experimentally infected by the i.p. route and immersion challenge to determine a median lethal dose were analyzed using the Cox Regression Test to conclude whether the dose

administered was significant in affecting survival between each group of 10 fish infected (Cox 1972). If the resultant *P*-value was less than <0.05, the difference was regarded as significant. Statistical Analysis Software (SAS 9.1, Cary, NC) was used to compute the data.

Results

Prior to their use in the experimental infection studies, a subsample of muskellunge was tested with cell culture and RT-PCR assay using VHSV-specific primers. Results of this testing demonstrated the freedom of the experimental fish from VHSV or any other virus that can replicate in EPC or FHM cell lines. Furthermore, clinical examination of these fish before experimental infection did not identify any suspicious lesions or overt signs of disease associated with VHSV infection.

Experiment 1: Intraperitoneal infection with VHSV IVb and Calculation of IP-LD_{50} in juvenile muskellunge

Many of the fish inoculated with high doses of VHSV exhibited clear lethargy with the first mortalities noticed as early as 4 d p.i. Fish that were inoculated with 7 x 10^4 pfu through 70 pfu experienced the first mortalities by 5 d p.i. and reached 100% mortality between 5-8 d p.i. depending on the dose injected. Similarly, fish inoculated with 7 pfu reached 100% mortality, albeit at 13 d p.i. On the contrary, fish that received less than 7 pfu dose^{-1} survived the experimental exposure to VHSV and were apparently healthy. No mortalities were noticed in the negative control group that received sterile MEM. Cumulative mortality of the fish groups are displayed in Figure 4-1. As noticed in Table 1, the mean time to death and days to maximum cumulative mortality correlated well with the injected dose. The mortalities recorded between doses were statistically significant ($P < 0.0001$) by

the Cox Regression, thus indicating that the dose administered correlated to the survival time.

The mean time to death varied from one dose group to the other. The mean days to death observed was shortest at a dose of 7 x 10^3 pfu and increased at each subsequent decrease in dose. Interestingly, fish infected at the highest dose of 7 x 10^4 pfu had a mean survival time of 4.7 days, which was only slightly higher than fish infected at the next lowest dose (7 x 10^3 pfu)

According to the equation developed by Reed and Muench (1938), the IP-LD_{50} of VHSV IVb in juvenile muskellunge was calculated to be 2.21 pfu.

The majority of infected fish exhibited haemorrhagic diathesis typical of VHSV infection such as marked dermal petechial haemorrhage, largely distributed mid-body (Figure 4-2a), with haemorrhages noted between fin rays (Figure 4-2b). Gills of almost all infected fish were pale and in some individuals turned completely white (Figure 4-2c). On dissection, livers were greenish, friable, and often enlarged. Areas of petechiation on the dermis were inspected further by transecting through the muscle, which revealed intramuscular haemorrhages (Figure 4-2d). Petechial and ecchymotic haemorrhages were noted on the swimbladder wall (Figure 4-2e). The kidneys were either degenerated or

congested. A few fish showed no abnormal internal or external disease signs prior to death.

Experiment 2: Disease following immersion exposure to a high VHSV-IVb dose

Following exposure of muskellunge to the high dose (1 x 10^5 pfu mL^{-1}), a peracute disease course developed with 100% mortality within 6 d p.i. (Figure 4-3). The first mortalities appeared as early as 3 d p.i. Mean day to death was 3.9 which was significantly lower than those that received the highest dose by i.p. injection. Despite the high mortality rate, no gross internal or external lesions were observed. From each dead fish, VHSV was re-isolated from kidney and spleen tissues and PCR-confirmed. There were no mortalities in the control group.

Experiment 3: Experimental Infection of juvenile muskellunge by immersion in VHSV containing water and calculation of the median lethal dose (IM-LD_{50})

As displayed in Table 2, fish immersed in 1 x 10^5 pfu mL^{-1} of MI03 experienced 100% mortality between 4 and 7 d p.i. Additionally, fish infected at the 1 x 10^4 pfu mL^{-1} experienced mortalities on days 5, 6 and 10 with 70% of the fish remaining after the duration of the study. Approximately 90% of the fish infected at the 1 x 10^3 pfu mL^{-1} dose survived with only one mortality occurring on day 10. Fish

infected at concentrations of 1 x 10^2 pfu mL^{-1} and 1 x 10^1 pfu mL^{-1} did not experience any mortality throughout the observed 28 d p.i. The control tank experienced no mortalities throughout the experiment. Cumulative mortality data is displayed in Figure 4-4.

As shown in Table 2, the days to maximum cumulative mortality increases as the concentration of virus decreases. Also, the mean day to death was only slightly higher in the group challenged at the concentration of 1 x 10^5 pfu mL^{-1} compared to 1 x 10^4 pfu mL^{-1}, but was otherwise inversely correlated to the concentration. From each dead fish, VHSV was re-isolated from kidney and spleen tissues.

Similar to fish infected by the i.p. route, fish challenged by immersion displayed clinical signs of disease by 4 d p.i. Overall, there were fewer clinical signs observed in mortalities collected when compared to individuals infected by the i.p. route. Subcutaneous haemorrhages were more pronounced in the nuchal area (Figure 4-5a) and the middle third of the body (Figure 4-5b). During necropsy, the pale, non-haemorrhagic viscera was removed which indicated that the mid-body haemorrhages were more localized to intramuscular and dermal tissues (not shown). The mortalities recorded between doses were statistically significant ($P < 0.0001$) by the Cox Regression Test indicating that the dose administered was correlated to mean day to death.

According to Reed and Muench (1938) equation, the IM-LD_{50} of VHSV IVb in juvenile muskellunge was calculated to be 1.7 x 10^4 pfu mL^{-1}, whereas the dose required to induce 25% mortality (IM-LD_{25}) was estimated to be 4 x 10^3 pfu mL^{-1}.

Experiment 4: Disease following immersion exposure to low-medium VHSV-IVb doses

Fish challenged with LD_{50} (1.7 x 10^4 pfu mL^{-1}) also experienced heavy mortalities albeit at a much slower rate than those observed at higher VHSV concentrations in experiments 2 and 3. While first mortalities were encountered by day 8 d p.i., a significant increase in mortalities was noted between 19-26 d p.i. with the cumulative mortality reaching >95% during the 60 d observation period. On the contrary, the fish group that received the LD_{25} (4 x 10^3 pfu mL^{-1}) experienced 47% cumulative mortality after 60 d p.i., with the majority of mortalities occurring in the last four weeks of the observation period (Figure 4-6). The mean day to death for this group was 26 days, whereas fish infected at the LD_{50} dose experienced a mean day to death of 23 days. The control group experienced no mortalities.

The overall clinical picture did not exhibit drastic variations between doses of 1.7 x 10^4 pfu mL^{-1} to 4 x 10^3 pfu mL^{-1}. In general, the gross clinical picture was

much less pronounced when compared to the severe haemorrhagic diathesis noticed in fish receiving high MI03 doe in experiments 1-3. Infected fish experienced lethargy and subdermal haemorrhages with uniformly pale gills. External lesions often appeared as profound intramuscular haemorrhages at the dorsal fin-muscle junctions of several fish. Furthermore, petechial haemorrhages were also noted on the serosa of the spleen and adjacent adipose tissue. As previously observed, the livers were consistently enlarged, pale, and friable. Extensive petechial and ecchymotic haemorrhages were also observed on the swim bladder wall. There was no correlation between the concentration of virus at which the fish were immersed and the severity of clinical signs noted.

Histopathologic Analysis

A number of histopathologic alterations have been observed in tissue sections of experimentally infected fish. These pathologic alterations were similar among infected fish regardless of the route of virus exposure.

The gills from fish experimentally infected with VHSV exhibited degeneration and necrosis of secondary lamellae that often sloughed (Figure 4-7). Most primary lamellae were massively necrotic to the extent that they were often unrecognizable and lacked erythrocytes (Figure 4-7). In fish displaying subdermal petechial haemorrhages, skin and underlying muscular tissues exhibited accumulation of erythrocytes at the muscle-skin junction (Figure 4-8). Moderate to severe haemorrhages were noticed in the intermuscular spaces. In

some instances, the haemorrhage was severe enough to fill spaces between muscle fibers in both cross and longitudinal sections (Figure 4-8).

Histologic sections of swimbladder with petechial and ecchymotic haemorrhages revealed massive accumulation of extravasated red blood cells in the submucosal layer of the swimbladder wall and lumen (Figure 4-9). Haemorrhagic lesions were also focal in location, which were also noted between the mucosa and submucosal layers (Figure 4-9).

The livers of infected fish exhibited a range of pathological changes including hepatocyte vacuolation and multifocal areas of necrosis with pyknotic nuclei (Figure 4-10). There were also subtle and mild changes of hepatocytes with indiscrete cell borders, but normal sinusoids (Figure 4-10). Splenic tissues from experimentally infected were devoid of erythrocytes and lacked lymphoid tissue (Figure 4-11).

Histologic examination of normal renal tissue revealed the presence of intact tubular epithelial cells and glomeruli, intermixed with areas of erythrocytes and lymphoid tissue (Figure 4-12a,b). These features were, however, altered in VHSV infected fish. Epithelial cells lining renal tubules in infected fish suffered hydropic degeneration and necrosis (Figure 4-12c). Other renal pathology noted in glomeruli of infected fish were shrunken and degenerated along with cellular debris and granular material accumulated in Bowman's capsule. Lymphoid

depletion in conjunction with interstitial congestion and edema were noted throughout areas of degenerated and necrotic tubular epithelia.

Discussion

The results of this study clearly demonstrate that muskellunge are susceptible to VHSV Genotype IVb following i.p. or immersion experimental infection. The combination of clinical observations, morbidity and mortality data, VHSV reisolation, PCR-confirmation, and histopathological alterations in experimentally infected muskellunge (experiments 1-4) fulfill Rivers' postulates (1937), thereby linking VHSV-IVb to muskellunge mortality observed from Lake Erie, Lake Ontario, and St. Lawrence River watersheds over the past three years. The data also demonstrates that juvenile muskellunge succumb to VHSV-IVb infection with high morbidity and mortality despite the fact that no mortality was reported in sub-adult muskellunge during natural outbreaks. This discrepancy in field observations could be explained by the ease at which dead and moribund fish of juvenile size can be predated upon. Other plausible reasons could include the lack of schooling behavior in muskellunge, which would decrease the likelihood of detecting massive fish kills while also minimizing the potential for horizontal transmission. If widespread mortalities are occurring in juvenile muskellunge, the future recruitment of the species in the Great lakes may be at a much greater risk than previously thought.

The calculated IP-LD_{50} of 2.2 pfu points to a relative high susceptibility of juvenile muskellunge to VHSV-IVb. In contrast, the IM-LD_{50} value was several orders of magnitude higher (7×10^4 pfu mL^{-1}) than the IP-LD_{50} in the same fish species. The difference between the two values could be the result of the age

class and/or the broodstock used for the year in which the fish were obtained. Interestingly the fish injected by i.p. were older and larger in size, but the LD_{50} value was extremely low. Also, the IC-LD_{50} was much higher in smaller and younger fish thus emphasizing the efficacy of muskellunge's natural barriers against virus invasion. It is believed that VHSV gains access to fish body through the gill epithelial cells (Brudeseth & Evensen 2002; Chilmonczyk 1980) and skin (Yamamoto, Batts & Winton 1992). In the same context, Novoa, Romero, Mulero, Rodríguez, Fernández & Figueras 2006, who calculated the LD_{50} value for VHSV-Ia in the zebrafish, *Danio rerio* (Hamilton) found a contrast between the IP-LD_{50} (~9.5×10^3 $TCID_{50}$ mL^{-1}) and the IM-LD_{50} (2×10^7 $TCID_{50}$ mL^{-1}); both of which are considerably far higher than our values. This difference must be interpreted with caution, given zebrafish are not a recognized host of VHSV and that the observed clinical and histopathologic alterations were observed under experimental conditions. Furthermore, the i.p. route clearly depicted a higher number of mortalities while fish infected by immersion had survivors from the lowest to second highest dose, perhaps indicating than infection via the i.p. route reduces incubation periods. Although the overall vulnerability of muskellunge to VHSV IVb appears to be high, the susceptibility of other Great Lakes fish species to the same virus strain remains to be elucidated. The LD_{50} values obtained in this study are however alarming, particularly to fishery managers as an extremely low concentration of VHSV-IVb can cause devastating effects to exposed fish

and underscores the need to design and implement stringent biosecurity methods.

Regardless of VHSV delivery method (i.p. or immersion), the range of VHSV-IVb concentrations used in experiments 1-4 produced acute, subacute, and chronic courses of infection. When higher doses of virus were used, the time to death was shortened and percent cumulative mortality was higher. These dose-dependent infection courses noted in our study were not very much different than those obtained by Castric and de Kinkelin (1984) using VHSV-Ia in turbot, *Scophthalmus maximus* (L.). On the contrary, when Kocan *et al.* (1997) infected juvenile Pacific herring, *Clupea pallasi* (Valenciennes) with a range of VHSV genotype IVa by immersion they noted 100% mortality by 7-10 days in all VHSV concentration. Like in the case of Pacific herrings and VHSV-IVa, we observed that the severity of infection and time to death at the individual level may not be dose dependent. For example, fish that received 7×10^3 pfu by i.p. injection demonstrated a higher percentage of mortality at 4 days p.i. than did fish infected at the next highest dose (7×10^4 pfu). Despite the relative high susceptibility that muskellunge exhibited, some experimentally infected fish were able to survive, not only after the initial exposure, but even after other fish in the same tank contracted the infection and died, a matter that may have increased the virus concentration in the tank. These individual differences propose that resistance in some individual fish may have developed after the initial exposure to the virus, a matter that is currently being investigated. This variability among VHSV genotypes, fish species, and individual variability in disease resistance

underscores the complex relationship between fish species and VHSV genotypes.

Based on both clinical and histopathologic analyses it was obvious that fish experimentally infected by the i.p route or water borne challenge suffered from haemorrhages, both external and internal. The massive infiltration of erythrocytes into the muscle and subcutaneous tissues suggest that an extravasation of blood into the tissues occurred leading to a significant loss of blood volume. These findings were consistent with those previously described by Yasutake (1975) who described similar haemorrhages between muscle bundles and fibers in VHSV infected rainbow trout. Other studies have indicated that haemorrhages in tissues were more severe in northern pike fry infected with VHSV than those of rainbow trout (Ahne 1980, Meier & Vestergard-Jørgensen 1980). The decreased blood volume along with the associated tissue hypoxia may explain the anemic gill appearance and the degenerative changes noted in almost every tissue examined of infected muskellunge. Haemorrhages may, however, not be the sole cause of death of infected fish, since fish in Experiment 2 ran an acute course and died within 6 days without exhibiting haemorrhages. Rather, they showed severe lymphoid depletion in hematopoietic tissues (Figure 4-12). VHSV-associated degenerative changes have also been described by a number of investigators such as the Northern pike, *Esox lucius* (L.) (Meier & Jørgensen 1980), sea bass, *Dicentrarchus labrax* (L.) (Castric & de Kinkelin 1984), turbot (Ross, McCarthy, Huntly, Wood, Stuart, Rough, Smail & Bruno 1994; Brudeseth *et al.* 2005), and Pacific herring (Kocan *et al* 1997), while Pacific

cod, *Gadus macrocephalus* (Tilesius) have only exhibited dermal lesions (Meyer, Sullivan, Emmenegger, Follett, Short, Batts & Winton 1992).

The information provided by these studies is significant in that muskellunge may be at greater risk to succumb to VHSV IVb than previously expected, which is disconcerting given the importance of captive production of these species as gamefish. While our studies identify VHSV as one threat to muskellunge populations, further investigations are warranted to determine what other environmental factors and pathogens may be involved in favoring disease. More importantly, the presence of survivors following infection, may also suggest the existence of a carrier state. Although viral concentrations in tank water of infected fish were not measured in our study, the potential for shedding p.i. has been suggested in previous experimental infections of Esocids with VHSV (Meier & Jørgensen 1980). The ability of fish to become carriers and active shedders was recently demonstrated in experimentally infected salmonids that were held in tanks for nine months following infection (Faisal and Shavalier, personal communication). Others have also commented on the ability of VHSV infected fish to shed virus and become sources of infections leading to epizootic mortality events (reviewed Wolf 1988). Future studies will require that the shedding potential and carrier status of muskellunge be evaluated, if these states of infection subsist in muskellunge.

In summary, this study gave evidence for the high susceptibility of muskellunge to VHSV-IVb. Another Esocid, the Northern pike, was reported to be especially vulnerable to VHSV-Ia, a strain that has caused widespread mortality

in a relatively short period of time in European rainbow trout farms (Meier & Jørgensen 1980; Meier *et al.* 1994). A replication of such events throughout the Great Lakes could prove costly for muskellunge fisheries, which constitute a large portion of a profitable economy throughout the Great Lakes basin. These threats must be met and reduced through muskellunge conservation programs, which are also at risk since wild, adult muskellunge often supplant the necessity for captive broodstock (Buss & Fox 1961; Johnson 1958). In the end, our data suggests that muskellunge populations may be dwindling and require the implementation of control and monitoring strategies to reduce the spread of VHSV IVb.

Acknowledgement

The authors would like to thank the Great Lakes Fishery Trust (Grant #08WRGR0006), the United States Department of Agriculture – Cooperative State Research, Education, and Extension Service (Grant #2007-37610-1383), and the National Institutes of Health for their generous support.

Table 4-1 Cumulative mortalities in juvenile muskellunge infected with viral hemorrhagic septicemia virus IVb by the intraperitoneal route.
The median lethal dose by i.p. injection was calculated by following the equation detailed in Reed and Muench (1938). The two bolded rows are indicators of where the median lethal dose should be located. The cumulative mortality reached 100% between 7– 70,000 pfu per injection, while injections of lesser amount have 0% mortality. The days to maximum mortality and mean day to death can be noted to increase as the dose decreases.

Dose (pfu injection^{-1})	**Maximum Cumulative Mortality %**	**Days to Maximum Mortality**	**Mean Days to Death**
70,000	100	5	4.7
7,000	100	6	4.4
700	100	6	5.2
70	100	8	5.7
7	**100**	**13**	**7.6**
0.7	**0**	**>28**	**>28**
0.07	0	>28	>28

Table 4-2 Cumulative mortalities in juvenile muskellunge infected with viral hemorrhagic septicemia virus IVb by immersion challenge.
The median lethal dose by immersion was calculated by following the equation detailed in Reed and Muench (1938). The two bolded rows are indicators of where the median lethal dose should be located. Fish immersed in water containing virus concentrations of 10^5 pfu mL^{-1} exhibited 100% mortality, while only the second and third highest doses reach 30% and 10% cumulative mortality, respectively. The maximum days to death and mean day to death steadily increase as the dose decreases.

Concentration (pfu mL^{-1})	**Maximum Cumulative Mortality %**	**Days to Maximum Mortality**	**Mean Days to Death**
100,000	**100**	**7**	**5.4**
10,000	**30**	**10**	**5.3**
1,000	10	10	10
100	0	>28	>28
10	0	>28	>28

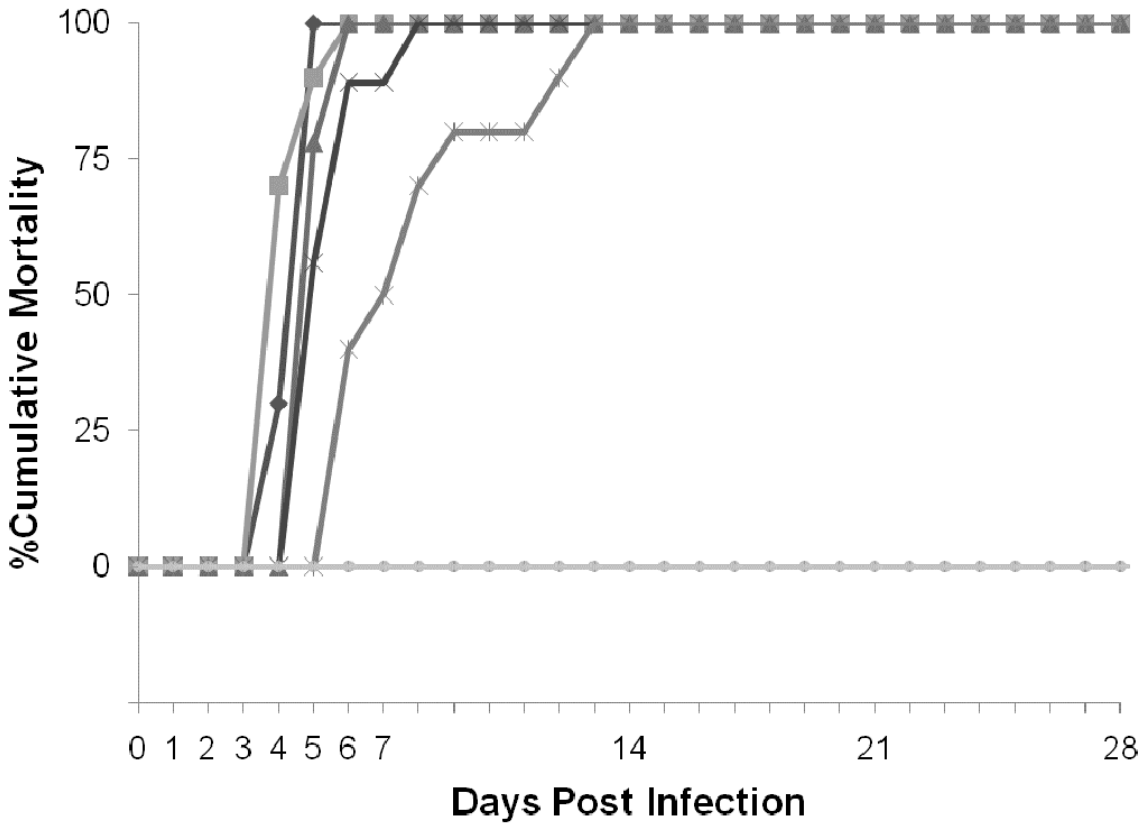

Figure 4-1 Cumulative mortality of juvenile muskellunge intraperitoneally injected with viral hemorrhagic septicemia virus IVb in calculation of a median lethal dose of infection. (-♦-, 7 x 10^4 pfu; -■-, 7 x10^3 pfu; -▲-, 7 x 10^2 pfu; -X-, 7 x 10^1 pfu; -~~X~~-, 7 pfu; -●-, 0.7 pfu; -+-, 0.07 pfu; -○-, Negative control). Daily monitoring for mortalities extended to a period of 28 d p.i. Mortalities occurred primarily between day 3 and 7 following experimental infection. The control tank did not experience any mortality throughout the experiment. Each dose, including the negative control, was administered to 10 fish that were an average weight of 17.5 ± 2.3 g and total length of 15.8 ± 1.0 cm.

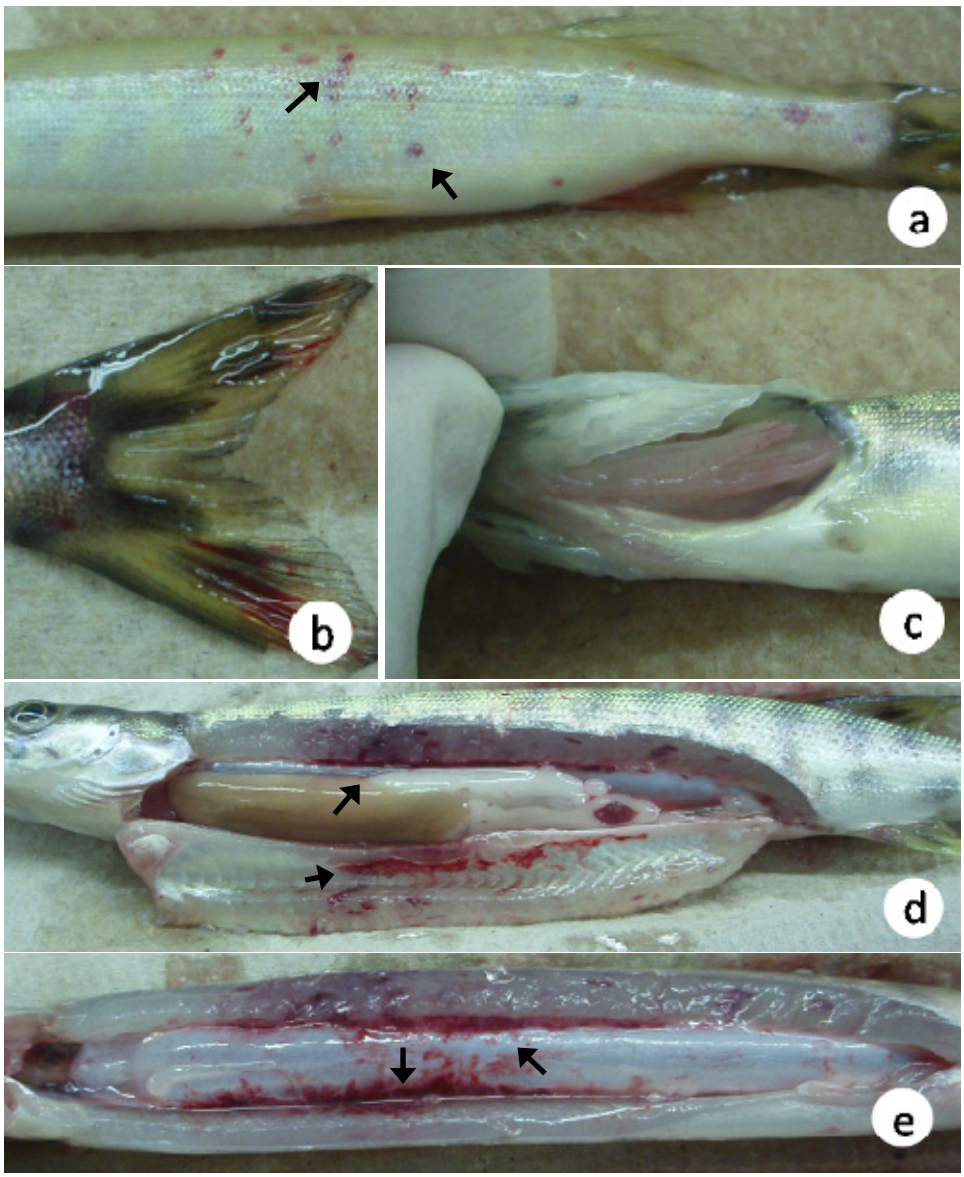

Figure 4-2 Clinical signs of juvenile muskellunge intraperitoneally injected with viral hemorrhagic septicemia virus IVb.
(a) Moderate to severe subdermal petechial haemorrhages (black arrows) (b) Haemorrhage between caudal fin rays (c) Pale and blanched gills (d) Intramuscular haemorrhaging (black arrows), pale friable liver (e) Swim bladder serosal petechia and ecchymoses (black arrows).

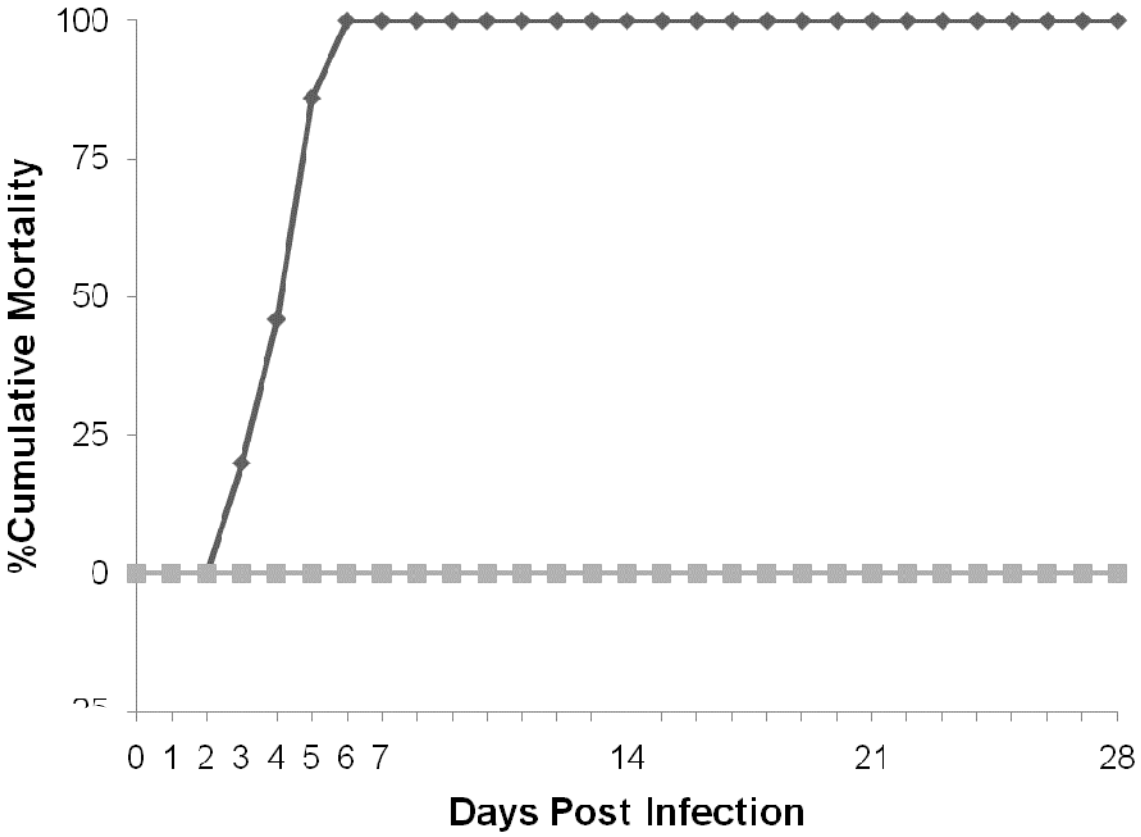

Figure 4-3 Cumulative mortality of juvenile muskellunge challenged by immersion using water containing 10^5 plaque forming units mL^{-1}. (-♦-, 100,000 pfu mL^{-1}; -■-, Negative control). Daily monitoring for mortalities extended to a period of 28 d post infection. Mortalities occurred primarily between days 3 and 6 following experimental infection. No mortalities were observed in the negative control tank. A total of 60 fish were exposed to VHSV and 25 fish served as a negative control. The fish were an average weight of 20.0 ± 7.6 g and total length of 18.9 ± 5.2 cm.

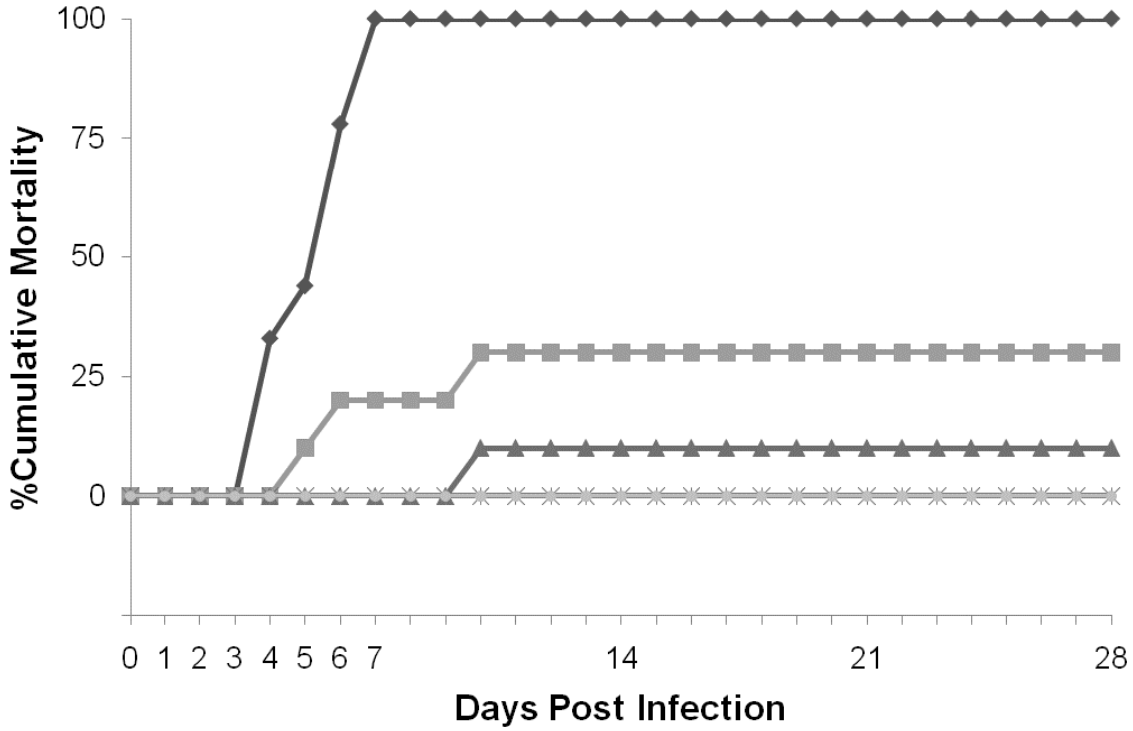

Figure 4-4 Cumulative mortality of juvenile muskellunge exposed to viral hemorrhagic septicemia virus IVb by immersion in calculation of a median lethal dose of infection. (-♦-,1 x 10^5 pfu mL^{-1}; -■-, 1 x 10^4 pfu mL^{-1}; -▲-, 1 x 10^3 pfu mL^{-1}; -X-,17 x 10^2 pfu mL^{-1}; -X-,10 pfu mL^{-1}; -□-, Negative control). Daily monitoring for mortalities extended to a period of 28 d p.i. Mortalities at the highest dose (10^5 pfu mL^{-1}) reached 100% mortality by 7 d post infection, while mortalities at 10^4 pfu mL^{-1} and 10^3 pfu mL^{-1} did not experience mortalities after 10 d. Each dose, including the negative control, was administered to 10 fish that were an average weight of 0.7 ± 0.2 g and total length of 5.6 ± 0.7 cm.

Figure 4-5 Clinical signs of juvenile muskellunge exposed to viral hemorrhagic septicemia virus IVb by immersion at 10^4 plaque forming units mL^{-1}.
The predominant clinical signs included (a) severe nuchal ecchymosis and (b) subdermal haemorrhages at the middle third of the body.

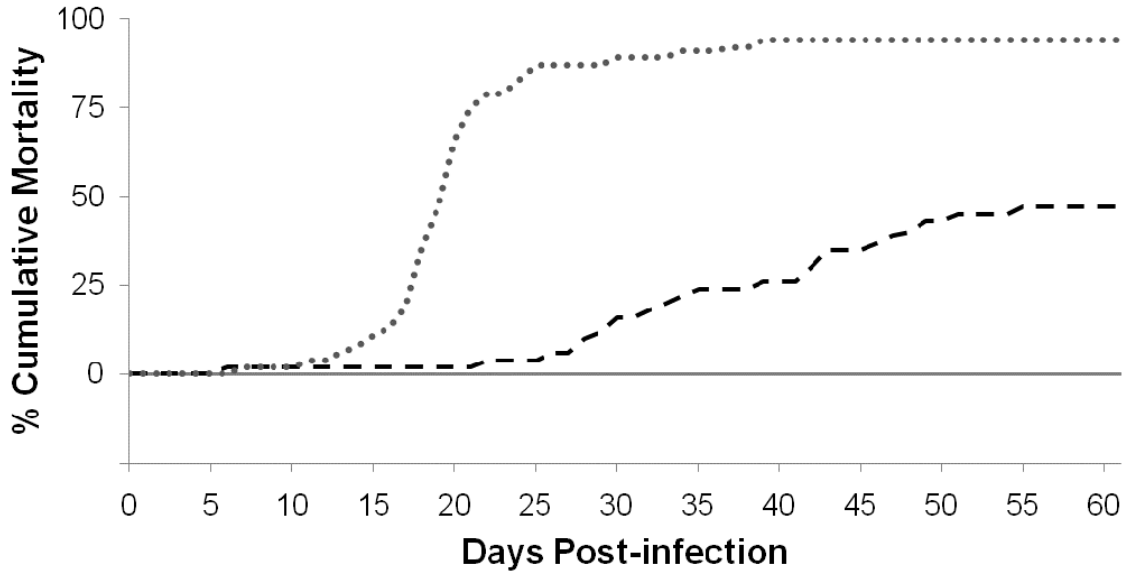

Figure 4-6 Cumulative mortality of juvenile muskellunge exposed to viral hemorrhagic septicemia virus IVb at a relatively high, medium, and low dose of infection by immersion. Percent cumulative mortality recorded in individual curves for each group of fish infected at calculated doses (dotted line, 1.7×10^4 pfu mL^{-1}; dashed line, 4×10^3 pfu mL^{-1}; solid line, Negative control). Daily monitoring for mortalities extended to a period of 60 d p.i. Mortality in fish infected at 1.7×10^4 pfu mL^{-1} exhibited an acute mortality from day 17 to 23 p.i., while fish infected at 4×10^3 pfu mL^{-1} experienced a prolonged mortality event throughout the 60 d observation period. Each dose of VHSV was administered to 50 fish, while the negative control contained 25 fish. Fish were an average weight of 1.8 ± 0.6 g and total length of 8.5 ± 0.7 cm.

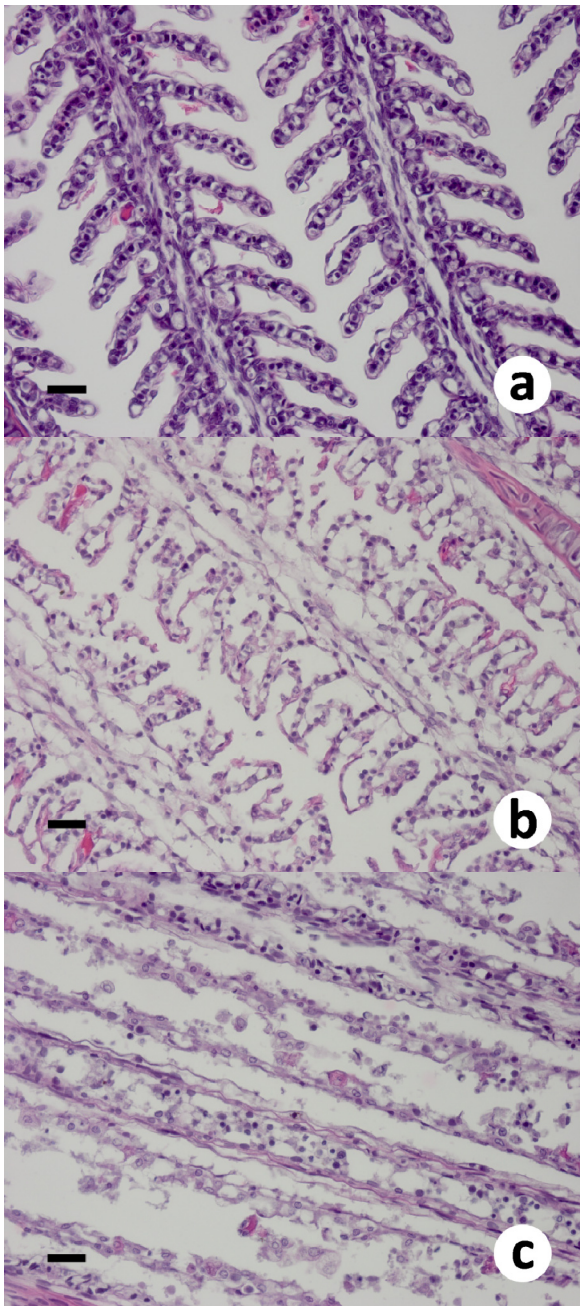

Figure 4-7 Histologic sections of gill from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control; (b) Necrosis, sloughing, and degeneration of secondary lamellae with some blunting; (c) Destruction and necrosis of primary lamellae. (scale bar = 50μm)

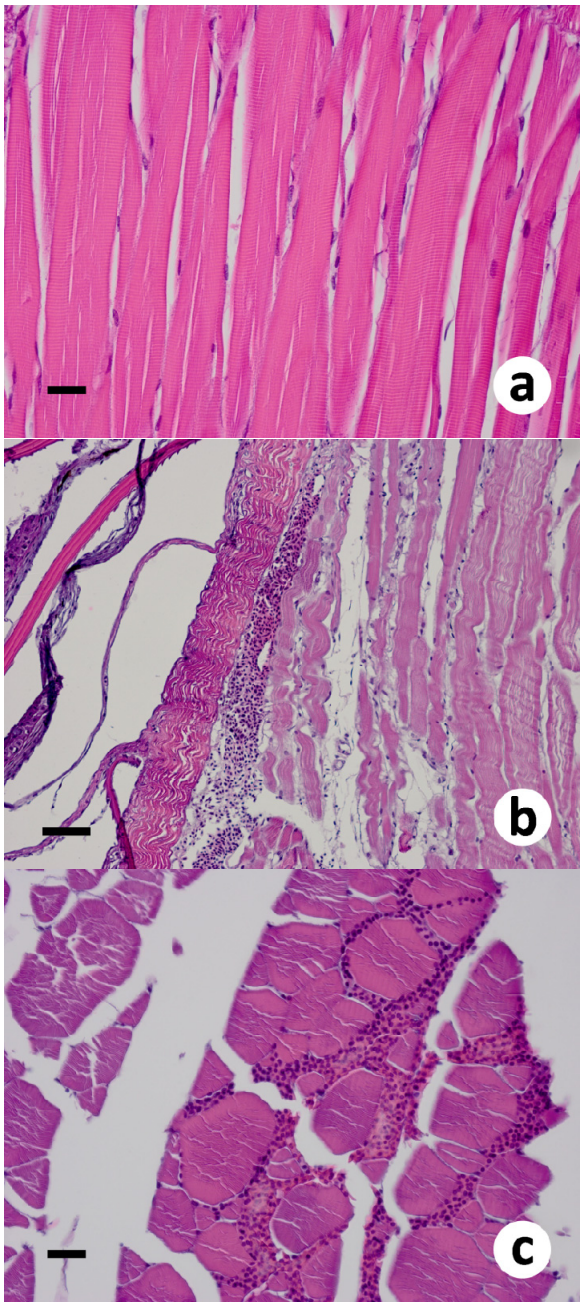

Figure 4-8 Histologic sections of muscle from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control; (b) Moderate haemorrhage at the skin-muscle junction; (c) Severe infiltration of erythrocytes between muscle fibers and bundles. (scale bar; a,c = 50μm, b = 100μm)

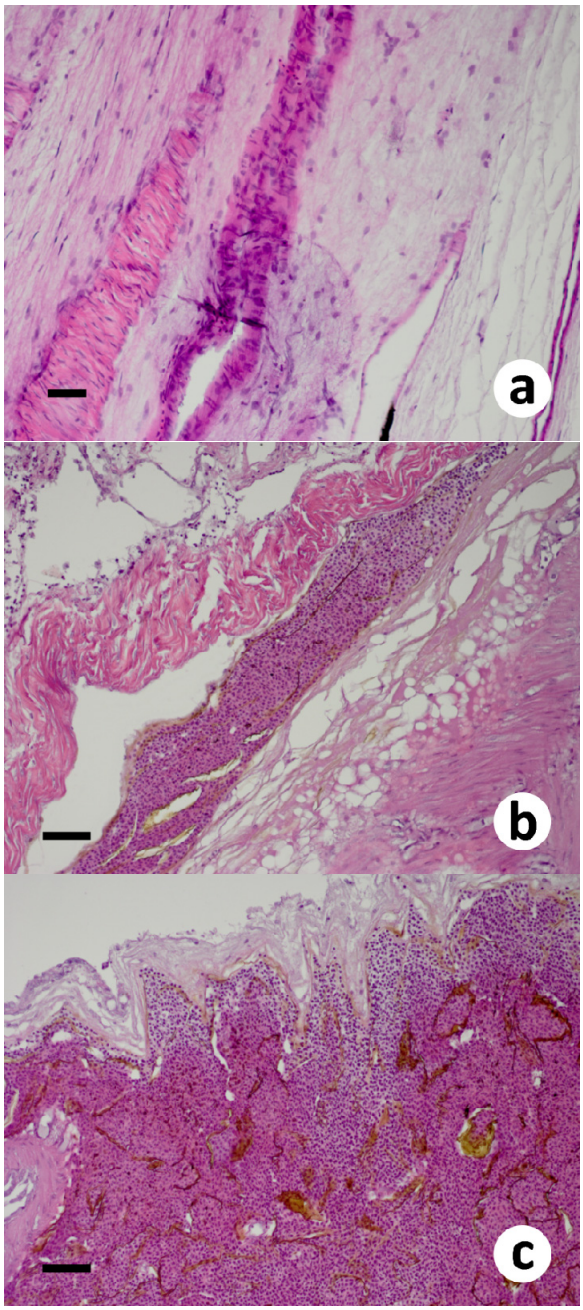

Figure 4-9 Histologic sections of swim bladder from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control swimbladder; (b) Mild haemorrhage within the swim bladder wall; (c) Massive accumulation of erythrocytes in the swimbladder in the outer wall. (scale bar; a = 50µm, b,c = 100µm)

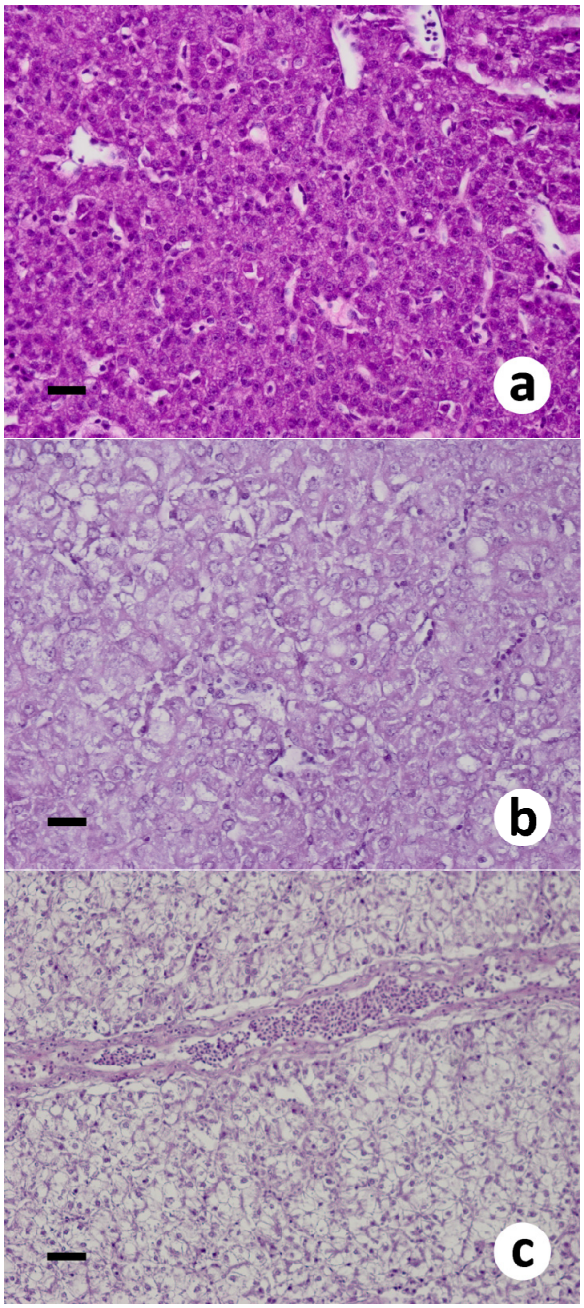

Figure 4-10 Histologic sections of liver from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control; (b) Mild intracellular changes with mild to moderate number of pyknotic nuclei and indiscrete cell borders; (c) Severe, diffuse vacuolation of hepatocytes and multifocal necrosis. (scale bar = 50µm)

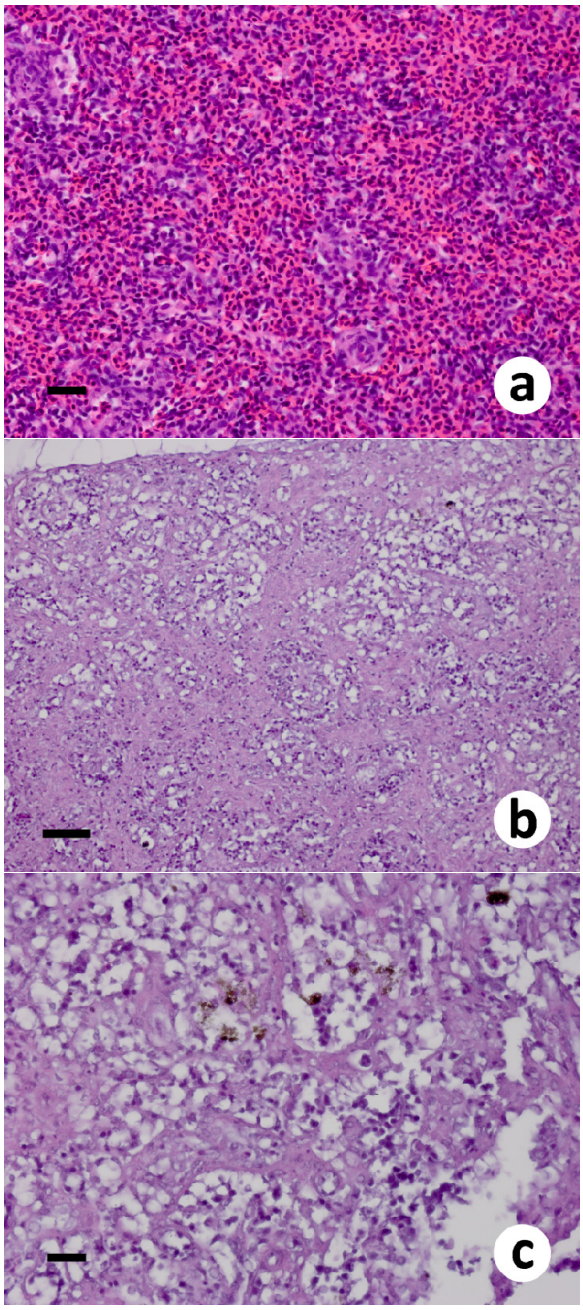

Figure 4-11 Histologic sections of spleen from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control; (b) Severe lymphoid depletion and necrosis; (c) Absence of erythrocytes and some connective tissue. (scale bar; a,c = 50μm, b = 100μm)

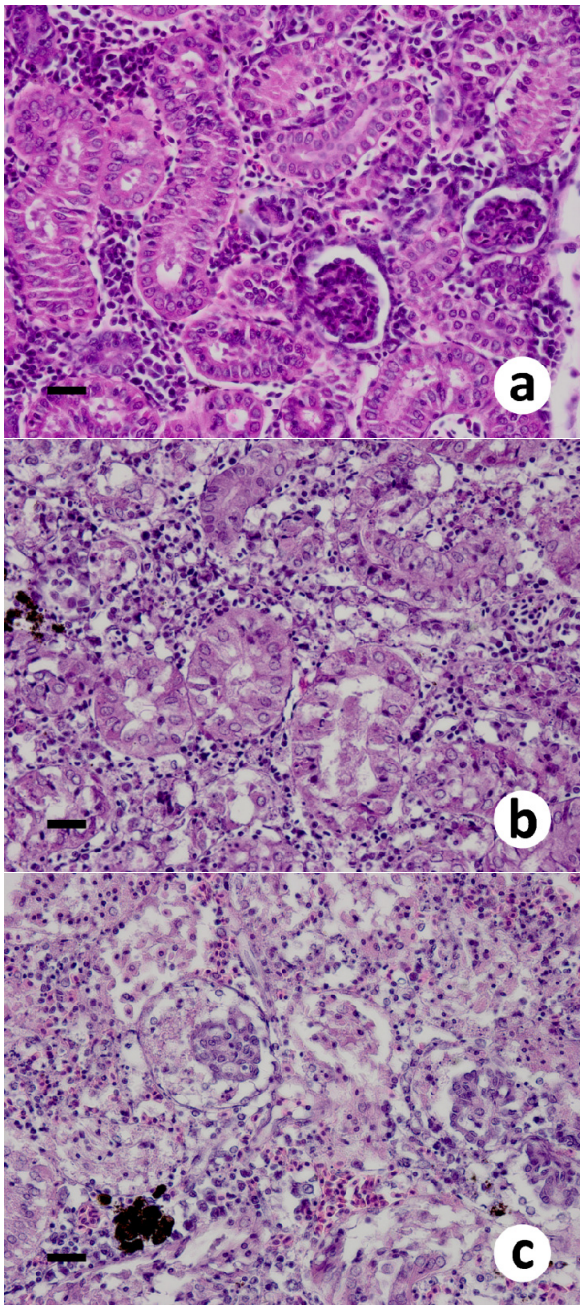

Figure 4-12 Histologic sections of kidney from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb and negative controls stained with hematoxylin and eosin (H&E). (a) Control; (b) Severe degeneration and necrosis of tubular epithelia; (d) Degeneration and necrosis of glomeruli with cellular debris and granular material filling Bowman's space. (scale bar = 50µm)

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CHAPTER 5

Sequential Distribution, Viral Load, and Tissue Alterations of Viral Hemorrhagic Septicemia Virus Genotype IVb

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Abstract

The emergence of a new sublineage of Viral hemorrhagic septicemia virus (VHSV), genotype IV, designated as VHSV-IVb in the Laurentian Great Lakes of North America necessitated investigations regarding its pathogenesis in susceptible host species. This study focused on following the sequence of pathologic changes induced by VHSV-IVb and its distribution within host organs. A total of 180 juvenile muskellunge were experimentally infected by immersion at 4×10^3 plaque forming units (PFU) mL^{-1}, followed by sequential sampling of the spleen, kidneys, heart, liver, gills, pectoral fin, posterior intestine, and skin/muscle from four fish at each predetermined timepoint. Organ samples were processed for VHSV IVb reisolaton on the *Epitheliosum papulosum cyprini* (EPC) cell line and quantification by plaque assay. Distribution and load of VHSV IVb within the organs was highly variable between individual fish and sampling point. The earliest reisolation of VHSV IVb occurred in one fish from pectoral fin samples at 24 hours post infection. By 6 days post infection (d p.i.), all organs sampled were positive for VHSV IVb. Of the organs sampled, the heart and skin/muscle displayed an increased frequency of VHSV IVb reisolation throughout the 64 day observation period, while spleen samples tended to be lower. Elevated virus loads were obtained from the heart and liver which were as high as 5.93×10^8 and 5.20×10^8 plaque forming units (PFU) gram (g) of tissue$^-$

[1], respectively. Furthermore, virus loads obtained from the heart, liver, gill, and pectoral fin were repeatedly higher than in other organs. Histopathologic changes were noted as early as 6 d p.i. in the heart, which exhibited progressive necrosis, degeneration, and inflammatory changes with each subsequent sampling, while mild and non-progressive lesions were observed in other organs. This study provided evidence that profound viremia occurs during VHSV IVb infection, resulting in the invasion and damage of multiple organs. Furthermore, high viral loads collected from a number of organs indicated that infected hosts were able to amplify VHSV IVb to titers that were several orders of magnitude above the original dose of infection. The sum of the data generated in this study fills a void of knowledge regarding the pathogenesis and disease course of VHSV IVb in muskellunge.

Introduction

Viral hemorrhagic septicemia virus (VHSV) is a notifiable pathogen under the World Organization for Animal Health (OIE), and is recognized for its ability to cause large scale mortality events within a number of freshwater and marine fish species in North America and Europe (Wolf 1988). More recently, a Great Lakes strain of VHSV, denoted genotype IVb (VHSV IVb) emerged and was associated with fish kill episodes in the Great Lakes basin (Elsayed et al. 2006; Gagné et al. 2007; Groocock et al. 2007; Lumsden et al. 2007). Clinical manifestations of VHSV infection reported in wild fish kills are similar to those described in the literature, which include gill palor, abnormal swimming behavior, and hemorrhages of the skin, fins, eyes (Wolf 1988). Despite these similarities, a major limitation in field samples is that fish are often found dead, which yield few clues into the progression of disease and tissue alterations induced by VHSV IVb. As a result, a dearth of knowledge currently exists regarding the pathogenesis and kinetics of VHSV IVb infection, which is pivotal to our understanding of the biologic and ecologic impacts of this emerging strain of VHSV.

The need to understand VHSV dissemination within hosts and its concomitant pathology in sequence was initially recognized by Neukirch (1984,1985) during a time when information regarding VHSV pathogenesis was scarce. As a result, a study was performed in which rainbow trout (*Oncorhynchus mykiss*) were experimentally infected with VHSV (F1 strain, genotype I; Jensen 1965) by waterborne immersion. Samples of the gills, brain, urine, feces, and

pools of spleen, kidney, and liver were collected in sequence from three fish to assess virus distribution and load. The study revealed that VHSV was able to reach all organs of infected fish, except the brain, by 2 days post infection (d p.i.). By the third day of infection, titers of VHSV increased sharply and remained elevated throughout the study. The authors noted profound differences in virus titers among collected samples.

The more recent studies of Evensen et al. (1994) and Brudeseth et al. (2002) have also shed light into the sequential pathology of VHSV infection. Through experimental infection of rainbow trout using VHSV (F1 strain), both research groups demonstrated that over the course of time VHSV (F1 strain) can cause progressive degeneration in multiple organ systems. A prevailing feature of both studies was that the kidneys were primary target sites for VHSV, followed by a more widespread distribution into other organs and tissues. Yet subtle differences were noted in that VHSV was found at 1 d p.i. in only the anterior kidney by Evensen et al. (1994), while Brudeseth et al. (2002) detected the virus in the gill and kidney by 2 d p.i. Despite these subtle differences, pathologic changes noted in other tissues were largely similar and thus fulfilled a major knowledge void regarding VHSV (F1 strain) pathogenesis.

In the same context, pathogenesis of VHSV IVb is largely unknown and has not been investigated in depth. To date, field observations (Elsayed et al. 2006; Gagné et al. 2007; Groocock et al. 2007; Lumsden et al. 2007) and experimental studies conducted by Kim and Faisal (2010a,b) have shown that VHSV IVb can infect and cause death in a broad range of freshwater fish, albeit

with differences in susceptibility and clinical signs. Such a wide host range has not been recognized as a feature of other VHSV strains and has therefore raised speculation with regard to whether virus-host interactions are comparable to other strains. The argument that VHSV disease course could vary between species and VHSV isolates is supported by the studies of Brudeseth et al. (2005), when turbot (*Scophthalmus maximus*) experimentally infected with VHSV herring isolate 4p168 (genotype III, Mortensen et al. 1999) demonstrated delayed mortality with virus distribution largely restricted to the kidneys, heart, and spleen, which was not similar to the generalized nature of VHSV (F1) infection in the rainbow trout (Evensen et al. 1994, Brudeseth et al. 2002). Such discrepancies led to the conclusion that pathology induced by VHSV likely depends on the strain (Skall et al. 2004) and host species, and should be considered along with other disease factors such as age of the host, initial exposure dose, and environmental parameters (i.e. water temperature).

As the list of species that are found to be susceptible to VHSV IVb continues to increase, a need to understand the progression of disease and pathologic alterations governing VHSV IVb infection has become critical. Therefore, a study was designed to establish the sequence of pathologic changes and virus distribution in a highly susceptible host, the muskellunge (*Esox masquinongy*). Data to be generated from this study will serve as a basis for future studies on other species of lesser or higher susceptibility to VHSV-IVb.

Materials & Methods

Cell culture and virus propagation & titration

The Great Lakes VHSV IVb index strain MI03, originally isolated in our laboratory in 2003 from muskellunge kidney and spleen tissues (Elsayed et al. 2006), was used. The virus was propagated in the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan et al. 1983). Quantification of virus was accomplished through the viral plaque assay using EPC cells, polyethylene glycol and methylcellulose overlay as described in Batts & Winton (1989) and Batts et al. (1991). Virus stocks were aliquoted in cryogenic vials (Corning, Lowell, MA) and kept at -80 $^{\circ}$C until used. Ten fold serial dilutions were applied to the virus stock beginning with 1:100 to $1:10^{12}$. Plaques were allowed to form for a period of six days, and the remaining cells of each well were fixed and stained with a crystal violet (0.5% w/v) and formaldehyde (1:1 dilution with water) solution. The virus stock was calculated to have approximately 7.32×10^{8} plaque forming units (PFU) mL^{-1}.

Cell lines were maintained and subcultured in 150 cm^2 tissue culture flasks (Corning, Lowell, MA) at 25 $^{\circ}$C using a growth formulation of Earle's salt-based Minimal Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 29.2 mg mL^{-1} L-glutamine (Invitrogen), Penicillin (100 IU mL^{-1}) and Streptomycin (0.1 mg mL^{-1}; Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich, St. Louis, MO).

Fish and Maintenance

Certified VHSV-free juvenile muskellunge (4 months post hatch) were obtained from the Rathbun National Fish Hatchery in August of 2009 and maintained as described in Kim & Faisal (2010). Prior to infection, a subsample of five fish were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) (25 mg mL^{-1}) and tested for the presence of VHSV. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen) supplemented with 12 mM tris buffer (Sigma-Aldrich), Penicillin (100 IU mL^{-1}), Streptomycin (100 µg mL^{-1}) (Invitrogen), and Amphotericin B (250 µg mL^{-1}) (Invitrogen) to produce 1:4 dilution (w/v) of original tissues. Samples were centrifuged at 2000 *g* and the supernatants inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Plates were incubated at 15 $^{\circ}C$ for 7 d, and observed for the formation of cytopathic effects (CPE). A second blind passage was performed and assessed for the presence of VHSV.

Fish were acclimated to temperatures from 16 $^{\circ}C$ to 11 $^{\circ}C$ over a three week period before the experiment was initiated. All fish were initially held in a 1,900 L circular fiberglass tanks in a continuous flow-through system supplied by oxygenated and facility chilled well water at Michigan State University - Research

Containment Facility (East Lansing, MI). The juvenile muskellunge were fed *ad lib* with 2.0 mm sinking feed (Silver Cup, Nelson and Sons, Murray, UT) and transitioned to VHSV-free certified fathead minnows, *Pimephales promelas (*Rafinesque) purchased from Robinson Wholesale, Inc (Genoa City, WI).

Experimental infection of juvenile muskellunge

A group of 180 fish (weight 20.0 ± 10.9 g; total length 17.1 ± 1.5 cm) were randomly assigned into two groups. Fish in Group 1 (90 fish) were challenged by immersion in water containing VHSV MI03 at a medium dose (4×10^3 PFU mL^{-1}), while fish in group 2 (90 fish) were immersed in water mixed with 1 mL of sterile maintenance-MEM, that served as a negative control group. The two experimental groups were held in static, oxygenated water for 90 min and subsequently divided into two tanks of equal numbers (45 fish). Calculated dose levels were in accordance to those described in Kim & Faisal (2010c).

Tissue Sampling

Samples of tissue were collected in parallel from four fish in each of the groups, including the negative control. Moribund and/or apparently healthy fish were euthanized at predetermined hours (0h, 6h, 12h, 24h, 36h) and days (2d, 4d, 6d, 8d, 15d, 22d, 29d, 36d, 43d, 50d, 57d, 64d), and subsequently dissected for collection of pectoral fin, gill, spleen, heart, liver, kidney, posterior intestine, and dorsal musculature with skin. Sampled tissues were divided into two groups, one of which would be stored at -80 $^{\circ}$C in 1.5 mL centrifuge tubes (Denville Scientific,

South Plainfield, NJ) for the viral plaque assay and the second group to be fixed in phosphate buffered formalin (10%) for 24h and embedded in paraffin wax.

Viral plaque assay

Tissues were weighed and diluted at a range of 1:4 to 1:250 (w/v) and homogenized by mortar and pestle (Fisher Scientific, Pittsburgh, PA). Homogenates were centrifuged at 2000 × *g* and inoculated onto 24 well plates of confluent monolayers of EPC. Quantification of virus was completed in accordance with the methods described above. Plates were incubated at 15 $^{\circ}$C for 6 d, and stained to quantify the number of plaques in order to calculate the number of plaques per tissue.

VHSV reisolation and confirmation

Supernatant from tissue homogenates were tested for the presence of VHSV using the EPC cell line. For PCR confirmation, total RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Reverse transcription was accomplished by a two step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR^{TM} (Stratagene, La Jolla, CA) following the manufacturer's instruction. The primer set used in this assay is recommended by the World Organization for Animal Health Manual of Diagnostic Tests for Aquatic Animals (2003) for detection of a 811 base pair sequence of the VHSV nucleocapsid gene: 5'-GGG-GAC-CCC-AGA-CTG-T-3' (forward primer) and 5'-TCT-CTG-TCA-

CCT-TGA-TCC-3' (reverse primer). Polymerase chain reaction was achieved by adding into each reaction tube 5 µL of viral RNA, 50 pmol of each primer, 25 µL of GoTaq® Green Mastermix DNA Polymerase (Promega), and the DNA-ase free water to create a final volume of 50 µL. The reverse transcriptase was inactivated by subjecting the mixture to 94 °C for 2 min, and 30 cycles of PCR (denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and polymerization at 68 °C for 1 min) in a Mastercycler Personal Thermal Cycler (Eppendorf, Hauppauge, NY). The polymerization was finalized by maintaining the mixture for a period of 7 min at 68 °C. The product was visualized by gel electrophoresis in 1.5% agarose gels.

Histopathology

Paraffin embedded tissue samples from all sampling times were sectioned (5 µm) and then stained with hematoxylin and eosin as detailed in Prophet, et al. (1992).

Results

Virus distribution

Detection of VHSV IVb occurred as early as 24 hours post infection (h p.i.) in the pectoral fin of one fish. However, virus was not reisolated in subsequent tissues sampled from 36 h p.i. to 4 d p.i. (Table 1). Virus was detected in all organs tested at 6 d p.i., with the majority of isolations occurring in the kidney, heart, liver, gill, pectoral fin, and skin/muscle. At 8 d p.i., virus was confirmed in skin and muscle tissue of one fish, whereas at 15, 29, and 43 d p.i. VHSV IVb was detected in all organs. All four posterior intestine samples collected at 29 d p.i. were also positive for VHSV IVb. By 36 d p.i., none of the organs collected from the four fish were identified as VHSV IVb positive. Interestingly, virus was found in the pectoral fin and skin and muscle tissue of one fish at 50 and 57 d p.i. Virus reisolation demonstrated the presence of VHSV IVb in tissues collected as far as 64 d p.i. By the end of the observation period, the total number of VHSV reisolations appeared to be more common in heart, skin/muscle, and gill, while less common in spleen samples. Non-infected control fish organs were free of virus by cell culture and RT-PCR at all sampling points.

Viral load

The amount of virus quantified within tissues differed amongst samples. A majority of tissues collected from 0 h p.i. to 4 d p.i., did not have quantifiable levels of virus by the plaque assay. A pectoral fin sample from one fish at 24 h p.i. contained 9.75×10^3 PFU g tissue^{-1}. From 6 to 29 d p.i., viral loads were

consistently higher in all tissues that were confirmed VHSV positive (Figure 1). The highest viral load was detected in the heart of one fish at 6 d p.i. at 5.93 × 10^8 PFU g tissue^{-1}, followed by the liver (5.20 × 10^8 PFU g tissue^{-1}) and gill (5.04 × 10^8 PFU g tissue^{-1}) of the same fish (Table 1) which were several folds higher than the original dose of infection. The lowest amount of virus was in the skin and muscle tissue, which was 1.31 × 10^2 PFU g tissue^{-1} at 8 d p.i. When assessing the viral load over the 64 d period, an overall decrease in virus load was noted in samples of the spleen, kidney, gill, and pectoral fin, while the heart, liver, posterior intestine and skin/muscle exhibited fluctuating levels of virus among individual fish.

Histopathologic analysis

Sequential histopathology was variable amongst tissues, and in most instances did not correspond to the viral load or sampling time. In a majority of the organs sampled, a correlation between the severity of lesion(s) and virus load was not observed.

Analysis of gill sections revealed mild branchitis with epithelial hyperplasia in two of the three VHSV IVb positive fish as early 6 d p.i. A moderate increase in inflammatory changes at the base of primary lamellae were noted at 15 and 21 d p.i. (Figure 2), but did not did not progress in severity or correlate to the quantified viral load. A mild branchitis was observed in at least one of the four

fish collected between 28 and 64 d p.i. with intermittent shortening or absence of the secondary lamellae, which included timepoints in which VHSV IVb was not reisolated.

Pathologic changes were noted in the pectoral fins of one fish collected at 21 and 28 d p.i. Lesions were located in the epithelial layers as focal aggregations of pyknotic and fragmented nuclei (Figure 3). All other histologic sections of the pectoral fin did not exhibit any lesions.

Progressive pathology was noted within histologic sections of the heart between 6 and 64 d p.i. The first observable lesions were noted as early as 6 d p.i., which were characterized by few foci of pyknotic nuclei intermixed throughout areas of cardiac myofibers (Figure 4a). By 29 d p.i. the heart of one fish exhibited advanced pathologic changes in the form of large foci of pyknotic and karyorrhectic nuclei amidst aggregates of lymphocytes and macrophages (Figure 4b). Subsequent histologic sections of heart collected from 36 to 64 d p.i., demonstrated severe destruction of cardiac myocytes in combination with a massive infiltration of inflammatory cells (Figure 4c). The dissolution of cardiac myofibers due to damage resulted in widespread mononuclear infiltrate primarily composed of lymphocytes and macrophages.

Lesions in histologic sections of the liver were seen as early as 6 d p.i., in which mild hepatitis appearing as small foci of inflammatory cells was observed.

Moderate enlargement of these foci coupled with hepatocellular necrosis was a repeated finding at 15, 28 and 43 d p.i. (Figure 5), and did not progress to a more widespread and generalized destruction of hepatocytes. No subsequent pathology was observed between 50 and 64 d p.i. despite the reisolation of VHSV IVb from the tissues.

The spleen of one fish sampled at 14 d p.i. was devoid of erythrocytes and lacked lymphoid tissue (Figure 6). All other spleen samples did not exhibit any pathologic changes. Histologic sections of the anterior kidney at all timepoints lacked pathology despite concomitant virus reisolation (*data not shown*).

Histologic sections of the large intestine exhibited mild endothelial hyperplasia and a trace of subserosal inflammation as early as 6 d p.i. By 14 d p.i., the smooth muscle of the intestinal wall was noticeably separated from the inner luminal and outer serosal epithelial layers due to infiltration of mononuclear cells of lymphoplasmacytic origin (Figure 7). Similar findings were noted in fish collected at 21, 28, 42, and 64 d p.i., but did not advance beyond a mild to moderate enteritis localized to the submucosal and subserosal layers of intestine.

The most prominent change observed in skin/muscle tissue was the separation of myocytes by massive infiltrations of erythrocytes at 6 and 14 d p.i. (Figure 8a). The degree of intramuscular hemorrhage did not vary and was less apparent by 28 d p.i., in which aggregates of swollen myofibers that were both necrotic and

hypereosinophilic were organized into large foci surrounded by masses of inflammatory cells (Figure 8b). All subsequent samples of skin/muscle tissue were normal and did not exhibit hemorrhage and necrosis observed at earlier timepoints.

Histopathologic changes are described in the appendix of Chapter 5 of this dissertation.

Discussion

Our studies confirm that VHSV IVb can be distributed at varying quantities in an array of organs throughout the course of infection, which provides new information into the pathogenesis of VHSV IVb in a highly susceptible host, the muskellunge. The ecologic relevance of these findings is significant in that VHSV IVb can be reisolated in cell culture beginning at 24 h p.i. to 64 d p.i indicating that virus could remain in host tissues for an extended period of time, without causing death or severe signs of morbidity. Tissue targets of VHSV IVb are largely unknown, but distribution and viral load data would suggest that VHSV IVb can subsist and amplify within tissues to levels well above the original exposure dose. Histopathologic changes were observed in all organ samples but were not associated with concurrent virus loads. The only tissue to demonstrate pathologic changes in sequence was the heart, while lesions in other organs were either consistent in appearance or could not be reproduced in subsequent samples.

To date, disease course studies remain vital to investigating entry and replication sites of VHSV. Since reisolation of VHSV IVb occurred in only the pectoral fin of one fish at 24 h p.i., it is plausible that the pectoral fins of muskellunge may be a site of virus entry and replication. These findings are corroborated by the studies of Harmache et al. (2010), in which the replication of a recombinant Infectious hematopoietic necrosis virus (IHNV) was visualized through bioluminescence in only the fins at 8 and 16 h p.i. However, in the pioneering studies of Neukirch (1985) and Neukirch & Glass (1984), VHSV (F1

strain) was reisolated from the gills of experimentally infected rainbow trout as early as 1 d p.i. In the case of muskellunge, VHSV IVb reisolation did not occur from the gills until 6 d p.i. Inherent differences in the virus strain, host species, and the detection methods may be contributing factors to such discrepancies. Other proposed sites of entry include epidermal tissues of salmonids (Yamamoto et al. 1992), while in some experimental infections VHSV transmission was achieved by feeding infected fish tissues implying the digestive tract as a portal of entry (Ahne 1980). While there are few studies to identify a definitive route by which VHSV gains access to susceptible hosts, virus attachment and entry likely occurs at more than one tissue site and could rely on a number of factors that may include the strain of virus and host species.

Once VHSV IVb gained access to juvenile muskellunge, viral distribution extended to all tissue types by as early as 6 d p.i. as was shown by cell culture reisolation (Table 1). In most instances, VHSV IVb was recovered from all tissue types, despite reports of other VHSV strains or rhabdoviruses such as spring viremia of carp virus that have demonstrated preferential distribution to the kidney early in infection (Faisal & Ahne 1984, de Kinkelin et al. 1979, Evensen et al. 1994). One may speculate that VHSV IVb replicates in a wide range of tissues other than the kidney and spleen, since quantities of virus were substantially higher in the heart, liver, and gills. However these findings should be interpreted with caution, since VHSV IVb was recovered from whole tissue homogenates, which by definition creates a mixture of different cell types. Given that VHSV is traditionally considered to be endotheliotropic (Chilmonczyk 1995, Brudeseth et

al. 2002) and can infect white blood cells such as macrophages (Estepa et al. 1992), VHSV IVb could have been present in a number of cell types that were not major constituents of the organ. More importantly, viremia as a result of viral replication at a site other than our sampled tissue also could have contributed to VHSV IVb reisolation. In the end, more detailed studies are warranted to localize replication sites of VHSV and differentiate the aforementioned discrepancies in VHSV cell preference.

High viral loads and increased VHSV IVb reisolation appeared to be associated with heart samples throughout the 64 d observation period (Table 1). These results indicate that VHSV IVb was able to remain in the heart for upwards of 64 d p.i. without outward clinical signs. More importantly, the heart may be a tissue site in which VHSV could replicate and remain at detectable levels, without compromising the longevity of the host. While prolonged VHSV presence in the heart may be alarming, persistence of VHSV has previously been demonstrated in the brain tissues of VHSV (F1 strain) exposed rainbow trout for up to 421 d p.i. (Neukirch 1986). Similarly, the muskellunge heart may be an organ in which VHSV resides once muskellunge are exposed to VHSV IVb. Such claims are uncertain, since histopathologic lesions observed in the heart progressed in severity with each subsequent sampling and may have led to mortality. The supposition that VHSV has a predilection for heart tissue has been supported by Brudeseth et al. (2005), when histologic sections of heart from turbot (*Scophthalmus maximus*) experimentally infected with marine isolates of VHSV demonstrated strong positive signaling by immunohistochemistry and

degenerative changes by histopathology. Whether the heart is an epicenter of viral replication is largely unknown, and therefore warrants further investigation to determine the inherent properties of the piscine heart that may support rhabdoviral replication. On the other hand, viremia may substantially increase the likelihood of infection susceptible cells in cardiac tissues.

In the current study, a broader range of tissues were sampled to identify additional sites in which VHSV IVb may be causing pathologic changes. In the case of the pectoral fin and posterior intestine tissues that were VHSV IVb positive, degenerative and necrotic changes were observed, suggesting that VHSV IVb may be able to cause damage in more tissues than previously thought. Knowledge of where VHSV IVb localizes and induces pathology may offer clues into extent of viral-host interactions and how the virus may be released into the surrounding environment. Reisolation of VHSV from urine collected by catheterization (Neukirch & Glass 1984) and concomitant histopathologic changes (Yasutake 1975) have strongly suggested that the kidneys are a target of VHSV whereby virus is shed via urine. Given that virus was obtained from the large intestine and pectoral fin, VHSV IVb may have the opportunity to be shed either in the feces or via the epithelium, respectively. The lack of VHSV detection in the feces of early studies (Neukirch & Glass 1984) and limited in vivo studies regarding VHSV infectivity of teleost fins (Estepa et al. 1993) warrant further investigation.

Despite intensive and prolonged sampling of muskellunge organs, a lack of sequential pathology was observed in histologic sections in conjunction with

the virus load. While obvious progressions in lesion severity were noted in the heart and muscle, tissue alterations noted over time in other organs were often mild or unvarying. The difficulty of assessing histopathologic changes that occur in sequence is likely due to a number of factors, which may rely on the fact that single histologic sections of organs may not provide a representative sample in providing an overview of the lesion(s) that are present. Secondly, VHSV IVb pathogenesis may be unrelated to levels of virus detected within tissues since VHSV cellular predilections may be localized to cell types other than the ones found in the major organ of interest (de Kinkelin et al. 1979, Hoffman et al. 1979, Faisal & Ahne 1984, Estepa et al. 1992, Evensen et al. 1994, Chilmonczyk 1995, Brudeseth et al. 2002) or that virus replication and exit may preceed pathology. In order to overcome such obstacles, the utilization of more specific and sensitive techniques such as immunohistochemistry (Evensen et al. 1994, Brudeseth et al. 2002, 2005), in-situ hybridization, or quantitative reverse transcriptase polymerase chain reaction combined with histopathology may offer a more complete approach to determining pathologic changes within tissues.

Altogether, our studies present an overview of tissues that likely contain high and low quantities of virus. Heart, liver and gill tissues yielded the highest levels of virus during the early and latter stages of infection. Given that protocols for virus isolation often recommend sampling of the kidney and spleen, inclusion of additional tissues that are known to contain high levels of virus may be a key element to improving current diagnostic methods and increase the likelihood of detecting and isolating VHSV IVb. With the exception of the heart, which is a

tissue sample of choice by the OIE (Manual of Diagnostic Tests for Aquatic Animals 2003), additional processing steps to reduce the inherent cytotoxicity of the liver and environmental flora of the gills could enhance virus reisolation.

Our findings expound on the differential distribution of VHSV IVb in muskellunge, which has not been previously described. Quantitative analysis and histopathology reveal that the heart of muskellunge may be a target of virus replication. Based on the limitations of our assays, the reported elevations in virus load raises speculation into whether additional tissues can support virus production. Studies are currently underway to utilize more sensitive and specific assays to visually localize sites of VHSV IVb replication. The ecologic importance of our study indicates that viral replication continues up to 64 d p.i., suggesting that the virus is maintained within susceptible hosts that can potentially serve as carriers. The fate of newly produced VHSV IVb virions is uncertain and therefore necessitates further study into viral shedding from infected hosts.

Table 5-1 Distribution of viral hemorrhagic septicemia virus IVb (VHSV IVb) in muskellunge tissues collected in parallel at predetermined timepoints. The number of VHSV IVb positive fish and range of virus load in each tissue is indicated below.

	VHSV Positive Samples (*n* = 4 per sampling time)																	**Range of Viral Load In Positive Tissues (PFU g^{-1} tissue)**	
	Hours					**Days**													
Tissue	0h	6h	12h	24h	36h	2d	4d	6d	8d	15d	22d	29d	36d	43d	50d	57d	64d	**Total**	**High**	**Low**
spleen	0	0	0	0	0	0	0	1	0	1	0	2	0	2	0	0	1	7	1.30×10^{8}	1.85×10^{4}
kidney	0	0	0	0	0	0	0	3	0	1	1	2	0	2	0	0	1	10	2.63×10^{7}	5.96×10^{2}
heart	0	0	0	0	0	0	0	3	0	1	2	3	0	3	0	0	3	15	5.93×10^{8}	5.72×10^{3}
liver	0	0	0	0	0	0	0	2	0	2	1	2	0	2	0	0	1	10	5.20×10^{8}	8.21×10^{2}
intestine	0	0	0	0	0	0	0	2	0	1	2	4	0	2	0	0	0	11	3.17×10^{7}	1.23×10^{3}
gill	0	0	0	0	0	0	0	3	0	2	2	3	0	2	0	1	1	14	5.04×10^{8}	1.49×10^{3}
pectoral fin	0	0	0	1	0	0	0	3	0	1	0	3	0	2	1	0	1	12	8.16×10^{7}	5.15×10^{3}
skin/muscle	0	0	0	0	0	0	0	3	1	3	0	3	0	3	1	0	1	15	2.82×10^{7}	1.31×10^{2}

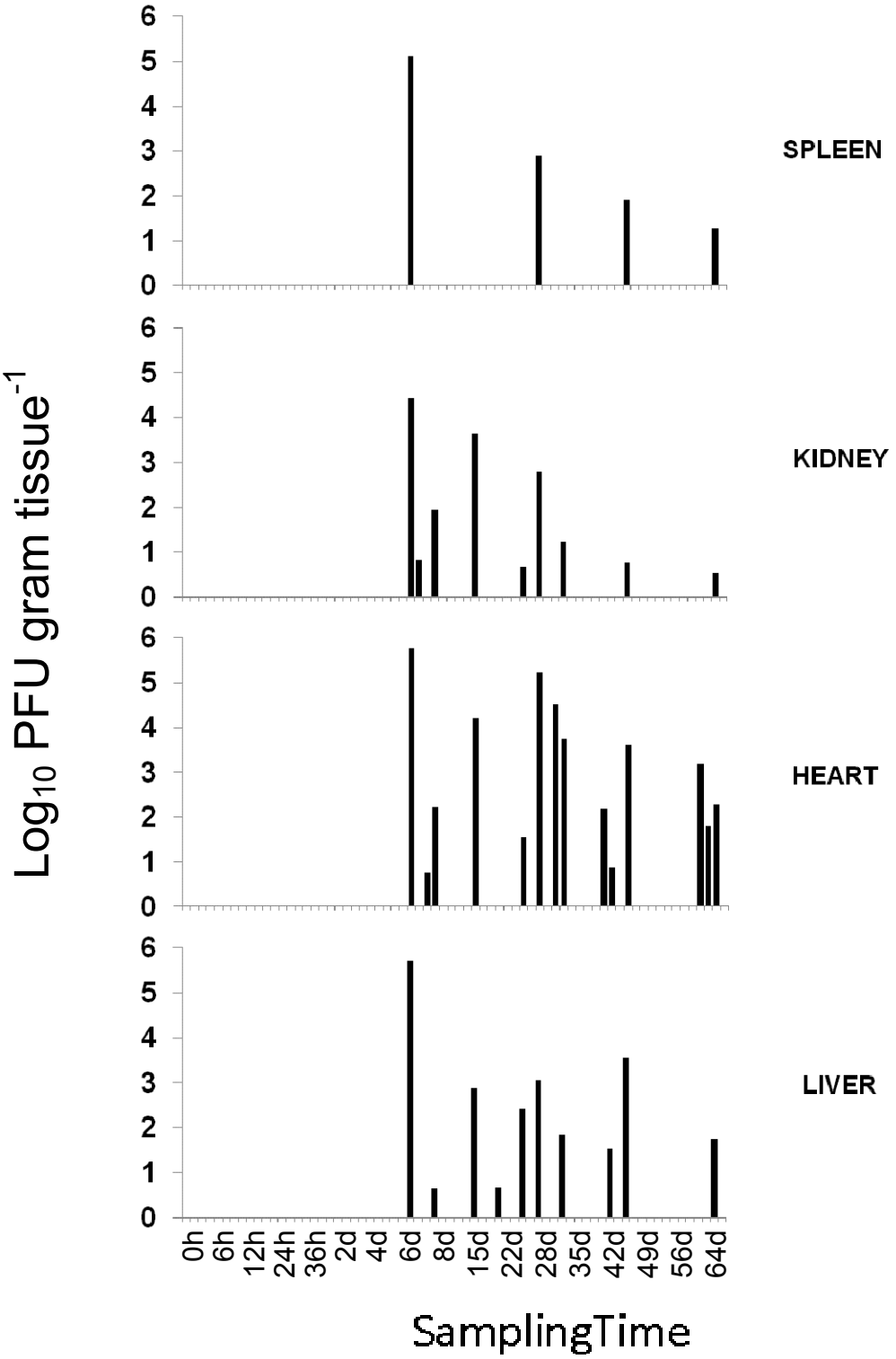

Figure 5-1 Load of viral hemorrhagic septicemia virus IVb determined by plaque assay, shown in $\log_{10}$ plaque forming units (PFU) gram tissue^{-1} in parallel organ samples from four fish at predetermined timepoints.

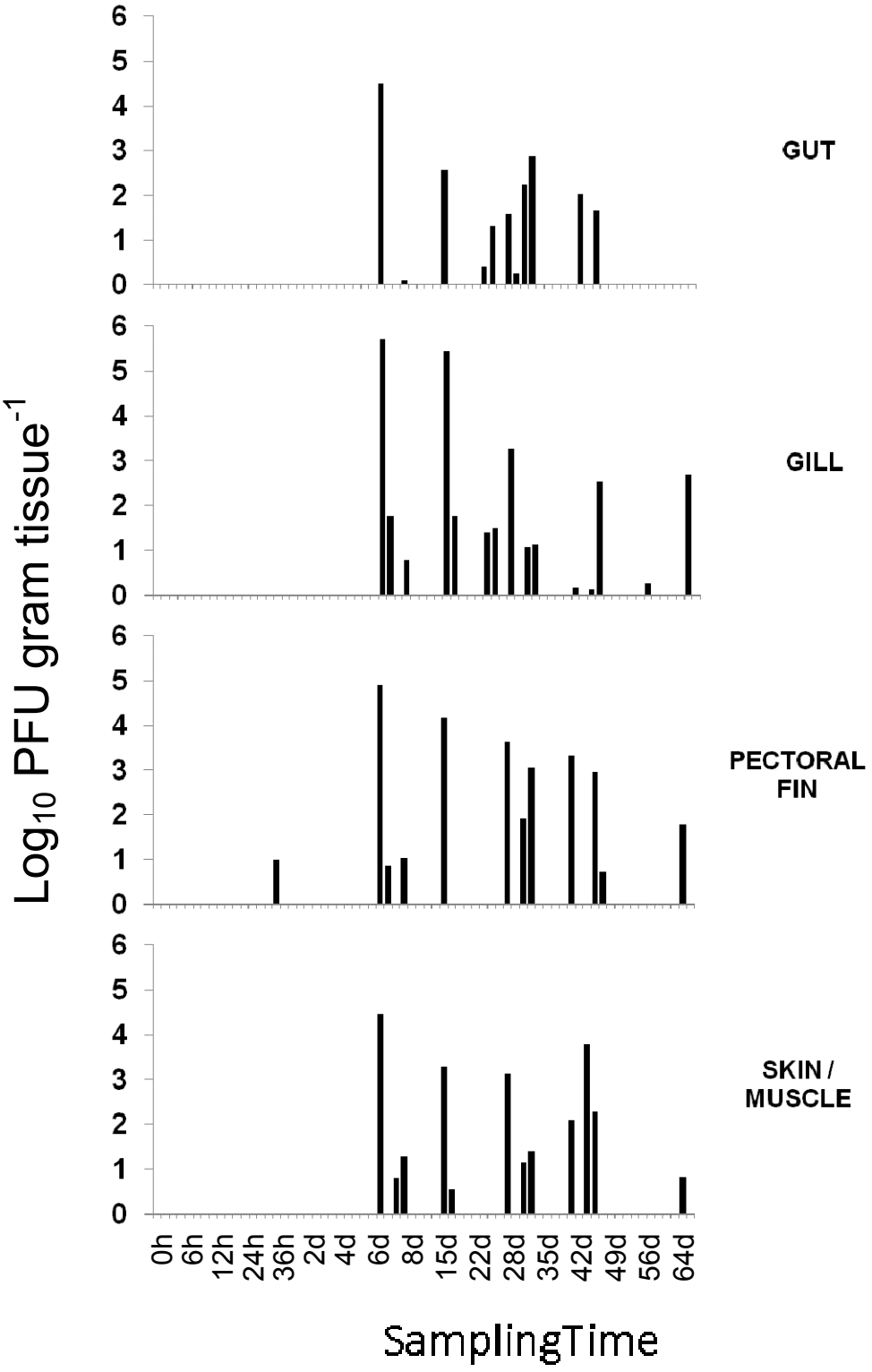

Figure 5-1 (cont'd) Load of viral hemorrhagic septicemia virus IVb determined by plaque assay, shown in $\log_{10}$ plaque forming units (PFU) gram tissue^{-1} in parallel organ samples from four fish at predetermined timepoints.

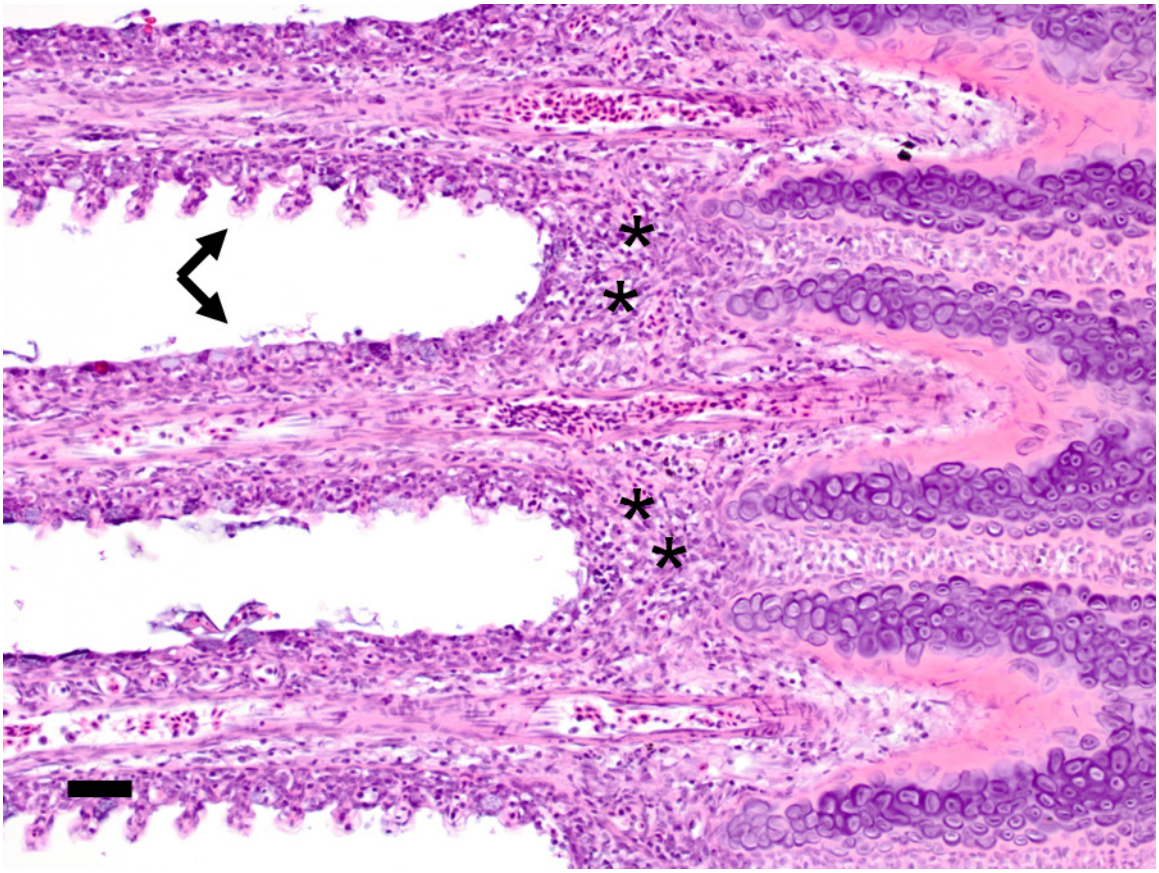

Figure 5-2 Gill tissue collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at 21 days post infection demonstrating mild to moderate epithelial hyperplasia at the base of primary lamellae (asterisks). Note that some of the secondary lamellae are either blunted or absent (arrows) along the axis of the primary lamellae. Tissue is stained with hematoxylin and eosin. (scale bar = 44µm)

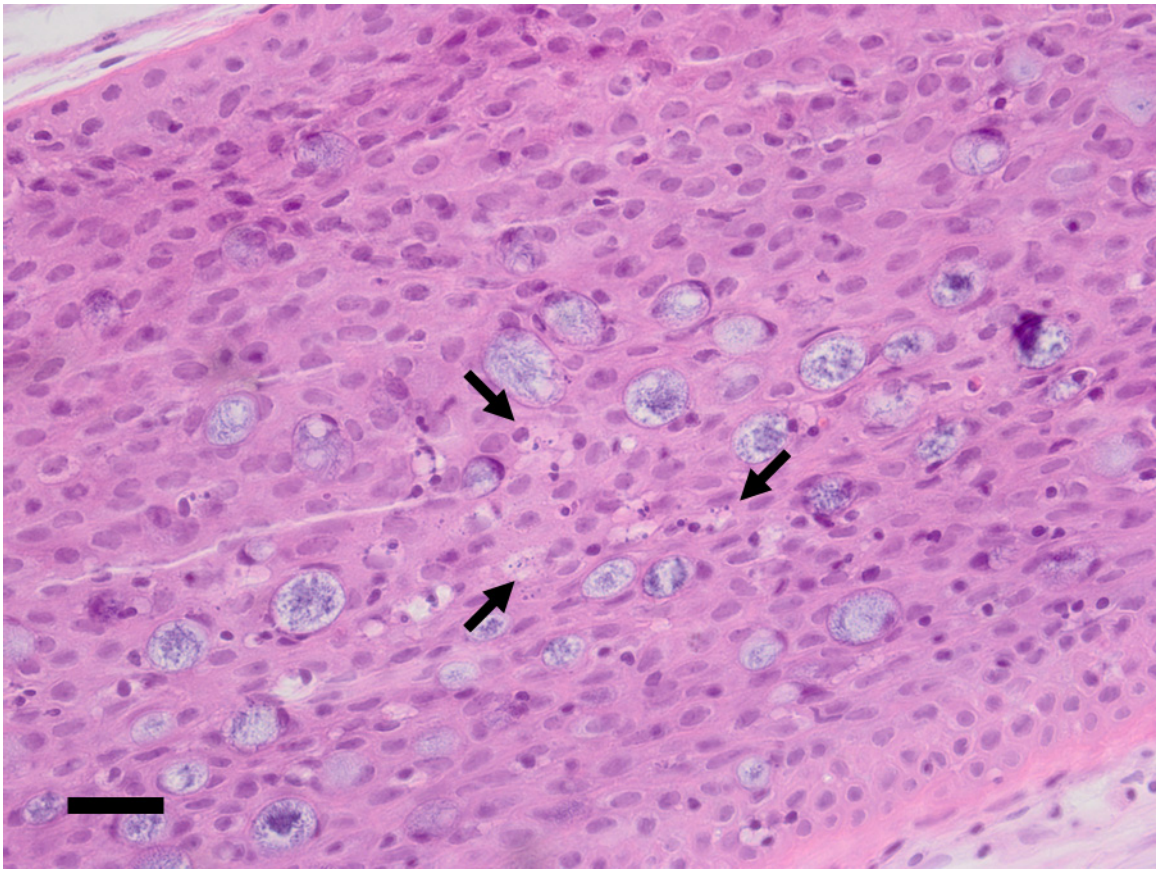

Figure 5-3 Pectoral fin collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at 21 days post infection. Epithelial nuclei are observed undergoing pyknosis and karyorrhexis (arrows). Tissue is stained with hematoxylin and eosin. (scale bar = 44µm)

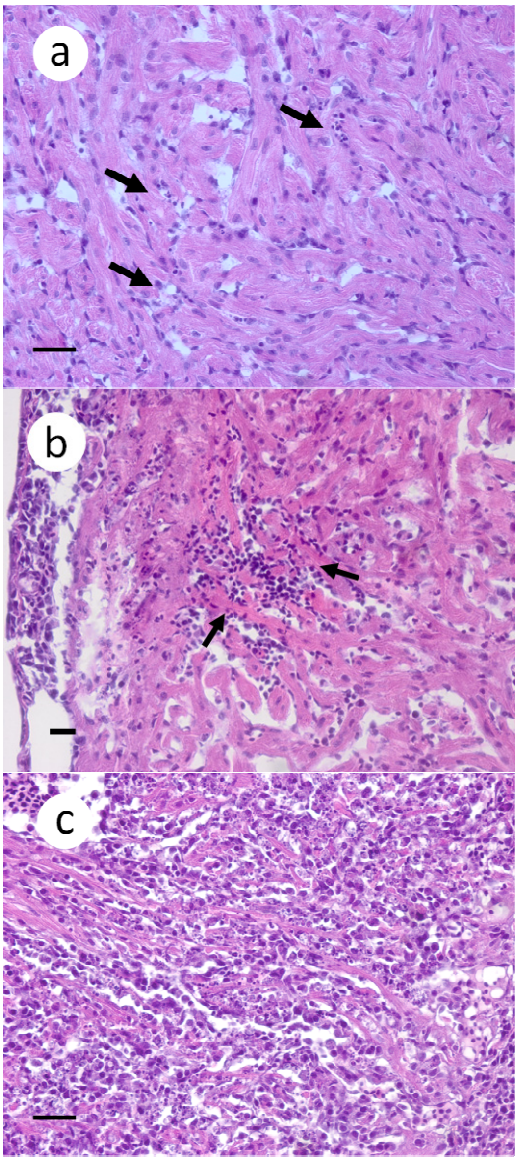

Figure 5-4 Heart tissues collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at varying timepoints. (a) Foci of dead and dying cardiac myofibers (black arrows) interspersed throughout unaffected tissue at 6 days post infection (d p.i.), (b) Focal mononuclear infiltration with myocardial degeneration and necrosis (arrows) at 28 d p.i., (c) Multifocal necrosis and degeneration of myofibers and severe inflammation at 43 d p.i., Tissues stained with hematoxylin and eosin (scale bar, a-c = 44µm)

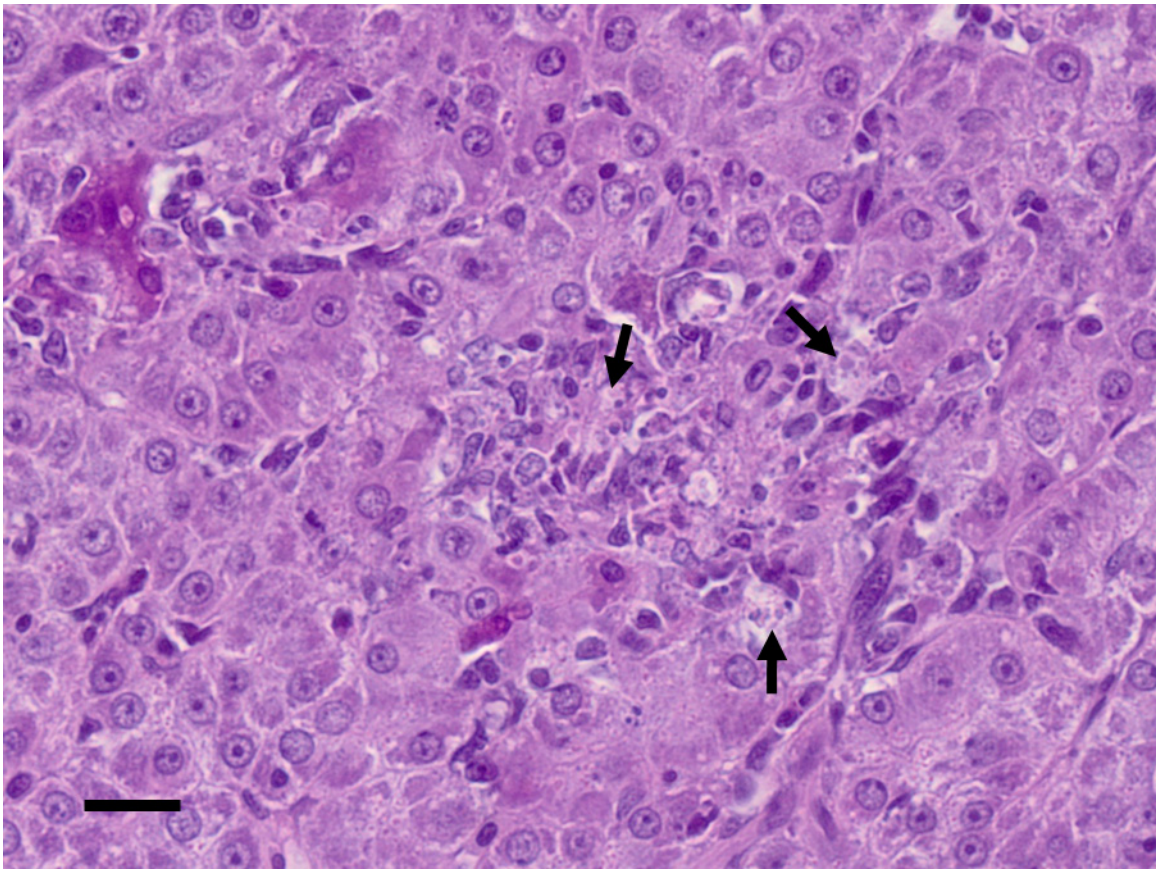

Figure 5-5 Liver collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at 15 days post infection. Focal hepatocellular necrosis (arrows) was noted throughout liver sectin. Tissue is stained with hematoxylin and eosin. (scale bar = 22μm)

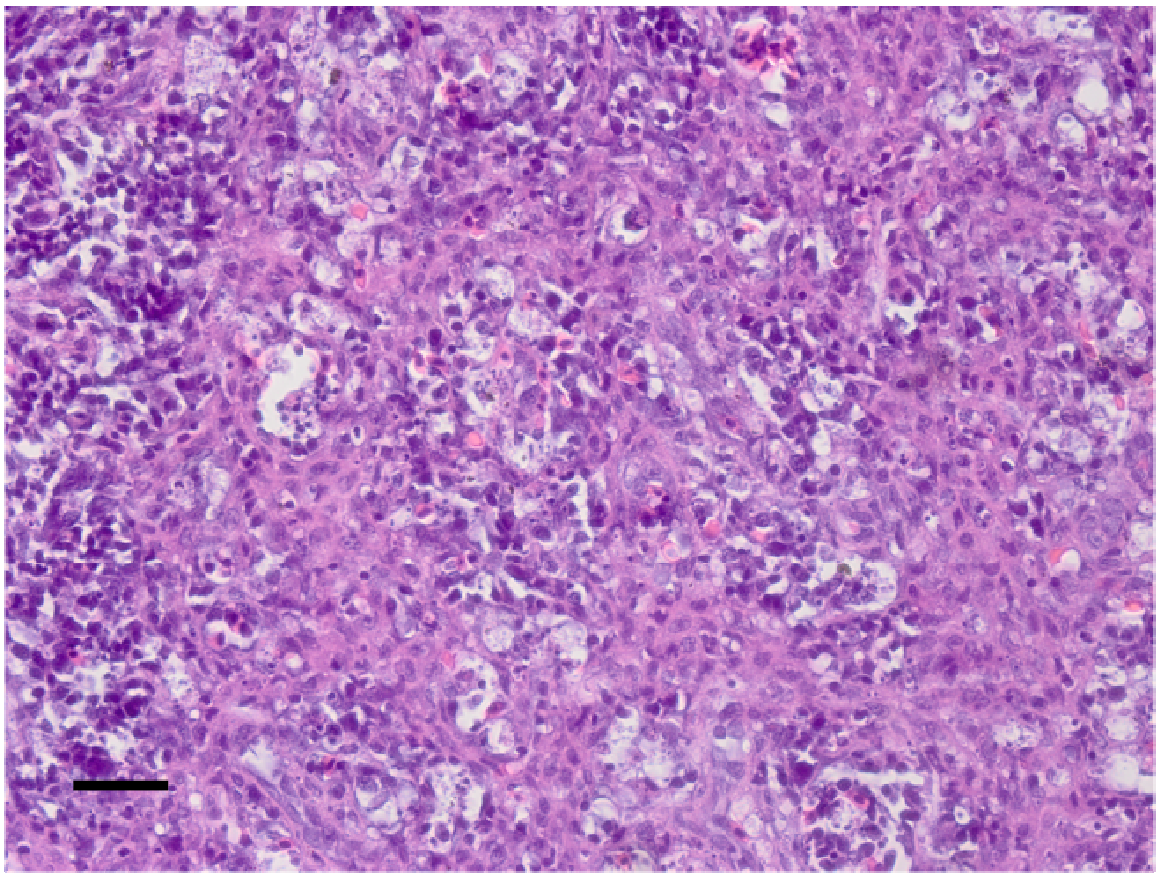

Figure 5-6 Spleen collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at 14 days post infection. Severe lymphoid depletion and lack of erythrocytes. Tissue is stained with hematoxylin and eosin. (scale bar = 44μm)

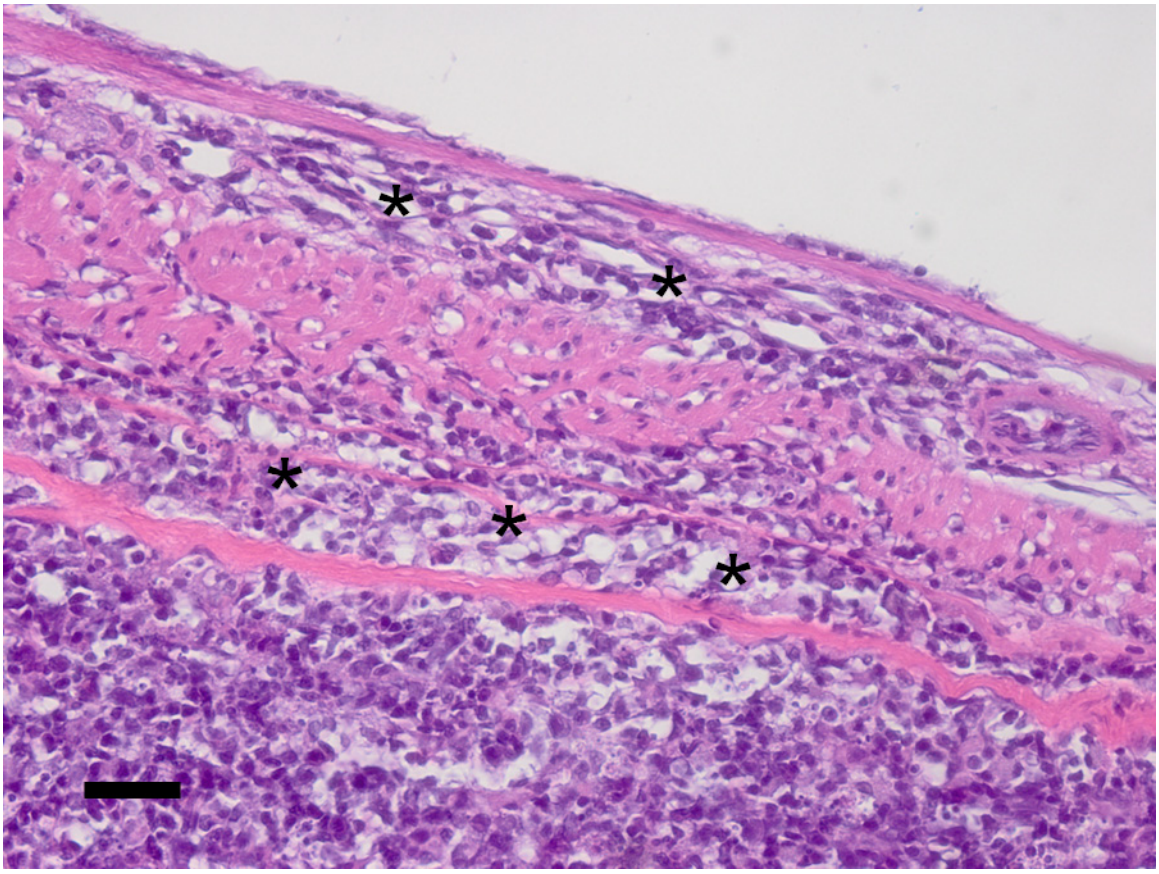

Figure 5-7 Posterior intestine collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at 14 days post infection. Subserosal and submucosal lymphoplasmacytic infiltration (asterisks). Tissue is stained with hematoxylin and eosin. (scale bar = 44μm)

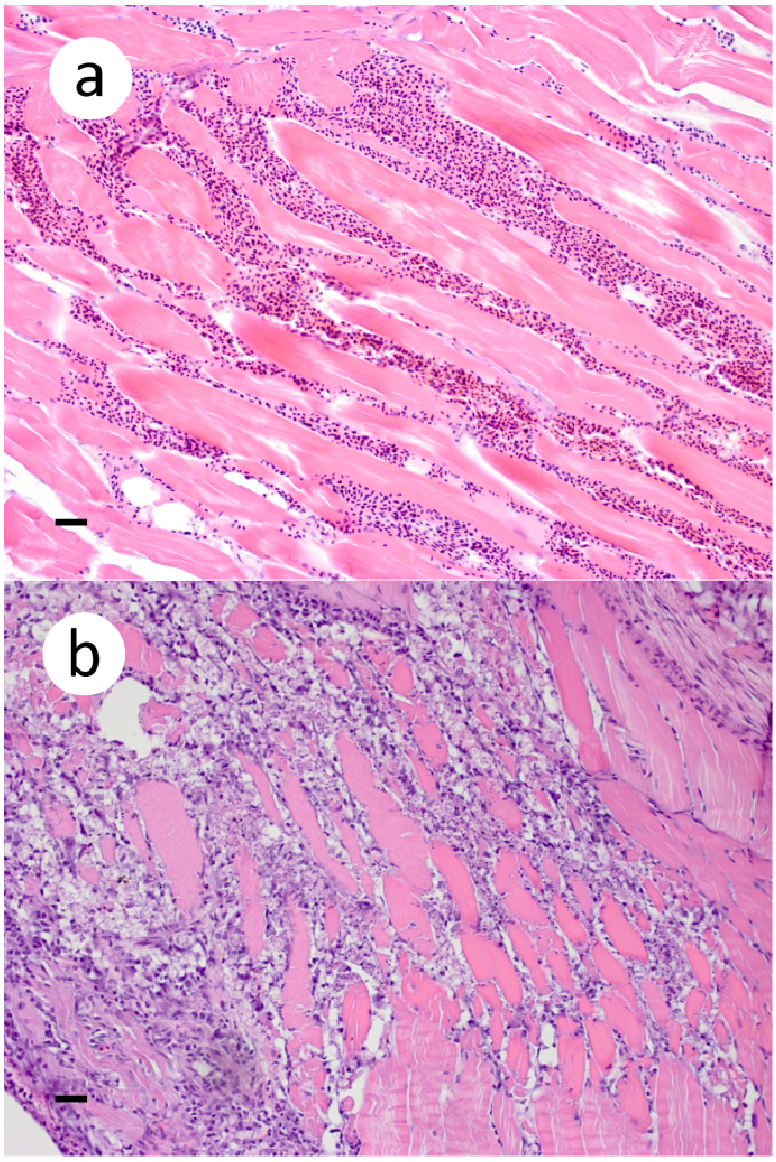

Figure 5-8 Muscle collected from juvenile muskellunge experimentally infected with viral hemorrhagic septicemia virus IVb at various timepoints.
(a) severe intramuscular hemorrhage at 14 days post infection (d p.i.) and (b) severe myofibers degeneration and necrosis surrounded by inflammation at 43 d p.i. Tissue is stained with hematoxylin and eosin. (scale bar = 44µm)

Appendix

Table 5-2 Summary of histopathologic changes at all timepoints.

Tissue		0h - 4d	6d	8d	15d	22d
Spleen	**# of fish**	0/4	0/4	0/4	1/4	0/4
	Description	n/a	n/a	n/a	lack of erythrocytes	n/a
Kidney	**# of fish**	0/4	0/4	0/4	0/4	0/4
	Description	n/a	n/a	n/a	n/a	n/a
Heart	**# of fish**	0/4	1/4	0/4	0/4	0/4
	Description	n/a	mild mononuclear infiltrate, basophilic stippling, mild myocardial degeneration and mineralization	n/a	n/a	n/a
Liver	**# of fish**	0/4		0/4	1/4	1/4
	Description	n/a	moderate hepatocellular vacuolation, mild peribiliary mononuclear accumulation	n/a	mild to moderate hepatocellular necrosis	mild plasmacytic infiltrate

Table 5-2 (cont'd 1) Summary of histopathologic changes at all timepoints.

Tissue		29d	36d	43d
Spleen	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a
Kidney	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a
Heart	**# of fish**		1/4	
	Description	mild-moderate perivascular inflammation	one small foci of myocardial degeneration and necrosis, combined with mild inflammatory changes	moderate to severe inflammation and degeneration of cardiomyocytes
Liver	**# of fish**		0/4	
	Description	moderate inflammation	n/a	mild lymphoproliferative infiltrate and necrotizing hepatitis

Table 5-2 (cont'd 2) Summary of histopathologic changes at all timepoints.

Tissue		50d	57d	64d
Spleen	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a
Kidney	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a
Heart	**# of fish**	1/4	1/4	1/4
	Description	one small foci of myocardial degeneration and necrosis, combined with mild inflammatory changes	one small foci of myocardial degeneration and necrosis, combined with mild inflammatory changes	severe myocardial necrosis and lymphoplasmacytic and granulocyte infiltration
Liver	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a

Table 5-2 (cont'd 3) Summary of histopathologic changes at all timepoints.

Tissue		0h - 4d	6d	8d	15d	22d
Intestine	**# of fish**	0/4	1/4	0/4	1/4	1/4
	Description	n/a	mild endothelial hyperplasia, traces mononuclear cells perivascularly	n/a	moderate subserosal and submucosal inflammation	mild subserosal inflammation
Gill	**# of fish**	0/4	2/4	0/4	2/4	1/4
	Description	n/a	mild mononuclear expansion at the base of primary lamellae, mild atrophic and proliferative branchitis	n/a	mild branchitis and epithelial hyperplasia	mild branchitis and epithelial hyperplasia
Pectoral fin	**# of fish**	0/4	0/4	0/4	0/4	1/4
	Description	n/a	n/a	n/a	n/a	mild-moderate multifocal epithelial necrosis
Skin / Muscle	**# of fish**	0/4	1/4	0/4	1/4	0/4
	Description	n/a	moderate-severe intramuscular hemorrhage and mild degeneration of myotomes	n/a	moderate-severe intramuscular hemorrhage and mild degeneration of myotomes	n/a

Table 5-2 (cont'd 4) Summary of histopathologic changes at all timepoints.

Tissue		29d	36d	43d
Intestine	**# of fish**	1/4	0/4	
	Description	mild subendothelilal hyperplasia, mild mononuclear infiltrate around vessels in the mesentery	n/a	mild degeneration and necrosis of smooth muscle and endothelial hyperplasia, severe inflammation of throughout mesentery
Gill	**# of fish**	1/4	0/4	
	Description	mild proliferative branchitis and atrophy of secondary lamellae	n/a	mild-moderate branchial atrophy and hyperplasia at the base of primary lamellae
Pectoral fin	**# of fish**	1/4	0/4	0/4
	Description	mild-moderate multifocal epithelial necrosis	n/a	n/a
Skin / Muscle	**# of fish**		0/4	0/4
	Description	moderate muscle degeneration and hypereosinophilia, swollen myofibers, moderate necrosis and inflammatory response	n/a	n/a

Table 5-2 (cont'd 5) Summary of histopathologic changes at all timepoints.

Tissue		50d	57d	64d
Intestine	**# of fish**	0/4	0/4	1/4
	Description	n/a	n/a	moderate focal infiltration of inflammatory cells within smooth muscle later
Gill	**# of fish**	0/4	0/4	1/4
	Description	n/a	n/a	mild proliferative branchitis and atrophy of secondary lamellae
Pectoral fin	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a
Skin / Muscle	**# of fish**	0/4	0/4	0/4
	Description	n/a	n/a	n/a

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CHAPTER 6

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Abstract

Our previous studies demonstrated that the Great Lakes strain of Viral hemorrhagic septicemia virus (VHSV) can induce a variable disease course depending on the species and dose of exposure. Herein, we report that juvenile muskellunge that survived experimental VHSV infection by immersion continue to shed the virus for extended periods of time post-infection (p.i.), despite being originally infected with a relatively low dose. Juvenile muskellunge were exposed to VHSV by immersion challenge at a dose of 1,400 plaque forming units (PFU) mL^{-1} and viral shedding extended for up to 15 weeks p.i. with the highest levels of shedding occurring between weeks 1 and 5 p.i. We estimated that each juvenile muskellunge can shed upwards of 1.36×10^5 PFU $fish^{-1}$ $hour^{-1}$ after initial exposure. Approximately 76% of the muskellunge survived low dose exposure and viral shedding could be detected between 5 and 14 weeks p.i. Surviving muskellunge were re-infected at week 22 p.i. with VHSV by immersion in water containing virus concentrations ranging from 1 to 10^6 PFU mL^{-1} to determine a median lethal dose of infection (LD_{50}). Viral shedding was detected in all re-exposed fish, including mock rechallenged controls. Rates of viral shedding were substantially higher in the first 5 weeks after reinfection but could not be predicted based on the dose at which the fish were rechallenged. The

highest rate of shedding measured was approximately 4.6×10^6 PFU $fish^{-1}$ $hour^{-1}$. The calculated LD_{50} was approximately 1.34×10^5 PFU mL^{-1} at 28 days p.i. for muskellunge previously exposed to VHSV. Shedding also appeared to increase in the first five weeks following reinfection and ceased between 35 and 37 weeks p.i. to the end of the study. When viral shedding was assessed at 60 weeks p.i. in muskellunge surviving double exposures to VHSV IVb, water samples were negative by plaque assay and RT-PCR. The results of this study shed new light into the dynamics of VHSV shedding in carrier hosts and offer insights into how management and regulatory agencies should proceed in minimizing the spread of VHSV in endemic regions.

Introduction

The recent emergence of *Viral hemorrhagic septicemia virus* genotype IVb (VHSV IVb) in the Laurentian Great Lakes poses one of the greatest challenges to fisheries managers in the region (Elsayed, Faisal, Thomas, Whelan, Batts & Winton 2006). This World Organization for Animal Health (OIE) - reportable virus, capable of causing widespread mortality events in a number of important fish species, has spread to all five North American Great Lakes as well as several landlocked water bodies. Compounding the dilemma has been the isolation of virus from macroinvertebrates in infected waterbodies (Faisal & Schulz 2009; Faisal & Winters 2010) indicating that VHSV-IVb has been broadly established in the Great Lakes ecosystem. As a result, there exists a dire need to better understand the biological properties of this particular VHSV strain and decipher its interactions with susceptible hosts. Previous experimental infection studies in our laboratory have demonstrated that the muskellunge, *Esox masquinongy* (Mitchill) is highly susceptible to VHSV IVb (Kim & Faisal 2010c), yet can survive exposure to low virus concentrations. This has raised questions regarding the fate of VHSV-IVb in survived muskellunge and whether the fish can clear their tissues of virus or continue to shed infectious virus into the surrounding environment thereby facilitating virus transmission to other susceptible hosts,

Present information on VHSV shedding stems from the pioneering studies of Neukirch & Glass 1984 and Neukirch 1985, in which farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) were experimentally infected with a genotype I strain of VHSV (F1). These studies demonstrated that VHSV exists at high titers

in urine samples collected from indwelling catheters. In the same context, the studies of Kocan, Bradley, Elder, Meyers, Batts & Winton (1997) and Hershberger, Gregg, Grady, Collins, & Winton (2010) demonstrated that juvenile Pacific herring, *Clupea pallasii* (Valenciennes) that survived VHSV-IVa experimental infections shed the virus into the surrounding water and may infect their cohorts. Since the pathogenicity and disease course varies between viral strains (Skall, Slierendrecht, King & Olesen 2004) and infected hosts (Kim & Faisal 2010a,b), a study was designed to characterize the shedding dynamics and patterns of a classically infected host, the muskellunge.

Benefits in characterizing the carrier state and kinetics of viral shedding in VHSV IVb infected juvenile muskellunge are twofold. Firstly, quantifying the amount and duration of viral shedding will equip fisheries managers with the information necessary to develop control strategies and regulatory guidelines to reduce further spread of the virus in a highly valued and aquaculture reared gamefish species. Secondly, the question of whether previous exposure to VHSV infection would increase survival in reinfection may provide clues into why variable and sporadic mass mortality episodes in the field have been reported.

Materials and Methods

Cell culture and virus

The Great Lakes VHSV IVb index strain MI03, originally isolated in our laboratory in 2003 from muskellunge (Elsayed et al. 2006), was used throughout. The virus was propagated in the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & de Kinkelin 1983). Virus concentration was determined by the plaque assay on EPC cells treated with polyethylene glycol and using a methylcellulose overlay as described in Batts & Winton (19890 and Batts, Traxler & Winton (1991). Virus stocks were aliquoted in cryogenic vials (Corning) and kept at -80 $^{\circ}$C until used. Ten fold serial dilutions were applied to the virus stock beginning with 1:100 to 1:10^{12}. Plaques were allowed to form for a period of six days, and the remaining cells of each well were fixed and stained with a crystal violet (0.5% w/v) and formaldehyde (1:1 dilution with water) solution. The virus stock was calculated to have approximately 7.32×10^{8} plaque forming units (PFU) mL^{-1}. Virus was titrated by inoculation onto 24-well cell culture plates. Cell lines were maintained and subcultured in 150 cm^{2} tissue culture flasks (Corning, Lowell, MA) at 25 $^{\circ}$C using a growth medium formulation that consisted of Earle's salt-based Minimal Essential Medium (MEM, Invitrogen) supplemented with 29.2 mg mL^{-1} L-glutamine (Invitrogen), Penicillin (100 IU mL^{-1}) and Streptomycin (0.1 mg mL^{-1};

Invitrogen), 10% fetal bovine serum (Hyclone), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich).

Fish and Maintenance

Certified VHSV-free juvenile muskellunge (4 months post hatch) were obtained from the Rathbun National Fish Hatchery in August of 2009. Prior to infection, five fish were euthanatized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories) (25 mg mL^{-1}) and tested for the presence of VHSV. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen) supplemented with 12 mM tris buffer (Sigma), Penicillin (100 IU mL^{-1}), Streptomycin (100 µg mL^{-1}) (Invitrogen), and Amphotericin B (250 µg mL^{-1}) (Invitrogen) to produce 1:4 dilution (w/v) of original tissues. Samples were centrifuged at 2000 x *g* and the supernatant was inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Plates were incubated at 15 $^{\circ}C$ for 7 d, and observed for the formation of cytopathic effects (CPE). A second blind passage was performed and assessed for the presence of VHSV.

Fish were acclimated to temperatures from 16 $^{\circ}C$ to 11 $^{\circ}C$ over a three week period before the experiment was initiated. All fish were initially held in a 1,900 L

circular fiberglass tanks in a continuous flow-through system supplied by oxygenated well water at Michigan State University - Research Containment Facility. The fish were fed *ad lib* with 2.0 mm sinking feed (Silver Cup, Nelson and Sons) and then transitioned over a 2 week period to VHSV-free certified fathead minnows, *Pimephales promelas (*Rafinesque) purchased from Robinson Wholesale, Inc (Genoa City, WI).

Immersion challenge

A group of 234 fish (weight 12.8 ± 3.1 g; fork length 14.9 ± 1.2 cm) were experimentally infected with the MI03 strain of VHSV-IVb via immersion at a dose of 1.4×10^{3} PFU mL^{-1}. This dose gave <25% mortality in juvenile muskellunge when challenged by immersion with MI03 in an earlier study (Kim & Faisal 2010c). An additional group of 52 fish was immersed in water mixed with 1 mL of sterile maintenance-MEM and was considered the negative control group. The virus challenge and negative control tanks were divided equally so that each tank contained 26 fish. All fish were held in 74 L polyethylene tank (Aquatic Eco-Systems) in continuous flow through system at a water temperature of 11 ± 1 $^{\circ}C$ and were monitored every 8-12 hours for mortality.

Assessment of viral shedding

Every 7 days post infection (d p.i.), all of the fish from three of the nine experimental tanks and one of the negative control tanks were removed and transferred to corresponding glass aquaria containing aerated static water from

the same source at 500 mL fish^{-1}. The fish remained in the water for a period of 90 min and were then transferred back to their respective tanks. Approximately 50 mL of water was collected from each of the tanks. An additional 500 µL of a 1:1 mixture of Streptomycin (100 µg mL^{-1}) (Invitrogen) and Amphotericin B (250 µg mL^{-1}) was added to each water sample and subsequently centrifuged at 2000 x *g* for 10 minutes. After centrifugation, a viral plaque assay was performed as previously described on water samples.

VHSV reisolation from water samples and confirmation of isolates

The water samples were tested for the presence of VHSV using EPC cell line. For Polymerase chain reaction (PCR) confirmation, total RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Reverse transcription was accomplished by a two step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR^{TM} (Stratagene) following the manufacturer's instructions. The primer set used in this assay is recommended by the World Organization for Animal Health (OIE; Manual of Diagnostic Tests for Aquatic Animals 2003) for the amplification of a 811 base pair sequence of the VHSV nucleocapsid gene: 5'-GGG-GAC-CCC-AGA-CTG-T-3' (forward primer) and 5'-TCT-CTG-TCA-CCT-TGA-TCC-3' (reverse primer). Reaction mixture contained 5 µL of viral RNA, 50 pmol of each primer, 25 µL of GoTaq® Green Mastermix DNA Polymerase (Promega), and the DNA-ase free water to create a final volume of 50 µL in each tube. The reverse

transcriptase was inactivated by subjecting the mixture to 94 °C for 2 min, and 30 cycles of PCR (denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and polymerization at 68 °C for 1 min) in a Mastercycler Personal Thermal Cycler (Eppendorf). The polymerization was finalized by maintaining the mixture for a period of 7 min at 68 °C. The amplicons were visualized by gel electrophoresis in 1.5% agarose gels.

Rechallenge of Surviving Fish

Fish that survived the first VHSV immersion challenge and ceased virus shedding for three consecutive weeks were pooled together and maintained for additional three weeks. For a second immersion rechallenge with MI03-VHSV-IVb, fish were divided in groups of 13 fish and exposed to virus concentrations that ranged from 1×10^6 PFU mL^{-1} (7.7×10^5 $TCID_{50}$) to 10 PFU mL^{-1} (7.7 $TCID_{50}$). Virus stocks were diluted and added accordingly to a glass aquarium containing 10 L of water to achieve the aforementioned doses at which time the fish were immersed for 1.5 h. A negative control immersion challenge was also conducted using an additional group of 13 fish that were kept in water mixed with 1 mL of maintenance-MEM. Unfortunately, a parallel virus exposure to naïve fish could not be performed due to the death of this group following an unexpected failure of the oxygenation pump supplying their tank. Dead or moribund fish were collected every 8-12 hours. Fish were maintained for an additional 19 weeks

under the conditions detailed above. The median lethal dose of VHSV in muskellunge that survived the first experimental infection was calculated at 28 d p.i. and the end of the observation period by the method of Reed and Muench (1938). Virus reisolation and confirmation and the viral plaque assay were completed every 7 d p.i. as described above.

Virus shedding following reinfection

Fish that survived the second virus challenge (22 muskellunge) were pooled and maintained in a 200 L circular fiber glass tank and maintained at the parameters described previously for an additional 60 weeks during which they became one year old (weight 128.18 ± 36.27 g; total length 29.31 ± 3.09 cm). The fish were then subjected to handling stress as reviewed by Bonga (1997) and Barton & Iwama (1991). Briefly, the fish were netted three consecutive times, then maintained in their respective tank. At 48 and 96 hr post stress VHSV shedding was evaluated. Stress was induced by a combination of handling (i.e. netting) and increased density by being placed in an aquarium at approximately 1.5 L water $fish^{-1}$ for 2 hr. The fish were placed into glass aquaria containing aerated static water from the same source at 1.5 L $fish^{-1}$. The fish were remained in the water for a period of 120 min and were then transferred back to their tank. Approximately 50 mL of water was collected from each tank for quantification by the viral plaque assay and confirmation by cell culture and RT-PCR. At weekly intervals, handling stress and shedding assessment were repeated twice.

Results

When juvenile muskellunge were exposed to VHSV IVb by immersion at 1,400 PFU mL^{-1}, approximately 76% of the fish survived (Figure 1). The first mortality occurred as early as day 5 p.i., and an increased number of mortalities were observed between days 5 and 20 p.i. The number of mortalities decreased beginning at day 21 p.i. through the end of the observation period (119 d p.i.) reaching a cumulative mortality of 23% (Figure 1). Dead and moribund fish exhibited external and internal lesions indicative of VHSV infection such as external hemorrhages on the nuchal area (Figure 2a) and sides of the body (Figure 2b). The gills were pale (Figure 2c). Internally, infected fish exhibited ecchymotic to petechial hemorrhages in the visceral organs (Figure 2d) and musculature (Figure 2e). Kidney and spleen tissues from dead and moribund fish were collected and confirmed VHSV positive by cell culture and RT-PCR. In the negative control group, the cumulative mortality did not exceed 3.8% (Figure 1), exhibited no abnormal internal or external signs of disease, and were VHSV negative by both cell culture isolation and RT-PCR.

As displayed in Figure 3, detection of VHSV in water varied greatly from among the fish groups and sampling events. Shedding rates observed in the three tanks of the first week p.i. ranged from 2.22×10^3 to 9.56×10^4 PFU $fish^{-1}$ $hour^{-1}$. By the second week, the viral shedding was low or undetectable with only one of the three tanks shedding at a rate of 1.11×10^3 PFU $fish^{-1}$ $hour^{-1}$. The highest rate

of shedding during the observation period occurred in one fish group at 3 weeks p.i. (1.36×10^5 PFU fish^{-1} hour^{-1}). Subsequent shedding rates observed in weeks 4 and 5 p.i. remained elevated in all groups, at which time the shedding rate steadily decreased until week 14 p.i. Viral shedding could not be detected from week 15 through week 18 (Figure 3). Throughout the 18 weeks p.i., shedding rates fluctuated within and among the nine fish groups, but often remained above the initial exposure dose (Figure 3). Overall, shedding appeared to increase in the first 5 weeks p.i., which is followed by fluctuations until 15 weeks p.i. at which time shedding appeared to cease.

When the VHSV-survived muskellunge were re-exposed to varying doses of VHSV, virus shedding resumed by all fish groups including the fish group that were not re-exposed to VHSV. Levels of viral shedding were substantially higher following re-exposure when compared to the initial challenge (Table 1). There was, however, obvious variability in rates of VHSV shedding and cumulative mortalities that did not correspond to the dose of re-exposure (Table 1, Figure 4). Interestingly, the highest rate of shedding of any of the doses was in the group of fish exposed at 1 PFU mL^{-1}, which was approximately 4.6×10^6 PFU fish^{-1} hour^{-1} at 2 weeks p.i. The highest shedding rates of all the infected groups occurred between weeks 1 and 5 p.i. (Table 1), with the exception of fish exposed to VHSV IVb at 1×10^4 PFU mL^{-1} which had a second peak of viral shedding at 10

weeks p.i. (Table 1). Fish continued to shed virus for as long as 14 weeks p.i. (Table 1) and as short as 4 weeks p.i. (Table 1).

Further analysis suggested the absence of any correlation between the dose of re-exposure and cumulative mortality. For example fish re-challenged with 10^6 PFU mL^{-1} had less cumulative mortality than fish re-exposed to 1 PFU mL^{-1} (Figure 4). By 40 d p.i. fish from the lowest dose (1 PFU mL^{-1}) reached 100% mortality. Other doses that resulted in 100% mortality during the experiment were 1×10^5 PFU mL^{-1}, 1×10^4 PFU mL^{-1}, and 100 PFU mL^{-1}, (Figure 4). While there were no acute mortality episodes noted in the challenged groups, fish infected at 1 and 100 PFU mL^{-1} exhibited an increased number of mortalities from 14 to 50 d p.i. (Figure 4). Also, the mock re-challenge group had approximately 42.9% of the fish die during the study with most of the mortalities occurring between 28 and 35 d p.i. (Figure 4)

At 28 days p.i., the LD_{50} in surviving, re-exposed muskellunge was calculated to be 1.34×10^5 PFU mL^{-1}. This value significantly decreased to 5.25 PFU mL^{-1} by the end of the 19 week observation period. All mortalities were the result of VHSV IVb, since the virus was reisolated and confirmed in homogenates of kidney and spleen tissues collected post-mortem.

The 22 fish that survived two virus exposures were assessed for viral shedding following each of three cycles of handling stress at 60, 61, and 62 weeks post re-exposure. No VHSV shedding was detected in these fish by the viral plaque assay or cell culture followed by nested PCR on RNA extracted from inoculated EPC cells.

Discussion

This study provided evidence that infected muskellunge shed the virus into surrounding water, at relatively high titers and for an extended period of time. The importance of this finding is multifold. Given that muskellunge are a VHSV-IVb highly susceptible fish species (Kim & Faisal 2010c), survivors of infections may contribute to spreading the virus. Secondly, it indicates that VHSV has been amplified within a highly susceptible host to the extent that the amount shed in one hour may exceed the original exposure dose, thereby increasing the virus load in the surrounding water to levels that can initiate infection in less susceptible fish species such as salmonids and percids (Kim & Faisal 2010b,c) inhabiting the same watershed. Lastly, the relatively long viral shedding period should be considered by fishery managers when implementing biosecurity measures and factored into the design of live fish transport and quarantine protocols.

It appears that muskellunge were able to shed VHSV for an extended duration, which lasted for 105 days. When Hershberger et al. (2010) followed the shedding of VHSV IVa in the highly susceptible Pacific herring, they failed to detect the virus in water after 20 d p.i. In the same context, Neukirch and Glass (1984) followed the shedding of VHSV genotype I strain (F1) in its susceptible host, the rainbow trout, and were able to detect VHSV in the urine of infected fish for an upwards of 40 d p.i. The relatively high-titered and long VHSV shedding by muskellunge, compared to Pacific herring, requires additional investigation. These observed differences in the duration of viral shedding support the idea that

the disease course is highly variable between species (Kim & Faisal 2010b; Skall et al. 2004) but should be assessed in light of other factors including virus genotype, sublineage (or strain), age of the host, and the prevailing environmental conditions, water temperature in particular. It was clear, however, that the disease course did vary among individual fish exposed to the same virus dose, as some died within the first week and exhibited major pathology, while others were apparently healthy and survived the infection.

The fact that VHSV is shed into the water from infected fish is not surprising, given that cohabitation has been used in experimental infection studies (de Kinkelin & Castric 1982; Snow, Cunningham & Bricknell 2000; Bowden 2003; Muroga, Iida, Mori, Nishizawa & Arimoto 2004; López-Vázquez, Dopazo, Barja & Bandín 2007). However, the specific tissue origin(s) from which the virus particles are released into the environment remains a subject of discussion. The early studies of Neukirch & Glass (1984) strongly suggested that the kidney and urinary tract, but not feces, were major routes by which VHSV is shed, when virus was reisolated from urine collected by indwelling catheters. The idea that the kidneys may be a major source of virus is corroborated by the studies of Brudeseth, Raynard, King, & Evensen (2005) and Al-Hussinee, Huber, Russell, LePage, Reid, Young, Nagy, Stevenson, & Lumsden (2010), when histologic sections visualized by immunohistochemistry (IHC) revealed the presence of virus in the interstitium and tubular epithelia of kidneys. Other tissues in which VHSV particles have been identified by IHC include the gills (Al-Hussinee et al. 2010), while others surmise that the post mortem degradation of

VHSV infected fish tissues may also be a source (Wolf 1988). As a result, a multitude of tissues are likely involved in the amplification and shedding of VHSV. Although infected fish may be primary contributors in spreading VHSV in the ecosystem, other aquatic organisms can also play a role in the spread of VHSV such as piscicolid leeches (Faisal & Schulz 2009) and *Diporeia* sp. (Faisal & Winters 2010).

Repeated exposure of muskellunge to VHSV IVb generated interesting observations related to VHSV shedding dynamics and potential protection conferred after surviving an initial infection. When VHSV shedding rates of individual tanks were evaluated in light of the re-exposure doses, there were no observable correlations, a finding that underscores the high variability among individual fish in their interactions with VHSV. Despite the increased mortalities noted in fish infected at lower doses, higher dose infected fish either survived or succumbed to mortality albeit at lower rates. This information in combination with an increased LD_{50} value (1.34×10^5 PFU mL^{-1}), suggest that protection can occur due to previous exposure and may lessen the severity or time to death in subsequent exposures to VHSV. Conceivably, the increased LD_{50} seen in the rechallenged group at 28 days, compared to the LD_{50} performed on naive muskellunge obtained from the same source (Kim & Faisal 2010c) may imply increased protection in VHSV survivors due to an adaptive immune response. Because of the age and size difference in the two fish groups, one cannot claim that surviving a VHSV infection may confer full protection against reinfection. On

the other hand, protection following an exposure to VHSV, was demonstrated in a study by Hershberger, Gregg, Pacheco, Winton, Richard, & Traxler (2007) in which larval Pacific herring, previously exposed to VHSV IVa, exhibited increased survival following a second exposure as juveniles when compared to naïve groups of fish. The increased survival of two highly susceptible, yet biologically dissimilar, species provides relevance to events that may be occurring under field conditions. Given that single exposure to VHSV in the wild populations cannot be circumvented, especially in VHSV endemic waters, wild fish may become less apt to succumb to infection following subsequent exposure. On the other hand, since our studies prove that re-exposure can reinitiate episodes of viral shedding at higher quantities, infected survivors may be a critical element to sustaining VHSV endemicity.

The magnification of viral shedding rates after rechallenge introduces new information into the persistence of VHSV infected fish. Previous studies using other VHSV genotypes demonstrate that VHSV can persist in fish tissues for up to 421 d p.i. (Neukirch 1986), without leading to clinical signs or mortality. The detection of shedding in the rechallenge group not exposed to VHSV (Table 1), suggest that a stressful stimulus, such as handling, may be sufficient to reignite viral shedding. However when survivors were not subjected to stress for an extended period of time, a total cessation of shedding may occur. How these observations relate to VHSV ecology in wild fish stocks requires further investigation into the pathophysiologic mechanisms that influence the production and release of virus.

The information presented in our studies not only confirms that VHSV is shed in juvenile muskellunge, but provides novel information into the overall course of shedding of this emerging VHSV strain. Not only are juvenile muskellunge highly susceptible to VHSV infection, but may play an important role in the amplification and subsequent spread of the virus to other more resistant fish species. More importantly, the study demonstrates that viral shedding is a complex event and should be considered a major factor in the expansion and spread of VHSV throughout the Great Lakes basin. The prolonged course of shedding and the ability to reinitiate shedding following a stressful event or re-exposure imposes new challenges for hatchery managers and aquaculturists in eliminating the persistence of virus in production facilities. Alternatively, these studies provide promising data that fish have the capacity to survive infection. However, this could be due to a number of factors such as the full development of host defense mechanisms and/or that more repeated exposure is needed to achieve full protection, a matter that should be considered in vaccination strategies. In the end, the findings of this study highlight the importance of regulatory guidelines to restrict the movement of fish and water from VHSV endemic areas.

Table 6-1 Shedding rates of juvenile muskellunge (*Esox masquinongy*) re-exposed to viral hemorrhagic septicemia virus IVb (MI03) at doses ranging from 1 to 10^6 plaque forming units mL^{-1} and rechallenge using sterile media. Shaded boxes indicate week(s) in which viral shedding was highest. Shaded areas indicate that all fish in that group died.

Week Post Challenge	VHSV Shedding Rates (pfu $fish^{-1}$ $hour^{-1}$) Exposure Dose							
	1×10^6 pfu mL^{-1}	1×10^5 pfu mL^{-1}	1×10^4 pfu mL^{-1}	1×10^3 pfu mL^{-1}	100 pfu mL^{-1}	10 pfu mL^{-1}	1 pfu mL^{-1}	Sterile Media
1	6.9×10^5	7.3×10^5	9.8×10^4	2.6×10^4	3.3×10^2	7.3×10^3	6.3×10^5	4.6×10^4
2	8.7×10^4	3.0×10^5	1.5×10^6	7.8×10^4	5.6×10^3	1.1×10^5	4.6×10^6	2.6×10^5
3	1.7×10^6	1.8×10^5	2.9×10^6	5.3×10^4	0	5.0×10^3	1.5×10^6	4.9×10^5
4	1.2×10^6	7.1×10^4	5.9×10^5	7.8×10^5	3.5×10^4	2.0×10^6	3.3×10^3	1.9×10^6
5	2.1×10^5	1.6×10^5	3.7×10^5	1.5×10^6	0	3.1×10^3	2.2×10^5	1.4×10^4
6	1.4×10^5	1.9×10^4	2.1×10^5	8.5×10^4	0	3.9×10^3		2.1×10^4
7	5.6×10^5	5.6×10^4	2.1×10^5	1.5×10^4	0	1.0×10^4		1.5×10^4
8	1.5×10^5	1.4×10^5	2.7×10^5	2.8×10^5	0	0		2.8×10^2
9	2.7×10^5	1.8×10^5	3.6×10^5	1.3×10^4		0		2.8×10^3
10	1.1×10^5	3.2×10^4	1.4×10^6	8.9×10^3		0		7.9×10^3

Table 6-1 (cont'd) Shedding rates of juvenile muskellunge (*Esox masquinongy*) re-exposed to viral hemorrhagic septicemia virus IVb (MI03) at doses ranging from 1 to 10^6 plaque forming units mL^{-1} and rechallenge using sterile media. Shaded boxes indicate week(s) in which viral shedding was highest. Shaded areas indicate that all fish in that group died.

	VHSV Shedding Rates (pfu $fish^{-1}$ $hour^{-1}$)							
	Exposure Dose							
Week Post Challenge	**1×10^6 pfu mL^{-1}**	**1×10^5 pfu mL^{-1}**	**1×10^4 pfu mL^{-1}**	**1×10^3 pfu mL^{-1}**	**100 pfu mL^{-1}**	**10 pfu mL^{-1}**	**1 pfu mL^{-1}**	**Sterile Media**
11	1.2×10^5	2.1×10^4	4.7×10^4	5.0×10^3		0		0
12	2.8×10^4	2.1×10^4	2.2×10^5	2.6×10^4		0		6.4×10^3
13	3.0×10^4	0	8.3×10^2	8.3×10^2		0		0
14	1.9×10^3		5.6×10^2	0		0		0
15	0		0	0		0		0
16	0		0	0		0		0
17	0		0	0		0		0
18	0		0	0		0		0
19	0		N/A[a]	0		0		0

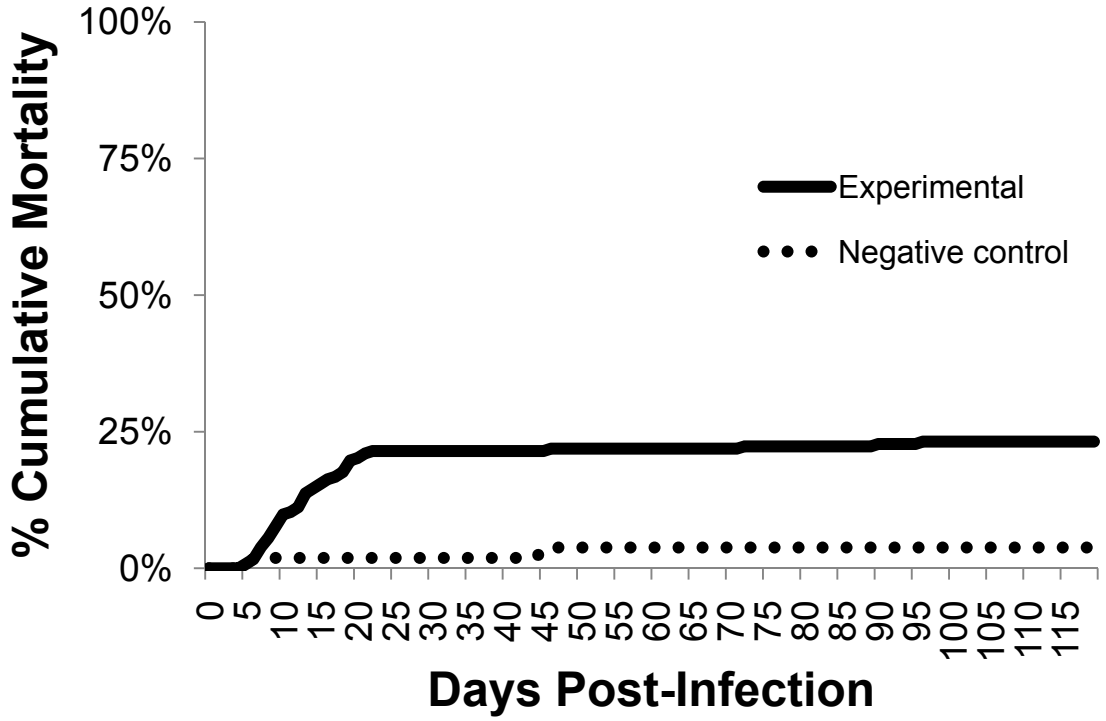

Figure 6-1 Cumulative mortality of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) by immersion in water containing 1.4×10^{3} PFU mL^{-1}.

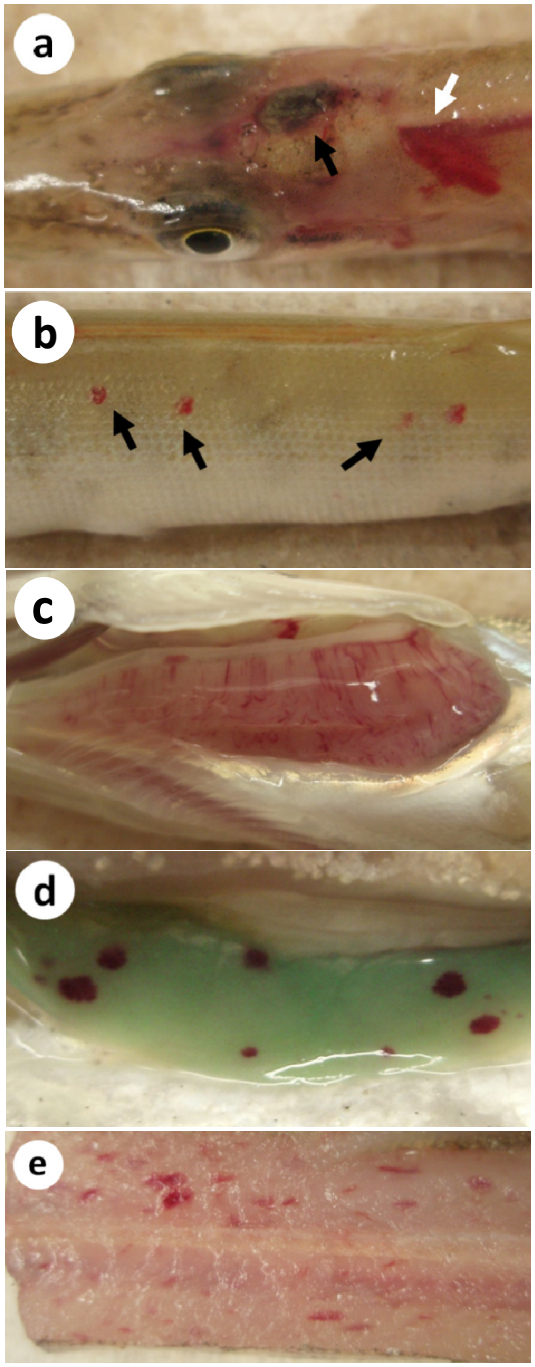

Figure 6-2 Juvenile muskellunge (*Esox masquinongy*) experimentally infected with viral hemorrhagic septicemia virus IVb (MI03) at 1,400 plaque forming units mL^{-1} exhibiting external and internal signs of disease.
(a) unilateral nuchal hemorrhage (black arrow) and intramuscular hemorrhages (white arrows), (b) severe gill palor, (c) dermal petechial hemorrhage, (d) petechia hemorrhages throughout liver, (e) severe intramuscular hemorrhage.

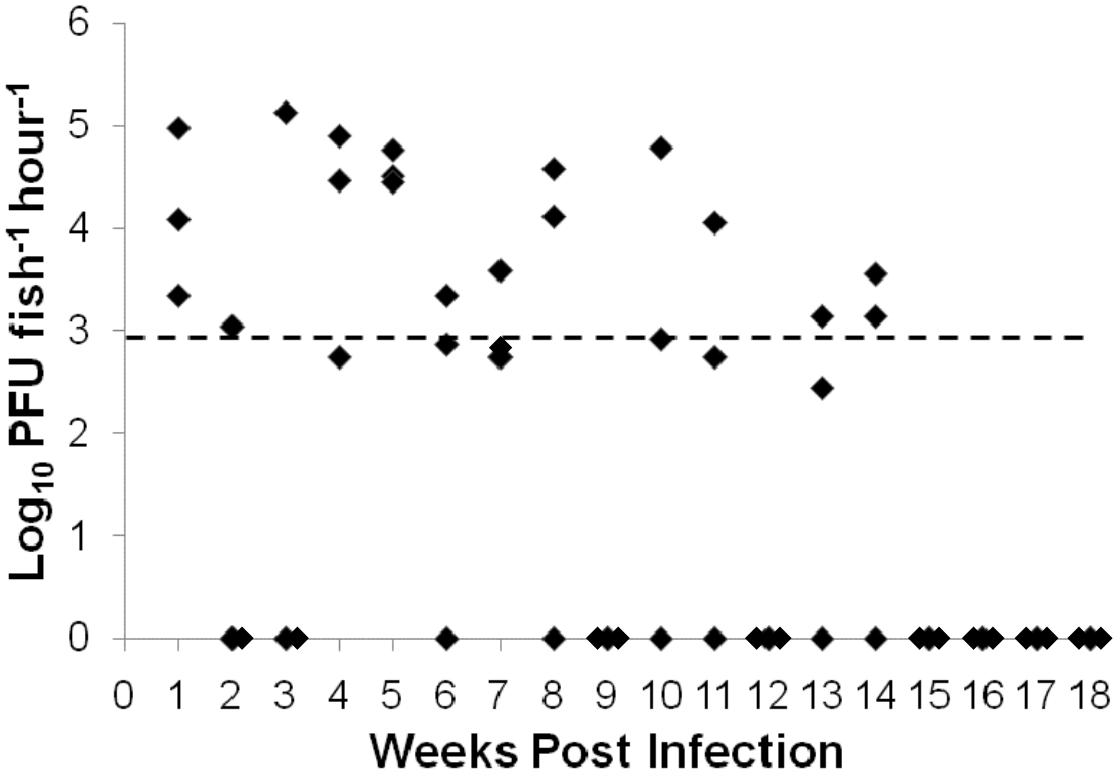

Figure 6-3 Shedding rates of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) at 1,400 PFU mL^{-1}.

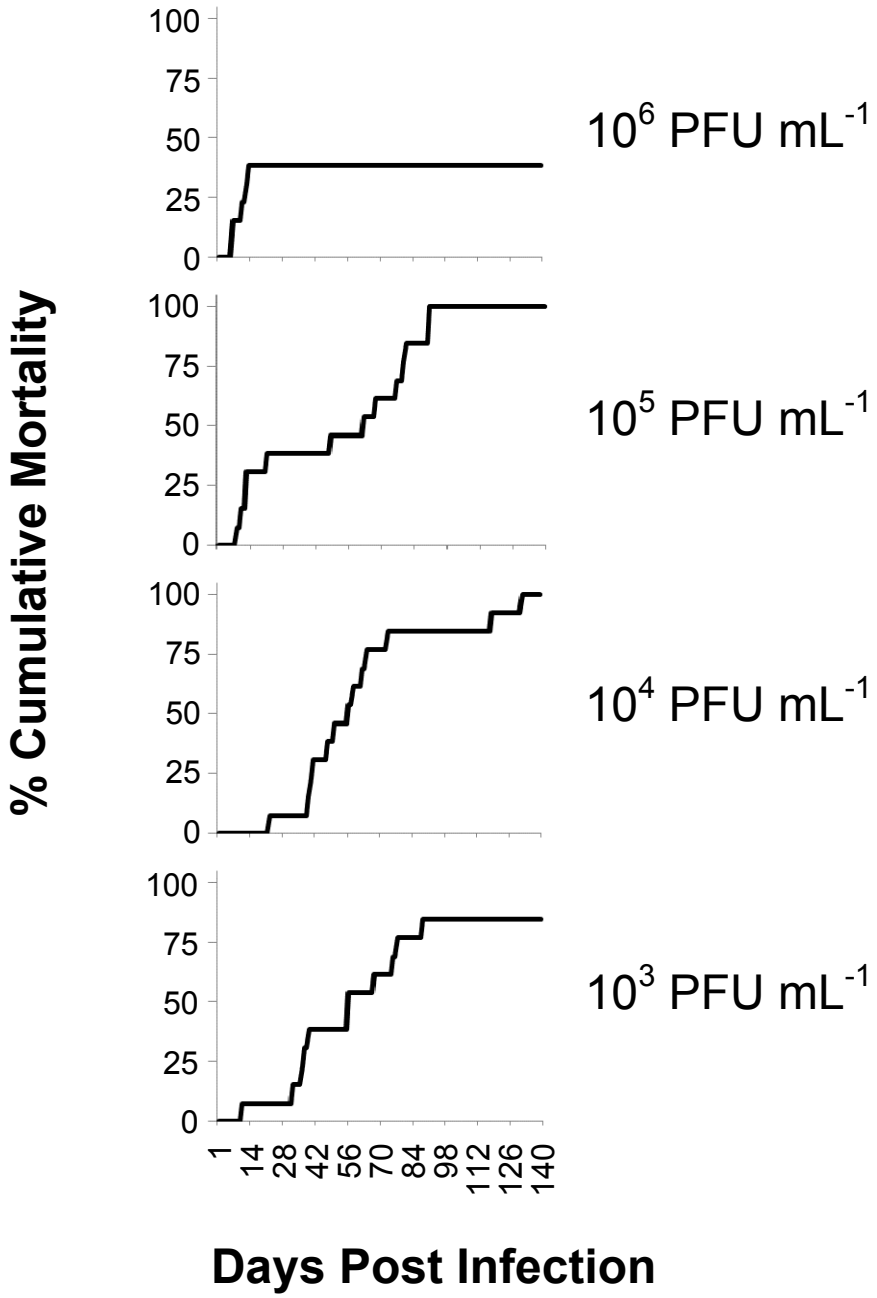

Figure 6-4 Cumulative mortality of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) over a 140 day period at varying dose levels in plaque forming units (PFU) mL^{-1}.

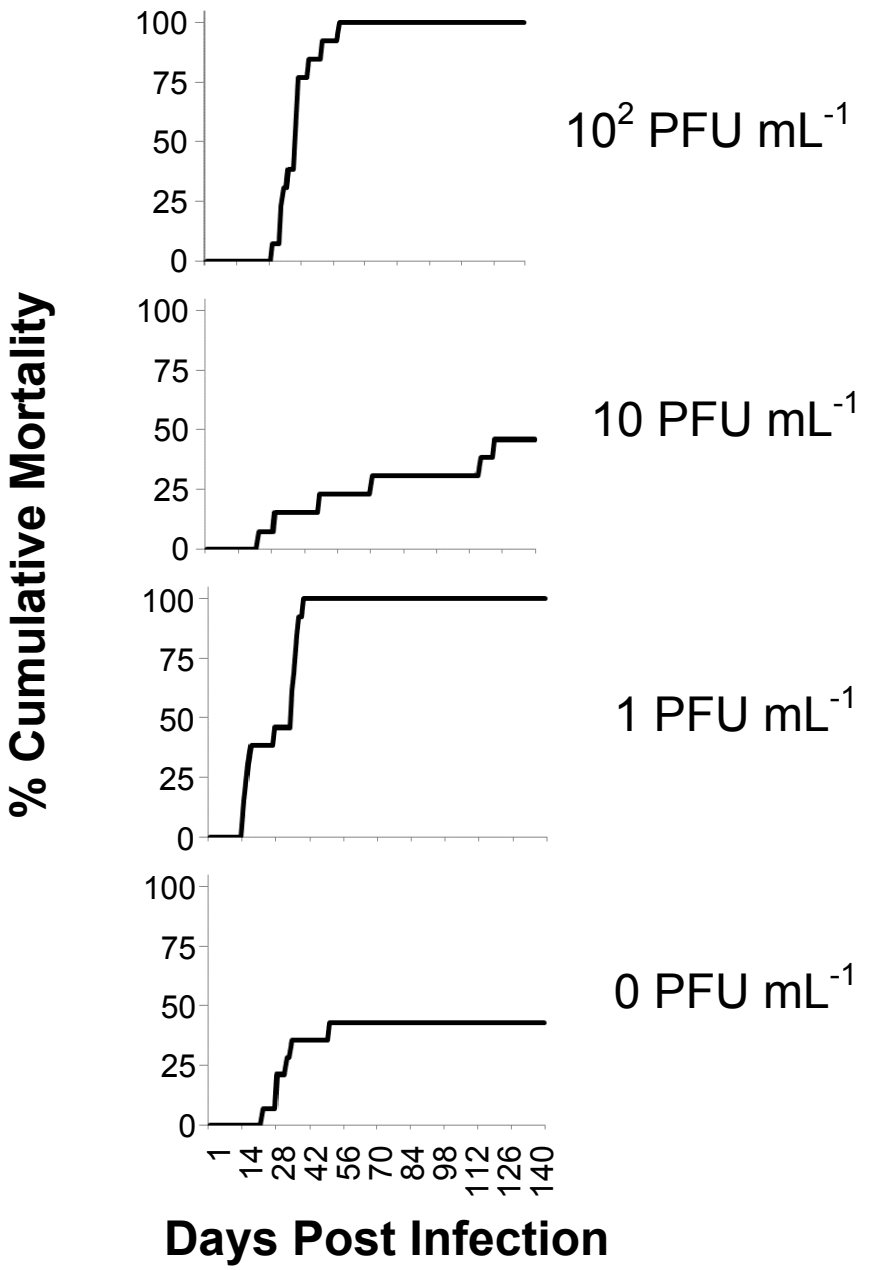

Figure 6-4 (cont'd) Cumulative mortality of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) over a 140 day period at varying dose levels in plaque forming units (PFU) mL^{-1}.

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CHAPTER 7

CONCLUSIONS AND FUTURE STUDIES

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Conclusions

Viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) can infect a wide range of freshwater fish, albeit at differing levels of susceptibility, and is similar, yet distinct, from other VHSV strains with regard to pathogenesis in susceptible hosts. These findings have been confirmed and detailed through a number of studies described in this dissertation.

In our first study, 11 VHSV-free fish species were experimentally infected using a single dose of VHSV IVb, which revealed that 1) virus could be reisolated from the tissues of VHSV infected fish thereby fulfilling River's postulates (1937), 2) juvenile fish species are susceptible to infection, and 3) clinical signs and gross pathology of dead and moribund fish of all species tested were similar among one another and to those described in the literature (Wolf 1988).

As a result, an additional study was designed to investigate the susceptibility of 8 of the 11 fish species used previously by calculation of median lethal dose by intraperitoneal injection (IP-LD_{50}). Differences among species in IP-LD_{50} values provided evidence that the species vary from highly, moderately, or mildly susceptible to VHSV IVb infection. Among the most susceptible fish species were the muskellunge (*Esox masquinongy*) with an IP-LD_{50} of 2.2 plaque forming units (PFU), followed by the largemouth bass (*Micropterus salmoides*) with an IP-LD_{50} value of 1.5×10^2 PFU. Conversely, salmonids were

moderately to minimally pathogenic to VHSV with LD_{50} values no less than 1.4×10^6 PFU. Our data revealed that while VHSV IVb could initiate infection, the dose of administration affected outcome and corresponded to whether a fulminant infection could be induced. However, the presence of survivors following experimental infection raised questions into whether the virus continued to persist or was cleared from the host tissues.

In subsequent studies, the disease course of VHSV IVb infection was evaluated using muskellunge, as the disease model of choice. Initially, groups of muskellunge were experimentally infected by immersion to determine the median lethal dose of infection (IM-LD_{50}), which resulted in an IM-LD_{50} of 1.7×10^4 PFU / mL. The difference between the IM-LD_{50} and IP-LD_{50} values were likely the result of differences in the age class and/or the broodstock used for the year in which the fish were obtained. On the other hand, the fish injected by intraperitoneal injection were older and larger in size, although the LD_{50} value was extremely low. The fact that the IM-LD_{50} value was much higher in smaller and younger fish, underscores the efficacy of the muskellunge's natural barriers against infection. When juvenile muskellunge were exposed by immersion to doses that were relatively high, medium, and low in virus concentration, an acute, subacute, and chronic trend in mortality was observed. The findings from this study were significant in that VHSV IVb revealed a dose dependency for mortality in this species and that juvenile muskellunge could survive VHSV IVb infection

upwards of 60 days post infection, suggesting a carrier state of at least 2 months duration can exist in this fish species.

A more detailed investigation into the disease course was completed by examining the sequential pathogenesis of VHSV IVb in host tissues of muskellunge. Tissues collected in parallel at predetermined timepoints were evaluated by cell culture reisolation and the viral plaque assay to determine the distribution and viral load in sequence. The study revealed that VHSV IVb can be detected as early as 24 hours post infection (h p.i.) in pectoral fin tissues, but tends to be highest in concentration and more frequently isolated in the heart tissues. Distribution of the virus varied and could be isolated in low quantities from one tissue, or found at relatively high levels in all tissues. Interestingly, the heart tissues were consistently higher in virus load as compared to other tissues indicating its importance as a diagnostic sample. Virus was isolated from all tissues in fish experimentally infected upwards of 64 d p.i. suggesting that VHSV IVb could exist in host tissues for extended time periods.

Subsistence of VHSV IVb in muskellunge surviving infection signified the presence of a carrier state, but whether the virus was being cleared from hosts or shed into the surrounding environment was unclear. Therefore, a final study was performed to assess shedding of VHSV IVb in muskellunge experimentally infected with a relatively low dose of virus. Our findings revealed that infected juvenile muskellunge shed the VHSV IVb into the surrounding environment at varying titers for up to 15 weeks p.i. In some instances, shedding rates of survivors were considerably higher than the original dose of infection, indicating

that VHSV IVb was amplified within host tissues. In the second component of our study, juvenile muskellunge surviving initial exposure to VHSV IVb were rechallenged by immersion to VHSV IVb at varying dose levels in determination of an LD_{50} and assessed for shedding. The LD_{50} for previously exposed muskellunge was approximately 1.34×10^5 PFU / mL, which was higher than the LD_{50} for naïve muskellunge (1.7×10^4 PFU / mL) demonstrating the potential for acquired immunity in this species. Rates of viral shedding and cumulative mortality trends were highly variable, and could not be correlated to the original dose of infection. However, the overall rates of shedding were substantially higher following reinfection than initial exposure. These studies confirm that survivors of VHSV IVb are in fact carriers and shedders of VHSV IVb and should be a major consideration in the development of biosecurity measures and quarantine protocols.

Future Studies

Viral hemorrhagic septicemia virus (VHSV) presents one of the greatest challenges to the Laurentian Great Lakes basin from a managerial standpoint. Our current understanding of VHSV has been largely provided by published data regarding European and Pacific VHSV isolates. Despite obvious similarities in disease manifestations to other VHSV strains, there is clear evidence that VHSV IVb is not only unique in its genome (Elsayed et al. 2006), but has the widest freshwater host-range of all VHSV isolates and functions under dissimilar environmental conditions. A dire need to elucidate its impacts within the Great Lakes is imperative to management decisions and has therefore remained the motive behind our studies to characterize VHSV IVb and assess the risk it imposes on the Great Lakes ecosystem.

While our studies reveal that VHSV IVb has a varied predilection for species listed by the Federal Order of the United States Department of Agriculture (http://www.aphis.usda.gov), the discrepancies observed in susceptibility noted within salmonids alone indicate that intra-family differences in susceptibility amongst fish species exists. Our studies compared the susceptibilities of only a fraction of the fish species inhabiting the Great Lakes basin. Therefore, future experimental infections involving species of unknown susceptibility are of paramount importance, especially with regard to surveillance and diagnostic efforts of fisheries managers and aquatic animal health practitioners.

Given that a large component of our studies involved experimental infections, our finding should be used as indices of comparison since naturally occurring fluctuations in environmental factors could not be reproduced. The extent to which these factors impact the ability of VHSV to cause disease is largely unknown and therefore should be considered important variables in future study. Additional factors that should also be included are the age of the fish used, observation period(s), tank density, strain or type of fish used number of fish infected and source of water.

Since the detection of VHSV IVb occurred in all sampled tissues, albeit at differing loads, it is unclear whether the virus was detected in transit through the blood stream, or whether active replication at these tissue sites was occurring. Although high loads of virus in one particular tissue would indicate viral replication, methods such as in situ hybridization or electron microscopy would allow visualization of where viral replication is occurring. Furthermore, sites of viral replication would also offer clues into locales from which virus is amplified and shed. The notion that urine is a major source of viral shedding is based on reisolation of VHSV from urine collected by catheter (Neukirch & Glass 1984) and that histopathologic alterations have been observed in the kidney of VHSV infected fish (Yasutake 1975). However given the pathology noted in the liver and pectoral fin of VHSV infected muskellunge, alternative routes of viral release into the environment could exist.

Aside from the alternatives to study design, one of the most important pieces of information gained from our studies is that muskellunge are capable of

surviving infection and re-exposure to VHSV IVb. Extended periods of viral shedding followed by cessation, provide promise into the recovery and survival of VHSV IVb infected fish. Although the immunologic status of the survivors is unknown, conferred protection to VHSV IVb would be of significant value from the standpoint of vaccine development and elucidating the immunologic response to VHSV IVb. Antibody development to VHSV has been widely studied and utilized in a number of serologic assays in the detection of antibodies against other VHSV genotypes through enzyme linked immunosorbent assay (ELISA) and the plaque neutralization assay (Brudeseth & Evensen 2002, Dopazo et al. 2002, Dixon & Hill 1984). Perhaps the most convincing rationale behind the utilization of serological examination stem from an investigation using serological evidence in rainbow trout previously infected with IHNV for screening purposes. Based on neutralizing antibody results, investigators determined that an immune response was both measurable and indicative of previous infection to a virus (Hattenberger et al. 1989). Similarly if a humoral response in Great Lakes fish could be detected and measured, prior exposure to VHSV IVb could be assessed and enhance surveillance efforts greatly.

As VHSV IVb continues to leave trails of mass mortality episodes in the wake of its spread, our studies lay promise and optimism for the Great Lakes. Efforts to mitigate the spread and expansion of VHSV IVb are currently underway and are improving with the implementation of policy and aquatic animal health programs worldwide. To this end, a major goal and function of our studies was to

fulfill existing knowledge voids of VHSV IVb and foster our current understanding of VHSV IVb infection in susceptible hosts.

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