DIAGNOSTIC EVALUATIONS OF A QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

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DIAGNOSTIC EVALUATIONS OF A QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

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The emergence of viral hemorrhagic septicemia virus (VHSV) genotype IVb is reviewed with a particular emphasis on New York State. The first detection of VHSV in New York State in round gobies, *Neogobius melanostomus*, collected in 2006 is described. The diagnostic ability of a qRT-PCR test to detect this new genotype of VHS is then evaluated.

Firstly, the qRT-PCR was used to retrospectively analyze results from a 2006 fish collection from the Saint Lawrence River. The qRT-PCR test detected a significantly higher sample prevalence of 37.0% compared to the 9.3% prevalence as determined by viral isolation. The negative predictive value of the qRT-PCR was determined to be 97.6%. The qRT-PCR testing also revealed positive detections in three additional species and three additional locations. Logistic regression models for disease outcome by both testing methods were compared.

Next experimental infection trials of VHSV IVb in four aquacultured species were performed. The fish were given a concurrent thermal shock to promote eliciting clinical signs. Periodic sampling was performed to detect VHSV using virus isolation and qRT-PCR. The results show that these aquacultured species have different susceptibilities to VHSV. Using the qRT-PCR test allowed us to detect infections in exposed fish at sub-clinical levels which were not detected by virus isolation.

Finally the persistence of VHSV on walleye, *Sander vitreus*, eggs was evaluated. Fertilized walleye eggs were exposed to VHSV and then treated with different concentrations of iodophor as a disinfectant. The effects of tannic acid, used for surface de-adhesion of eggs and an inhibitor of qRT-PCR testing, was also evaluated. Virus was isolated in 0 mg/L iodophor treated eggs up to three days post infection (DPI). Virus was also isolated in the 50 mg/L iodophor treated group at 1 DPI. Testing by qRT-PCR detected VHSV at many time-points throughout the experiments, including the end of the experiment. Inhibition of the qRT-PCR testing was found in many samples at earlier time-points.

BIOGRAPHICAL SKETCH

Geof Groocock was born July 22nd, 1975 in Bryn Mawr, Pennsylvania and soon moved to Cutchogue, on the east end of Long Island. When he was six years old, his family moved to Nairobi, Kenya. After navigating the transition to the British system of education, he attended Oundle School in Peterborough, England for his high school education.

After high school, he returned to the United States and began college at the State University of New York at Stony Brook. After two years, he transferred to Cornell University and obtained his Bachelors of Science in Biology in 1998. He was accepted into the College of Veterinary Medicine at Cornell University and completed his Doctor of Veterinary Medicine in 2003. As a veterinary student, he found his calling in investigating emerging infectious disease in wildlife and spent two summers researching Rinderpest in East African wildlife.

After graduating from veterinary school, he worked in a small animal and exotics practice on Long Island for a year and a half. In 2005 he returned to Cornell as a Postdoctoral Associate in Dr. Paul Bowser's lab. He has been working for Dr. Bowser ever since, was promoted to an Extension Associate in 2006, and enrolled in the Employee Degree Program in 2007. Soon after he started, a new emerging fish disease was found in the Great Lakes. He has been privileged to be working with the Aquatic Animal Health Program during a very exciting time and has learnt an enormous amount about fish and disease surveillance and looks forward to continuing to work with infectious disease in wildlife, particularly fish.

For Sandy, the best friend a kid could have had.

Safari salama.

For my wife, my parents, my family, and my friends.

Asante sana.

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CHAPTER ONE:

THE EMERGENCE OF VIRAL HEMORRHAGIC SEPTICEMIA IN THE GREAT LAKES

1.1 A Brief History of Viral Hemorrhagic Septicemia Virus

Each of the further chapters of this dissertation include a detailed review of VHSV specific to the chapter material and thus only a brief review of VHSV will be presented here.

Viral Hemorrhagic Septicemia (VHS) is a highly pathogenic disease of fish caused by the *Novirhabdovirus* Viral Hemorrhagic Septicemia virus (VHSV) (Walker et al. 2005). The disease continues to cause large fish kills in Europe, such as in rainbow trout, *Oncorhynchus mykiss*, in the United Kingdom (Stone et al. 2008) and turbot, *Psetta maxima,* in Turkey (Nishizawa et al. 2006). It is listed by the World Organization of Animal Health (Office International des Epizooties, OIE) as one of only nine reportable diseases of finfish (OIE 2009) because of its broad host range and its potential to cause economic losses to aquaculture.

Originally described by Schäperclaus in 1938, VHS was first reported in Europe in cultivated rainbow trout and described as "infectious kidney swelling and liver degeneration" (Schäperclaus 1938). The disease was responsible for large mortality events in aquaculture, particularly in younger age classes of fish (Wolf 1988). It was not until the early 1950's that VHS was first reproduced in the laboratory with injected fish using a bacteria-free filtrate. In 1963 the virus was isolated and identified by Jensen (1963) and initially named "Egtved disease", but was later renamed VHS by the OIE. It is annually responsible for significant economic losses in the aquaculture sector in many European countries (Subasinghe et al. 2000).

VHS was predominantly considered a problem in cultured rainbow trout in Europe until 1988 when VHSV was isolated for the first time from returning adult Chinook, *Oncorhynchus tshawytscha*, and Coho, *Oncorhynchus kisutch*, salmon in Washington State, USA (Winton et al. 1991). Subsequently, VHSV was detected in multiple marine species in the Northwest American and Japanese waters of the Pacific and the European Atlantic (Meyers et al. 1992, 1994, 1999;

Kent et al. 1998; Smail et al. 2000; Isshiki et al. 2001; Dopaz et al. 2002; Hedrick et al 2003; and Gagné et al. 2007).

1.2 Viral Hemorrhagic Septicemia in the Great Lakes

In 2005 VHSV emerged in the Laurentian Great Lakes and caused a large-scale fish kill of freshwater drum, *Aplodinotus grunniens*, in the Bay of Quinte, Lake Ontario (Lumsden et al. 2007). The earliest known detection of VHSV in the Great Lakes was then isolated from muskellunge, *Esox masinquongy*, tissues collected in 2003 (Elsayed et al. 2006). In subsequent years, VHS continued to cause disease in fish in the Great Lakes. In 2006 a large fish kill of round gobies, *Neogobius melanostomas*, due to VHS was reported in the Saint Lawrence River (Groocock et al. 2007). This was the first report of VHSV in New York State and in round gobies and is fully described in Chapter 2. Since the initial detection of VHSV in the Great Lakes, the virus has continued to spread into many new species and locations throughout the Great Lakes basin and surrounding waterways and was recently reviewed by Kim and Faisal (2011). Currently confirmed isolations of VHSV have been found from four of the Great Lakes (Huron, Michigan, Erie, and Ontario) and detected in Lake Superior (Cornwell et al. 2011).

VHSV is broadly defined by geographic range, but partial sequence analysis of the nucleoprotein (N) gene of VHSV has designated four distinct genotypes I–IV (Snow et al. 2004). Genotypes I–III are widespread in freshwater and marine fish of Europe. There have also been isolations of genotypes I and III in Japan in Japanese flounder, *Paralichthys olivaceus*, (Isshiki et al. 2001 and Nishizawa et al. 2002). Genotype IV is presently restricted to the marine and freshwater environment of North America, Japan and Korea (Kim et al. 2009, Kim and Faisal 2011). Further sequence analysis focused on the full glycoprotein (G) gene confirmed that the

North American Pacific type IVa isolate of VHSV was widely divergent from other isolates (Einer-Jensen, 2004). Sequence analysis of the entire N and G genes of the recently emerged VHSV in the Great Lakes was shown to be related to the North American Pacific isolates, but sufficiently divergent (3.6–3.7% nucleotide diversity) to propose its classification as Type IVb (Elsayed et al. 2006). More recently, sequencing of the partial G gene showed that VHSV IVb in the Great Lakes has a low viral genetic diversity, with over 90% of the isolates confined to two dominant sequence types (Thompson et al. 2011).

Fish mortality events caused by VHS peaked in 2007 in New York State, yet detections of the virus in new species and locations continued through 2008 and 2009 in healthy fish (Bain et al. 2009, Kane-Sutton et al. 2010, and Cornwell et al. 2011). The genotype IVb of VHSV appears to have a lower pathogenicity in some of the traditionally affected species of salmonids (Al-Husinee et al. 2010). Conversely the type IVb isolate appears to be highly pathogenic in some native Great Lakes species such as muskellunge, *Esox masquinongy*, (Kim and Faisal 2010a, 2010b).

The emergence of VHSV in New York State resulted in significant interest from state and federal agencies. A quantitative real-time polymerase chain reaction (qRT-PCR) test was developed at Cornell University which is more analytically sensitive and highly specific for the detection of VHSV type IV in fish tissues (Hope et al. 2010). This test has already been widely used in surveillance for VHSV in wild fish collected from the Great Lakes (Bain et al 2009, Frattini et al. 2010, and Cornwell et al. 2011). This dissertation focuses on evaluating its use in testing diagnostic samples for VHSV.

Chapter 2 describes the first isolation of VHSV in round gobies in New York State in 2006. Chapter 3 details the investigation of a 2006 collection of fish from the Saint Lawrence

River and compares the qRT-PCR test results to virus isolation methods. Chapter 4 compares the results of qRT-PCR, virus isolation and histopathology testing in 4 aquacultured species experimentally infected with VHSV. Finally, in Chapter 5, the use of qRT-PCR testing of walleye eggs experimentally exposed to VHSV is examined.

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CHAPTER TWO:

*DETECTION OF VIRAL HEMORRHAGIC SEPTICEMIA IN ROUND GOBIES IN NEW YORK STATE (USA) WATERS OF LAKE ONTARIO AND THE ST. LAWRENCE RIVER

*G. H. Groocock, R. G. Getchell, G. A. Wooster, K. L. Britt, W. N. Batts, J. R. Winton, R. N. Casey, J. W. Casey, and P. R. Bowser. 2007. Detection of viral hemorrhagic septicemia in round gobies in New York State (USA) waters of Lake Ontario and the St. Lawrence River. Diseases of Aquatic Organisms 76:187–192.

2.1 Abstract

In May 2006 a large mortality of several thousand round gobies, *Neogobius melanostomus*, occurred in New York waters of the Saint Lawrence River and Lake Ontario. Necropsies of sampled fish from these areas showed pallor of the liver and gills, and hemorrhagic areas in many organs. Histopathologic examination of affected tissues revealed areas of necrosis and hemorrhage. Inoculations of fathead minnow, *Pimephales promelas*, cell cultures with dilutions of tissue samples from the necropsied gobies produced a cytopathic effect within five days post inoculation. Samples of cell culture supernatant were tested using reverse trancriptase polymerase chain reaction (RT-PCR) and confirmed the presence of viral hemorrhagic septicemia virus (VHSV). Sequence analysis of the VHSV isolate resulted in its assignment to the genotype IVb subgroup. The detection of VHSV in a relatively recent invasive fish species in the Great Lakes and the potential impact of VHSV on the ecology and economy of the area will require further investigation and careful management considerations.

2.2 Introduction

Lake Ontario and the Saint Lawrence River are economically and ecologically important water systems supporting a large variety of fish species. Round gobies, *Neogobius melanostomus*, are a recently invasive species to the Great Lakes, first detected in the early 1990's (Jude et al. 1992). Since that time, their population has expanded rapidly and they have become an important part of the food chain in these ecosystems.

In early May 2006 the New York State Department of Environmental Conservation (NYSDEC) received reports of large scale fish kills involving several thousand round gobies on the St. Lawrence River near Cape Vincent, New York. Over the course of subsequent weeks, the

NYSDEC received additional reports of locations in US waters on the St. Lawrence River and Lake Ontario where hundreds of dead and dying round gobies were observed. Collections of round gobies from the initial fish kill event and from a subsequent fish kill that occurred in the mouth of Sandy Creek, Lake Ontario, were submitted by the NYSDEC to the Aquatic Animal Health Program (AAHP) at the College of Veterinary Medicine, Cornell University, for diagnostic evaluation.

2.3 Materials and Methods

Samples of round gobies were received by the AAHP at Cornell University from 2 locations. The first sample consisted of 23 moribund and dead fish from the St. Lawrence River near Cape Vincent, New York. On 4 May 2006, divers from the NYSDEC collected dead and moribund gobies from this area and shipped them by courier immediately to the AAHP at the College of Veterinary Medicine, Cornell University. The fish arrived at Cornell in moribund or freshly dead condition and were processed for diagnostic evaluation. The second sample consisted of nine dead round gobies collected on 15 May 2006 from Sandy Creek, Lake Ontario, west of Rochester, New York. These fish were transported on ice to the AAHP.

Cape Vincent, St. Lawrence River samples. All moribund fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate, Western Chemical Inc.) in water. Eight fish were processed for diagnostic evaluation. The remaining 15 fish were frozen whole at –20 degrees Celsius (°C) for future evaluation if necessary. The procedure for diagnostic evaluation is described by Noga (1996) and included collecting skin scrapings and gill clip samples, sterile collection of posterior kidney samples for bacteriology, gross pathology and collection of tissues for histopathology and virology. Samples of liver, kidney, spleen and gonad were collected in

two pooled samples for detection of viral agents. The first pooled sample (Sample A) included tissues from gobies that were dead at the time of presentation. The second pooled sample (Sample B) included fish that were moribund at the time of presentation. These samples collected for virus isolation were processed as described in the following sections. Attempts at bacterial isolation consisted of cultures taken from the posterior kidneys that were streaked onto blood agar (TSA II 5% SB, BBL™, Becton Dickinson). These cultures were incubated for seven days at 21 °C. Samples of intestines, gut contents and liver were collected to test additionally for type-E botulism by polymerase chain reaction (PCR) (Getchell et al. 2006).

Sandy Creek, Lake Ontario samples. Five fish were processed for diagnostic evaluation. Skin scrapings, gill clips and histopathology were not performed on these fish as the samples were moderately autolyzed and the diagnostic significance of these tissues was considered marginal for these samples. Due to the condition of the specimens further processing of these fish was limited to attempts at virus isolation. No attempts at bacterial isolation were performed. Samples of liver, kidney, spleen, swim bladder and gonad were collected from each of the 5 fish for this purpose and processed as described in the following sections.

Gross pathology and histopathology. Fish were examined for the presence of external and internal lesions. Tissues for histopathology were fixed in 10% neutral buffered formalin. Bony tissues, such as gill arches, were additionally decalcified for five to seven days in 14% disodium EDTA (Sigma). All tissues were sectioned at 5 micrometers (μm) and stained with hematoxylin and eosin (H&E) for microscopic evaluation (Luna 1968). Immunohistochemical stains were performed on select tissue sections using the standard procedures for the streptavidin-biotin immunoperoxidase technique.

Virus isolation. The protocols used followed the standard guidelines published by the American Fisheries Society (2004) and the World Organization for Animal Health (Office International des Epizooties, OIE) (2003). Tissue samples were prepared at 1:10 dilutions by aseptically weighing 0.5 grams (g) of tissue and adding 4.5 milliliters (mL) Minimal Essential Medium with Hanks' salts (Gibco®, Invitrogen) prepared with 5% fetal bovine serum (Gibco®, Invitrogen), penicillin (200 International Units(IU)/mL), streptomycin (200 micrograms (μg)/mL), glutamine (0.584 mg/mL) (Gibco®, Invitrogen) and HEPES buffer (1 Molar 0.015 mL/mL) (Gibco®, Invitrogen), hereafter referred to as HMEM-5% 2× PSG + HEPES. The tissue and media were homogenized using a Stomacher® 80 homogenizer (Stomacher®, Seward, London, England) for 2 minutes (min). The homogenized media were kept on ice for 10 min, then centrifuged (Multifuge™, American Scientific Products) at 500 × gravity for 5 min. Inocula were prepared by pipetting off the homogenized media, avoiding the pellet, and sterile filtering through 0.2 μm biological filters (Acrodisc®, Pall Life Sciences). One milliliter of the inocula was placed on fresh monolayers of fathead minnow (FHM), *Pimephales promelas*, cell cultures (American Type Culture Collection CCL-42; Gravell & Malsberger 1965) in 50 mL cell culture flasks (Corning, NY). Flasks containing inoculations were placed on a slow-speed orbital rocker (model PS-M3D, Grant Instruments) at 8 revolutions per min at 21 °C for 45 min. Finally, an additional 4 mL of the medium, HMEM-5% 2× PSG + HEPES, was added to each flask. The inoculated cells were incubated at 15 °C and examined for the presence of cytopathic effects (CPE) daily for 21 days post-inoculation. Initial identity of the virus was confirmed using RT-PCR using viral hemorrhagic septicemia virus (VHSV) specific primers followed by sequencing of the glycoprotein (G) gene as described by Elsayed et al. (2006). Samples of supernatant from cell cultures exhibiting CPE were sent to the OIE reference laboratory for

VHSV at the Danish Institute for Food and Veterinary Research, Community Reference Laboratory for Fish Diseases, Denmark, for VHSV PCR evaluation.

2.4 Results

Gross pathology. On gross necropsy all the fish examined showed a wide range of signs indicative of a hemorrhagic disease (Table 2.1). Most notable were signs of anemia indicated by pale coloration to the gill lamellae and pale livers seen in many of the fish examined.

Table 2.1: Summary of gross pathology observed in round gobies involved in mortality events and submitted for disease diagnostic evaluation. F = female, M = male; Y = presence in sample of pathology described.

					Pathology									
Fish no.	Sex	Length (mm)	Weight (g)	Body Condition	Pale gills	Pale liver	Pale spleen and other internal organs	Hemorrhagic areas in liver	Hemorrhages on omentum or abdominal fat	Intramuscular hemorrhages	Hemorrhagic gonads	Hemorrhages on skin or fins	Hemorrhagic spleen	Empty gastrointestinal tract
Cape Vincent Fish														
1	F	166	73.5	Good		Y					Y			Y
2	F	182	101.6	Good	Y	Y			Y		Y			Y
3	F	167	72.1	Good	Y	Y			Y					Y
4	F	98	19.8	Good	Y	Y			Y	Y		Y		Y
5	F	100	15.6	Good		Y						Y		Y
6	M	163	65.0	Thin		Y						Y		Y
7	M	131	36.5	Thin	Y	Y			Y	Y		Y		Y
8	M	108	21.5	Good				Y						Y
Sandy Creek Fish														
1	M	189	109	Good				Y		Y	Y		Y	Y
2	M	180	85	Good		Y					Y			Y
3	M	150	54	Good				Y				Y		Y
4	M	158	61	Good		Y								Y
5	M	149	57	Good		Y	Y					Y		Y

Hemorrhagic areas were seen in many organs, in particular the omentum, abdominal fat, gonads, liver, muscle tissue (Figure 2.1), skin and fins. None of the fish examined had food in their gastrointestinal tracts. No parasites were observed on examination of skin scrapes and gill clips from the Cape Vincent fish.

Figure 2.1: Gross pathology image of a round goby with intramuscular petechial hemorrhages.

Histopathology results. The affected hepatocytes appeared severely vacuolated with occasional pyknotic and karyolytic nuclei. The posterior renal tissue was similarly affected with evidence of scattered individual cell necrosis. There was moderate melanin deposition on the renal periphery, which may be associated with a prolonged period of stress. There was severe splenic parenchymal necrosis and depletion characterized by abundant numbers of individual cell necrosis (Figure 2.2). In intramuscular hemorrhagic areas, extravasated erythrocytes were seen between intact muscle fibers. The gonadal and gill tissue examined appeared normal. Immunohistochemistry staining for vonWillebrand's factor and CD31 to differentiate endothelial cells was inconclusive.

Bacteriology results. Cultures for bacterial pathogens collected from the posterior kidneys and streaked onto blood agar (TSA II 5% SB, BBL™, Becton Dickinson) media produced no significant bacterial growth after seven days incubation at 21 °C. PCR testing of the Cape Vincent fish for botulinum type-E toxin gene produced a negative result.

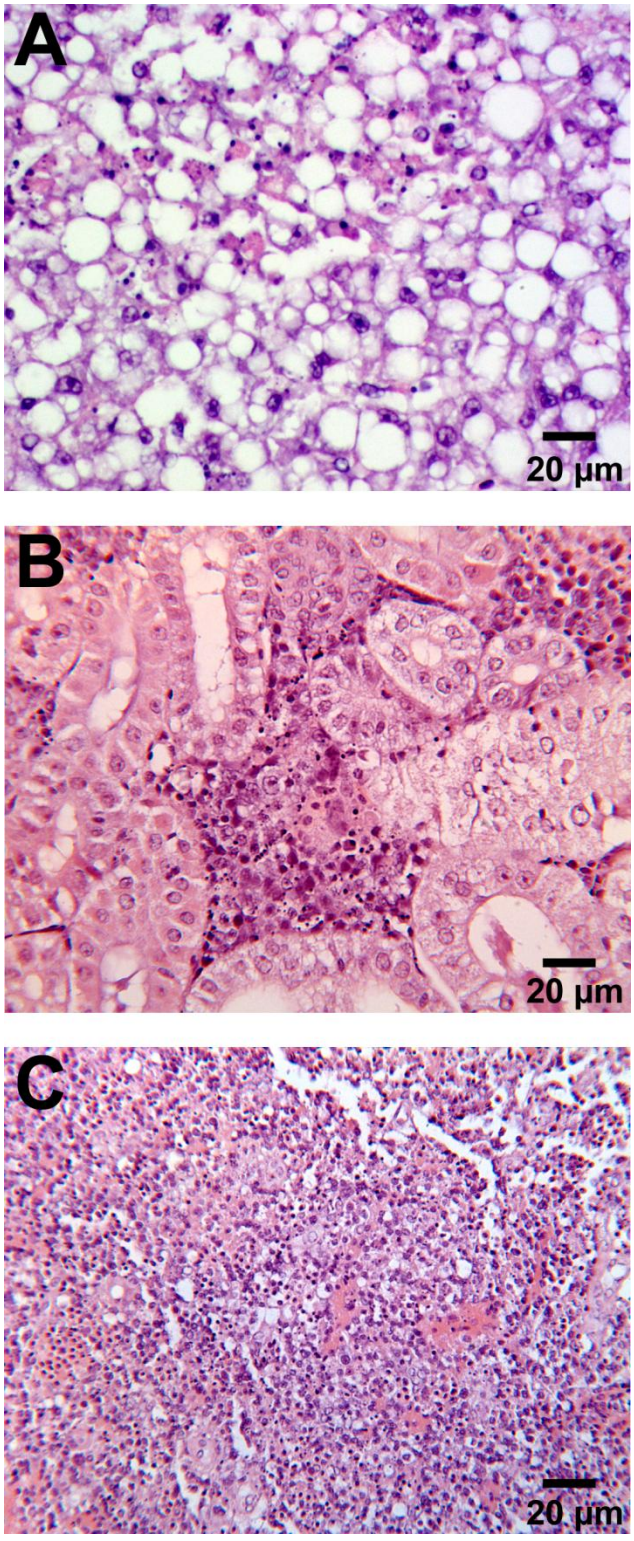

Figure 2.2: Microscopic images (H&E stain, 40×) of histological tissue sections. (A) Vacuolated hepatocytes with occcasional pyknotic and karyolitic nuclei. (B) Renal individual cell necrosis and melanin deposition. (C) Severe splenic parenchymal necrosis and depletion.

Virology results. CPE were detected in all FHM cultures five days post inoculation. Visible effects varied from destruction of the monolayer (Figure 2.3) to areas of rounded refractile cells. Subsequent passages of cells showed attenuation of the CPE. In some cases second passage inoculations, which destroyed the monolayer, only produced areas of focal CPE in the third passage. Samples of supernatant from first passage flasks with CPE were collected, subjected to RT-PCR testing for VHSV and subsequently sequenced as described by Elsayed et al. (2006). A positive RT-PCR result was obtained in all cases.

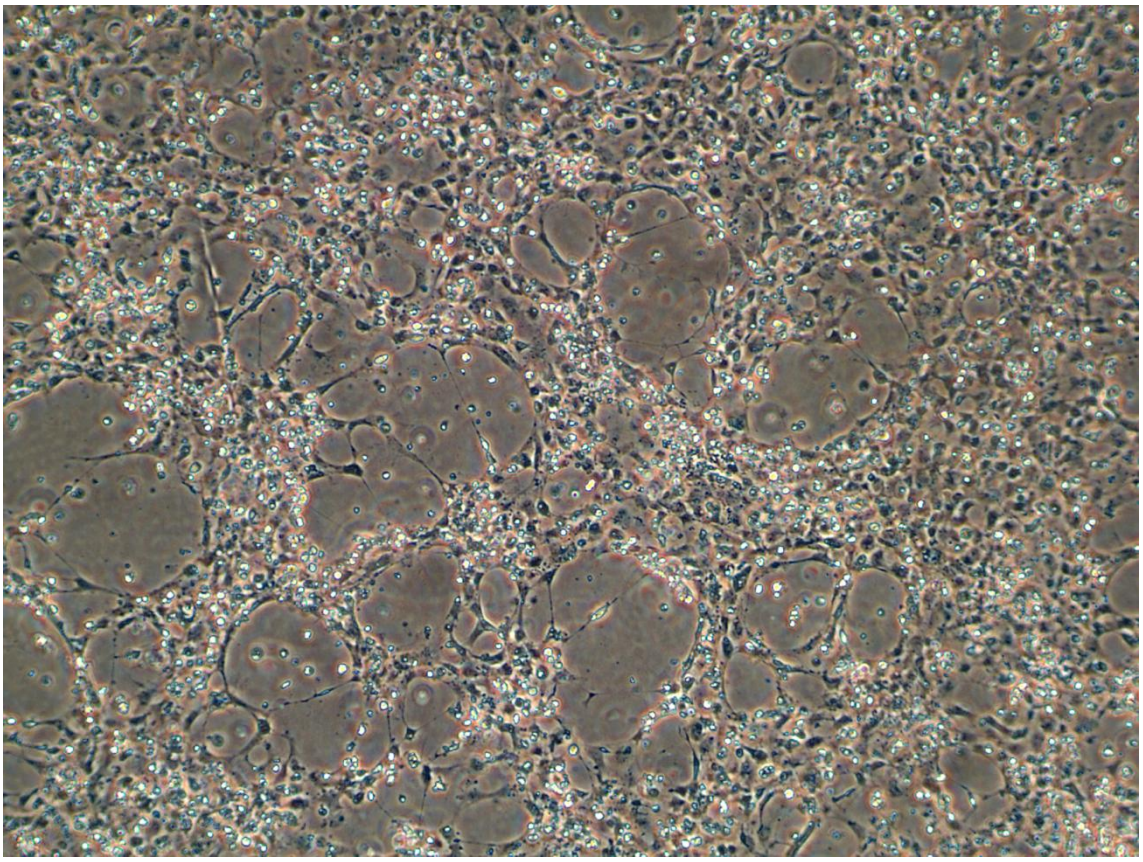

Figure 2.3: Cytopathic effect observed in fathead minnow (FHM) cell monolayers at 4 days post-inoculation.

The nucleotide sequence of the central region of the G-gene of the round goby isolate differed by only a single nucleotide from the VHSV isolate recovered from muskellunge, *Esox masquinongy* (Mitchill, 1824), in Lake St. Clair in 2003 (Elsayed et al. 2006). Culture fluids from flasks inoculated with Samples A and B from Cape Vincent were submitted to the OIE reference laboratory in Denmark. A positive result was obtained by PCR testing for these samples, confirming the presence of VHSV in these fish.

2.5 Discussion and Conclusions

The gross pathological changes seen in these round gobies were consistent with the internal signs of VHSV infection described for salmonids (Wolf 1988). PCR testing for VHSV by two independent laboratories confirmed the identification of this virus. Viral hemorrhagic septicemia virus is a *Rhabdovirus* well known for causing mortalities in both rainbow trout, *Oncorhynchus mykiss*, (Castric & de Kinkelin 1980) and turbot, *Scophthalmus maximus*, (Schlotfeldt et al. 1991) in Europe. The disease caused by VHSV is classified as reportable to the OIE. VHSV has been detected in the USA as early as 1988 in anadromous salmonids in the Pacific Northwest (Winton et al. 1991, Meyers et al. 1992, 1994). In 2005, VHSV was detected in freshwater in muskellunge collected in 2003 from Lake St. Clair, Michigan (Elsayed et al. 2006) and in freshwater drum, *Aplodinotus grunniens*, in 2005 from the Bay of Quinte, Lake Ontario, Canada (Canada Cooperative Wildlife Health Centre 2005).

The isolate found in these round gobies (GenBank accession number EF564588) was essentially genetically identical to the VHSV type-IVb isolate described in muskellunge from Lake St. Clair (Elsayed et al. 2006). The North American VHSV type-IV isolate is known to infect a wide range of fish species in the Pacific Northwest (Hedrick et al. 2003). It is apparent

from the wide genetic diversity between round gobies and muskellunge that the type-IVb isolate has a similarly broad host species range amongst freshwater fish. The 2006 mortalities in round gobies are probably part of a basin-wide VHSV type IVb (Elsayed et al. 2006) epizootic affecting multiple fish species.

The detection of VHSV in round gobies is an important finding in that it identifies another species of susceptible hosts in the Great Lakes. It also confirms the presence of VHSV in fish in the US waters of the Lake Ontario and St. Lawrence River. The mortality event that occurred in association with this virus is very disconcerting as it demonstrates the potential effect that VHSV can have on the ecology of these water systems. With large numbers of round gobies moribund or dead, the potential to transmit the virus to other species is greatly increased. As of June 2006 reports of suspect VHSV affecting other species of fish in the Lake Ontario and St. Lawrence River area included muskellunge, burbot, *Lota lota*, and smallmouth bass, *Micropterus dolomieu*; however, as of this time, no reports of affected salmonids have been received. Particularly important is the infection of muskellunge with VHSV, which may have a serious economic impact on the area as the St. Lawrence River is renowned for its muskellunge sport fishery.

It is not known how viral VHSV entered the St. Lawrence River, but it is probably a combination of factors that played a role, including natural fish movement and movement of fish due to human influence. The close gene sequence similarity between this round goby isolate and the previously described muskellunge isolate (Elsayed et al. 2006) may indicate that this is a newly emerging viral strain affecting a naïve population. Finally, the presence and establishment of an OIE reportable disease in a major connecting waterway will require careful management to prevent further spread of this disease to naïve areas.

2.6 Acknowledgments

The authors would like to thank the fisheries biology staff of the New York State Department of Environmental Conservation for supplying fresh specimens for diagnostic evaluation. Particular thanks to Stephen Frattini, AAHP for technical assistance and Dr. Ana Alcaraz, Section of Anatomic Pathology, Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, for assistance with histopathology interpretation. Kelly L. Britt was the recipient of a Summer AQUAVET Research Fellowship, through the AQUAVET Program administered by the School of Veterinary Medicine, University of Pennsylvania, and the College of Veterinary Medicine at Cornell University.

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CHAPTER THREE:

*A RETROSPECTIVE ANALYSIS OF VIRAL HEMORRHAGIC SEPTICEMIA TYPE IVB IN FISH COLLECTED FROM THE ST. LAWRENCE RIVER, USA, IN 2006.

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3.1 Abstract

Between 11 May and 12 June 2006, a collection of 298 apparently healthy fish was taken from the St. Lawrence River to test for a newly emerging genotype of viral hemorrhagic septicemia virus (VHSV). The samples were tested at the time using traditional virus isolation methods and VHSV was confirmed by independent laboratories. A quantitative reverse transcriptase polymerase chain reaction test (qRT-PCR) has since been developed to specifically detect this genotype of VHSV. The samples from the 2006 collection were tested by the qRT-PCR test and the results from both diagnostic testing methods were compared. The qRT-PCR test revealed a significantly higher sample prevalence of 37.0% compared to the 9.3% prevalence as determined by virus isolation. The negative predictive value of the qRT-PCR compared to virus isolation was determined to be 97.6% when specifically testing for VHSV. The qRT-PCR testing also revealed three additional species that tested positive, namely northern pike, *Esox luscious*, shorthead redhorse, *Moxostoma macrolepidotum*, and white perch, *Morone americana*, and an additional three locations tested positive. Logistic regression models for disease outcome by both testing methods were compared. The only significant predictor for testing positive by virus isolation was the species smallmouth bass, *Micropterus dolomieui*. The model for testing positive for VHSV by qRT-PCR reveals the additional significant predictors channel catfish, *Ictalurus punctatus*, and black crappie, *Pomoxis nigromaculatus*, as well as smallmouth bass.

3.2 Introduction

In the mid 2000's large-scale fish kills of wild freshwater fish occurred in the lower Laurentian Great Lakes. A large die-off of tons of freshwater drum, *Aplodinotus grunniens*, happened in the Bay of Quinté in 2005 (Lumsden et al. 2007). One year later, thousands of dead round gobies, *Neogobius melanostomus*, were found in the St. Lawrence River (Groocock et al. 2007). In the years that followed, increasing reports of large fish kills occurred across the Lower Great Lakes (Kim and Faisal 2011). These fish kills were a result of viral hemorrhagic septicemia (VHS) outbreaks caused by a newly emerged genotype of viral hemorrhagic septicemia virus (VHSV).

VHSV is a *Novirhabdovirus* of the family Rhabdoviridae (Walker et al. 2005) and is considered to be one of the most serious fish pathogens worldwide (Wolf 1988). The World Organization for Animal Health (Office International des Epizooties, OIE) has listed it as a reportable disease because of its broad host range and potential to cause large scale die-offs in fish populations. Currently the OIE lists 82 species as susceptible to VHSV worldwide (OIE 2009).

The disease was first described as the cause of mortality in rainbow trout, *Oncorynchus mykiss*, (Schäperclaus 1938, Smail 1999), however its viral etiology was not confirmed until decades later when it was isolated in rainbow trout gonad (RTG-2) cells (Jensen 1963). For many years after this VHS was considered predominantly a pathogen of rainbow trout, and caused large losses of aquacultured rainbow trout (Wolf 1988). In 1988, VHSV was detected for the first time outside Europe in spawning Chinook salmon, *Oncorynchus tshawytscha*, (Hopper 1989) and Coho salmon, *Oncorynchus kisutch*, (Brunsen et al. 1989) in Washington, USA. VHSV was soon after found to be responsible for wild fish kills of Pacific herring, *Clupea*

pallasii, Pacific hake, *Merluccius productus*, and walleye pollock, (*Theragra chalcogramma*, in 1998 off the coast of Alaska, USA (Meyers et al. 1999). In the years that followed VHSV was isolated from an ever-increasing range of marine and freshwater hosts across the northern hemisphere (Einer-Jensen et al. 2004, Kim and Faisal 2011). The isolates of VHSV were grouped into four genotypes I–IV that broadly corresponded with geographic location (Snow et al. 2004). Genotype I is composed of several sublineages and include the European freshwater isolates and marine isolates. There is a single Japanese isolation in this group from an aquaculture facility. Genotype II includes a group of marine isolations from the Baltic Sea. Genotype III is predominantly marine isolates form the European North Atlantic to the North Sea, yet also includes a single isolation from Japan in aquaculture. Group IV contains all the North American, Korean and remaining Japanese isolations (OIE 2009).

In the Great Lakes, the earliest detection of VHSV was found in archived muskellunge tissues saved from fish collected in 2003 (Elsayed et al. 2006). Sequencing of the entire nucleoprotein (N) and glycoprotein (G) genes showed this VHSV isolate was most closely related to Western USA genotype IVa, but distinct enough from these isolates to be considered a separate subgroup (Elsayed et al. 2006). Independent sequencing of the of the N and G genes showed this first Great Lakes isolate was also closely related to VHSV isolated from mortalities in wild mummichog, *Fundulus heteroclitus*, three-spined stickleback, *Gasterosteus aculeatus aculeatus*, brown trout, *Salmo trutta*, and striped bass, *Morone saxatilis*, in New Brunswick and Nova Scotia, Canada (Gangé et al. 2007). These two studies showed that the freshwater Great Lakes isolate of VHSV was sufficiently different from previous isolates of VHSV to be considered its own subgroup and was named VHSV genotype IVb.

In 2006, a large number of diagnostic samples were tested by the Aquatic Animal Health Program (AAHP) at Cornell University after the initial detection of VHSV in round gobies in the St. Lawrence River. Specifically, in the spring of 2006, samples of apparently healthy fish of various species were collected from locations in the St. Lawrence River by researchers at the State University of New York, College of Environmental Sciences and Forestry (SUNY-ESF), Thousand Island Biological Station. VHSV was isolated from a number of these fish by the AAHP and confirmed by independent testing by both the United States Geological Survey Western Fisheries Research Center (USGS-WFRC) and the United States Department of Agriculture - Animal and Plant Health Inspection Service (USDA-APHIS). Most importantly, these were the first positive detections of VHSV in the following species: bluegill, *Lepomis macrochirus*, brown bullhead, *Ameiurus nebulosus*, channel catfish, *Ictalurus punctatus*, largemouth bass, *Micropterus salmoides*, smallmouth bass, *Micropterus dolomieui*, and pumpkinseed, *Lepomis gibbosus*. The confirmation of VHSV in a wide range of new host species detected in this collection contributed to the enactment of the USDA Emergency Order in October 2006, which restricted the movement of fish from the eight US states surrounding the Great Lakes (USDA 2007).

Concurrent to the initial virus isolations of VHSV a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was being developed for the specific detection of VHSV genotype IVb (Hope et al. 2010). The qRT-PCR technique has since been used successfully in a number of targeted surveillance and research efforts basin-wide (Bain et al. 2010, Eckerlin et al. 2011, Cornwell et al. 2011). It was shown that the qRT-PCR test is significantly more analytically sensitive for the detection of VHSV (Hope et al. 2010) than traditional virus isolation in cell culture.

We were interested in applying the qRT-PCR test to the samples collected in 2006 and comparing the results to the traditional virus isolation results. Specifically we determined the prevalence of VHSV by both testing methods in asymptomatic fish collected during a concurrent VHSV outbreak in round gobies. We also performed preliminary measures of association between the qRT-PCR test and virus isolation and between recorded variables and disease status as determined by qRT-PCR.

3.3 Methods

Sample collection of fish in 2006. Researchers at the Thousand Islands Biological Station sampled the fish community at 11 sites along approximately 25 km of the St. Lawrence River (Figure 3.1). Traps were set in embayments at depths of 1–2 m near key vegetation and bathymetric morphology habitat features known to attract a wide variety of other fish species. Traps were checked daily from 11 May to 12 June 2006 and fish submitted for diagnostics were humanely euthanized with an overdose of MS-222 (tricaine methanesulfonate, Western Chemical Inc., Ferndale, WA) buffered with sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), placed on ice and frozen at –20 °C on land. Key species from each trap included black bass and sunfish (Centrarchidae), northern pike *Esox luscius* and Ictalurids including brown bullhead and channel catfish. A random selection (n = 6–10) of apparently healthy fish from each of these key groups were retained along with a few individuals from less common species like bowfin, *Amia calva*, and shorthead redhorse, *Moxostoma macrolepidotum*.

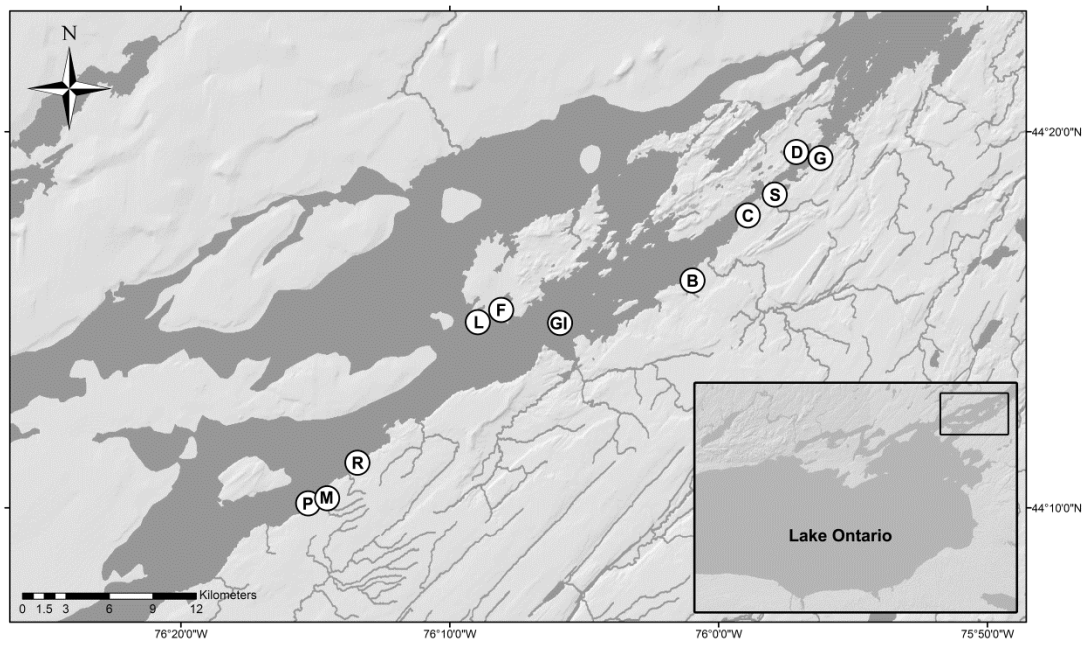

Figure 3.1: Map of the 2006 St. Lawrence River Sampling Locations. Locations are as follows from West to East:

P =	Peos Bay	(44° 10' 08.73" N, 76° 15' 03.43" W)
M =	Millen's Bay	(44° 10' 12.54" N, 76° 14' 41.88" W)
R =	Rose Bay	(44° 11' 08.76" N, 76° 13' 26.94" W)
L =	Lindley Bay	(44° 14' 57.99" N, 76° 08' 57.90" W)
F =	Flynn Bay	(44° 15' 15.03" N, 76° 08' 07.53" W)
GI =	Governor's Island	(44° 14' 51.24" N, 76° 05' 51.90" W)
B =	Blind Bay	(44° 16' 00.66" N, 76° 00' 46.56" W)
C =	Cobb Bay	(44° 17' 53.52" N, 75° 59' 02.10" W)
S =	Swan Bay	(44° 18' 20.41" N, 75° 57' 54.80" W)
D =	Densmore	(44° 19' 26.52" N, 75° 56' 55.08" W)
G =	Garlock	(44° 19' 18.00" N, 75° 56' 21.48" W)

In total, 298 fish of 15 different species were collected from a total of 11 geographic locations along the St. Lawrence River. After transport to the AAHP the fish were thawed in cool water and a necropsy was performed for the aseptic collection of selected tissues for viral assay following the standard protocol guidelines published by the American Fisheries Society (AFS 2010). Lengths and weights of fish were recorded at the time of necropsy. At the time of dissection, sex or gonadal maturity was not recorded, which limited the statistical analysis as discussed below.

Virus isolation in cell culture and viral identification. Samples of spleen, liver, heart, posterior and anterior kidney were collected for virus isolation. Tissue samples were initially prepared at 1:9 dilutions in a WhirlPak bag (Nasco, Fort Atkinson, WI) by aseptically weighing 0.5 g of tissue and adding 4.5 mL Minimal Essential Medium with Hanks' salts (Gibco®, Invitrogen, Carlsbad, CA) prepared with 5% fetal bovine serum (Gibco®, Invitrogen, Carlsbad, CA), penicillin (200 IU/mL), streptomycin (200 µg/mL), glutamine (0.584 mg/mL) (Gibco®, Invitrogen, Carlsbad, CA) and HEPES buffer (1M 0.015 mL/mL) (Gibco®, Invitrogen, Carlsbad, CA), hereafter referred to as HMEM-5. Tissues were homogenized in media using a Stomacher® 80 homogenizer (Seward, Bohemia, NY) for 2 min. The homogenized tissues were kept on ice for 10 min, and then centrifuged at 5000 × gravity for 10 min. Further dilution was achieved by pipetting off 1.0 mL of the homogenate, while avoiding the pellet, into 2.0 mL HMEM-5. Final inoculations were prepared by sterile filtration of the 1:29 dilution through 0.2 µm biological filters (Acrodisc®, Pall Life Sciences, Port Washington, NY). A 250 µL volume of the inoculum was pipetted on fresh monolayers of *epithelioma papulosum cyprini* (EPC) cell cultures (American Type Culture Collection CRL-2872; Fijan et al. 1983, Winton et al. 2010) in

48-well cell culture plates (CoStar-3548, Corning Life Sciences, Lowell, MA). Each well contained 250 µL HMEM-5 for a final tissue:homogenate inoculation dilutions of 1:59.

Inoculated cells were incubated at 15 °C and examined for the presence of cytopathic effects (CPE) at least twice a week for thirty days post inoculation, including at least one subsequent blind passage. Identity of the virus was confirmed with RT-PCR using VHSV specific primers followed by sequencing of a portion of the glycoprotein (G) gene (OIE 2009). When positive CPE was observed, samples of homogenates and original tissues when available were sent for further confirmation to USGS-WFRC and USDA-NVSL.

RNA Isolation and qRT-PCR. Total RNA was isolated from fish tissues that had been kept frozen and stored at –80 °C. The protocol used was a modification of the RNA Bee protocol (Tel-test Inc., Friendswood, TX) described by Hope et al. (2010). Briefly; the modifications were that all samples, the unknowns, the standards, and the no template control were run in at least duplicate on a MicroAmp Optical 96-well Reaction plate (Applied Biosystems, Invitrogen, Carlsbad, CA). A VHSV relevant RNA standard was made from an isolate of VHSV IVb extracted from a round goby exhibiting VHS pathogenesis in 2006 (Groocock et al. 2007) and was calibrated to the absolute values of T7-prepared viral RNA (Hope et al. 2010). Ten-fold serial dilutions of the isolated round goby standards were then used in all assays as the standard curve for determining the copy number of the VHSV IVb N gene in diagnostic samples. Positive samples were recorded as those samples that returned a positive result in the majority of replicates.

The primers and probe used were:

forward: 5'–ACC–TCA–TGG–ACA–TCG–TCA–AGG–3'

reverse: 5'–CTC–CCC–AAG–CTT–CTT–GGT–GA–3'

probe: 5'–/56–FAM/CCC–TGA–TGA–CGT–GTT–CCC–TTC–TGA–CC/36–TAMSp/–3'.

Statistical analysis. The prevalence values for each diagnostic test for VHSV in healthy fish were calculated as a direct proportion of positive samples over total number of samples tested with corresponding binomial exact confidence intervals (CI). Statistical analysis of the data obtained from this sampling effort was performed using R (R Core Development Team 2008). Univariate analyses of measured variables were first performed to examine any potential association between variables and cell culture and qRT-PCR results. After examination of the univariate results, logistic regression models were prepared to more closely examine any significant association between measured variables and disease outcome as determined by virus isolation or qRT-PCR test results.

Logistic regression models were made for predicting disease status as determined by both test methods. In both cases an initial full model was made using the variables and all biologically significant interactions. Forward, backward and stepwise selection was used to give a most parsimonious final model. In preparing final logistic regression models to further examine any potential association between measured variables, some variables were removed from the analysis. There is a confounding interaction between the variables length, weight and species, particularly when examining many fish of widely different species and body conformities. Initial attempts to stratify the data into clades of similar species resulted in no significant associations between species × length and disease status. The length and weight

variables give us little additional biological information for determining risk factors that cannot be explained by species alone in our data set.

The only extra information that would have been useful to determine from the length and weight data would be an indicator for age. Age-length keys have not been established for many species within a reasonable temporal and geographic proximity surrounding the recent introduction of round goby, which has apparently altered the length-at-age relationship in piscivores in Lake Erie (Steinhart et al. 2004, Stepanian et al. 2007). At the time of sample collection, sex or maturity was not recorded as a variable for these fish.

3.4 Results

Prevalence. VHSV was detected in 26 samples by virus isolation and subsequent RT-PCR confirmation of viral identity. Eighteen samples were not tested by virus isolation. The qRT-PCR test detected 97 positive samples out of a total of 262 tests performed. Thirty-six samples were not evaluated by qRT-PCR. This incongruity was due to the small size of some fish leading to insufficient tissue for both diagnostic tests. In the first cases there was insufficient tissue or homogenate saved for later RNA extraction and qRT-PCR analysis. In the second cases, the fish were of such small size that only RNA extraction was performed.

The prevalence results obtained by standard virus isolation methods indicate that there is a detectable prevalence of 9.3% (CI: 6.2–13.3%) of the fish sampled. The use of qRT-PCR reveals a significantly higher sample prevalence of 37.0% (CI: 31.2–43.2%).

Diagnostic test evaluation. The cross-tabular frequencies for diagnostic test results by sample and the resulting conditional probabilities were calculated and 70 samples were positive for qRT-PCR while negative for cell culture. Four samples; a bluegill, a rock bass, *Ambloplites*

rupestris, a brown bullhead, and a pumpkinseed all from the Garlock location collected on 4 June 2006, were positive by virus isolation and negative by qRT-PCR (Table 3.1). Repetitions of qRT-PCR on the RNA from these samples also returned negative results, and there was insufficient material from these fish to repeat cell culture or re-extract RNA from original tissue samples at a later date.

Conditional probabilities were calculated for prediction of test results based on the data collected. Only the 254 samples which contained information for both testing types were used. The highest probability of 97.6% was obtained for the negative predictive value; however the positive predictive value is very low at 22.2%. Sensitivity (83.3%) and specificity (69.6%) values obtained are low, however these results must be interpreted with the knowledge that the qRT-PCR test is more analytically sensitive than the "gold standard" virus isolation test (Hope et al. 2010). Hence the estimates for sensitivity and specificity were skewed by the large number of positive qRT-PCR tests which were negative by virus isolation.

Table 3.1: Cross-Tabular Frequencies by Diagnostic Test and Conditional Probabilities

		qRT-PCR		
		POS	NEG	Total
Virus isolation	POS	20	4	24
	NEG	70	160	230
	Total	90	164	254

Condition	Probability
Diagnostic Sensitivity	83.3%
Diagnostic Specificity	69.6%
Positive Predictive Value	22.2%
Negative Predictive Value	97.6%

Host range. VHSV was detected by both virus isolation and qRT-PCR tests. Species in which VHSV was detected by both tests (Figure 3.2) included black crappie (*Pomoxis nigromaculatus*), bluegill, brown bullhead, channel catfish, largemouth bass, pumpkinseed, rock bass, smallmouth bass, and yellow perch (*Perca flavescens*). In northern pike, shorthead redhorse, and white perch VHSV was detected by qRT-PCR only. All species that tested positive for VHSV by virus isolation also tested positive by qRT-PCR.

Geographic range. Seven of the 11 locations tested positive for VHSV by cell culture. An additional three locations, Densmore, Governor's Island, and Peos tested positive by qRT-PCR only. All locations that tested positive by cell culture also tested positive by qRT-PCR. The Garlock location contains the four fish described previously that have cell culture positive results but negative qRT-PCR results (Figure 3.3). Two samples which tested qRT-PCR positive did not have location information recorded and are not displayed in Figure 3.3.

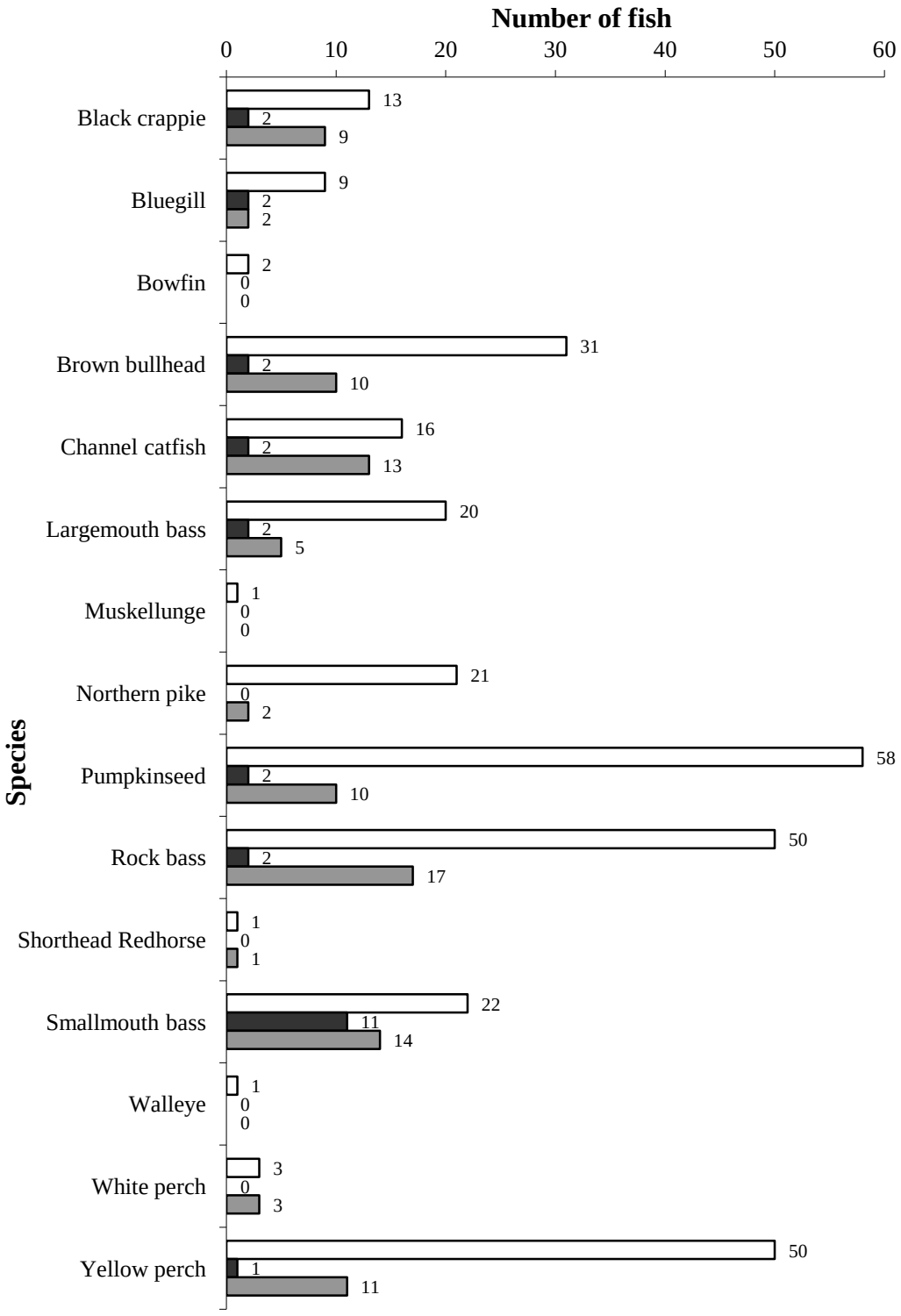

Figure 3.2: Species and number of VHSV positive fish by test.

Key: Fish collected
Cell culture positive samples
qRT-PCR positive samples

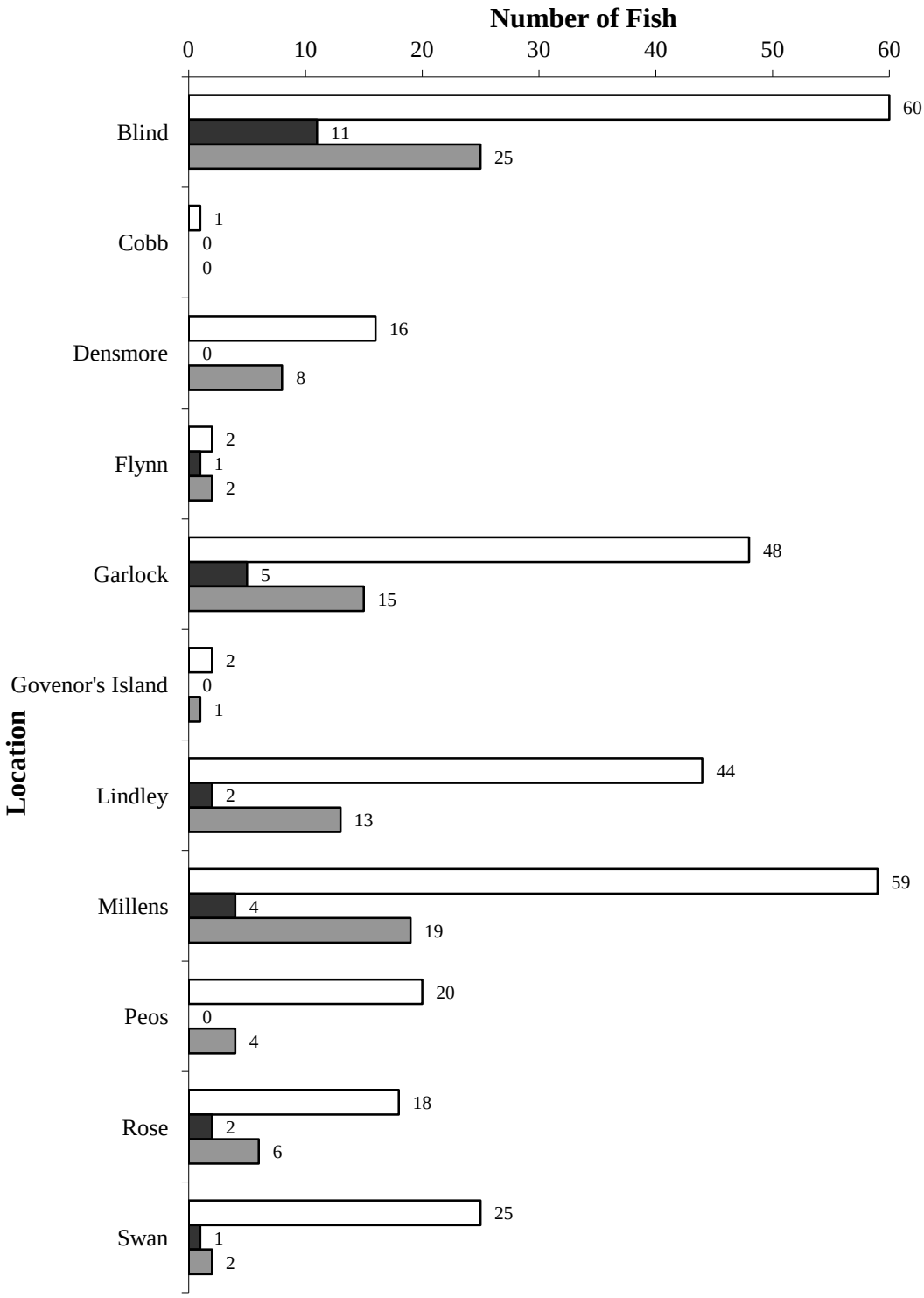

Figure 3.3: Location and number of VHSV positive fish by test.

Key: Fish collected
Cell culture positive samples
qRT-PCR positive samples

Statistical analysis and measures of association. Initial univariate analysis of the variables species, location and length showed that, when individually considered, there appears to be a correlation between some of these variables and disease status. Length and weight were not determined to be a significant predictor of disease status for either testing type when considered as individual univariates.

When disease status is determined by virus isolation, univariate analysis shows that only smallmouth bass is a significant predictor of positive disease status (Z-value 3.208, $p = 0.002$). In contrast when disease status is determined by qRT-PCR, more variables are significant when considered individually. Specifically, smallmouth bass (Z-value 3.2, $p = 0.002$), channel catfish (Z-value 3.3, $p = 0.001$), pumpkinseed (Z-value 3.4, $p = 0.001$) and the location Swan Bay (Z-value 2.4, $p = 0.02$) are significant.

The final most parsimonious logistic regression model for disease status as determined by virus isolation included only smallmouth bass as a predictor with a formula of:

$$\textit{Logit}(\textit{Positive test by virus isolation}) = -2.79 + 2.89(\text{smallmouth bass}) + \text{Error}$$

The residual deviance in this model is 143.6 on 278 degrees of freedom with an Akaike information criterion **(**AIC) of 147.6. The odds ratio determined from this model for smallmouth bass is 17.9 ($CI_{95\%} = 6.6, 49.9$).

The final most parsimonious logistic regression model for disease status as determined by qRT-PCR testing included more predictors and has a formula of:

Logit(Positive test by qRT-PCR) = –0.78 +2.65(channel catfish)
–0.49(pumpkinseed) –1.40(Swan) +2.47(smallmouth bass) +2.47(black crappie)
–1.06(Rose) +17.35(white perch) +17.35(Flynn) + Error

The residual deviance in this model is 282.4 on 253 degrees of freedom, which is not significant at the 5% level, so there is no evidence that this model does not fit. The AIC of the model is 300.4. The significant odds ratios determined from this model are channel catfish 14.3 ($CI_{95\%}$ = 3.8, 77.2), smallmouth bass 7.5 ($CI_{95\%}$ = 2.6, 25.8) and black crappie 11.8 ($CI_{95\%}$ = 2.8, 83.3).

3.5 Discussion

This retrospective analysis of the 2006 sampling effort reveals important information concerning the disease status of apparently healthy fish collected from the St. Lawrence River at a time when a concurrent outbreak was ongoing in round gobies. Traditional virus isolation methods had already elicited some information from these samples, but by using a more sensitive qRT-PCR test more insights can be determined.

The initial isolations of VHSV in bluegill, brown bullhead, channel catfish, largemouth bass, smallmouth bass and pumpkinseed that are now more fully described here were highly important for implementing regulations for VHSV in the USA. The qRT-PCR positive testing of these samples supports the confirmation of VHSV in these species.

The qRT-PCR test results clearly show that the prevalence of VHSV may be much higher than was initially determined by virus isolation. The species range for VHSV was expanded to include shorthead redhorse, northern pike, white perch and three additional locations were determined to be positive by qRT-PCR testing. The effects of a VHSV outbreak may have a greater impact on a wild population than was initially apparent. The exact effect of the virus on individual species has not yet been fully determined for many of the species collected in this survey. It is likely that there are differing levels of susceptibility to VHSV in different species and some fish may be asymptomatic carriers of the disease (Kim and Faisal 2010, Al-Hussinee et al. 2011) Since 2006, the AAHP has continued to detect VHSV in fish collected from the St. Lawrence River (Bain et al. 2010, Eckerlin et al. 2011).

Cross-tabular frequency results (Table 3.1) are often used as a simple measure of diagnostic sensitivity and specificity when comparing a new diagnostic technique to an established "gold-standard" technique. However, the advent of powerful molecular techniques has made it more difficult to interpret these results in this way due to the very high sensitivity and specificity of the molecular techniques. The detection of 70 additional samples by qRT-PCR compared to cell culture likely reflects the analytical power of the qRT-PCR test. Although other methods to determine diagnostic sensitivity and specificity in the absence of a gold standard are increasingly being used, including Bayesian methods (Enøe et al. 2000), these estimations would require a more extensive data set than is available from this one sampling collection from 2006. Thus, we report the diagnostic sensitivity and specificity as determined by this study, but the real values are likely much higher for the qRT-PCR test and require further evaluation.

The 98% negative predictive value for virus isolation in a sample testing negative by qRT-PCR is a significant finding of these results. It is important to consider though that cell culture assays for many potential viral pathogens, while this qRT-PCR specifically targets the VHSV type IVb genome. The practical implication of this high negative predictive value is that this retrospective analysis provides strong support for the use of qRT-PCR as a negative screening tool in surveillance efforts directed specifically for VHSV type IVb. Using qRT-PCR as a negative screening tool will allow for high-throughput testing for disease and allow preliminary results to be returned within a much quicker time-frame than traditional virus isolation methods. Typically a negative result for VHSV virus isolation can take 28 days, while qRT-PCR can return results within hours. In terrestrial animals, using quantitative PCR as an initial screening tool has become more commonly accepted (Pestana et al. 2010) and the application of this methodology to VHSV surveillance in wild fish populations will be very valuable.

The logistic regression models developed from this sampling effort shed more light on the potential for detecting VHSV within a wild fish population. The initial predictor of smallmouth bass having higher odds of being positive by virus isolation led to an increased focus on sampling smallmouth bass in the St. Lawrence River (Eckerlin et al. 2011) between 2007 and 2008. With the knowledge that there may be other additional species that have higher odds of testing positive for VHSV by qRT-PCR, including channel catfish and black crappie, it may be valuable to further investigate the disease status of these species in the wild population or through experimental studies. An experimental infection of channel catfish is described in Chapter 4.

In summary, this retrospective analysis has shown that examining previous collection data using more sensitive testing methods can be a highly useful strategy for adding more resolution to the dynamics of a VHSV infection in the wild. As the use of molecular surveillance for disease becomes increasingly more accepted, the potential for more retrospective analyses of a similar nature could lend valuable insight into the early years of the emergence of VHSV into the Great Lakes.

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CHAPTER 4:

*EXPERIMENTAL INFECTION OF FOUR AQUACULTURED SPECIES WITH VIRAL HEMORRHAGIC SEPTICEMIA VIRUS TYPE IVB.

*G. H. Groocock, S. A. Frattini, E. R. Cornwell, L. L. Coffee, G. A. Wooster, R. G. Getchell, and P. R. Bowser. *In review.* Experimental Infection of Four Aquacultured Species with Viral Hemorrhagic Septicemia Virus Type IVb. Journal of the World Aquaculture Society.

4.1 Abstract

Viral hemorrhagic septicemia virus (VHSV) is the etiologic agent of a disease of finfish found worldwide and is of great concern to aquaculture. In the last decade, a new VHSV genotype, IVb, was first detected in the Laurentian Great Lakes. We performed experimental infection trials with intraperitoneal (IP) injections of 10^6 pfu/fish of VHSV IVb in four aquacultured species: tiger muskellunge, ♂*Esox lucius* × ♀*Esox masquinongy*, Atlantic salmon, *Salmo salar*, channel catfish, *Ictalurus punctatus,* and walleye, *Sander vitreus*. Fish from each species were exposed to a thermal shock concurrent with the infection to cause suppression of their immune system and increase the probability of eliciting clinical signs of disease. Each species was observed for morbidity and mortality and periodic sampling was performed to detect virus using virus isolation in cell culture and a real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Histology was used to confirm lesions in tiger muskellunge and Atlantic salmon. Using immunohistochemistry, virus was found associated with some of these lesions in these two species. Tiger muskellunge were found to be highly susceptible to VHSV IVb and high levels of virus were detected in exposed fish. A single mortality was observed in exposed Atlantic salmon along with a short viral replication period. Channel catfish and walleye were found to be resistant to developing clinical signs of VHS. The results show that these species vary in the development of clinical VHS when infected IP with a known dose of virus and subjected to a concurrent thermal stress event. Using a sensitive qRT-PCR test allowed us to detect virus in exposed fish at sub-clinical levels.

4.2 Introduction

Viral hemorrhagic septicemia (VHS) is one of the most serious diseases of finfish in the Northern Hemisphere (Wolf 1998). Since its discovery in Europe in 1938 (Schäperclaus 1938), it has had a serious economic impact in farmed European rainbow trout, *Oncorhynchus mykiss*, (Skall et al. 2005). Until the late 1970s it was believed that VHS was principally a disease of farmed rainbow trout in Europe. However in the late 1980s, VHS virus (VHSV) was discovered in returning Chinook, *Oncorhynchus tshawytscha*, and Coho salmon, *Oncorhynchus kisutch*, in the Pacific Northwest of the United States (Brunson et al. 1989, Hopper 1989). Subsequently the geographic and host range of VHSV has expanded dramatically to include many marine and freshwater species and span the northern hemisphere from Japan to Europe (Snow et al. 2004, Skall et al. 2005, Kim and Faisal 2011). Currently genotypes I–III are widespread in freshwater and marine fish of Europe. Genotypes I and III have also been isolated in Japan from Japanese flounder, *Paralichthys olivaceus*, (Isshiki et al. 2001, Nishizawa et al. 2002). Genotype IV is presently restricted to the marine and freshwater environment of North America, Japan and Korea (Kim et al. 2009, Kim and Faisal 2011). In Europe, VHS continues to be a major threat to aquaculture causing fish kills, for example, in rainbow trout in the United Kingdom (Stone et al. 2008) and turbot, *Psetta maxima*, in Turkey (Nishizawa 2006).

In the last decade a new VHSV genotype, IVb, has been detected in the Laurentian Great Lakes and has caused large-scale fish kills in wild freshwater fish. In 2005 , a large die-off of freshwater drum, *Aplodinotus grunniens*, in the Bay of Quinte, Lake Ontario, was attributed to VHSV IVb (Lumsden et al. 2007). The virus was first detected in New York State during a large fish kill of round gobies, *Neogobius melanostomus*, in the St. Lawrence River in May 2006 (Groocock et al. 2007). The earliest known detection of VHSV IVb in the Great Lakes is from a

muskellunge, *Esox masquinongy*, collected in 2003 (Elsayed et al, 2006). Sequence analysis of the full glycoprotein gene showed that the Great Lakes isolate of VHSV is related to the North American Pacific isolates, but sufficiently divergent (3.6–3.7% nucleotide diversity) to propose its classification as Type IVb (Elsayed et al. 2006). Due to the increasing frequency of mortality events and detections of the virus in expanding geographic and host ranges, the United States Department of Agriculture (USDA) enacted in October 2006 an emergency order limiting the transport of live fish from the states and provinces bordering the Great Lakes.

Currently the USDA lists 28 susceptible species (USDA 2007) affected by the VHS Federal Order. The OIE lists 82 species from 16 orders affected by at least one genotype of VHSV throughout the world. Since 2006, VHSV IVb has been detected in fish from all the Great Lakes and some surrounding waterways (Bain et al. 2010, Kane-Sutton et al. 2010, Frattini et al. 2011, Cornwell et al. 2011). Although reports of large-scale fish kills in New York State received by Cornell University have subsided in recent years, VHSV remains a significant concern for some regulatory agencies. The New York State Department of Environmental Conservation (NYSDEC) has enacted regulations to limit the spread of VHSV and other fish pathogens in the wake of the emergence of VHSV (NYSDEC 2010).

The emergence of this new genotype IVb of VHSV in the Great Lakes has caused much concern for aquaculture. To date, VHSV type IVb has not been isolated in any aquaculture facility; however the threat of a positive isolation is significant and would have major repercussions in the USA. VHS is a reportable disease according to the World Organization for Animal Health (Office International des Epizooties, OIE), and there are specific responses that would go into effect if it was detected in an aquaculture facility in the United States. The detection of a related rhabdovirus that is also a reportable disease, Spring Viremia of Carp Virus,

in North Carolina in 2002 (Goodwin 2002), resulted in complete depopulation and fallowing of the ponds.

In wild freshwater fish, VHSV IVb has been detected in channel catfish, *Ictalurus punctatus,* walleye, *Sander vitreus,* and the parent species of tiger muskellunge, ♂*Esox lucius* × ♀*Esox masquinongy*, muskellunge and northern pike, *Esox lucius*. As a result, these four species are included on the list of susceptible species by both the OIE (OIE 2009) and the USDA (USDA 2007). Channel catfish and northern pike were included on the USDA list as a result of diagnostic evaluations performed at Cornell University on fish collected from the Saint Lawrence River, New York, USA, in 2006. Samples of fish tissues and virus isolation fluids from these two species were sent to the USDA and the United States Geological Survey, Western Fisheries Research Center (USGS-WFRC) for additional confirmation. VHSV IVb was independently identified by both USDA and USGS-WFRC. Isolations of VHSV in walleye were obtained from fish collected both from the Saint Lawrence River and Conesus Lake, New York, USA, in 2006 and also subsequently identified by the USDA and USGS-WFRC. To date VHSV IVb has not been detected in Atlantic salmon, *Salmo salar,* however, VHSV IVa has been isolated from this species (Traxler et al. 1995). Atlantic salmon were not susceptible when experimentally infected with European marine isolates of VHSV genotypes I–III (King et al. 2001).

To our knowledge, no previous experimental studies have been published using VHSV IVb in channel catfish, walleye and tiger muskellunge; however some infection trials have been conducted in similar species. Kim and Faisal (2010a) performed infection studies in muskellunge and found them to be highly susceptible to VHSV. They also tested Atlantic

salmon and found a low to moderate susceptibility, however, virus levels were not quantified after infection in Atlantic salmon in their study.

The goal of these studies was to determine if clinical signs of VHS disease could be elicited in four species: tiger muskellunge, Atlantic salmon, channel catfish, and walleye. We aimed to encourage the development of clinical disease by bypassing the natural immersion exposure route of VHSV and directly injecting a specific dose of VHSV into the coelomic cavity of the fish. Concurrently, the fish were subjected to a thermal shock event to cause immunosuppression. Using a temperature shock effectively suppresses a fish's immunity (Bly and Clem 1992, Le Morvan et al. 1998) and has previously been used to promote disease in fish. For example, saprolegniasis in channel catfish can be promoted by using low-temperature shock in addition to physical abrasion stress (Howe et al. 1998).

These species were chosen because their susceptibility to VHS is of particular interest to both the USDA and the NYSDEC. Channel catfish and Atlantic salmon are important commercial aquacultured species in North America. Channel catfish remain the most cultured fish species in the United States. In 2008 catfish comprised 81% of the total tons of finfish produced in the United States and 61% of the economic value (FAO 2011). Atlantic salmon production worldwide exceeded 1 million tons in 2008 with farmed Atlantic salmon accounting for >90% of the farmed salmon market (FAO 2011). In the United States, Atlantic salmon aquaculture has maintained a small (approximately 5%) but significant portion of annual finfish production (FAO 2011). Walleye and tiger muskellunge are of particular importance to the NYSDEC and other state and national natural resource agencies as both fish are intensively cultured for stocking into rivers and lakes to support recreational fishing. The tiger muskellunge hybrids are popular as an aquaculture fish for natural resource agencies for a number of reasons

including faster growth rate, ease of feeding, increased survival after hatching, and disease resistance (Hessner 1978).

In addition to investigating whether VHS could be elicited in these species, we used a recently developed real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) test (Hope et. al. 2010) to track the development of the disease. By using periodic sampling of fish exposed under conditions to promote viral replication and disease, we were able to quantify the amount of virus detected during the course of the experiment.

4.3 Materials and Methods

For all experiments the temperature of the tanks was recorded daily and water chemistry for the facility was checked at least weekly to assure normal water chemistry values. The flow rate for all tanks was maintained at 1–3 L/min for the duration of the experiments. Wastewater from the tanks was disinfected with 12.5% sodium hypochlorite to maintain a free residual chlorine concentration of at least 1.0 mg/L for at least 10 minutes before discharge into the municipal sewage lines

A stock of Michigan 2003 type VHSV isolate (isolate MI03; Elsayed et al. 2006) was previously prepared by inoculating *epithelioma papilosum cyprini* cells (Fijan et al. 1983, Winton et al. 2010) and the stock titer determined by plaque assay methods as described in the American Fisheries Society Fish Health Section bluebook (AFS-FHS 2010) using a 2% methocel overlay. The 2003 isolate of VHSV type IVb has been used in other experimental studies (e.g.: Kim and Faisal 2010a) and is the index isolate for VHSV IVb. One mL aliquots of the VHSV stock were stored at –80 °C until needed for each experiment. Sufficient aliquots to create the final 10^7 pfu/mL volume needed for giving a 0.1 mL injection to each fish for an infective dose

of 10^6 pfu/fish were thawed immediately before the start of an experiment, combined together to ensure homogeneity of the titer and diluted with Minimal Essential Medium with Hanks' salts prepared with 5% fetal bovine serum, penicillin (200 IU/mL), streptomycin (200 μg/mL), glutamine (0.584 mg/mL) and HEPES buffer (1M 0.015 mL/mL) (Gibco, Invitrogen, Carlsbad, CA), hereafter referred to as HMEM–5. A sample of the diluted VHSV in HMEM–5 used for injection was saved in each trial and a further plaque assay performed to confirm the 10^6 pfu/fish infective dose.

Tiger Muskellunge. Tiger muskellunge were obtained from the NYSDEC South Otselic Fish Hatchery, South Otselic, New York, USA. The fish had been tested as specific pathogen free for viral hemorrhagic septicemia, bacterial furunculosis, enteric redmouth, infectious pancreatic necrosis and spring viremia of carp by the NYSDEC.

Initial pilot studies were performed to assess experimental conditions for a full experimental challenge. In the first pilot experiment, forty 7-month old tiger muskellunge (total length 187–292 mm, median = 229 mm) were divided into two equal groups and maintained at 15 ± 1 °C for the duration of the pilot experiment. One group received 0.1 mL intraperitoneal (IP) injection of VHSV MI03 in HMEM-5 at a dose of 10^6 pfu/fish. The second group received a control 0.1 mL IP injection of HMEM-5. The control and exposed fish were separately maintained in a single 700 L flow-through tank for each group at 15 ± 2 °C for 6 weeks. At the end of the experiment the fish were processed as described below and samples taken for virus isolation in cell culture and histology only.

In the second pilot study ten 10-month old tiger muskellunge (total length 255–350 mm, median = 311 mm) were injected IP with a 0.1 mL dose of 10^6 pfu/fish VHSV MI03 in HMEM-5. Five tiger muskellunge were injected IP with HMEM-5 as controls. The control and exposed

fish were separately maintained in a single 700 L flow-through tank for each group at 15 ± 2 °C for one week. After one week the temperature of the water flowing into the tank was reduced to 9 ± 2 °C. Moribund and dead fish were processed as described below and samples taken for virus isolation and qRT-PCR testing only.

The full experimental trial was performed on 6-month old juvenile tiger muskellunge (total length 164–233 mm, median = 204 mm), which had been acclimatized to 15 ± 1 °C and held in 700 L flow-through tanks for two months. The fish were randomly assigned to two treatment groups of 55 fish each before the trial was initiated. Fish in the control group received a 0.1 mL IP control injection of HMEM-5. The fish then received a rapid temperature shock immediately after injection by being placed into 10 ± 1 °C water. Five fish were immediately euthanized and processed as described below for the 0 days post infection (DPI) sample. The remaining 50 control fish were randomly assigned to two 700 L flow-through tanks in equal groups of 25 fish each where they were maintained for the duration of the experiment.

The 55 fish in the exposed group were IP injected with 0.1 mL of VHSV MI03 in HMEM-5 at a dose of 10^6 pfu/fish. After receiving a similar temperature shock by being placed in 10 ± 1 °C water, five fish were immediately euthanized and processed for the 0 DPI sample. The remaining 50 exposed fish were randomly divided into equal groups of 25 fish, and placed in two 700 L flow-through tanks in a separate room from the control fish, where they were maintained for the duration of the experiment. Periodic sampling of fish was performed at 0, 1, 3, and 6 DPI until the first deaths were observed in order to track viral levels in fish with no clinical signs of disease and moribund fish. A similar number of control fish were also sampled at each time point. All fish were processed for virus isolation, qRT-PCR and selected fish from

both control and exposed groups were processed for histology and immunohistochemistry (IHC) as described below.

Atlantic Salmon. Atlantic salmon fingerlings (Little Clear strain) were obtained from the NYSDEC Adirondack Hatchery, Saranac Lake, New York, USA and acclimatized to 17 ± 2 °C for 3 months. The broodstock, from which the fish were spawned, were maintained and certified as free from specific pathogens including all those described for the tiger muskellunge as well as whirling disease, bacterial kidney disease and infectious hematopoietic necrosis.

No pilot study was performed with this species. For the experimental trial, one hundred 10-month old fish (total length 120–206 mm, median = 171 mm) were randomly assigned to two treatment groups of 50 fish and injected with a media control or 10^6 pfu/fish VHSV in HMEM-5 as described above. After injection, the fish in each group were thermal shocked by being placed immediately in 10 ± 1 °C water. Five fish were immediately euthanized and processed for the 0 DPI samples from both the control and exposed groups. The remaining 45 fish in each group were placed in a single 700 L flow-through tank for each group for the duration of the experiment. Samples were collected from all fish for virus isolation and qRT-PCR and selected fish for histology and IHC. Periodic sampling was performed at 0, 1, 3 and 7 DPI and then every 7 days until all fish were removed.

Channel Catfish. Channel catfish fingerlings were obtained from Owens and Williams Fish Farm Inc., Hawkinsville, Georgia, USA. Upon arrival, ten fish were euthanized and checked for external and internal pathogens according to AFS Blue Book methods (AFS 2010). Viral diseases, including VHSV, were tested by inoculation of diluted homogenates on cell cultures. No significant pathogens were detected in any of the fish tested, and there is also currently no history of VHSV in Georgia.

In a pilot study ten 5-month old channel catfish (total length 112–158 mm, median = 127 mm) were randomly divided equally into two groups of five fish. The exposed group was injected IP with a 0.1 mL dose of 10^6 pfu/fish VHSV MI03 in HMEM-5, while the control group were injected IP with HMEM-5. The control and exposed fish were separately maintained in a single 700 L flow through tank for each group at 15 ± 2 °C for one week. After one week the temperature of the water flowing into the tanks was reduced to 9 ± 2 °C and the colder water replaced the existing water. After one month, all the fish were euthanized with buffered MS-222 (tricaine methanesulfonate, Western Chemicals Inc., Ferndale, WA), processed as described below and samples taken for virus isolation and qRT-PCR testing only.

For the full experimental trial, 4-month old channel catfish (total length 82–145 mm, median = 105) were acclimatized at 17 ± 2 °C for 6 weeks prior to the start of the infection. A total of 62 fish were divided into a group of 32 fish which received 10^6 pfu/fish virus isolate MI03 and 30 fish which received control injections as described previously. Due to the presence of sharp fin spines these fish were lightly sedated with 50 mg/L MS-222 until minimal manual restraint was needed for injection. After injection, the fish were immediately thermal shocked by being placed in water at 10 ± 1 °C. Five fish were immediately removed from both the control and exposed groups for the 0 DPI samples. The remaining fish in each group were placed in a single 700 L flow-through tank for each group and kept there for the duration of the experiment. Periodic sampling was performed at 0, 1, 3, 7, 14 and 28 DPI. At the end of the experiment all remaining fish were euthanized and processed as described below. Organs were collected for VHSV testing by virus isolation and qRT-PCR only.

Walleye. A pilot study was performed using forty 6-month old walleye (total length 92–155 mm, median = 132 mm) that were randomly divided into two equal groups of 20 fish each and maintained at 15 ± 1 °C for the duration of the pilot experiment. The exposed group received 0.1 mL IP injection of VHSV MI03 in HMEM-5 at a dose of 10^6 pfu/fish. The control group received a 0.1 mL IP injection of HMEM-5. The control and exposed fish were separately maintained in a single 700 L flow-through tank for each group at 15 ± 2 °C for one month. At the end of the experiment the fish were processed as described below and samples taken for virus isolation in cell culture and histology only.

In the full experimental trial, one year old fish (total length 120–190 mm, median = 160 mm) fish were acclimatized for 2 weeks at 15 ± 1 °C, and then randomly divided into two equal treatment groups of 30 fish each and received a control IP injection or 10^6 pfu/fish virus isolate MI03 IP injection as described above. Fish were immediately thermal shocked by being placed in water at 10 ± 1 °C. Five fish were immediately removed from both the control and exposed groups for 0 DPI sample. The remaining fish in each group were placed in a single 700 L flow-through tank for each group and kept there for the duration of the experiment. The fish were maintained at 10 ± 1 °C for the duration of the experiment. Periodic sampling at 0, 1, 3, 7, 14 and 28 DPI and diagnostic testing was performed in the same manner as in the channel catfish trial.

Sample Collection and Processing. Clinically affected fish were classified as having significant behavioral changes associated with VHS, such as inability to maintain proper equilibrium in the water column, and visible external lesions consistent with VHS, such as external hemorrhages. Clinically diseased fish matching these criteria were euthanized with an overdose (250–300 mg/L) of MS-222 buffered with sodium bicarbonate (Sigma, St. Louis, MO). Dead fish were removed from the tank as soon as possible. Fish were then dissected and samples collected for diagnostic evaluation. Length, weight, and any visible signs of disease were recorded. Liver, spleen, heart, anterior and posterior kidney were collected for diagnostic evaluation according to the methods described in the American Fisheries Society Blue Book (AFS 2010). Individual sterile instruments were used for each fish to eliminate potential cross-contamination. The instruments were previously washed, soaked in a 10% solution of bleach (6% sodium hypochlorite, KIK International Inc., Concord, Ontario) for at least 10 minutes to degrade nucleic acids and then sterilized by autoclaving. Organ samples were divided into two or three portions for each of the diagnostic techniques used to determine VHSV status.

Virus Isolation in Cell Culture. Organs samples for cell culture and viral identification were collected as described above and processed for virus isolation with modifications as described in Groocock et al. (2007). Identity of the virus after observation of cytopathic effect (CPE) in EPC cells was confirmed with RT-PCR using VHSV-specific primers (OIE 2011).

Nucleic Acid Extraction and qRT-PCR. Organ samples were collected at dissection and immediately placed into 200 µL of RNA*Later* (Ambion, Applied Biosystems, Carlsbad, CA) in a bead-beater tube (Biospec Products Inc., Bartlesville, OK) and frozen at –80 °C until the conclusion of the experiment. The weight of the bead-bead beater tube with and without samples was recorded to determine the weight of organs sampled. At the end of the trial, total RNA from

tissues was isolated using a Qiagen RNAeasy (Qiagen Inc., Valencia, CA) kit as described in Frattini et al. (2011). Total RNA from virus isolations exhibiting CPE were extracted using the same Qiagen RNAeasy kits and following manufacturer's guidelines for cell lysates.

The qRT-PCR assay was performed on an Applied Biosystems-PRISM model 7500 sequence detector (Applied Biosystems, Carlsbad, CA) using the conditions and reaction components described in Hope et al. (2010). For each of the samples, the no template control, and at least three standards, 5 μL of the extracted RNA was loaded into three wells on a 96 well plate (Axygen, Union City, CA). A positive sample was determined as a sample that returned a result of ≥2/3 positive wells. Extracted RNA was measured using a spectrophotometer (Nanovue, GE Healthcare, Piscataway, NJ) and quantities per reaction were standardized to 1 μg of the total RNA present in each sample for the qRT-PCR testing in the full experimental trials. In the pilot studies the extracted RNA concentration was not measured and quantities per reactions are reported.

Immunohistochemistry. In fish that were sampled for IHC, portions of all five organs from each fish were placed into a histology cassette and fixed in a methanol free 3% aqueous formaldehyde solution prepared from paraformaldehyde (EMS, Hatfield, PA) which was buffered with 0.1 M sodium phosphate (Sigma, St. Louis, MO) (Ramos-Vara 2005, Webster et al. 2009). The fixed organ samples were then rinsed with phosphate buffered saline (Gibco, Invitrogen, Carlsbad, CA) and placed into 70% ethanol. Fixed organ samples were dehydrated in graded ethanol baths, cleared with xylene, embedded into paraffin blocks, sectioned at 4 μm and mounted onto positively charged Poly-l-lysine treated slides. Slides were deparaffinized in xylene, rehydrated through graded alcohols and irradiated in 0.1 M citrate solution at 800 W for 5 min after which the slides were rinsed liberally with deionized water. Endogenous peroxidases

were blocked by soaking the slides in 0.5% hydrogen peroxide in methanol for 10 min. Slides were incubated at room temperature for 5 min with successive blocking agents including avidin and biotin (Invitrogen, Camarilla, CA) and 10% goat serum with 10% non-fat dry milk, each followed by three washes in Tween 20 in tris-buffered saline (TBS; 0.01 M, pH 7.4). Slides were then incubated at 37 °C for 90 min with polyclonal rabbit anti-VHSV serum (Al-Hussinee et al. 2010) at a 1:200 dilution in phosphate buffered saline (PBS). The slides were rinsed with TBS, incubated at room temperature for 20 min with a secondary biotinylated goat anti-rabbit IgG (H+L) antibody (Vector Laboratories, Burlingame CA) at a 1:200 dilution in PBS, and given a final rinse with Tween 20 in TBS (0.01 M, pH 7.4). Slides were stained with streptavidin-peroxidase (Invitrogen, Carlsbad, CA) followed by application of AEC KIT (Invitrogen, Carlsbad, CA) then Gill's hematoxylin (Fischer Scientific, Fair Lawn, NJ) counterstain. Coverslips were applied and sealed with Fluoromount G (Southern Biotech, Birmingham, AL) aqueous mounting reagent.

Duplicate slides were treated with rabbit IgG (Vector Laboratories, Burlingame CA) at a 1:200 dilution in PBS in lieu of the primary antibody for negative IHC controls. Immunohistochemistry for VHSV was performed using the methods described on tissues from non-VHSV infected control fish (confirmed by VHSV qRT-PCR) and collected with infected fish at each DPI sampling event.

4.4 Results

Water quality parameters in the facility during all experiments were as follows; pH: 7.2–7.4, Hardness: 110–120 mg/L, Akalinity: 110–120 mg/L. Total ammonia, total chlorine, nitrite and nitrates were below detection levels. All plaque assays performed on the injection doses of VHSV in HMEM-5 confirmed the 10^6 pfu/mL infection dose.

Tiger Muskellunge. There were no mortalities observed in either the exposed or control groups of fish in the first pilot study. A single moribund fish was observed at both 7 and 15 DPI, however, no virus was detected by virus isolation in either of these two fish. No virus was isolated from any fish in the control and the exposed groups euthanized at the end of the experiment at 43 DPI. On microscopic examination, lesions were mild and included melanomacrophage hyperplasia in the spleen and kidney; lymphocytolysis in the splenic white pulp; multifocal, random, lymphocytic hepatitis; and nephrogenesis characterized by abundant compact tubules distributed throughout the renal interstitium and lined by crowded, deeply basophilic epithelial cells with high nuclear to cytoplasmic ratios. These lesions are consistent with chronic antigenic stimulation and previous necroinflammatory insults in the liver and kidney. No significant lesions were observed in the control fish.

In the second pilot study, two fish died at 11 DPI and another 3 fish died at 12 DPI. Gross lesions consistent with VHS included multifocal dermal hemorrhages, hemorrhages at the base of fins, intramuscular hemorrhages in the skeletal muscle of the body walls, and hemorrhages on the internal organs including liver, gastrointestinal tract and lining of the swim bladder. VHSV was isolated and identified from all 5 dead fish and one of the remaining 5 exposed fish that were euthanized at the end of the experiment. Results of the qRT-PCR testing showed high levels of viral RNA detected in the 5 dead fish (8.2×10^5–1.4×10^7 viral RNA

copies detected, mean = 6.6×10^6). The five remaining exposed fish euthanized at the end of the experiment also had detectable viral RNA but at much lower levels (3.5–382.1 viral RNA copies detected, mean = 178.7).

In the full experimental trial, the first death in the exposed group of fish occurred at 6 DPI. Mortality increased in both tanks over the next several days, with 16 fish dead in tank 1 and 17 dead in tank 2 by 10 DPI. By 14 DPI, cumulative mortality had reached 85% and the experiment was ended as all remaining fish showed signs of clinical disease (Figure 4.1).

Kaplan-Meier survival estimates at 13 DPI indicated a tank 1 survivorship of 20% (95% confidence interval: 8.9%, 48.1%) and a tank 2 survivorship of 10% (95% confidence interval: 2.7%, 37.2%) (R version 2.13.0, 2011). The overlapping confidence intervals indicate no significant difference in survivorship between the two tanks.

Gross lesions consistent with VHS were similar to those elicited in the second pilot study and included hemorrhages along the ventral skin and at the base of the fins (Figure 4.2A). Internally, foci of hemorrhage were scattered throughout the gill lamellae, skeletal muscle, swim bladder, peritoneum (Figure 4.2B), and less commonly within the gastrointestinal mesentery and retrobulbar connective tissue. The posterior kidney was swollen and friable in 15 fish which died between 6 and 9 DPI. No clinical signs, gross lesions or deaths were observed in the control group.

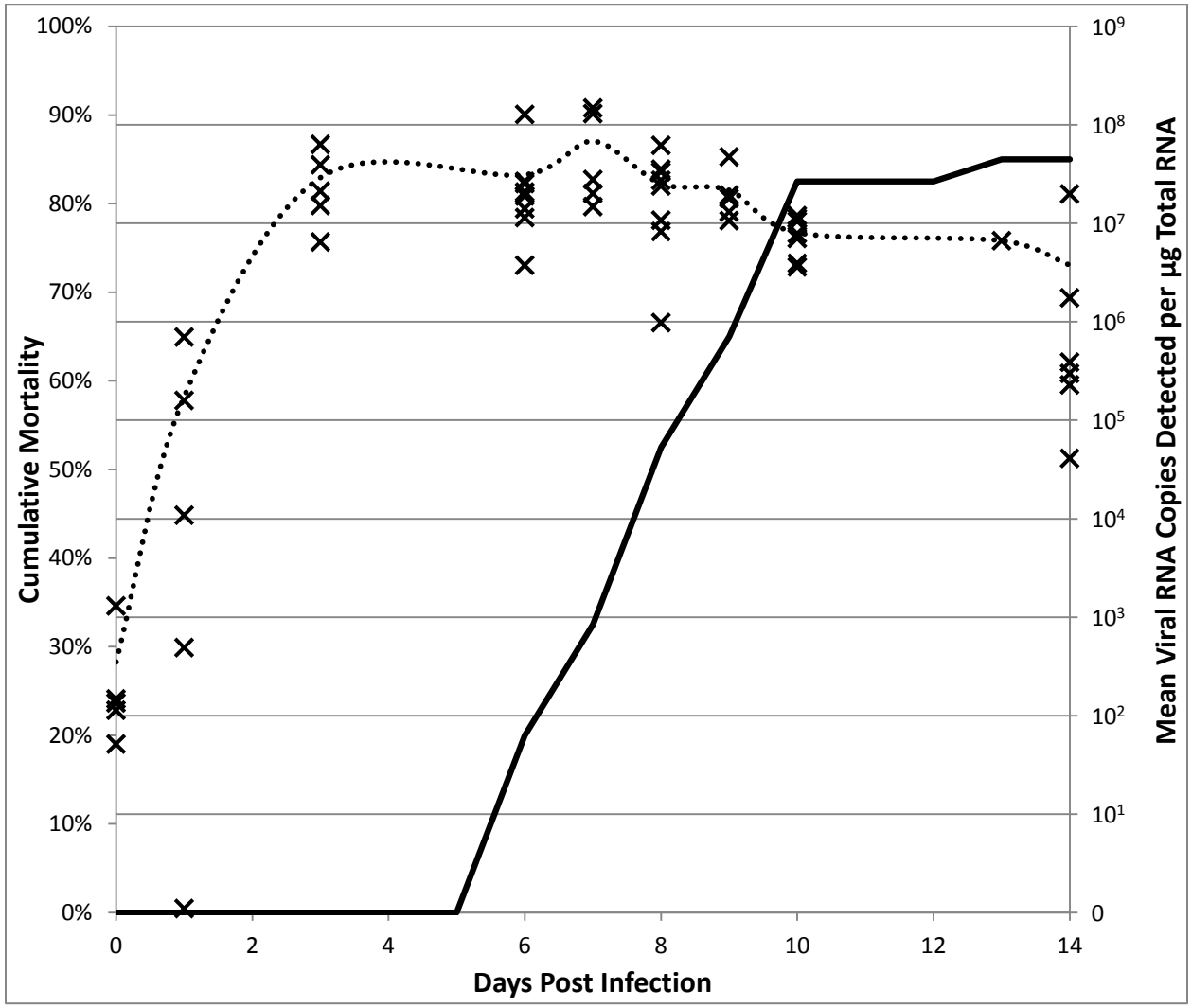


Figure 4.1: Mortality curve of tiger muskellunge VHSV challenge. The primary axis plots the cumulative mortality in exposed fish (solid line). The secondary axis plots the mean viral RNA copies detected per µg total RNA in each fish (X) and the mean over time (dotted line). Virus was successfully isolated from all exposed fish. No mortalities were observed and no virus was isolated from any control fish.

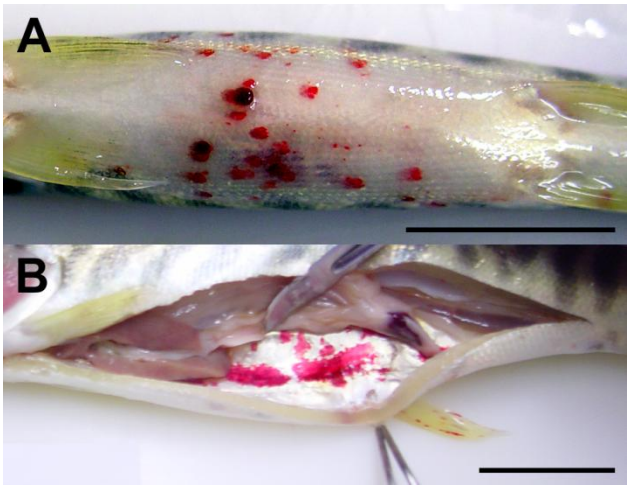


Figure 4.2: Tiger muskellunge lesions.

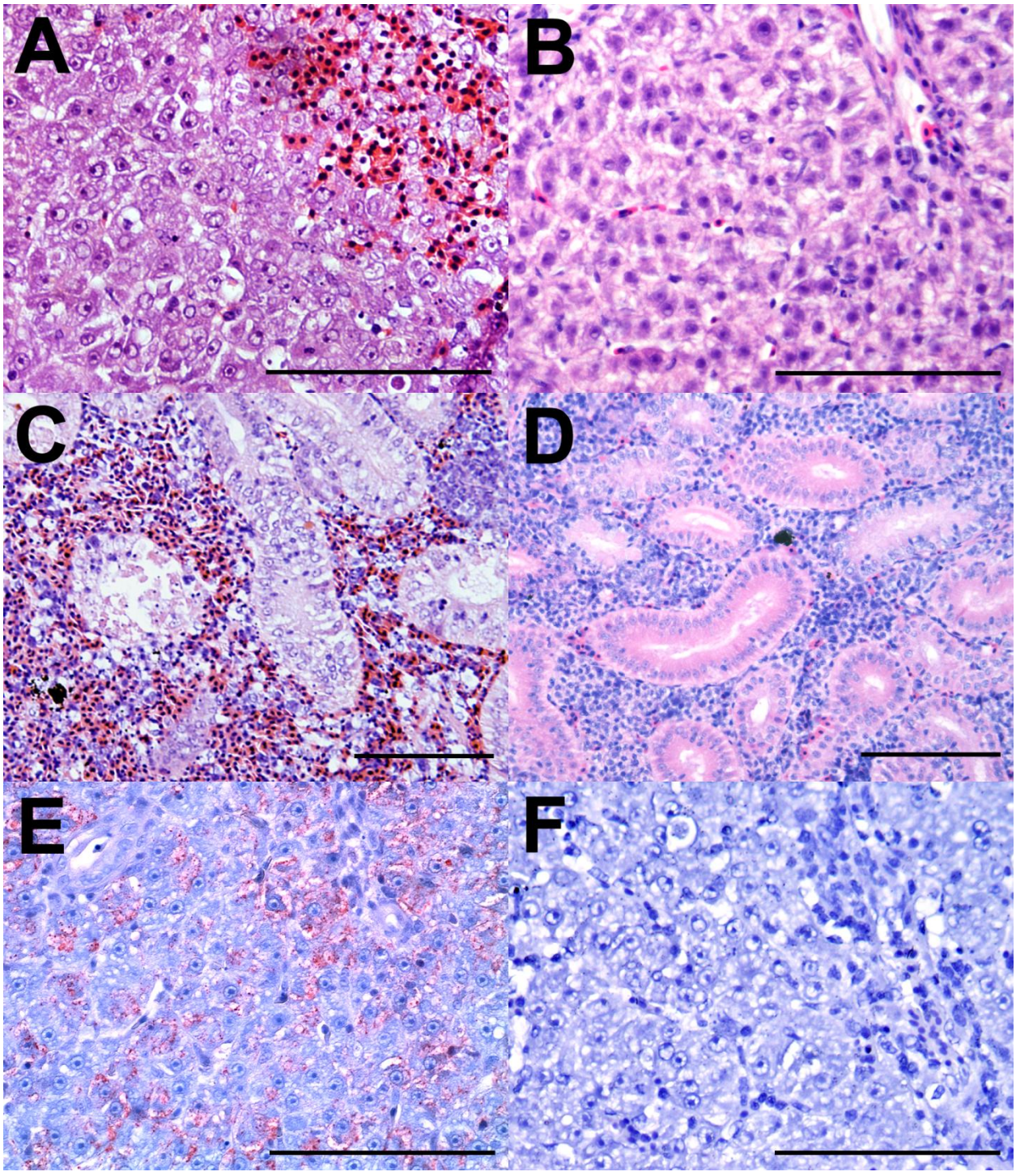
A) Hemorrhages on the ventral and lateral surfaces of a tiger muskellunge exposed to VHSV; bar = 2 cm.
B) Multiple hemorrhages on the peritoneal lining in a tiger muskellunge exposed to VHSV; bar = 2 cm.

The dominant microscopic lesion in VHSV infected tiger muskellunge was systemic necrotizing panvasculitis with hemorrhagic necrosis in multiple organs. Vascular lesions were characterized by endothelial damage, subintimal edema and fibrinoid necrosis of vessel walls characterized by minimal karyorrhectic leukoclastic debris. In the liver, regional foci of hepatocellular necrosis and parenchymal loss were frequently replaced by pools of hemorrhage (Figure 4.3A). Marked lymphocyte depletion and lymphocytolysis effaced the splenic white pulp. The posterior renal interstitium was diffusely necrotic and contained large areas of hemorrhage that entrapped necrotic renal tubules (Figure 4.3C). Generalized lymphocytic and granulocytic epicarditis was present in varying degrees of severity and infrequently progressed to pancarditis with scattered necrotic cardiac myocytes characterized by loss of internal cross-striations and pyknotic to karyorrhectic nuclei. No significant microscopic lesions were observed in the control fish (Figure 4.3B and Figure 4.3D).

The most reliable organ for immunohistochemical detection of the virus was liver, where strong immunostaining in endothelial cell cytoplasm and sloughed intrasinusoidal cells corresponded with previously described areas of parenchymal necrosis (Figure 4.3E). In all fish tested by IHC, positive immunostaining correlated to high levels of viral RNA detected by qRT-PCR (1.76×10^6–1.28×10^8 viral RNA copies detected per μg RNA, mean = 4.82×10^7). In duplicate sections, immunostaining was not detected using rabbit IgG with AEC label and hematoxylin counterstain (Figure 4.3F).

Figure 4.3: Microscopic lesions in tiger muskellunge and VSHV immunohistochemistry.

A) In a section of liver from a VHSV infected tiger muskellunge collected at 10 DPI regional hepatic necrosis is characterized by islands of degenerative hepatocytes isolated in areas of hemorrhage; hematoxylin and eosin, bar = 100 μm.
B) A section of liver from a control tiger muskellunge collected at 10 DPI shows no significant lesions; hematoxylin and eosin, bar = 100 μm.
C) In a section of kidney from a VHSV infected tiger muskellunge collected at 10 DPI, multiple necrotic tubules are surrounded by areas of interstitial hemorrhage containing karyorrhectic debris and devoid of recognizable hematopoietic cells (hematopoetic necrosis); hematoxylin and eosin, bar = 100 μm.
D) A section of kidney from a control tiger muskellunge shows no significant lesions; hematoxylin and eosin, bar = 100 μm.
E) Viral immunostaining in a section of liver from a VHSV infected tiger muskellunge collected at day 10 DPI in areas of hepatic necrosis is indicated by red, punctuate staining in the cytoplasm of both hepatocytes and sinusoidal endothelial cells and throughout intrasinusoidal debris; rabbit-anti-VHSV antibody with AEC label and hematoxylin counterstain, bar = 100 μm.
F) In a duplicate control section of the same fish above (3E), immunostaining is not detected; rabbit IgG with AEC label and hematoxylin counterstain, bar = 100 μm.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The periodic sampling qRT-PCR results showed an increase in the VHSV quantity detected in the fish prior to the onset of clinical signs and mortality. Initial viral quantities were low and were variable in the 5 fish sampled at 1 DPI. By 3 DPI, all 5 fish sampled had very high levels of virus, but no deaths or clinical signs had been observed. During the acute mortality phase of the experiment, all fish continued to have very high levels of virus. At the conclusion of the experiment, virus quantities were still high in all fish (Figure 4.1). No virus was detected by qRT-PCR in any of the control fish. Virus was detected by virus isolation in cell culture in all the exposed fish and none of the control fish. Viral identity of VHSV was confirmed by RT-PCR and electrophoresis in all CPE positive cell cultures (data not shown).

Atlantic Salmon. A single death was observed in the exposed group at 8 DPI (Figure 4.4). Kaplan-Meier survival estimates at the end of the experiment indicated a survivorship of 97.5% (95% confidence interval: 92.8%, 100%; R version 2.13.0, 2011). Gross lesions consistent with VHSV were observed in 54% of exposed fish sampled between 7 DPI and 14 DPI. At necropsy, these fish had serosanguinous ascites, moderate to severe, coalescing hemorrhages in skeletal muscle and throughout the peritoneum, and diffuse hepatosplenomegaly with marked congestion and variable loss of stromal architecture (necrosis) in both organs. A single fish at 35 DPI showed similar gross lesions. No clinical signs of disease, gross lesions or deaths were observed in the control group.

Periodic sampling qRT-PCR results showed a variable amount of detectable virus in the fish sampled (Figure 4.4). At 0 and 1 DPI virus was detected in all 5 fish and virus levels were relatively low. At 3 DPI, virus was detected in 4 of the 5 fish and levels remained low. At 7 DPI, virus was detected in 4 of 5 fish again, and levels had increased in 3 of these fish to

approximately 10^7 copies/μg total RNA. After 7 DPI, virus levels decrease with only low levels being detected at later sample points.

In exposed fish with gross lesions consistent with VHSV, microscopic lesions included massive splenic necrosis and hemorrhage with significant loss of resident myeloid and lymphoid progenitor cell populations (Figure 4.5A). Moderate cell-poor, necrotizing phlebitis was prominent throughout liver and posterior kidney, where affected venules were centered within zones of parenchymal necrosis and mild hemorrhage (Figure 4.5C). No lesions were observed in the anterior kidney or heart in any of the fish examined by histopathology. No significant lesions were seen in any control fish examined microscopically (Figure 4.5B and Figure 4.5D).

In exposed fish with VHSV-associated lesions, moderate but consistent positive viral immunostaining was detected in the cytoplasm of endothelial cells and pigmented macrophages lining remnant splenic cords (Figure 4.5E). In duplicate sections, immunostaining was not detected using rabbit IgG with AEC label and hematoxylin counterstain (Figure 4.5F).

Virus was isolated from fish taken at each sampling day except the final 49 DPI sampling. Successful recovery of virus from these fish, however, was variable in that not all fish sampled at a time-point were positive by virus isolation and it did not necessarily correlate with the qRT-PCR result in the same fish (Figure 4.3). All qRT-PCR and virus isolation results were concordant until 14 DPI. After 14 DPI there were four fish that tested positive by qRT-PCR only and six fish that tested positive by virus isolation only. Viral identity of VHSV was confirmed by RT-PCR and electrophoresis in all CPE positive cell cultures (data not shown).

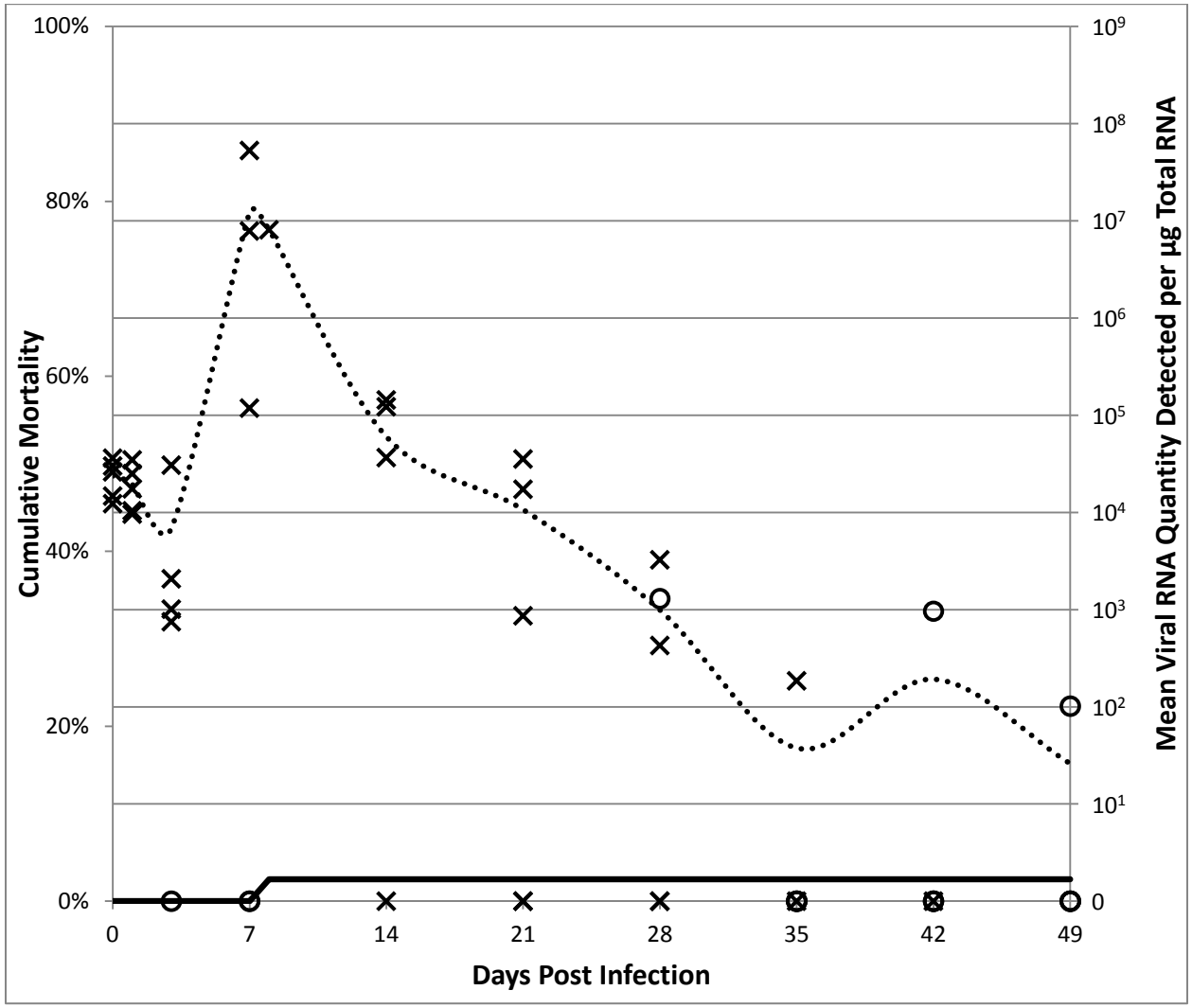
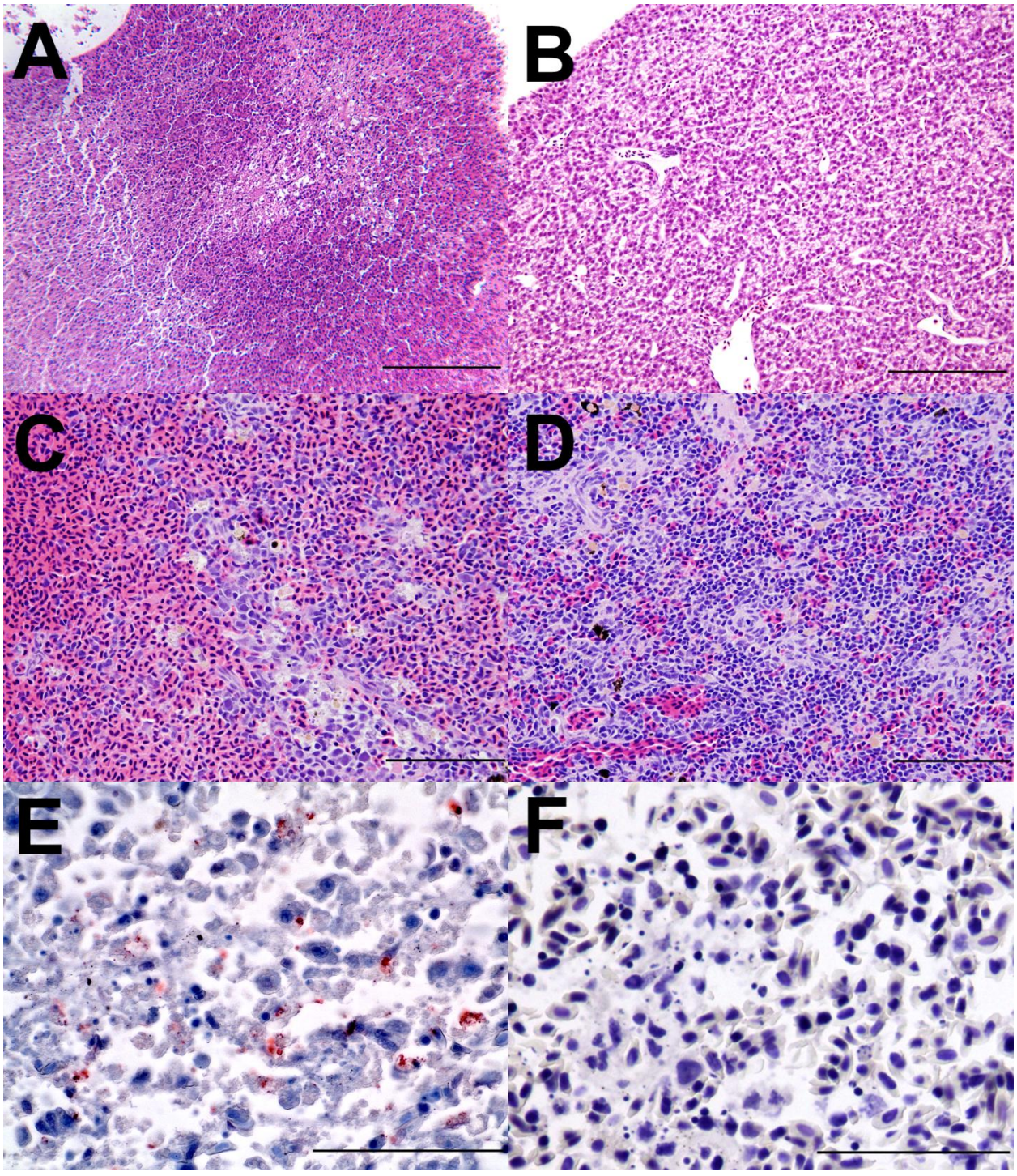


Figure 4.4: Mortality curve of Atlantic salmon VHSV challenge. The primary axis plots the cumulative mortality in exposed fish (solid line). The secondary axis plots the mean viral RNA copies detected per µg total RNA in each fish from which virus isolation was successful (X) or not (O) and the mean over time (dotted line). No mortalities were observed and no virus was detected from any control fish.

Figure 4.5: Microscopic lesions in Atlantic salmon and VSHV immunohistochemistry.

A) In a section of spleen from a VHSV infected Atlantic salmon collected at 7 DPI, replacing the lymphoid follicles, red pulp cords and hematopoetic progenitor compartment are coalescing areas of hemorrhage containing granular, eosinophilic and karyorrhectic debris (splenic necrosis); hematoxylin and eosin, bar = 100 μm.
B) A section of spleen from a control Atlantic salmon collected at 7 DPI shows no significant lesions; hematoxylin and eosin, bar = 100 μm.
C) In a section of liver from a VHSV infected Atlantic salmon collected at 7 DPI there is a well-demarcated area of coagulative necrosis typical of a vasculitis-associated lesion; hematoxylin and eosin, bar = 200 μm.
D) A section of liver from a control Atlantic salmon collected at 7 DPI shows no significant lesions; hematoxylin and eosin, bar = 200 μm.
E) Positive viral immunostaining in a section of spleen from a control Atlantic salmon collected at 7 DPI is indicated by red, punctuate staining in the cytoplasm of remnant interstitial and endothelial cells and throughout the cellular debris; rabbit-anti-VHSV antibody with AEC label and hematoxylin counterstain, bar = 100 μm.
F) In a duplicate section of the fish above (5E), immunostaining is not detected; rabbit IgG with AEC label and hematoxylin counterstain, bar = 100 μm.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Channel Catfish. In the pilot study no clinical signs, gross lesions, or deaths were observed in either the control or exposed group. At the end of the experiment, VHSV was isolated from a single fish from the exposed group. Low levels (182–3,300 viral RNA copies) of VHSV were detected in all five exposed fish. No VHSV was detected in any of the control fish by qRT-PCR or virus isolation.

In the full experiment, no deaths were observed in either the exposed or the control groups of fish. A single exposed fish, sampled at 3 DPI, had generalized mesenteric edema and multifocal congestion throughout the gastrointestinal serosa. This fish was positive for VHSV detection by both virus isolation and had a high level of detectable viral RNA by qRT-PCR (2.16×10^5 viral RNA copies per 50 ug total RNA).

Periodic sampling qRT-PCR results showed a non-significant (ANOVA $p = 0.6$) increase in the mean quantity of virus detected at 1, 3 and 6 DPI. Virus quantities subsequently decreased and by 28 DPI no virus was detected (Figure 4.6). Virus was only isolated and confirmed from two fish in the experimental group, a single fish at 1 DPI and the previously described 3 DPI fish.

Walleye. In the pilot study, no clinical signs or deaths were observed in any fish in either the control or the exposed groups. No VHSV was isolated from any fish in either group. No significant lesions were observed by histological examination in either the exposed or control groups.

In the full experimental trial clinical signs of disease were not observed in any fish from either group. Periodic sampling qRT-PCR results returned a negative result for the majority of fish at the sampled time points. Only two fish at 7 DPI returned positive results, but both fish had high levels of virus. No virus was isolated from any fish at any time point (Figure 4.7).

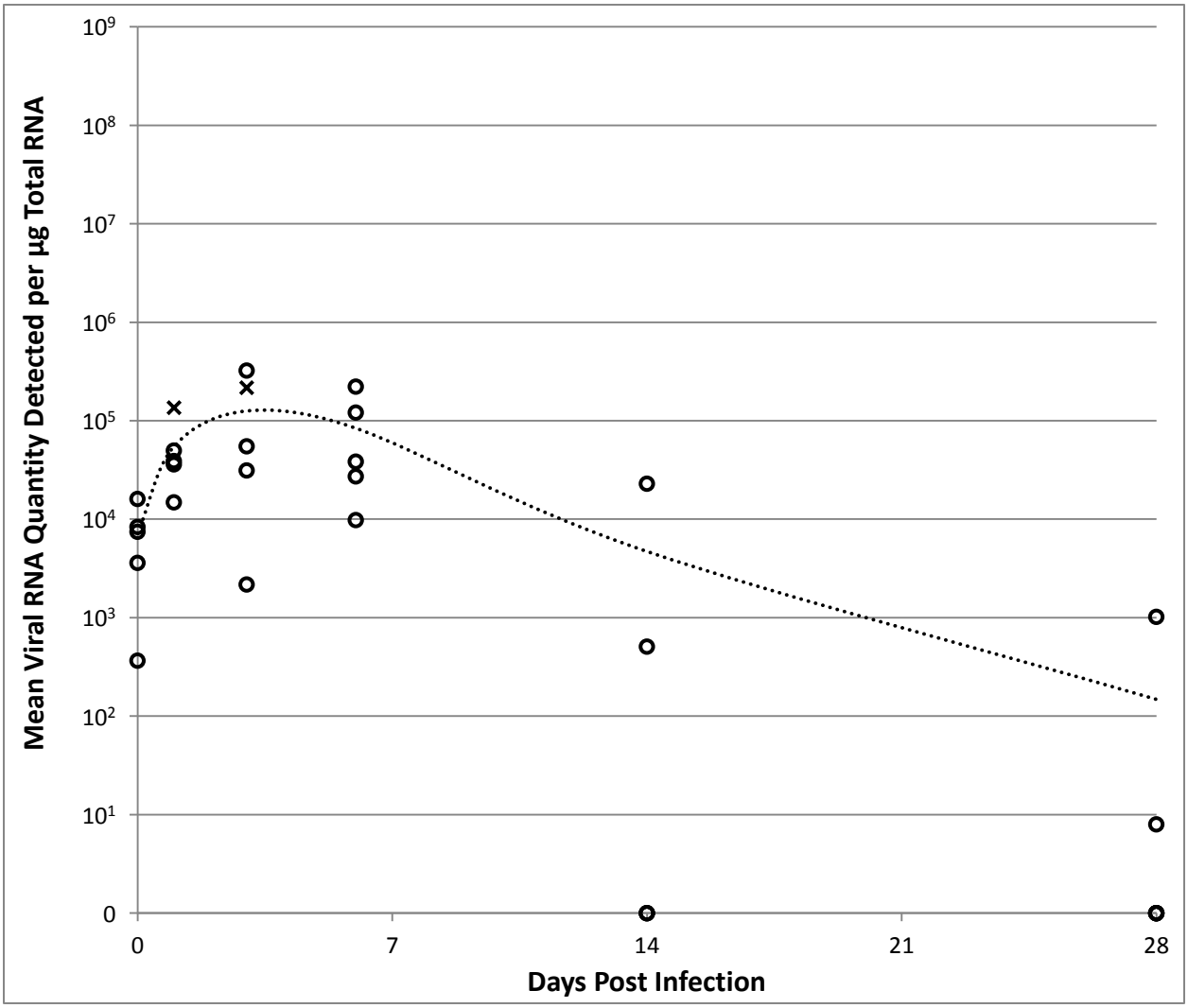

Figure 4.6: Mean viral RNA copies detected per µg total RNA in exposed channel catfish when virus isolation was (X) and was not (O) successful. No virus was detected from any control fish. The dotted line shows the mean change in virus quantity over time.

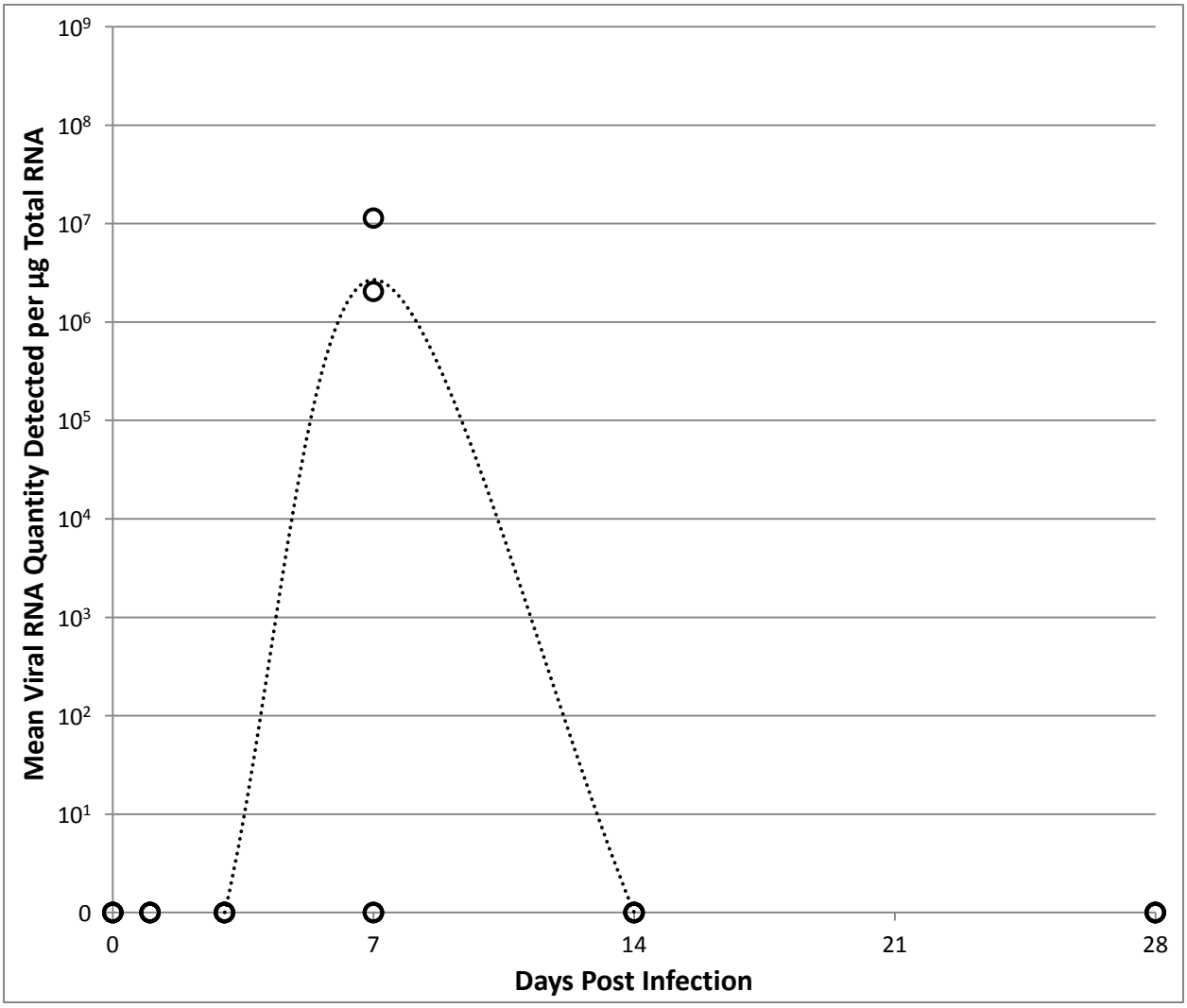

Figure 4.7: Mean viral RNA copies detected per µg total RNA in exposed walleye. No virus was isolated from any fish. The dotted line shows the mean change in virus quantity over time. No virus was detected from any control fish.

4.5 Discussion

These experiments show that the ability to elicit VHS varies by species when given an injection of a known dose of VHSV. Of the four species tested, VHSV was most pathogenic in tiger muskellunge. These results are not surprising considering that previous studies performed with muskellunge (Kim and Faisal 2010a, Kim and Faisal 2010b) showed that this species is very sensitive to VHSV genotype IVb. Based on the results of these studies, tiger muskellunge can be considered highly susceptible to VHSV type IVb under specific experimental conditions.

The tiger muskellunge experiments described here had the highest mortality, the greatest number of positive VHSV detections by both virus isolation and qRT-PCR tests, and the most severe gross and histopathological signs of VHS. Microscopically, lesions were most severe in the liver and kidney. Using IHC we were able to confirm the presence of the virus in areas of hepatic necrosis. In these areas, positive viral immunostaining was detected in the cytoplasm of sinusoidal endothelial cells and hepatocytes. Detection of viral immunostaining in the kidney, however, was impeded by the presence of background staining. There was apical immunostaining in the renal tubules in areas without tubular damage. This type of background staining implies the persistence of cytoplasmic biotin in renal tubules despite the use of mechanisms to control endogenous biotin.

The first pilot study performed showed that the tiger muskellunge were much more resistant to IP injection with 10^6 pfu/fish when maintained at 15 C and without experiencing a thermal shock. When a thermal shock was added into the experimental design in the second pilot study and the full experiment then the mortality rate increased. Unfortunately, due to facility limitations on the number of tanks needed, a concurrent study to the full experimental

trial without using a thermal shock was not performed, so we cannot elaborate on the role that the thermal shock is playing on the development of VHS in tiger muskellunge.

By using a very analytically sensitive test such as qRT-PCR we were able to obtain additional information about the disease status in these fish even in the absence of clinical signs of disease or positive results by virus isolation testing. Atlantic salmon appear to be only slightly susceptible to VHSV IVb; however, pathologic changes were noted both grossly and microscopically in some fish (Figure 4.5A and Figure 4.5C). Interestingly there was evidence of viral replication in these fish early in the course of the infection trial, but this replication did not persist at high levels and the amount of detectable virus diminishes over time. Virus isolation and qRT-PCR results were not always concordant. For the samples that were qRT-PCR positive but virus isolation negative, the low levels detected (101–1295 viral RNA copies detected per μg RNA) by qRT-PCR may be below the limit of detection by virus isolation. In the samples that were virus isolation positive but qRT-PCR negative, there may be inhibition of the enzymes used in the qRT-PCR test. Inhibition of this qRT-PCR test has been reported previously and may cause false negatives results (Cornwell et al. 2011).

Both channel catfish and walleye appear to be much more resistant to VHSV infection. In channel catfish, there is evidence that virus replicates and persists in the fish for a few weeks (Figure 4.6). The viral levels detected in channel catfish by qRT-PCR in the first 7 DPI showed that a higher amount of viral RNA was recovered from the fish tissue than was injected at the initial exposure. Further experiments to examine shedding of virus in catfish and replication within the fish would be necessary to conclusively prove replication in channel catfish. The walleye experiment is more difficult to interpret, however, these experiments provide evidence

that the virus may be replicating in the fish at an early (7 DPI) time point, but that the infection is quickly cleared and does not persist (Figure 4.7).

In these experiments light microscopy was limited to the organs collected for VHSV testing by both virus isolation and qRT-PCR testing. At the time of the experiments we were particularly interested in correlating the results of virus detection between the two testing types and supporting these detections with histopathological findings in the organs tested. To fully examine the pathology of VHS in these species, additional histopathology would be needed of other organs, particularly the brain, where pathology due to VHSV IVb has been described (Lumsden et al. 2007). Further experiments would be needed to fully determine the pathology of VHSV in these species. Additionally, histopathology was not used to determine pathology in the channel catfish and the walleye. This has limited our interpretation of the results of both these experiments. Replicate tanks were not used for the Atlantic salmon, channel catfish and walleye experiments, which limit the interpretation of the results since there are no controls for in-tank effects. The results of the experiments as we have reported them here emphasize the need for further more intensive studies to fully investigate the pathogenesis of VHSV genotype IVb in these species.

It is clear that when high morbidity and mortality are seen in a fish population, virus isolation testing methods are equally as sensitive as qRT-PCR testing. In our tiger muskellunge experiment there was complete concordance between the virus isolation results and the qRT-PCR results (kappa = 1). As the severity of disease and the quantity of virus in the tissue decrease, the ability of virus isolation to detect viable VHSV decreases significantly while qRT-PCR methods are still able to detect low levels of viral RNA. In the Atlantic salmon trial which had the most variability between results, the testing methods showed moderate agreement

between testing methods (kappa = 0.5, SEM: 0.1). Concordance between the two testing methods continues to decrease as clinical signs and detectable virus decreases, as shown in the channel catfish (kappa = 0.04 SEM: 0.03) and walleye (kappa = 0) experiments. These trials have demonstrated in the early stages of infection, virus isolation is equally proficient at detecting VHSV, but as infection progresses then VHSV is detected more by qRT-PCR. It is unknown whether the virus detected by qRT-PCR at this stage is inactivated or at such a low concentration that it is unable to be detected by virus isolation. These experiments highlight the advantage of using sensitive molecular testing in detecting low levels of VHSV viral RNA before the onset of clinical disease and in clinically normal fish.

These experimental studies reinforce the need for further testing of species susceptibility to VHSV IVb in controlled experimental trials. The detection of VHSV in exposed fish that were clinically normal and the evidence from our qRT-PCR results that the virus is replicating in these species emphasize the need for specific species susceptibility studies to examine both the pathogenesis of VHSV IVb in the fish and the ability of VHSV IVb to replicate and shed in these species.

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CHAPTER 5:

*IODOPHOR DISINFECTION OF WALLEYE EGGS EXPOSED TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS TYPE IVB

*G. H. Groocock, R. G. Getchell, E. R. Cornwell, S. A. Frattini, G. A. Wooster, P. R. Bowser, and S. R. LaPan. *In review*. Iodophor Disinfection of Walleye Eggs Exposed to Viral Hemorrhagic Septicemia Virus type IVb. North American Journal of Aquaculture.

5.1 Abstract

Two experiments were performed to determine the persistence of viral hemorrhagic septicemia virus (VHSV) genotype IVb on walleye, *Sander vitreus*, eggs. Fertilized walleye eggs were exposed for 30 min to 10^5 plaque forming units/mL VHSV genotype IVb (isolate Michigan 2003) and control eggs exposed to a media placebo. The exposed eggs were then treated with 0 (control), 50 and 100 mg/L iodophor as a disinfectant. The eggs were incubated at 12 ±1 °C in a constructed hatching assembly until the first fry emerged. Periodic samples were taken during the development of the embryos and tested for the presence of VHSV by both virus isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The effects of tannic acid, used for surface de-adhesion of eggs and an inhibitor of qRT-PCR testing was evaluated in the second experiment. In both experiments no virus was detected in any placebo exposed eggs by virus isolation or qRT-PCR. In the first experiment, virus was isolated in 0 mg/L iodophor treated eggs up to three days post infection (DPI). Virus was also isolated in the 50 mg/L iodophor treated group at 1 DPI. Testing by qRT-PCR detected viral RNA at many time-points throughout the first experiment, including the end of the experiment. In the second experiment, no VHSV was isolated after the initial 0 DPI sample. Viral RNA was detected again at many time-points throughout the second experiment and at samples collected at the end of the experiment. Tannic acid inhibition of the qRT-PCR testing was found in many samples at earlier time-points. These experiments show that VHSV may persist on exposed walleye eggs for longer than was previously known and reinforces the need for careful disinfection of eggs.

5.2 Introduction

Viral hemorrhagic septicemia (VHS) is a significant disease of finfish worldwide (Wolf 1998). It is a reportable disease to the World Organization for Animal Health (Office International des Epizooties, OIE) because of its potential to cause large economic losses in aquaculture facilities (OIE 2009). The earliest detection of VHS virus (VHSV) in the Laurentian Great Lakes was isolated from a muskellunge, *Esox masquinongy*, collected in 2003 (Elsayed et al. 2006) and sequencing confirmed its allocation to a new genotype denoted VHSV IVb. The subsequent emergence of VHSV in the Great Lakes and surrounding waterways (Kim and Faisal 2011) was very concerning for regional natural resource agencies. This new VHSV genotype IVb was responsible for large fish kills in wild freshwater fish in the Great Lakes (Lumsden et al 2007, Groocock et al. 2007). In October 2006, the United States Department of Agriculture (USDA) implemented a federal order restricting the transport of live fish of susceptible species into other states in the USA from the states and provinces surrounding the Great Lakes (USDA 2007). This list of susceptible species included walleye, *Sander vitreus*, based on isolations and identification of VHSV from affected fish collected from the St. Lawrence River and Conesus Lake (USDA 2007).

The emergence of VHSV IVb highlighted the need to determine whether current disinfection protocols were effective in preventing the introduction of VHSV into a hatchery through egg collections. Currently there is no direct evidence of true vertical transmission of VHSV of any genotype. In rainbow trout, *Oncorynchus mykiss*, VHSV genotype Ia exposed eggs failed to have any detectable VHSV at 4 weeks post-infection (Munro and Gregory 2010). Separately, VHSV was found to replicate in the efferent duct and some interstitial cells of the ovary. This viral replication triggered a strong chemokine response in the ovary, which has been

suggested as a reason why VHSV is unable to be vertically transmitted (Chaves-Pozo et al. 2010). However, the virus has been shown to be detectable and stable in shed ovarian fluid (Kocan et al. 2001) and is implicated as a source of horizontal transmission. In freshwater drum, *Aplodinotus grunniens*, VHSV IVb was detected in the oocytes by immunohistochemical staining specific for viral antigen (Al-Hussinee et al. 2011a). Further studies by Al-Hussinee et al. (2011b) using in-situ hybridization have detected VHSV IVb RNA in the ovaries of fathead minnows, *Pimephales promelas*, rainbow trout, and the gonads of muskellunge. Viral RNA was detected mainly surrounding yolk vacuoles or adjacent to the germinal vesicle. In the muskellunge, viral RNA was detected surrounding the primary and secondary spermatocytes. Additionally, VHSV is known to be very stable in unfiltered freshwater, with an average time for a three log reduction in infectivity of 21 d at 10 °C and 16 d at 15 °C (Hawley and Garver 2008). We have speculated a possible egg-associated transmission method for VHSV, in which infected broodstock shed virus with eggs or sperm at spawning and the virus sticks to the surface of the egg. Upon hatching, the fry may be infected by the virus present on the egg surface. In this scenario the importance of proper egg disinfection for VHSV is paramount.

Walleye culture is an important component of sport fishing population maintenance for many natural resources agencies in the USA. In many facilities, wild brood walleye are obtained, gametes collected and the fertilized eggs raised in a hatchery facility. To prevent the introduction of fish pathogens into the aquaculture facility, various disinfection protocols have been recommended. Most involve the use of soaking the eggs in a concentration of disinfection solution that provides anti-viral, anti-bacterial and anti-fungal properties (Leary and Peterson 1990, Fowler and Banks 1991, Erdahl 1994). In New York State, the New York State Department of Environmental Conservation (NYSDEC) instituted an egg disinfection protocol

requiring placing fertilized walleye eggs in a 50 mg/L solution of iodophor for 60 min immediately following fertilization. Low levels of iodine concentration were sufficient to disinfect other rhabdoviruses, such as infectious hematopoietic necrosis virus (Batts et al. 1991).

Recent studies have more closely evaluated the use of iodophor disinfection on walleye eggs survival. The survival of walleye embryos and viability of walleye larvae were not significantly compromised when using a disinfection protocol of 250 mg/L treatment of iodine for 15 min (Dabrowski et al. 2009). However, using 100 mg/L iodine and a 30 min treatment resulted in a reduced survival compared to controls or 15 min treatment (Dabrowski et al. 2009). In another study, the use of 100 mg/L iodine for 30 min resulted in no significant difference in survival compared to control walleye eggs (Bowzer et al. 2011).

Walleye eggs, similar to many species, have a sticky jelly layer which allows the eggs to stick to each other and the substrate. In naturally reproducing populations of walleye, this is advantageous as it promotes the formation of a defined egg mass. In egg culture, however, this sticky layer prevents eggs tumbling freely in hatching jars and promotes fungal infections (Bouchard & Aloisi 2002). Common de-adhesive compounds used in egg culture are tannic acid and Fuller's Earth. Current protocols for walleye egg culture in New York State include a 400 mg/L tannic acid treatment for 2.5 min immediately post-fertilization. The use of tannic acid and its interaction with iodophor and VHSV in vitro has previously been investigated by Cornwell et al. (2011). The important conclusions relevant to these experiments are that tannic acid neutralizes the disinfection properties of iodophor and inhibits qRT-PCR and virus isolation detection of VHSV. Thus thorough rinsing between tannic acid treatment and iodophor disinfection is highly recommended.

The primary goal of these studies was to determine if VHSV on experimentally exposed walleye eggs persists during the course of development until hatching. A previous study by Tuttle-Lau et al. (2010) showed that iodophor disinfection at a concentration of 100 mg/L for 30 min was effective at disinfecting VHSV when eggs were tested using virus isolation in cell culture. The secondary goal of these studies was to evaluate the use of a sensitive molecular quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) test in addition to testing for VHSV by virus isolation (Hope et al. 2010). Molecular testing for viral pathogens is becoming more widespread in the detection of disease in aquatic animals (Purcell et al. 2011) and is advantageous in being able to determine the presence of a pathogen at much lower levels and much more quickly than virus isolation, which may take weeks.

5.3 Methods

Broodstock collection and processing. Eight adult wild walleye (total length = 395–515 mm, median = 495 mm) were obtained from the NYSDEC Fish Cultural Station, Oneida Lake, NY, USA, and transported alive to Cornell University on the day the experiments started. Four breeding pairs of adult spawning walleye were used in both experiments. The fish were randomly assigned to pairs containing one of each sex and gametes were stripped from the broodstock without anesthesia into a spawning pan and fertilized. The eggs from each pair were assigned groups denoted A–D to distinguish parent brood pair. Immediately after fertilization a sample of eggs was collected for VHSV testing as described below. The adult fish were then euthanized with an overdose of MS-222 (tricaine methanesulfonate, Western Chemical Inc., Ferndale, WA) buffered 1:1 with sodium bicarbonate (Sigma, Carlsbad, CA). The brood fish were necropsied and examined for pathogens using the methods in the American Fisheries

Society Blue Book (AFS 2010). Samples of heart, liver, kidney, spleen, posterior and anterior kidneys were collected using sterile and nucleic acid free instruments and tested for the presence of viral pathogens using both virus isolation in cell culture and qRT-PCR specific for VHSV.

Virus preparation and titration. The Michigan 2003 VHSV IVb isolate (isolate MI03, Elsayed et al. 2006) was used for both experiments. A stock of VHSV was prepared by inoculating *epithelioma papilosum cyprini* (EPC) cells (Fijan et al. 1983, Winton et al. 2010) and the stock titer determined by plaque assay methods as described in the AFS Blue Book (AFS 2010) using a 2% methocel overlay. Aliquots of this stock were stored at –80 °C until needed for the experiments. Before the start of the experiment a dilution of 10^5 pfu/L VHSV in water was prepared from this stock. A subsample of this dilution was saved and further plaque assays used to confirm the viral dose.

Water quality parameters. Eggs were reared at 12 ± 1 °C for the duration of both experiments. The temperature of all tanks was recorded daily. Normal water chemistry values for the water in the facility were checked at least weekly and were maintained at; pH: 7.2–7.4, Hardness: 110–120 mg/L, Alkalinity: 110–120 mg/L. Total ammonia, total chlorine, nitrite and nitrates were below detection levels. The flow rate for all hatching assemblies was maintained at 0.08–0.15 L/min for the duration of the experiments to achieve gentle but thorough tumbling of the eggs in each hatching assembly. Waste water from the exposed tanks was disinfected with 12.5% sodium hypochlorite to maintain a free residual chlorine concentration of at least 1.0 mg/L for at least 10 min before discharge into the municipal sewage lines. Waste water from the control tanks was drained into the municipal sewage lines.

First experiment. Fertilized eggs from each breeding pair were first treated for 2 min 30 s with a 400 mg/L solution of tannic acid (Sigma, St. Louis, MO) to prevent the eggs from clumping. The pH of this solution was measured to ensure that the tannic acid did not significantly change the pH of the water. The fertilized eggs from each breeding pair were then thoroughly rinsed with at least 10 volumes of fresh water to remove as much residual tannic acid as possible. At this point a sample of each group of fertilized egg was taken to confirm that no VHSV was present before experimental exposure and the eggs from each pair were separated into two experimental groups. The infected group was exposed to 10^5 pfu/mL VHSV for 30 min. A control group was exposed to a placebo inoculation of the same volume of sterile phosphate buffered saline in water. The exposed and control groups from each breeding pair were then further subdivided into two disinfection groups and treated for a further 30 min at 0 and 50 mg/L iodophor (Ovadine®, Western Chemical Inc, Ferndale, WA). At the end of disinfection, a single day 0 sample was taken from each treatment group. Finally, the eggs in each subgroup were separated into 3 replicate hatching jars which were sampled during the course of the experiment. This resulted in a total of 48 egg groups for this experiment. Approximately 0.5 mL of eggs were collected from each group at 1, 3, 7, 14 and 19 d post infection (DPI) using a new, sterile transfer pipette (VWR International, Bridgeport, NJ) for each sample. At 19 DPI, a sample of hatched fry and eggs was taken and the experiment was ended. Samples from the three replicate egg groups from a single brood pair in each treatment type were pooled for virus isolation testing in a 15 mL polypropylene culture tube top section (BD Biosciences, Bedford, MA) and processed as described below. Samples from each egg group for RNA extraction and qRT-PCR testing were collected in bead-beater tubes (Biospec Products Inc., Bartlesville, OK) and frozen at –80 °C until the conclusion of the experiment.

Second experiment. Fertilized eggs from each breeding pair were treated following the same methods as the first experiment up to the point where the exposed and control groups were subdivided for disinfection. The exposed and control groups from each pair were then subdivided into three disinfection groups and treated for a further 30 min at 0, 50 and an additional treatment of 100 mg/L iodophor. During disinfection, the concentration of iodophor was measured at time 0, 10, 20 and 30 min using test strips (LaMotte Co., Chestertown, MA). Finally the eggs in each subgroup were equally divided into three replicate hatching jars which were sampled during the course of the experiment. This resulted in a total of 72 groups for this experiment. Eggs were collected at 0, 1, 3, 7, 9, 12, 14, 18, 21, and 24 DPI. At 24 DPI, a sample of hatched fry and eggs was taken and the experiment was ended. At each sampling time point, 0.5 mL of eggs was collected into a bead-beater tube (Biospec Products Inc., Bartlesville, OK). The eggs were collected using a new, sterile transfer pipette (VWR International, Bridgeport, NJ) for each sample. The bead beater tubes were previously weighed so that the exact weight of eggs was known. Samples of each individual egg group replicate were immediately processed for virus isolation testing as described below. The resulting homogenate was saved at –80 °C for RNA isolation and qRT-PCR testing at the end of the experiment.

Virus isolation testing. In the first experiment, pooled egg samples from each brood pair in each treatment group were processed for virus isolation using the methods described in Frattini et al. (2007). In the second experiment, a different method was used which enabled material to be processed for both virus isolation and qRT-PCR testing from the same homogenized sample. The excess water from each sample in the bead beater tube was removed with a pipette. The tube was weighed to determine the weight of eggs in the tube. A volume was added equal to four times the egg mass of Minimal Essential Medium with Hanks' salts

prepared with 5% fetal bovine serum, penicillin (200 IU/mL), streptomycin (200 µg/mL), glutamine (0.584 mg/mL) and HEPES buffer (1M 0.015 mL/mL) (Gibco, Invitrogen, Carlsbad, CA), hereafter referred to as HMEM-5. Approximately 50 µL of silica-carbide 0.1 mm particles (Biospec Products Inc, Bartlesville, OK) were then added to the tube. The eggs were homogenized in the HMEM-5 using a Mini-Beadbeater 16 (Biospec Products Inc, Bartlesville, OK) for 1 min. The tubes were then centrifuged at 5000 × gravity for 2 min. A 200 µL sample of the supernatant was taken and added to 1.8 mL HMEM-5. Final inoculations were prepared by sterile filtration of this egg homogenate dilution through 0.45 µm biological filters (Acrodisc®, Pall Life Sciences, Port Washington, NY). A first inoculation at 1:99 egg:HMEM-5 was made by pipetting 250 µl of this filtered homogenate into three wells on a 48-well plate (Corning Life Sciences, Lowell, MA) containing fresh monolayers of EPC cells in 250 µL HMEM-5. The second inoculation was prepared by further diluting 250 µL of the filtered homogenate with 1 mL HMEM-5 and inoculating 250 µL into three wells on a 48-well plate for an in-well dilution of 1:499.

Inoculated cells were incubated at 15 °C and examined for the presence of cytopathic effects (CPE) at least twice a week for thirty days post-inoculation, including at least one blind passage after two weeks. When CPE was observed, RNA was isolated by the methods described below and the identity of the virus was confirmed with RT-PCR using VHSV specific primers (OIE 2009).

RNA extraction and qRT-PCR testing. In the first experiment, total RNA from tissues was isolated using a Qiagen RNAeasy (Qiagen Inc., Valencia, CA) kit as described in Frattini et al. (2011). In the second experiment, RNA was extracted using a MagMAX Express-96 (Applied Biosystems, Carlsbad, CA) magnetic bead nucleic acid extraction system. Fifty

microliters of the initial 1:4 egg:HMEM-5 homogenate was used for RNA extraction. The manufacturer's guidelines for RNA extraction were followed and the manufacturer's program 1836_NVSL_DW_50_v2 was used. The system was internally validated to be equally effective at isolating VHSV type IVb RNA as the Qiagen kits.

The qRT-PCR assay was performed on an Applied Biosystems PRISM model 7500 sequence detector (Applied Biosystems, Carlsbad, CA) using the conditions and reaction components described in Hope et al. (2010). For each of the samples, the no template control, and at least three standards, 5 µL of the extracted RNA from each egg group sample was loaded into at least three wells on a 96 well plate (Axygen, Union City, CA). A positive sample was determined as a sample that returned a result of at least two positive wells.

In the second experiment, the effect of inhibition on the qRT-PCR results was examined by differentiating between true target negative results and negative results by tannic acid inhibition. Tannic acid binds to RNA during extraction and it inhibits the polymerase in both reverse transcription and PCR reactions (Kreader 1996). The TaqMan® Exogenous Internal Positive Control Reagents were used according to manufacturer's guidelines. The qRT-PCR assay was performed on an Applied Biosystems ViiA 7 model sequence detector (Applied Biosystems, Carlsbad, CA). The same amount (5 µL) of sample RNA was used in a 384 well plate (Axygen, Union City, CA). The total reaction volume was 15 µL per well and the assay was performed using the same reaction conditions as previously described. Sample inhibition was determined by those samples that showed a significant increase in the mean cycle threshold compared to the mean cycle threshold of the internal positive control standards. Significant differences were measured using a Student's t-test with a 95% confidence level in R (R Core Development Team 2008).

Hatching assembly construction. Walleye eggs were incubated, hatched and raised in small hatching bottles (Figure 5.1A). Each hatching bottle was made from a 237 mL plastic water bottle (Poland Spring, Nestlé Waters North America Inc, Louisville, KY) inverted and supported in a polypropylene cup. The bottom was removed from the bottle approximately 10 cm from the bottle top. A 16 mm diameter hole was drilled in the bottle cap to allow a threaded polypropylene barb elbow (Hardware World, Redmond, WA) with a 10 mm National Pipe Thread (NPT) barb then attached. This barb elbow had a circle of mesh polyester/fiberglass screen (Cat#: 70587, New York Wire Inc., Hanover, PA) covering the 16 mm threaded opening and attached with clear silicone to retain 2.5 mm glass beads (Biospec Products Inc., Bartlesville, OK) and walleye eggs. The barb elbow was threaded through the hole in the cap and the cap was reattached to the bottle with the barb assembly. To support the inverted bottle an 11.11 mm diameter hole was drilled on the side of a 220 mL polypropylene sample cup (BD Biosciences, Bedford, MA) approximately 15 mm on center from the bottom of the cup. Through this hole the elbowed NPT barb was inserted to hold the hatching bottle. An additional hole of identical size was drilled through the side of the polypropylene sample cup about 90 degrees from the porthole for the NPT barb to allow overflow water to drain. For egg retention and water overflow drainage of the egg hatching cup a 45 mm strip of mesh screen (Cat#: 70587, New York Wire Inc., Hanover, PA) encircled the cut bottom and was attached by gluing with clear silicon using approximately 15 mm of screen on the outside bottle surface.

For the second experiment, the hatching jars were modified to include an additional overflow drain tube (Figure 5.1C). This was added to the hatching bottle assembly by melting a 17 mm hole approximately 18 mm from the cut edge of the bottle bottom. Through this hole a drain tube was made by cutting a 15 mL polypropylene culture tube top section (BD Biosciences,

Bedford, MA) at the 8 mL measure mark and then inserting from the inside of the bottle using the flared tube top as a stop ridge that prevented the tube from passing through the hole. This overflow tube was used to direct water into a fry catching bottle. The fry-catching bottle was made similarly to the egg-hatching bottle except a standpipe tube made from a 5 mL polypropylene tube (BD Biosciences, Bedford, MA) with the round bottom cut off was inserted into the 16 mm threaded opening of the polypropylene barb elbow. A small cylinder of screen mesh (New York Wire Inc., Hanover, PA) was rolled and inserted into this standpipe tube to prevent loss of fry. The fry-catching bottle was attached to the egg-hatching bottle by a rubber band and positioned so that spill water poured into it from the egg-hatching bottle.

A linear array of 12 hatching bottle assemblies (Figure 5.1B) was attached to a rigid support of plastic composite lumber (Trex Company Inc., Winchester, VA) that spanned the length of a 530 L fiberglass trough tank (Frigid Units, Toledo, Ohio). Water to each assembly was delivered with a 12-place manifold valve unit (Aquatic Habitats Inc., Apopka, FL). The blue tubing provided with the manifold was snugly inserted into the NPT opening of the egg-hatching bottle to provide an upwelling current for egg incubation. Approximately 130 glass beads (2.75 g) covered the mesh screen cover of the barb elbow assembly to break up water current jets and allow the added walleye eggs to tumble more evenly. Water from the egg-hatching bottle overflowed and, in the second experiment, into the fry catching bottle. Water from the fry catching bottle was discharged through the overflow standpipe and drained into the tank.

Figure 5.1: Hatching assembly construction. Figure 5.1A depicts the initial assembly used for hatching the eggs in the first experiment. Figure 5.1B shows the linear array of hatching assemblies connected to a manifold and placed over a flow-through tank. Figure 5.1C shows the modifications to the hatching assembly made for the second experiment.

5.4 Results

Broodstock and viral titration. All brood fish from both experiments showed no signs of disease on necropsy. No bacteria or viruses were isolated from any of the brood fish. VHSV was not isolated or detected by qRT-PCR in any of the brood fish used or in any of the egg samples taken immediately after fertilization but prior to exposure to VHSV. The viral concentration for the exposed groups of eggs was confirmed to be 10^5 pfu/mL by plaque assay. Iodophor concentrations measured in the second experiment were confirmed to be at the correct concentration for the 30 min duration of the iodophor treatments and the pH of the solutions of both tannic acid and iodophor remained within a 7.1–7.3 range.

First experiment. All control egg groups that were not exposed to VHSV returned negative results by both virus isolation and qRT-PCR at all sampling times. In the exposed egg groups VHSV was detected in both 0 and 50 mg/L iodophor treated by both virus isolation and qRT-PCR (Table 5.1). In the 0 mg/L iodophor exposed group VHSV was isolated in two egg groups, A and B at 0 DPI, and in a single egg group, A, at 1 and 3 DPI. In the 50 mg/L iodophor exposed group, virus was isolated in 3 egg groups A, B, and D, at 0 DPI and in a single egg group, A, at 1 DPI. The egg groups where virus was isolated are bolded in Table 5.1. Detection of VHSV was confirmed by RT-PCR (data not shown).

In contrast, qRT-PCR detected viral RNA at many time points, although the levels of viral RNA were low at all times detected. In the 0 mg/L iodophor exposed group there was still a positive detection at 14 DPI in egg group D1. Similar low levels of viral RNA were detected in some egg groups in the 50 mg/L iodophor exposed groups at all time points, including two egg groups, C3 and D1, at the 19 DPI sampling.

Table 5.1: Virus isolation and qRT-PCR results from the first experiment. Viral quantities detected by qRT-PCR are reported in quantities of viral RNA detected ± standard deviation. The numbers appear in bold for those samples in which VHSV was isolated. The 0 DPI samples were pooled by group and the results are given for the pooled group.

		Egg Group											
DPI	$[I_3]$ (mg/L)	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
0	0	**0 (A1–A3 pooled)**			**530 ± 61 (B1–B3 pooled)**			108 ± 56 (C1–C3 pooled)			204 ± 37 (D1–D3 pooled)		
	50	**1693 ± 841 (A1–A3 pooled)**			**544 ± 12 (B1–B3 pooled)**			686 ± 41 (C1–C3 pooled)			**118 ± 16 (D1–D3 pooled)**		
1	0	**227 ± 95**	**51 ± 20**	**20 ± 4**	65 ± 17	722 ± 123	48 ± 50	66 ± 46	136 ± 35	0	0	58 ± 8	62 ± 27
	50	**45 ± 24**	**28 ± 10**	**165 ± 15**	0	48 ± 11	46 ± 30	19 ± 9	15 ± 8	17 ± 2	32 ± 32	0	0
3	0	**20 ± 14**	**0**	**0**	45 ± 17	16 ± 4	60 ± 10	0	0	16 ± 4	8 ± 3	47 ± 49	57 ± 33
	50	0	0	0	13 ± 2	0	25 ± 12	0	0	12 ± 6	9 ± 6	16 ± 19	0
7	0	0	0	0	37 ± 12	0	11 ± 14	0	0	22 ± 14	22 ± 4	0	15 ± 12
	50	0	0	54 ± 49	0	17 ± 23	0	34 ± 36	0	0	25 ± 12	39 ± 12	6 ± 2
14	0	0	0	0	0	0	0	0	0	0	82 ± 24	0	0
	50	0	0	0	4 ± 1	0	503 ± 813	0	0	0	5 ± 1	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	16 ± 15	8 ± 7	0	0

Second experiment. Similar to the first experiment, all control egg groups that were not exposed to VHSV returned negative results by both virus isolation and qRT-PCR at all sampling times. In the exposed egg groups VHSV was detected in 0, 50 and 100 mg/L iodophor treated egg groups by both virus isolation and qRT-PCR (Table 5.2). In the 0 mg/L iodophor exposed eggs VHSV was isolated and identified from egg groups A1, A3 and B3 at 0 DPI. In the 50 mg/L iodophor exposed eggs, virus was isolated and identified in two egg groups, C2 and C3 at 0 DPI. In the 100 mg/L iodophor exposed eggs, virus was only isolated and identified from a single group A3. Detection of VHSV was confirmed by RT-PCR (data not shown). The egg groups where virus was isolated are bolded in Table 5.2.

As in the first experiment, qRT-PCR testing detected viral RNA at many time points in many egg groups. The quantities detected were low, especially in the later sampling time-points. Results of the inhibition assay showed that full or partial qRT-PCR inhibition was detected in many of the samples at the early time points. In all except two of the 0 DPI samples, C2 and D2 at 50 mg/L iodophor, full or partial inhibition was detected. At 1 DPI, the number of inhibited samples was reduced to 7, 8 and 6 at 0, 50 and 100 mg/L iodophor respectively. The number of inhibited samples continued to decrease with time and at 7 DPI and beyond, only an occasional inhibited sample was detected. The inhibited samples at each time-point are denoted with an asterisk in Table 5.2.

Table 5.2: Virus isolation and qRT-PCR results from the second experiment. Viral quantities detected by qRT-PCR are reported in quantities of viral RNA detected ± standard deviation. The numbers appear in bold for those samples in which VHSV was isolated and identified. Samples in which inhibition was detected by the inhibition assay are marked with an asterisk (*).

DPI	$[I_3]$ (mg/L)	Egg Group A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
0	0	**240 ± 118***	64 ± 4*	**156 ± 45***	0*	0*	**0***	89 ± 33*	45 ± 19*	54 ± 0*	109 ± 12*	358 ± 116*	245 ± 1*
	50	90 ± 56*	156 ± 58*	264 ± 106*	0*	0*	0*	238 ± 84*	**165 ± 2**	**625 ± 39***	1639 ± 215*	947 ± 387	417 ± 6*
	100	0*	31 ± 6*	**28 ± 19***	54 ± 35*	0*	0*	132 ± 47*	129 ± 39*	109 ± 5*	134 ± 42*	58 ± 28*	65 ± 22*
1	0	0*	0*	0*	0*	0*	0	48 ± 3*	79 ± 36	56 ± 28	0	0*	48 ± 19
	50	0*	52 ± 10	32 ± 18*	79 ± 6*	0*	0*	37 ± 14	53 ± 36*	86 ± 20	30 ± 12*	93 ± 36	55 ± 14*
	100	0	45 ± 5*	0*	351 ± 44	137 ± 2	0*	0*	40 ± 16*	187 ± 49	0	47 ± 19*	282 ± 18
3	0	0*	0	0	0	0	0	0*	0*	0*	0	0	0
	50	0*	0	0	0*	0	0	0	19 ± 2	0	0	0	18 ± 5
	100	0	32 ± 19	33 ± 2*	0	0	0	212 ± 34	0	0	40 ± 9	45 ± 7	0
7	0	0	0	0	0	0	0	0	0	0	24 ± 1	0	0
	50	0	0	0	0	26 ± 14	0	30 ± 16	0	25 ± 13	0	39 ± 10	0
	100	0	0	0	28 ± 10	20 ± 2	0	0	0	0	49 ± 24	22 ± 3	99 ± 7
9	0	0	0	36 ± 13	0	0	0	24 ± 3	6 ± 5	0	0	0	0
	50	23 ± 2	37 ± 14	0	55 ± 23	0	0	186 ± 84	67 ± 30	70 ± 2	58 ± 30	69 ± 6	51 ± 5
	100	46 ± 23	81 ± 56	36 ± 33	33 ± 22	71 ± 40	41 ± 2	53 ± 35	100 ± 6	34 ± 12	51 ± 5	88 ± 2	82 ± 13
12	0	0	0	0	0	0	0	0	14 ± 7	18 ± 6	0	0	0
	50	22 ± 13	36 ± 30	39 ± 23	58 ± 11	35 ± 16	36 ± 1	29 ± 3	145 ± 37	128 ± 5	70 ± 20	65 ± 14	122 ± 73
	100	0	16 ± 9	13 ± 7	26 ± 15	11 ± 5	25 ± 6	170 ± 10	106 ± 39	60 ± 3	0	26 ± 7	20 ± 8
14	0	0	0	0	0	0	0	33 ± 25	35 ± 11	0	41 ± 19	29 ± 32	0
	50	0	0	0	28 ± 23	0	0	23 ± 11	90 ± 21	40 ± 1	0	28 ± 23	0
	100	0	0	0	0	40 ± 20	0	0	18 ± 12	29 ± 9	26 ± 16	57 ± 26	32 ± 0
18	0	0	0	0	0	0	0	0	0	0	0	0	0*
	50	0	0	0	0	17 ± 0	0	31 ± 9	35 ± 2	28 ± 2	0	20 ± 3	22 ± 5
	100	0	0	0	17 ± 3	0	17 ± 1	0	47 ± 27	12 ± 1	41 ± 18	28 ± 9	51 ± 30
21	0	0	0	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	19 ± 4	0	0	0	0	0
	100	0	0	0	0	0*	0	0	0	0	0	19 ± 4	0
24	0	0	0	0	0	0*	0	21 ± 0	0	0	20 ± 2	0	0
	50	0	0	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0	0	0*	0*	29 ± 23

5.5 Discussion

Determining whether VHSV persists on exposed walleye eggs in these experiments was dependent on the testing methods used to detect the virus. Using virus isolation to test for VHSV produced results that were similar to those previously published by Tuttle-Lau et al. (2010). In the first experiment, VHSV persisted to 3 DPI in the eggs that were not disinfected, but beyond that point was not detected in any other sample. Even without disinfection, VHSV was undetectable by virus isolation beyond 3 DPI. Interestingly, in one group that was disinfected with 50 mg/L iodophor, virus was isolated at 1 DPI. This result was not repeated in the second experiment, where no virus was isolated beyond Day 0. From these results we confirm that iodophor disinfects the eggs from VHSV at the 100 mg/L dose, but the 50 mg/L dose may be insufficient to destroy the virus immediately. When free virus in water is exposed to 50 mg/L iodophor, VHSV is destroyed as shown by Cornwell et al. (2011). When in the presence of eggs as well as water, the virus may be protected from iodophor disinfection by the jelly coating of the eggs or residual tannic acid may be mitigating the disinfection properties of iodophor. It is important to note that virus was not isolated in all exposed egg groups in both experiments at 0 mg/L iodophor. This may be due to the virus being reversibly inactivated by tannic acid alone (Cornwell et al. 2011) or the virus being unable to disassociate from the eggs and infect EPC cells.

In contrast, using qRT-PCR testing, virus was detected at many time-points in both experiments, and persisted to the end of both experiments in at least some groups. The amount of viral RNA detected at all time points beyond 0 DPI was low and in general declined over the course of the experiment. The amount of viral RNA detected at many time-points approached the limit of detection of the qRT-PCR assay, but these low levels were consistently reproducible

in replicate testing. The qRT-PCR results provide evidence that VHSV is not replicating in the eggs, which corresponds to the current knowledge of VHS infection in eggs. We would expect an increase in the viral RNA quantity detected if replication was occurring, which we did not observe. Even though we were able to detect viral RNA at much later time-points than virus isolation, we were unable to determine whether this was viable virus. It is possible that VHSV was still viable, but at such a low concentration that it was undetectable by virus isolation. Alternatively, VHSV may have been rendered inactive by adhesion to the egg surface, trapped within the egg or surface disruption of the viral envelope by iodophor disinfection has occurred; however the RNA is still detected by qRT-PCR. Thus these qRT-PCR results must be interpreted with caution. These experiments clearly show that at least some part of VHSV does persist in the exposed egg groups, but they cannot conclusively determine that the virus is viable and in sufficient concentration to be infective at the hatching stage.

In the first experiment, there were some initially perplexing results, most notably the positive isolation of VHSV from early egg groups without concurrent detection by qRT-PCR. We were also surprised at the low detection levels in the initial samples at 0 DPI. Considering the high infective dose of 10^5 pfu/mL we expected to detect much higher levels of virus. In the second experiment, modifications were made to try to clarify some these results. By performing virus isolation on individual replicates instead of pooled groups we could directly compare the test results. Also, by performing the inhibition assay, we could determine if the qRT-PCR results were being affected by residual tannic acid. In directly comparing the second experimental results of the two testing methods at 0 DPI, there are still instances were virus was isolated but not detected by qRT-PCR. The inhibition assay demonstrates that in these early samples there is significant inhibition of qRT-PCR in many samples (Table 5.2). Thus despite our efforts to

thoroughly remove tannic acid by rinsing many times before iodophor disinfection, there was still sufficient residual tannic acid or some other unknown inhibitor to affect the results. This may explain our low and negative detections of VHSV by qRT-PCR in some samples, particularly the early time-points. Over the course of the experiment, the number of inhibited samples declined, likely due to residual tannic acid being washed away from the eggs over time.

Our results confirm that using higher concentrations of iodophor effectively eliminates viable VHSV from walleye eggs. We strongly recommend the use of iodophor for its antiviral, bactericidal and fungicidal properties. As tannic acid neutralizes the disinfection properties of iodophor, it is critical that hatchery practitioners thoroughly rinse tannic acid-treated eggs prior to water hardening in iodophor solution. These experiments show that VHSV may persist in some form for much longer than previously known, but it is unknown whether the remaining VHSV is still infective, and as such, proper disinfection is extremely important. Iodophor disinfection at 100 mg/L for 30 min sufficiently disinfected VHSV as detected by virus isolation and we recommend this concentration be used instead of 50 mg/L. This must be considered with the fact that hatching survival is lower in walleye eggs in a 30 min treatment (Dabrowski et al. 2009). We did not investigate the effect of a 15 min treatment of iodophor, and further experiments should be considered to investigate the effect of 15 min iodophor disinfection.

These experiments highlight the risks and rewards of using sensitive molecular tools for the detection of viral pathogens in a practical aquaculture procedure. The advantage of being able to detect low-levels of virus quickly and efficiently is weighed against the facts that these tests do not differentiate between viable virus and viral RNA. Also, using a common treatment such as tannic acid may have serious implications for the results from these PCR tests. Without investigating the inhibitory effect of tannic acid on the samples in these experiments, it would

have been very difficult to interpret the qRT-PCR results in a meaningful biological way. By performing the inhibition assay, we can show that some of the unexpected negative results must be considered as false negatives due to sample inhibition. In general, using qRT-PCR testing for VHSV in eggs can provide valuable and timely information, but careful attention is needed to interpreting both positive and negative results.

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SUMMARY

The research presented in this dissertation was particularly focused on evaluating the use of the qRT-PCR test for VHSV type IVb as a tool for diagnostic investigations. Particular emphasis was placed on areas of interest to the NYSDEC. After the emergence of VHSV type IVb in New York State, the need for an accurate, efficient and rapid test for VHSV was an area of critical interest to the NYSDEC and other regional and national agencies. While large-scale fish surveillance projects were immediately implemented, there was also a need to evaluate this qRT-PCR test in controlled laboratory settings and compare it to the current accepted standard for VHSV testing, namely virus isolation.

This dissertation has shown that the use of a qRT-PCR test for VHSV IVb has advantages for the rapid and sensitive detection of the virus, but specific considerations must be made depending on the situation in which it is used. In wild fish surveillance, as detailed in Chapter 3, the qRT-PCR test is extremely valuable as a negative screening tool for surveillance for VHSV. With a high negative predictive value, large numbers of fish and locations can be rapidly tested for the presence of the virus. Thus positive detections can be focused on and a more limited number of samples tested by conventional virus isolation techniques and subsequent viral identity confirmation. The higher sensitivity of qRT-PCR detection allows more elaborate predictive models to be made and reveals other potential susceptible species.

However, the limits of detection of the qRT-PCR test also need to be taken into account. The analytical sensitivity of the test is very high and is reproducibly able to detect 100 copies of viral RNA in a sample. On the other hand, the amount of sample that is being tested is very small, and unless there is uniform homogeneity of virus within the sample, there is a possibility

of not detecting the virus. This is mitigated by using a subsample of the pooled organ homogenate for RNA extraction. Later experiments that we have performed in our lab have detected viral RNA in samples of encephalon while concurrently not being detected in the pooled organ sample from the same fish. In evaluating the overall diagnostic sensitivity of the qRT-PCR, all these factors, such as which organs were selected for testing, must be taken into account.

In VHSV trials in experimentally exposed fish, qRT-PCR is highly valuable in detecting VHSV in fish that are not showing clinical signs of disease as described in Chapter 4. By measuring viral levels by qRT-PCR during the course of the experiments, the amount of viral replication in the fish can be inferred, but in order to specifically test this, strand specific primers would need to be made. The qRT-PCR test detects both mRNA positive and genomic negative RNA, and to specifically analyze replication a test would need to be designed to differentiate between these.

The qRT-PCR test is very useful in determining the progression of VHS disease in fish. In susceptible species like tiger muskellunge, the qRT-PCR test can be used to detect the onset of clinical VHS before signs of disease occurred. In refractory species such as channel catfish, the qRT-PCR can show evidence that the virus is replicating in these fish. This highlights the need for intensive and specific examination of the pathogenesis of VHSV IVb in these species, and to determine if these fish may be asymptomatic carriers of VHSV, but able to transmit the virus to other more susceptible species. There is significant interest in the development of a laboratory model for VHSV type IVb infection. Although the species used in this dissertation would not necessarily be as suitable as smaller, more easily managed laboratory fish, the findings of viral

replication in sub-clinical fish presented in this dissertation are important considerations that should be evaluated in any further trials.

Finally, the sample type that the qRT-PCR test is being performed on is very important to the proper determination of disease status by this test. In the walleye eggs that were exposed to routine de-adhesive and disinfection procedures, the qRT-PCR test was hindered by the presence of the tannic acid inhibitor. In samples where inhibition is suspected, the qRT-PCR results must be interpreted with a concurrent evaluation of the potential PCR inhibition in the sample. Without examination of inhibition, there is a potential for false negatives to be reported by the qRT-PCR test. As shown in Chapter 5, VHSV IVb can persist in some form on eggs for much longer than was previously known. Careful evaluation of negative results must be made to ensure that they are true negative results.

The qRT-PCR test used in this dissertation is currently being evaluated as part of a national ring trial to determine the analytical and diagnostic sensitivity and specificity for all genotypes of VHSV. This collaboration involves multiple laboratories working on replicates of the same samples and determining test results by four different real-time PCR methods. While the results of this ring trial are still being processed, preliminary results have shown that the qRT-PCR test used in this dissertation performed equally well or better than any of the other three tests when specifically looking for VHSV genotype IVb. Additionally, although inhibition was seen in some sample types, particularly ovarian fluids, the amount of inhibition was similar to those seen by the other qRT-PCR tests. The full results of this ring trial study are still being analyzed and will be presented in a future publication authored by all collaborators in the ring trial. The preliminary results described in this summary are provided to show how investigation is continuing into the validation of our qRT-PCR as a diagnostic tool for VHSV.

Validation according to the OIE for this qRT-PCR test will require much further work, of which the ring trial is a major component. Currently, the OIE does not accept real-time PCR alone for targeted surveillance and presumptive diagnostics in the detection of fish pathogens. As real-time PCR is increasingly becoming available and used by diagnostic agencies, its use as a surveillance tool is increasingly becoming accepted. This dissertation provides more evidence for the validation of this qRT-PCR as a surveillance tool specifically for VHSV IVb.

All animals used in this dissertation were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care accredited laboratory facility and all experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee.