THE DEVELOPMENT AND APPLICATION OF A ZEBRAFISH INFECTION MODEL FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IVB

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THE DEVELOPMENT AND APPLICATION OF A ZEBRAFISH INFECTION MODEL FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IVB

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Viral hemorrhagic septicemia virus (VHSV) is the causative agent of a highly lethal, World Organization for Animal Health (OIE) reportable fish disease. With a broad host range and a long-term persistence, VHSV has become a worldwide threat, particularly to both fish farming and recreational fishing. As a result of the massive mortality events between 2005-2007 followed by disease disappearance in the Great Lakes, research began to focus on understanding the mechanisms of transmission and persistence of this new genotype of VHSV, IVb. In this study, a robust quantitative RT-PCR assay was first developed to provide a more sensitive tool to survey fish in the Great Lakes and has allowed the detection of persistent sub-clinical VHSV IVb infections throughout the region. The mechanisms of viral entry, replication, shedding, and transmission are only partially understood due to the lack of a suitable laboratory model host for VHSV IVb. A second phase of this work is the development a zebrafish model for VHSV IVb immersion infection that reflects many parameters governing infection of fish in the wild. This model has been used to evaluate temperature, dose and age effects on disease susceptibility. Using this model we have shown that VHSV IVb RNA and infectious virus are readily detected in infected post-mortem zebrafish for more than 100 days at 4°C in air or water suggesting that

deceased fish may contribute to viral persistence. Data are presented to show that direct physical contact with an infected host greatly enhances VHSV IVb transmission. Together data from the study will allow for a better understanding of the mechanisms of infection and transmission of VHSV IVb and provide a platform for further studies and translation to VHSV IVb transmission and pathogenesis in natural hosts.

BIOGRAPHICAL SKETCH

Kristine Marie Hope was born in Seattle, Washington in January of 1983. Kristine remained in Seattle throughout her childhood, attending Seattle Preparatory School for high school. For her senior year, Kristine joined the Matteo Ricci College at Seattle University, allowing her to begin her undergraduate education a year earlier. She graduated from Seattle University with a B.S. in Chemistry and a B.A. in Humanities. During her time at Seattle University, she performed and published on diabetes research under the mentorship of Dr. Paul Robertson and the Pacific Northwest Research Institute in Seattle. Following graduation in 2004, Kristine then joined the graduate school at Cornell University, eventually joining the microbiology and immunology department with an emphasis in virology. Under the mentorship of Dr. James Casey, Kristine has had the opportunity to work on the development and application of a zebrafish model for viral hemorrhagic septicemia virus, as well as being allowed to mentor four incredible undergraduate students, all who will achieve publications from their work. Upon completion of her Ph.D., Kristine will continue in academia with the intent of teaching undergraduate students both in the classroom and in the laboratory.

I dedicate this work to all of the graduate students out there who have not had a smooth ride, as an example of resilience to overcome all obstacles even when the world seems against you.

ACKNOWLEDGMENTS

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my fiancé and love of my life, Steven Petesch, with whom I have shared this journey in graduate school and now beyond! I will never be able to fully thank him for his steadfast support and faith in me.

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LIST OF ABBREVIATIONS

ATCC = American Type Cell Culture

CC = Cell culture cell and media mixture

CCID50 = 50% cell culture infectious dose

CPE = Cytopathic effects

EMEM = Eagle's minimal essential media

EPC = Epithelioma papulosum cyprini

dpi = Days post infection

dpm = Days post-mortem

FBS = Fetal bovine serum

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMEM = Hank's minimal essential media

HMEM-5FPSH = HMEM with 5% FBS, penicillin, streptomycin, and HEPES

HMEM-10FPSH = HMEM with 10% FBS, penicillin, streptomycin, and HEPES

hpf = Hours post fertilization

HT = Horizontal transmission

IO = Instant Ocean

MI03 = 2003 isolate of VHSV IVb

OIE = World Organization for Animal Health

pfu = Plaque forming units

qRT-PCR = Quantitative reverse transcription polymerase chain reaction

RO = Reverse osmosis

SVCV = Spring viremia of carp virus

VHS = Viral hemorrhagic septicemia

VHSV = Viral Hemorrhagic Septicemia Virus

VT = Vertical Transmission

CHAPTER 1

INTRODUCTION

History of Viral Hemorrhagic Septicemia

Viral hemorrhagic septicemia (VHSV) is a fish disease that affects a variety of fish species. External symptoms of this disease include hemorrhaging at the base of the fins and abdomen, exopthalmia, and lethargy. Internal symptoms primarily include hemorrhaging in the capillary and sinusoid endothelia, pale gills, and pale livers (2, 49). VHSV is routinely isolated from the anterior and posterior kidneys, liver, heart, and spleen, although VHSV has also been isolated from the blood, gills, skin, brain, and fins of fish (47, 73, 74). Both external and internal symptoms vary dramatically from species to species (2, 49).

VHS was first identified in the 1930's in European rainbow trout farms (47, 104). The cause of this disease was unknown at the time; however, researchers hypothesized that the disease was caused by a yet to be identified infectious agent. Although the causative agent of VHS, viral hemorrhagic septicemia virus (VHSV), was first isolated in 1963 (Jensen 1963), VHSV was considered for decades to be a virus specific to European rainbow trout, particularly those located in fish farms.

However, the isolation of VHSV from returning salmon on the West Coast of the United States in 1988 incited the discovery that VHSV is worldwide, pervasive virus (68, 105). VHSV was also isolated from the east coast of the United States as well as the coasts of Canada (9, 41, 67), Japan (14), Korea (48), and various locations around Europe (70, 88, 92), thus expanding the known geographical regions that VHSV infected. Not only did VHSV infect more than just rainbow trout it was spread throughout the marine environment.

In 2005 through 2007, VHSV was isolated from many different fish species in the Great Lakes region causing massive fish mortalities and extreme economic losses to the states surrounding the lakes (37, 61). With this discovery, VHSV could no longer be considered to be isolated to marine environments. Interestingly, these mass mortality events were seasonal, focused mainly in the spring, when the lakes experience the largest changes in temperature and the fish are taxed with the stresses due to spawning. To better understand how and where VHSV entered the Great Lakes, how it spread through the area, and what potential threat of VHSV in the future would be, survey work sampling various sites around the Great Lakes concurrent with laboratory challenge models were developed to test how changes in temperature and other conditions affected infectivity of VHSV (49). From 2008 to 2010, infectious VHSV was isolated from various fish from the Great Lakes region in the absence of clinical signs of disease or massive mortalities (6), leaving researchers wondering how VHSV persists in this environment.

The first identified VHSV positive samples came from four muskellunge collected in 2003 from Lake St. Clair, Michigan (25), indicating that VHSV was present before the major outbreak of disease in the Great Lakes. Although the casual element for the severe outbreaks beginning in 2005 is still unknown, many hypotheses including viral mutations, environmental changes (e.g. temperature, increased presence of other pathogens, or presence of other toxins), and impacts from increased shipping are each under consideration. In addition to having a broader geographic range of infection than initially thought, VHSV was also found to have a much broader host range than initially expected. VHSV is able to infect fish from rainbow trout, to flounder, to muskellunge, to emerald shinners. Over 28 species of fish have been found to be susceptible to VHSV (96). Interestingly, VHSV has also been isolated from a small invertebrate, *Diporeia ssp*, a leech, a river lamprey, and even an

aquatic turtle (28, 29, 31, 34). Not only does the susceptibility to VHSV differ dramatically, but also with the advent of a highly sensitive assay for VHSV detection, qRT-PCR (18, 33, 44), the presence of asymptomatic infections has become increasingly important to understand the mechanisms of both transmission and persistence of VHSV in the environment.

Viral Hemorrhagic Septicemia Virus

VHSV is a rhabdovirus in the genus novirhabdovirus, the same family containing the rabies virus. Several other rhabdoviruses have been found to infect fish, including most notably, infectious hematopoietic necrosis virus (IHNV), hirame rhabovirus (HIRRV), snakehead rhabdovirus (SHRV), and spring viremia of carp virus (SVCV) (1, 4, 10, 78). VHSV is an envelope virus with a bullet-shaped structure characteristic of its family (Figure 1.1). It has approximately an 11 kb single strand, negative sense RNA genome, encoding six genes: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), the non-viron (NV), and the polymerase (L).

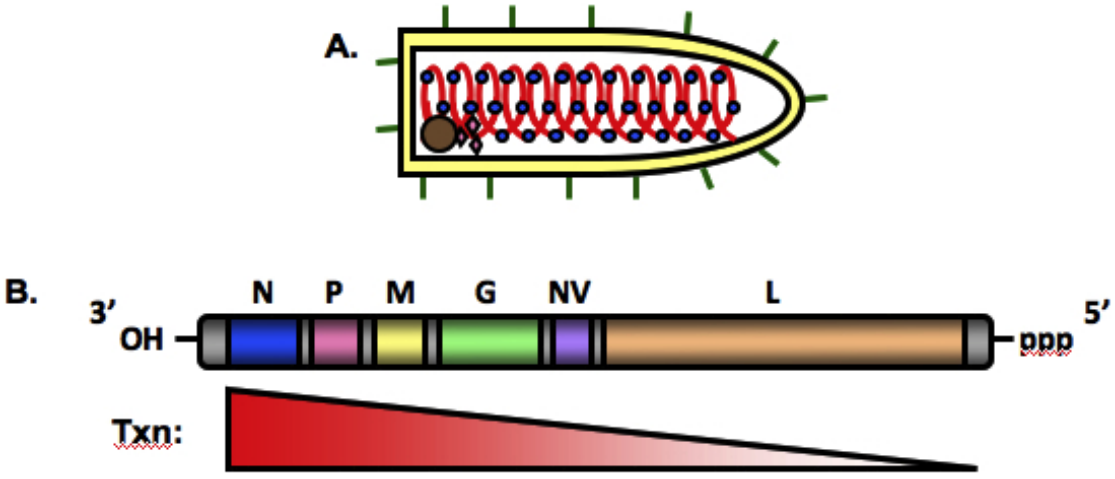

Figure 1.1 Diagrams of VHSV. (A) Viral particle representation of VHSV. (B) Transcriptional regulation of VHSV. Red triangle indicates the reduction of transcripts of the genes as the polymerase moves from the 3' end to the 5' end of the genome. Blue: nucleoprotein. Pink: phosphoprotein. Yellow: matrix protein. Green: glycoprotein. Purple: nonviral protein. Brown: polymerase. Red: single-stranded RNA genome.

Based on nucleotide sequencing of the N and G genes, VHSV isolates have been categorized into four distinct genotypes. Interestingly, each genotype generally correlates with geographical location of the isolation (9, 22, 89). Isolates categorized as genotype I have mainly been isolated from rainbow trout farms in continental Europe, but isolations from marine species from the Baltic Sea also fall into this genotype. Genotype II isolates also include those from wild marine fish from the Baltic Sea. Genotype III isolates come from wild marine fish, mainly from the North Sea near the United Kingdom. Genotype IV isolates have been divided into two distinct classes, either IVa, which include isolates from wild marine fish from the coasts of North America, Japan, and Korea, and IVb, which include isolates from both wild freshwater fish from the Great Lakes and wild marine fish from the eastern Atlantic coast of Canada (32, 104).

VHSV follows a similar lifecycle as its fellow rhadbdoviruses, entering the cell through attachment to extracellular receptors and then endocytosed into the cytoplasm. Phosphatidylserine is thought to be the primary receptor for VHSV; however, this might not be the case as more recent studies have disproven that phosphatidylserine is the primary receptor for the related vesicular stomatitis virus (VSV), as it was believed to be for a decade (19). Following endocytosis, the viral envelope fuses to the endosome membrane in response to a decrease in pH within the endosome, releasing the nucleocapsid into the cytoplasm. Here, the genome is transcribed and processed into messenger RNAs that encode for each of the viral proteins. Interestingly, transcriptional attenuation occurs as the polymerase moves from the 3' to the 5' end of the genome, resulting in the most copies of the N gene produced per cycle of transcription (7). Once transcribed, the viral messenger RNAs are then translated into proteins using the host ribosomes and complete protein processing occurs in the host's endoplasmic reticulum and the golgi apparatus. It is thought that the switch from

transcription of individual messenger RNAs to transcription of the complete template for genomic replication occurs in response to N protein accumulation on the genome templates. Upon completion of protein processing and genome replication, both are shuttled to the plasma membrane for packaging. With an accumulation of glycoprotein in the plasma membrane and the matrix proteins bridging the nucleocapsid to the glycoproteins, the mature virion is then exocytosed into the cytosol, in search of a new cell to infect.

Host Immune Response to VHSV

In response to infection by VHSV, several host mechanisms are triggered. Initially, several different pathways trigger the innate immune system (19). First, RIG-1 plays a key role in recognizing a variety of negative sense RNA viruses, including rhabdoviruses such as VSV. RIG-1 recognizes 5' triphosphates that are present on the viral leader as well as the negative and positive strand RNAs of the viral genome. Since 5' triphosphates of the host cell's messenger RNAs are capped before export from the nucleus, any host RNA that is packaged into the virus is protected from this anti-viral mechanism. Interestingly, the more defective interfering particles present, the more targets RIG-1 has to bind. RIG-1 activation through a phosphorylation cascade activates IFN production and ultimately the transcription of interferon-stimulated genes. Since rhabdoviruses cannot grow in IFN-alerted cells, a likely result of co-evolution of virus and host, VSV has developed countermeasures to prevent IFN production by 1) inhibiting the basal transcription factor TFIID, and 2) inhibiting nuclear export of host mRNA. Both of these actions have been attributed to the viral matrix protein. Interestingly, host cells have also learned to deal with the later of these actions by increasing production of the nuclear export proteins that the viral matrix protein binds. Second, toll-like receptors play an ancillary role in the anti-viral response to rhabdoviral infections. TLR3 recognizes dsRNA, while TLR7/8

recognizes ssRNA. Once triggered, these receptors initiate a signaling cascade resulting in production of interferons. TLR7 has been shown to recognize VSV; however, this seems to occur through recognition of cytosolic viral RNAs that are transported to the lysosome by autophagy. Interestingly, TLR4, expressed on the cell surface of many cell types including epithelial cells, fibroblasts, and monocytes, was also been shown to respond to VSV infection. The VSV glycoprotein was found to trigger a CD14/TL4-dependent pathway ultimately leading to the activation of IRF7 in memory dendritic cells and macrophages. Interferon stimulated genes of primary importance include dsRNA-dependent protein kinase (PKR), and Mx proteins (81), and IL-8 (69).

After recognition of infection, the host adaptive immune system is also induced. VHSV has been shown to trigger the production of neutralizing antibodies, exclusively targeted to the glycoprotein. Furthermore, DNA vaccines targeting the glycoprotein, but not the matrix or nucleoprotein have had significant affects in protecting fish from VHSV challenge for up to 6 months post vaccination (94); however, the immune mechanisms responsible for this are still unknown. Furthermore, this observed protection after vaccination does not always correlate with antibody titers (99) and a recombinant glycoprotein DNA vaccine only provided very low protective efficiency (14), implying a potential role for cellular mediated protection post vaccination. Cytotoxic T lymphocytes and natural killer cells have also been indicated as players in the immune response to VHSV; however, T cell mediated responses are not well understood (14, 99).

Tools for the Analysis of VHSV

As a World Organization for Animal Health (OIE) reportable disease, the protocols for the detection of VHSV in endemic or naïve bodies of water has been crucial and limiting as to understanding the mechanism of VHSV in its environment.

Cell culture, followed by classical RT-PCR, has been used to identify infectious VHSV in fish and water samples, with a detection limit for VHSV IVb around 10^3 pfu (37). With the increased awareness of the sub-clinical nature of VHSV, more sensitive methods became necessary with a focus specifically on qRT-PCR (44). Although qRT-PCR cannot replace cell culture due to its inability to define infectivity, its increased sensitivity by 2 to 3 orders of magnitude has been instrumental towards better defining where VHSV is located, both within a body of water as well as within its host, how it is moving in its environment, and where it may travel next. Also, with its increased processing time and ease of use, this method is rapidly changing the diagnostic field for fish viruses as it already has for human viruses (5, 53, 66). Several qRT-PCR assays have already been developed for VHSV genotype I through III (18, 20, 57, 63, 64), IHNV (79), SVCV (79, 110), snakehead rhabdovirus (78), and the recent addition of VHSV genotype IVb (33, 44). Other techniques that are redefining the VHSV research landscape include more sensitive immunohistochemistry (2), live host viral tracking (26, 39), and other host challenge model developments focusing on differences in susceptibility to VHSV (43, 58, 80).

Challenge and Natural VHSV IVb Infections

Within the Great Lakes regions, over 28 species are known to be susceptible to VHSV IVb (46), with varying degrees of susceptibility (49). However, although isolations of VHSV IVb have been taken from several species around the Great Lakes, the mechanisms of transmission and persistence have been difficult to understand due to a number of other factors that could lead to these susceptibility differences, such as current immune status, duration and exposure to VHSV, and environmental differences, including temperature and chemical differences.

Thus, to better understand these differences and to be able to control outside variables, several challenge experiments with natural fish species have been done in a

laboratory setting exposing fish to VHSV via immersion or an intraperitoneal (IP) injection (58, 80, 85, 90, 91). Ideally, immersion challenges are preferable they are a natural route of exposure to VHSV; however, in cases where this is not possible, IP injection has been used. From a recent comparison of 11 Great Lakes fish species, Kim et al. found that exposing the fish to either 10^4 or 10^5 pfu/fish, depending on the species, the mortalities ranged anywhere from 100% for muskellunge, 40% for yellow perch, brook trout, and brown trout, and 1 or 0% for Coho and Chinook salmon respectively (49). The average median time to death was approximately 9.6 days; however, this also varied significantly with species and did not always correlate with percent mortality. Interestingly, using a similar dose with IP injection, although not a natural route of viral entrance, the species differences to susceptibility are still very apparent, implying that not all of these differences can be attributed to entrance differences. It is likely that a combination of both viral entrance and internal differences, such as where the virus may be sequestered or how the immune system handles VHSV, contribute to the species-specific differences to VHSV susceptibility and disease.

Mechanisms of entry, internal movement, disease, exit, and transmission of VHSV

Another important aspect of VHSV infection involves entry into the host. Although the primary mode of entrance has yet to be identified, evidence indicates likely transmission through the gills, the skin, orally, or even vertically transmitted (47, 73, 74). Most likely a combination of these occur in nature; however, the most well documented route is entry through the fin bases.

Since the early 1980's, work with VHSV genotype I has indicated the importance of VHSV in the gills (73). Studies with VHSV genotype I in rainbow trout have shown high levels of VHSV in the gills and organ pools, around 10^5 CCID50/g, as early as 3 dpi, 6 days prior to the first mortality (73). For VHSV IVb, high levels of

virus have been detected from the organ pools, fin bases, with lower levels in the gills, and no detection in the blood.

VHSV has also been isolated from ovarian fluids, indicating the potential for vertical transmission. Prior work looking at VHSV and INPV found VHSV to be present around, but not in the oocytes, whereas INPV was inside the oocytes hypothesizing that, unlike INPV, VHSV cannot be vertically transmitted (17). More recent work, however, indicates that VHSV is present in mature oocytes, but not immature oocytes, indicating a likely possibility of vertical transmission of VHSV (2, 47). However, direct evidence for VHSV vertical transmission has yet to be shown due to complications in breeding, whether having access to enough fish when breeding or having appropriate facilities to breed natural hosts of VHSV.

Another potential site of entrance includes ingestion of infected fish, as is likely the case for infected predatory fish. Understanding the predator-prey relationship may help to explain some of the species susceptibility differences to VHSV IVb. For example muskellunge, which are large predatory fish are highly susceptible to VHSV infection and disease, whereas fathead minnows, a baitfish, are highly susceptible to VHSV infection, but not to disease, acting as a potential carrier fish. Indeed, high levels of VHSV IVb have actually been found in the minnows. However, it is not known if the differences in disease are due to an oral route of exposure. VHSV has now been isolated from a variety of other species, including leeches, *Diporeia spp*., and lampreys (28, 29, 31), and in a challenge model has been found to even infect freshwater turtles, which were found to feed off of VHSV positive fish (34).

The most highly accepted primary site of entry includes the fin base junctions for several reasons. First, severe hemorrhaging on the fin bases has been observed both in natural and laboratory challenge infections on numerous occasions and is an

accepted clinical sign of VHSV infection (47). Second, using bioluminescence and live host imaging, the spread of IHNV from the fin bases into the internal cavities of the host fish has also been documented (39). And third, at 2 days post infection, the immune response in VHSV genotype I infected zebrafish has been shown to be higher in the fins then in the internal organs (26).

Once inside the fish, the virus eventually invades several different organs, as VHSV IVb has been isolated from various organs, including brain, kidney, liver, heart, and spleen. Thus VHSV most likely moves through the circulatory system. Although earlier studies have isolated genotype I virions from blood samples and the brain (73, 74), VHSV IVb has yet to be detected in the blood (unpublished work). However, VHSV has also been shown to invade macrophages (12), and thus the lymphatics system could also be a major transporter of VHSV within the host.

Understanding viral spread may be crucial in elucidating species susceptibility differences; especially knowing that viral entry alone does not determine differences in susceptibility. It is possible that VHSV infects different primary cells in different hosts, or is sequestered in different tissues in different hosts, or simply that the host immune response is different in different hosts. Most likely a combination of these occurs in a natural host fish. Furthermore, different stressors such as rapid temperature shifts, spawning conditions, other pathogens, or environmental chemicals or toxins may also affect the fate of the virus in the host.

Zebrafish as an infection model

Zebrafish have long been used as a model organism, primarily in developmental research due to their near-transparent embryos (95). As a result of this, the zebrafish genome has almost entirely been sequenced, allowing for microarray or high-throughput sequencing technology to be adapted to zebrafish.

Although zebrafish have been used in developmental studies, their use as

infection models have only recently become of increasing interest (3, 65, 93). Fish viruses including SHRV (78), SVCV (84), infectious spleen and kidney necrosis virus (108), nervous necrosis virus (59), infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus (55), and VHSV genotype I (76) have already been used in zebrafish infection models. This growing trend is due to not only the ease of maintenance of these fish and their genetic knowledge, but also as a result of their immunological similarity to other fish species as well as mammals (3, 65, 93, 109). Unlike *C. elegans* and *Drosophila,* which have limited immune systems, zebrafish have a complete immune system, both innate and adaptive (100).

Our choice for the zebrafish model for VHSV IVb infection included the following parameters: 1) large numbers are easy to maintain, allowing us to do population based studies both to avoid individual variation or to exploit our understanding of the significance of this variation and to achieve biological replicates, thus gaining strength for our conclusions; 2) breeding can be performed year round, allowing us to directly test vertical transmission; 3) the sequenced genome allows us to look at the host response through the course of infection as well as the virus; 4) the small size of the fish allow us to use whole sections in both histology and immunohistochemistry, to study the entrance and movement of VHSV in fish at different stages during infection; and 5) the large dynamic temperature range of zebrafish allow us to halt virus replication in the host, essentially as a temperature sensitive mutant.

With these advantages of a zebrafish model in mind along with the newly developed qRT-PCR assay for VHSV IVb developed in the lab (Chapter 2), the following work can be divided into two segments. The first segment of work focused on developing a dynamic model while testing the effects of temperature acclimation prior to infection, duration of exposure, length of exposure, and age on the

susceptibility to VHSV IVb. Furthermore, we also tested the ability of fish to recover from VHSV using shifts up to higher temperatures (Chapter 3). In the second segment, we focused on applying our newly developed model to study the mechanisms of VHSV transmission in the following manners: 1) from post-mortem hosts (Chapter 4), 2) through vertical transmission (Chapter 5), and 3) through horizontal transmission (Chapter 6). I have concluded this work with an analysis of future directions for the VHSV IVb zebrafish model, expanding it to further address environmental conditions and their affects on the susceptibility to VHSV IVb (Chapter 7).

CHAPTER 2

COMPARISON OF QUANTITATIVE RT-PCR WITH CELL CULTURE TO DETECT VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IVB (VHSV IVB) INFECTIONS IN THE GREAT LAKES *

Hope KM, Casey RN, Groocock GH, Getchell RG, Bowser PR and Casey JW

ABSTRACT

Viral hemorrhagic septicemia virus (VHSV) is an important pathogen of cultured and wild fish in marine and freshwater environments. A new genotype, VHSV IVb, was isolated from a fish collected from the Great Lakes in 2003. Since the first isolation, VHSV IVb has been confirmed in 28 species, signaling the early invasion and continued spread of this Office International des Epizooties–reportable agent. For surveillance of this virus in both wild and experimental settings, we have developed a rapid and sensitive one- step quantitative real-time polymerase chain reaction (qRT-PCR) assay that amplifies a 100-base-pair conserved segment from both the genomic negative strand and the mRNA positive strand of the nucleoprotein (N) gene of VHSV IVb. This assay is linear over seven orders of magnitude, with an analytical capability of detecting a single copy of viral RNA and reproducibility at 100 copies. The assay is approximately linear with RNA input from 50 to 1,000 ng per assay and works equally well with RNA prepared from a column-based or phenol-chloroform-based method. In wild-caught fish, 97% of the cases were found to be more than three orders of magnitude more sensitive using qRT-PCR than using cell culture. Of the 1,428 fish

from the Great Lakes region tested in 2006 and 2007, 24% were positive by qRT-PCR whereas only 5% were positive by cell culture. All of the fish that were positive by cell culture were also positive by qRT-PCR. Importantly, qRT- PCR sensitivity is comparable to that of cell culture detection when comparing VHSV viral RNA levels with viral titer stocks, confirming that the high qRT-PCR signals obtained with diagnostic samples are due to the accumulation of N gene mRNA by transcriptional attenuation. The qRT-PCR assay is particularly valuable for rapid and high-throughput prescreening of fish before confirmatory testing by cell culture or sequencing tissue-derived amplicons and especially in detecting infection in fish that do not show clinical signs of VHS.

* Hope KM, Casey RN, Groocock GH, Getchell RG, Bowser PR and Casey JW. 2010. Comparison of quantitative RT-PCR with cell culture to detect viral hemorrhagic septicemia virus (VHSV) infections in the Great Lakes. Journal of Aquatic Animal Health 22(1):50-61. Copyright American Fisheries Society, reprinted with permission. Hope wrote the manuscript and provided data for Figures 1 through 6. Casey RN processed qRT-PCR samples for Figure 7 and ran the initial qRT-PCR assays for VHSV IVb. Groocock and Getchell dissected tissue samples and performed cell culture on the indicated samples for Figure 7. Bowser contributed intellectual direction pertaining to fish pathology. Casey JW is the primary investigator and contributed intellectually, particularly relating to qRT-PCR.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is both highly pathogenic for fish and spreading to new geographic locations. There are four distinct genotypes of VHSV based on the sequence of either the glycoprotein or nucleoprotein genes of various isolates of VHSV and these generally correlate with geographic location (9, 22, 89). Isolates from genotypes I, II, and III have been found mainly in Europe and Japan, whereas genotype IVa predominantly contains marine isolates from the west coast of North America. After its initial isolation in the 1960s, VHSV was thought to be both retrospectively associated with mass mortality in European fish farms as early as the 1930s and primarily a disease of rainbow trout, *Oncorhynchus mykiss* (87, 106). In 1988, a new variant of VHSV, now known as VHSV genotype IVa**,** was isolated from pacific salmon retuning inland for spawning (68) and later in mortality events affecting Pacific herring, *Clupea pallasi*, Pacific hake, *Merluccius productus*, and walleye Pollock, *Thergra chalcogramma* (41). In the summer of 2003, a new genotype, VHSV IVb, was isolated from dead muskellunge, *Esox masquinongy*, and freshwater drum, *Aplodinotus grunniens,* in the Great Lakes basin (25, 61). Significant fish kills and surveillance efforts in this region from 2006 to 2008 found the virus to be infecting a wide variety of fish. Currently twenty-eight fish species are susceptible to VHSV infection and are regulated by the USDA VHSV Federal Order (97). Furthermore, the virus has been found throughout the Great Lakes basin with the exception of Lake Superior. Importantly, VHSV isolations were made from clinically normal fish in 2008 from Clear Fork Reservoir, Ohio, a region outside of the Great Lakes basin, suggesting that VHSV may spread to southern regions of the United

States.

The currently used and accepted international standard for detecting VHSV is detailed in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE) and includes an initial cell culture to identify the presence of an infectious agent. This is followed by RT-PCR and nucleotide sequencing to confirm the presence of VHSV as the infectious agent. In endemic regions, cell culture can be circumvented by RT-PCR and amplicon sequencing (107). Although cell culture methods are essential for the confirmation of infectious VHSV, cell-culture isolation is limited by its lack of sensitivity, speed, and throughput (18, 38, 56, 77, 86). Molecular approaches, including qRT-PCR, are more sensitive in pathogen detection, can be accomplished in a few hours, and are being rapidly employed as preliminary screening tools to aid in confirmation by laboratories in research settings and more slowly by international regulatory agencies.

In a research setting, quantitative RT-PCR (qRT-PCR) is common practice for the detection of many fish RNA viruses, including other VHSV genotypes as well as other fish rhabdoviruses, like SVCV and IHNV (27, 62, 64, 79, 110). From these reports, qRT-PCR has been shown to be a reproducible, highly specific assay to evaluate varying levels of virus both from tissues and water samples, as well as significantly less time consuming than many other more commonly accepted cell culture methods. In our study, a TaqMan probe-based qRT-PCR system was designed to amplify a 100-bp segment of the N gene of VHSV genotype IVb. The sensitivity and specificity were evaluated and found to be similar to those in other reports of viral detection using qRT-PCR assays. This assay was extensively evaluated on fish

brought in from the Great Lakes region either showing signs of disease or clinically normal, and compared to the cell culture protocols for VHSV detection.

METHODS

Fish collection and tissue extraction.

Between 2006 and 2007, fish were collected in and around the Great Lakes Basin either live or frozen within 24 h of collection. Following collection, the fish were delivered to the Cornell Fish Disease Diagnostic Laboratory where they were categorized as normal or suspected of having VHS, based on gross external and internal examination. All live fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate, Western Chemical Inc, Ferndale, Washington). Fish were processed for diagnostic evaluation as previously described (75). This included collecting skin scrapings and gill clips, sterile collection of posterior kidney samples for bacteriology, gross pathology, and collection of tissues for histopathology and virology. Samples of liver, anterior and posterior kidney, spleen, and heart were collected and pooled for the isolation of VHSV in cell culture or by qRT-PCR. Fish tissues were most commonly stored at -20°C or -80°C until the tissues could be lysed and homogenized.

Tissue lysis and homogenization.

For lysis and homogenization, pooled fish tissues (liver, anterior and posterior kidney, spleen, and heart) were mixed with 1 mL of sterile phosphate-buffered saline (1×PBS) solution and applied either to a Bead-Beater (Bio-Spec Products) or manually processed using a mortar and pestle. Samples were kept on ice throughout this process. Briefly, the PBS-suspended tissues were mixed with 100 to 150 mg of

0.1 mm zirconia/silica beads in the designated sterile 2 mL screw-cap tubes (Bio-Spec Products), and homogenized for 10 s in the BeadBeater (Bio-Spec Products). Following homogenization, the samples were centrifuged at 10,000 revolutions per min (rpm) for 5 min at room temperature in an Eppendorf Model 54-15 centrifuge. The supernatant was transferred to a sterile 1.5 mL microcentrifuge tube and kept on ice until further processing. For samples that were manually processed using a mortar and pestle, 100 to 200 µL of PBS were added to the fish tissue in a sterile 1.5 mL microcentrifuge tube and homogenized for 2 min with a sterile plastic pestle. After this process, samples were centrifuged for 10,000 rpm for 5 min at room temperature. After the cells were lysed, homogenized, and centrifuged, a portion of the supernatant (0.02 to 0.06 g tissue weight) was used to isolate total RNA for qRT-PCR and the remaining supernatant (0.10 to 0.50 g tissue weight), frozen or direct application, was further diluted for cell culture. The dilutions ranged from 1:50 to 1:100 depending on the sample.

Cell culture.

Epithelioma papulosum cyprini (EPC) cells used for virus isolation were obtained from ATCC (30). These cells were routinely grown in 75-cm^2 or 25-cm^2 tissue culture flasks (Corning, Inc.) using Eagles Minimum Essential Medium with HEPES buffer containing 10% Fetal Bovine Serum (FBS) with penicillin, streptomycin, and L-glutamine (EMEM-10FPSH) as described (37). Cells were sub-cultivated through the use of a 0.05% trypsin wash to dislodge the cells from the surface of a flask, followed by re-suspending the cells in an appropriate volume of EMEM-10FPSH prior to seeding in new culture vessels. Virus isolations were

performed in 48-well tissue culture plates (Corning Inc.). One confluent 25-cm^2 tissue culture flask was used to seed each 48 well tissue culture plate, for an approximate 1:2 split. Cells were harvested as described above for cell passage, but were then re-suspended in EMEM-5 (same formulation as EMEM-10 but with 5% FBS). To each well of the 48 well plate, 500 µL of cell suspension was seeded. Sample homogenate was then thawed, in most cases within 2 weeks of dissection out of the fish, and used to inoculate triplicate wells of the 48 well plate for virus isolation before 48 h of cell growth time had passed so that the cells might be in a state of active division. During this inoculation process, 100 µL of filtered inoculum was carefully placed in each well to avoid disrupting the EPC monolayer. After inoculation, EPC cells were incubated at 15^{o}C for a maximum duration of 28 d. Cells were examined for cytopathic effects (CPE) at 1, 3, 7 and 14 days post inoculation. If at any time CPE was noted, cells and media were removed and passaged to fresh EPC cell monolayers after being filtered through a 0.2 micron porosity filter. Passage zero (p0) (the initial inoculation) was always passaged to P1 after 14 d even if no CPE was observed. Passage P1 however was only passaged if CPE was noted. Otherwise, the cells were scored as VHSV-negative at 28 d. Under the criteria used, a sample must show CPE for three consecutive passages and be confirmed using RT-PCR to be considered VHSV-positive.

Total RNA isolation.

Total RNA was isolated from the homogenized samples using one of two methods: a phenol-chloroform-based extraction process, RNA Bee reagent (Tel-Test) or a column-based extraction process, RNeasy mini kit (Qiagen). For the RNA Bee

process, total RNA was isolated according to the manufacturer's protocols with the following modifications. All samples and reagents were kept on ice or chilled to 4°C. To increase its performance, additional guanidine thiocyanate at a concentration of 0.2 g/mL was added to the RNA Bee reagent. One milliliter of this reagent was added to the homogenized tissue, followed immediately by 0.2 mL of chloroform. The tube was then inverted twenty times and left on ice for 10 min. After centrifugation (Eppendorf) at room temperature for 10 min at 13,000 rpm, the upper, aqueous layer was removed and added to 800 μL of isopropanol. The tube was vortexed briefly and then stored on ice for a minimum of 20 min or kept at –20°C overnight. The sample was then centrifuged at room temperature for 10 min at 13,000 rpm and the supernatant decanted. The pellet was washed with 1 mL of 75% ethanol, followed by a final centrifugation at room temperature for 10 min at 13,000 rpm. Ethanol was carefully removed with a pipette and the RNA pellet was allowed to semi-air-dry. RNA pellets were re-suspended in 100 to 200 μL of water treated with diethyl pyrocarbonate (DEPC) and incubated for 10 min in a 65°C water bath. The concentration of each sample was determined using a spectrophotometer, either a Beckman DU-40 (Beckman-Coulter) or a NanoVue (GE Healthcare Bio-Sciences Corp.). In preparation for qRT-PCR, dilutions were made to achieve 50 ng of total RNA per well of a 96-well plate.

For Qiagen's RNeasy kit, a modified version of the manufacture's protocols were used as follows. All centrifugation steps were performed at 10,000 rpm unless otherwise stated below. Six hundred microliters of both Buffer RLT and sterile 70% ethanol was added to the cleared lysate from the BeadBeater procedure. The mix was

applied to the RNeasy column and centrifuged for 15 s twice to allow for the full volume to be added to the column. To fully remove the wash buffers, an additional 2 min centrifugation step into a clean sterile collection tube was included immediately following the final RPE centrifugation step. To elute the total RNA from the column, 50 μL of RNase-free water was added and centrifuged for 1 min at 13,000 rpm. To allow for a more concentrated sample, this step was repeated, eluting the same 50 μL back through the column. The final concentration of RNA was quantified by an OD_{260} reading using a spectrophotometer, either a Beckman DU-40 (Beckman-Coulter) or a NanoVue (GE Healthcare Bio-Sciences Corp.). In preparation for qRT-PCR, dilutions were prepared to achieve 50 ng of total RNA per well of a 96-well plate.

Design of primers and probe for qRT-PCR.

The primers and probe were designed to target the N gene of the VHSV IVb MI03 isolate (25). The 400 base-pair conserved region of the N gene was selected for primer and probe design in order to produce an amplicon of approximately 100bp (83). The primers and probe were designed using ABI software.

The following were used: forward- 5'-
ACCTCATGGACATCGTCAAGG – 3', reverse- 5' -
CTCCCCAAGCTTCTTGGTGA - 3', and probe- 5' - /56-
FAM/CCCTGATGACGTGTTCCCTTCTGACC/36-TAMSp/ - 3'.

Standard curve for VHSV.

A VHSV-relevant RNA standard made from a VHSV-infected round goby, *Neogobius melanostomus*, was calibrated to the absolute values of T7-prepared viral RNA (35, 37). Briefly, 420 bp of the N gene of an isolate of VHSV IVb, previously

sequenced (25), was amplified using conventional PCR and then cloned in both orientations into pTopo-TA. Utilizing the vector's T7 promoter, viral RNA was transcribed using the MAXIscript T7 Kit (Ambion, Austin, Texas), precipitated, and re-suspended according to the manufacturer's protocol. The T7 transcribed RNA was digested with Turbo DNAase (Ambion) according to the manufacturer's protocol. The efficiency of the qRT-PCR using T7 transcribed RNA was evaluated using the slope of the amplified standards, which at -3.75 was comparable to the range shown in Figure 2.1. The final concentration of RNA was quantified by an OD_{260} reading. To assess the presence of remaining plasmid template DNA, an aliquot (3×10^6 copies) of T7 transcribed RNA was digested with RNase both before and after Turbo DNase digestion. Before Turbo DNase digestion, plasmid DNA template accounted for 0.1% of the RNA preparation (2.3×10^3 copies). After Turbo DNase digestion, plasmid DNA template was reduced to 0.0001% (5 copies). To generate a standard curve for use in qRT-PCR, 10-fold serial dilutions were made based on copy number per 50 ng of total RNA starting at 1×10^6 copies.

In order to avoid the burdensome process of continually making T7 RNA and to create a standard with an environment more similar to the viral RNA found in fish tissues, total RNA was isolated from the tissues of a VHSV infected round goby in 2005, further purified using Qiagen's RNA Easy Purification Kit, and then calibrated to the T7-prepared viral RNA standard curve in a qRT-PCR assay. A 10-fold serial dilution of this round goby isolate was then used in all future assays as the standard curve for determining the copy number of the VHSV IVb N gene starting at 3×10^7 copies.

qRT-PCR assay.

The assay was performed according to the manufacturer's protocols using their TaqMan One-Step RT-PCR Master Mix Reagents and run on an Applied Biosystems-Prism model 7700 sequence detector (ABI). Briefly, the unknowns, the standards, and the no-template controls (which contained either DEPC treated or RNase-free water in place of template) were run in duplicate on a MicroAmp Optical 96-well reaction plate from ABI. Each 25 μL per well reaction was comprised of 15 μL from the master mix solution, (final amounts per reaction: 1×Multiscribe, 1×TaqMan Universal PCR Master Mix with No AmpErase® UNG, 200 nM forward primer, 200 nM reverse primer, and 200 nM probe), and 10 μL of sample at a concentration of 50 ng total RNA/10 μL unless stated otherwise. The polymerase chain reaction conditions were as follows: 30 min at 48°C for reverse transcription; 10 min at 95°C for AmpliTaq activation; 15 s at 95°C for denaturing followed by 1 min at 60°C for annealing and extension, repeated for 42 cycles. Absolute copy numbers in unknown samples were determined from a standard regression fit using the supplier's software, SDS (ABI).

Isolation of viral RNA from VHSV IVa and SVCV.

Total RNA was prepared as described above for viral stocks of both the Makah isolate of VHSV IVa (NCBI Ac#: X59241) and the 2002 Wisconsin isolate of SVCV (NCBI Ac#: NC_002803). These isolates were obtained from Jim Winton and Gael Kurath via the U.S. Geological Survey's Western Fisheries Research Center in Seattle, WA.

RESULTS

Analytical sensitivity and reproducibility of VHSV N gene primers and probe

A VHSV-relevant RNA standard made from an infected round goby was calibrated to the absolute values of T7-prepared viral RNA. The standard curve, a 10-fold dilution series of this RNA, showed excellent reproducibility and high sensitivity. Amplification is observed to a single copy level. An example of a representative standard curve is shown both as an amplification plot (Figure 2.1A) and as a standard plot (Figure 2.1B) where the standards were run in duplicate on a 96-well plate.

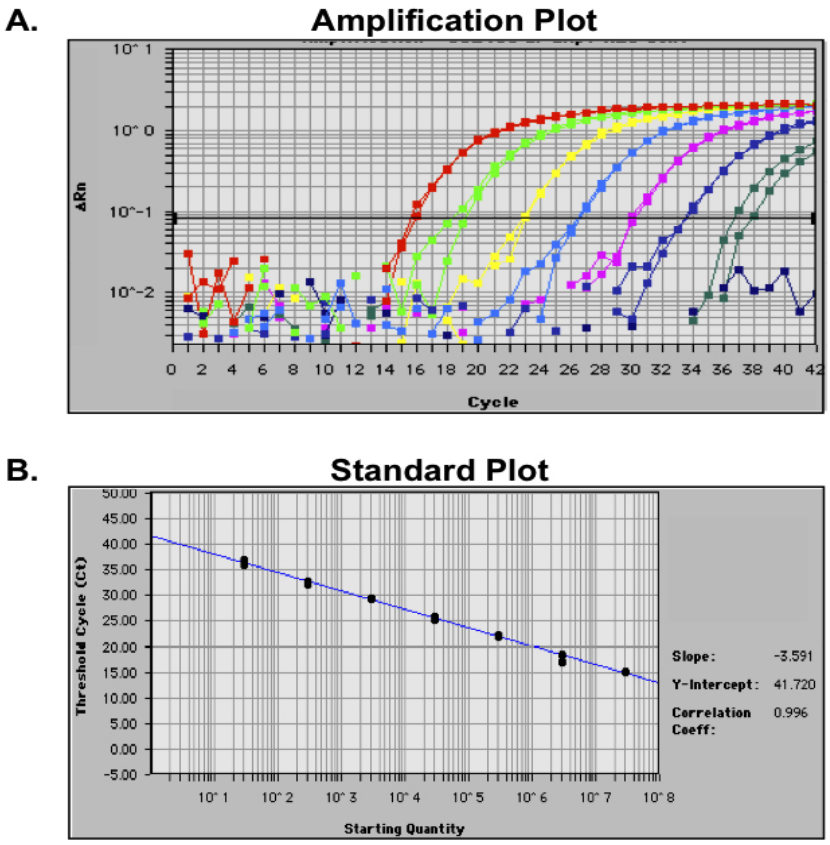

Figure 2.1 Quantification of a representative VHSV standard curve using the Taqman probe-based qRT-PCR. (A) Amplification plot of 10-fold serial dilutions of standards ranging from 10^1 to 10^7 copies. Bold line indicates threshold used to create the standard plot. (B) Standard plot showing the threshold copies (C_t) versus viral copy number.

The amplification of VHSV RNA serial dilutions was linear over seven orders of magnitude. Based on regression analysis, the correlation coefficient was 0.996 and the slope was –3.6. The qRT-PCR assay is approximately linear with RNA input from 50 to 1,000 ng per reaction (Figure 2.2). An observed 2-fold reduction in the expected signal (20-fold), based on the 50 ng C_t value, was observed at the highest input sample (1 μg). Independently run assays at higher RNA input (4–8 μg) result in an increase signal but more dramatically reduced from the expected signal.

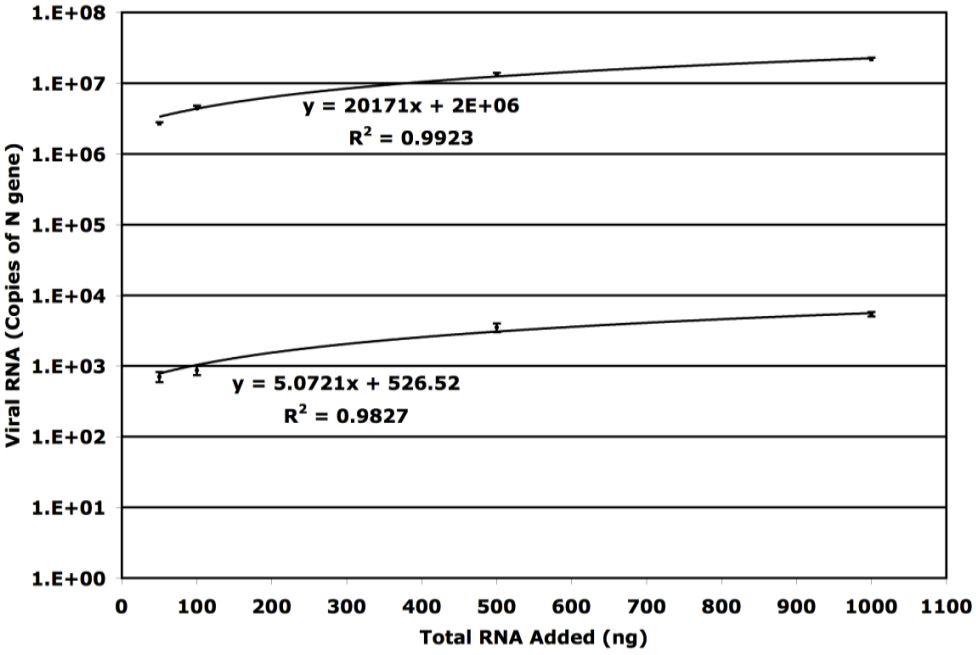

Figure 2.2 Effect of RNA concentration on the amplification of VHSV. Increasing amounts of total RNA extracted from two infected fish, ranging from 50 ng to 1 μg, were assayed by qRT-PCR. Upper curve represents a fish carrying 4×10^6 copy numbers (squares) and the lower curve represents a fish carrying 9×10^2 (circles). Averages of duplicate samples are shown at each experimental point.

By comparing several standard curves run on different plates and at different times, inter-assay variations were evaluated (Figure 2.3). Each point on the graph

represents an average of 23 different values from 23 different standard curves. The standard error associated with these averages ranged from 0.1 to 0.4, with the higher variations occurring with the more dilute samples. Although the assay detected as low as one copy of viral RNA per 50 ng of total RNA, it is reproducible with a 95%

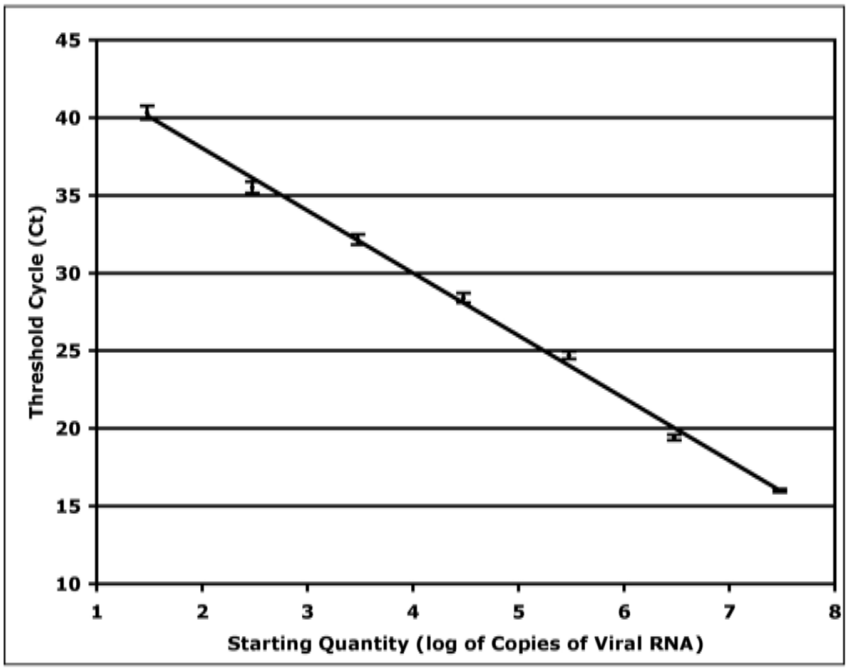

Figure 2.3 Variation in standard curves for detecting VHSV using qRT-PCR. The cumulative average of 23 individual standard curves is shown with a slope of – 4.02, a y-intercept of 46.1, and a correlation coefficient of 0.997. Error bars represent standard error of the mean.

confidence on the order of 10^2 copies of viral RNA per 50 ng of total RNA based on technical duplicates on a single plate (data not shown). For the standard on the order of 10^2 copies of viral RNA, one of two wells was amplified 100% of the time, whereas both wells were amplified 96% of the time. When evaluating the standard on the order of 10^1 copies of viral RNA, one of two wells was detected only 65% of the time and both wells were only detected 17% of the time.

Analytical specificity of VHSV N-gene primers and probe.

The primers and probe used in this assay were targeted to the central region of the N gene of the Michigan isolate (MI03) of VHSV genotype IVb (Table 2.1). These regions, which show the highest degree of similarity to many isolates of VHSV

Table 2.1 Sequence alignment of N-gene amplicon regions of various fish rhabdoviruses compared to VHSV genotype IVb primers and probe. Genbank accession numbers: VHSV IVb DQ427105; VHSV IVa X59241; VHSV I Z93412; VHSV III AB179621; IHNV NC_001652; SVCV NC_002803. The sequence for this region for genotype II has not been published.

```
                        Forward Primer
                      ACCTCATGGACATCGTCAAGG
VHSV IVb    …480-_____________________-500…
VHSV IVa    …454-_____________________-474…
VHSV I      …367-__________A_____T____-387…
VHSV III    …535-_____________________-555…
IHNV        …539-_TG__C_T__GG_T__G_CC_-559…
SVCV        …663-GTT_T_CAAC___TTCA__AA-683…

                             Probe
                      CCCTGATGACGTGTTCCCTTCATGACC
VHSV IVb    …504-_____________________Δ_____-529…
VHSV IVa    …478-_____________________T_____-503…
VHSV I      …392-__T_______A__C_______Δ_____-417…
VHSV III    …559-_____________________TΔ_____-584…
IHNV        …562-T___CT_C__C__CG____A_Δ____T-587…
SVCV        …688-_AT_A_A_CAACA____GG_GGG__A_-713…

                        Reverse Primer
                      TCACCAAGAAGCTTGGGGAG
VHSV IVb    …561-_____________________-580…
VHSV IVa    …535-_____________________-554…
VHSV I      …449-__________A__C______-468…
VHSV III    …616-_____________________-635…
IHNV        …617-G_CAA__C_____C_A_CGT-636…
SVCV        …747-C____CT_GGA_A__TT_TT-766…
```

genotype IVa, are significantly different from most other genotypes of VHSV, as well as other fish rhabdoviruses, such as SVCV and IHNV. Using the probes and primers designed for the MI03 isolate of VHSV in the qRT-PCR assay, total RNA extracted from the Makah IVa isolate amplified well, whereas no amplification was observed from total RNA extracted from an isolate of SVCV (Figure 2.4). SVCV RNA did

amplify with a similar C_t value to VHSV IVa using qRT-PCR primers and probe specifically targeting the N gene of SVCV.

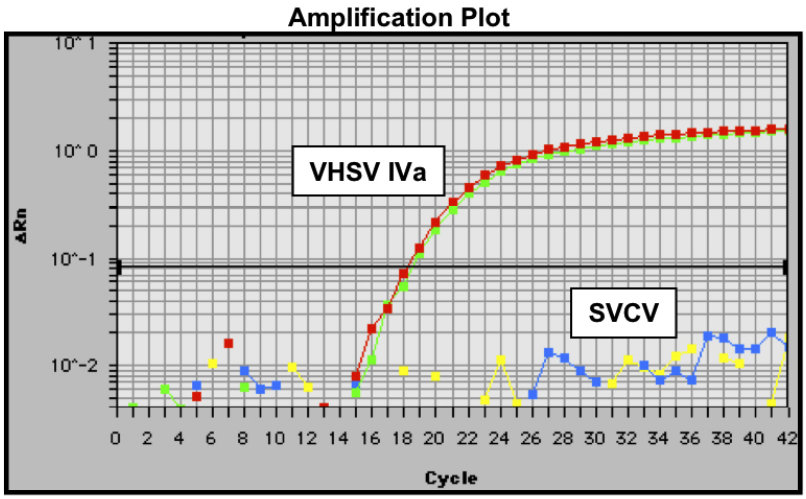

Figure 2.4 Evaluation of the specificity of the VHSV MI03 primers and probe in qRT-PCR. The amplification plot of RNAs from SVCV and VHSV IVa is shown. RNA representing the equivalent of 10^7 PFU per well was run in duplicate.

Comparison of RNA-purification protocols.

RNA purity and integrity are major concerns for this assay. Two RNA-isolation procedures were compared to optimize the isolation of total RNA (Figure 2.5), a phenol-chloroform-based assay using the Tel-Test RNA-Bee reagent and a column-based assay using Qiagen's RNeasy Mini Kit. To thoroughly compare these two procedures, fish tissue was spiked using a viral stock of the Michigan isolate of VHSV to get final concentrations of 10^2, 10^4, and 10^6 plaque-forming units (PFU) per 30 mg of tissue and a negative control. Total RNA purity, after isolation by either method, was not significantly different as measured by the A260/A280 ratio, ranging from 2.0 to 2.1. The total yields of RNA were also not significantly different between the two

procedures. To evaluate the quality of the RNA, the spiked samples were run in the qRT-PCR assay (Figure 2.5). The negative-control RNA did not show amplification in

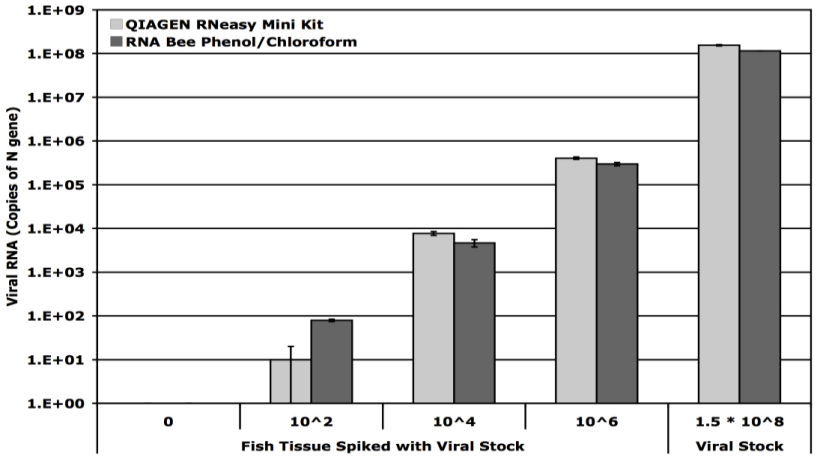

Figure 2.5 Comparison of RNA-isolation procedures on qRT-PCR amplification of VHSV. Thirty milligrams of fish tissue spiked with 0, 10^2, 10^4, or 10^6 PFU was extracted using either the Qiagen column-based method or the phenol-chloroform-based method and assessed by qRT-PCR. Control viral stock RNA is shown on the right.

this assay using either RNA isolation method, whereas all three spiked samples amplified at their predicted copy numbers regardless of the isolation method.

The sensitivity of qRT-PCR was compared to a viral titer stock. By definition, RNA amplified by qRT-PCR in this experiment represents primarily minus-strand genome transcripts (82). In eight independent experiments over a 4-month period, the variation between these two parameters ranged from 0.4 to 1.3 logs more in the qRT-PCR assay than in the viral titer stock (Figure 2.6).

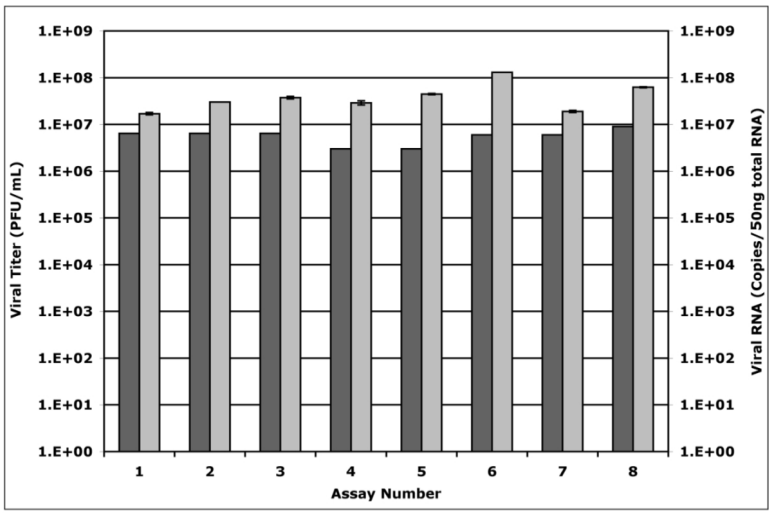

Figure 2.6 Viral RNA levels determined by qRT-PCR correlate closely with viral titer. Eight independent assays over a 4-month period were evaluated in duplicate. The expected viral copy number is shown in dark bars (left y-axis) and the actual viral copy number obtained is shown in the lighter bars (right y-axis).

Sensitivity of the qRT-PCR assay is significantly higher than cell culture when evaluating VHSV in fish tissues.

Tissues from 1,428 individual fish collected from several different locations in the Great Lakes region were evaluated independently by both cell culture and by qRT-PCR. For this data set, cell culture entails primary identification of the presence of a filterable agent that produces a cytopathic effect (CPE) in EPC cells, followed by RT-PCR, and in some cases nucleotide sequencing, to identify VHSV as the specific agent causing the CPE. In order to be cell culture positive for VHSV, a sample must produce CPE in three successive passages and be identified as VHSV using RT-PCR. In comparison to this cell culture assay, we are using the qRT-PCR assay that has been described in this manuscript. As applied to this data set, a qRT-PCR positive sample is defined as any sample with at least one well that results in a C_t value with an

amplification curve following the formula 2^n, which is the formula that results in a theoretical amplification of a known target sequence.

Of these 1,428 samples, 69 were cell culture positive, whereas 1,359 were negative (Figure 2.7A). From the 69 cell culture positive fish, all were qRT-PCR positive (Figure 2.7A). From the 1,359 samples negative by cell culture, 1,034 were also negative by qRT-PCR; however, 325 were positive by qRT-PCR (Figure 2.7A). When evaluating the relative threshold of sensitivity, VHSV was detected by cell culture from the majority of samples that displayed at or above 10^4 copies of viral RNA (Figure 2.7B). However, below 10^3 viral RNA copies, only 3 to 18% were cell culture positive, whereas all of these samples were detected by qRT-PCR.

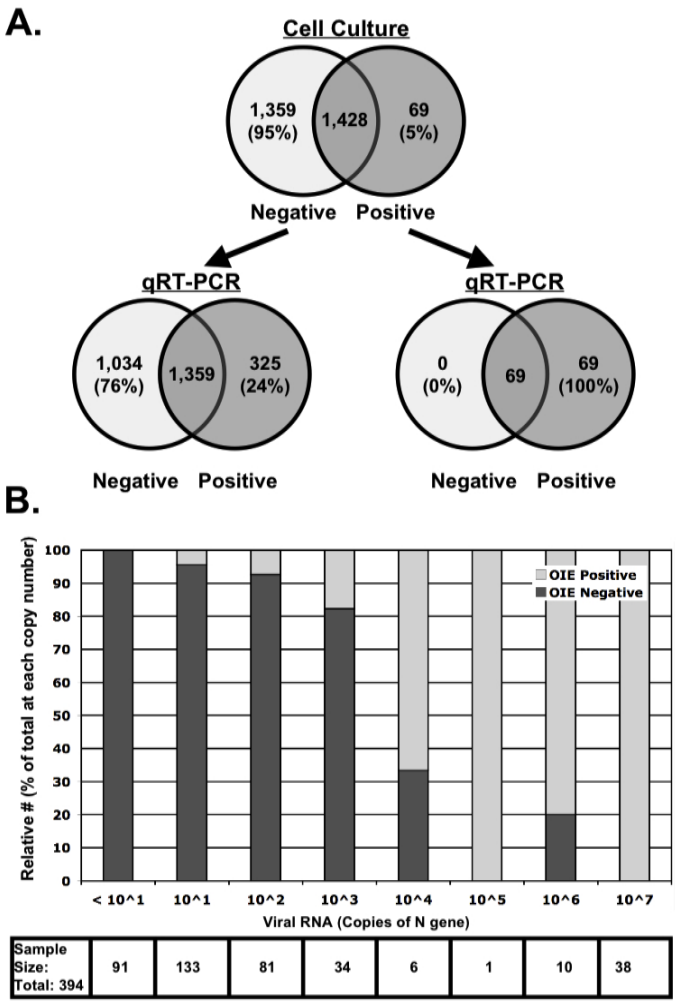

Figure 2.7 Comparison of qRT-PCR to cell culture from fish collected in the field. (A) Venn diagrams of 1,428 individual fish samples. (B) Distribution of the 395 qRT-PCR positive samples according to copy number and OIE status.

To evaluate the presence of clinical signs of VHS, all of the fish in the data set were grossly examined both externally and internally for the presence of hemorrhaging. Of the 1,359 fish that were negative by cell culture, 32 fish (2%) had varying signs of clinical disease, including internal or external hemorrhaging or both, whereas the other 1,327 fish were clinically normal (98%). Of these 32 fish with clinical signs that were cell culture negative, only 2 were positive for VHSV by qRT-

PCR. This was on the order of 10^6 copies of viral RNA. Of the 69 VHSV-cell culture positive fish, 32 (46%) had visible clinical signs of disease, whereas the other 37 were clinically normal.

The prevalence of VHSV in cell culture was sixty-nine out of 1,428 samples (4.8%) with a binomial exact confidence interval of 3.8% to 6.0%. The use of qRT-PCR determines the prevalence at 395 out of 1,428 samples (28%) with a binomial exact confidence interval of 25% to 30%. There is complete separation of the confidence intervals, which shows that qRT-PCR detects a significantly higher prevalence than standard cell-culture methods. Additionally, cross-tabular frequency distributions for the two tests show that there is a strongly significant association (Fisher's Exact Test p-value < 0.001) between the results of the cell-culture and the qRT-PCR testing.

DISCUSSION

Evaluation of the qRT-PCR assay for VHSV IVb.

Due to high-throughput sampling, sensitivity, reliability, and speed, qRT-PCR methods utilizing the TaqMan system are now being developed routinely for pathogen diagnosis and gene-expression studies in aquaculture, including those for rhabdoviruses such as IHNV, SVCV, and other VHSV genotypes (27, 62, 64, 79, 110). In the case of these fish rhabdoviruses, the development of nucleic acid based assays have targeted different viral genes to achieve high sensitivity. In an attempt to detect various isolates in the same genotype, a highly conserved gene, such as the polymerase, is often chosen as the target for the qRT-PCR assay (27). However, the process of transcriptional attenuation that occurs during rhabdovirus replication results

in high copy numbers of the nucleoprotein (N) gene mRNA, ranging from 50- to 200-fold higher than that of viral polymerase mRNA (54, 101). Thus, detection of the N gene in our qRT-PCR assay to detect VHSV IVb instead of the viral polymerase gene has greatly improved the overall sensitivity of the assay. More specifically, the central portion of the N gene of VHSV was targeted for amplification in the qRT-PCR assay due to its conserved RNA-binding function (82, 83). The one-step TaqMan approach described here amplifies both the negative-strand genomic RNA and the positive-strand mRNA transcripts of VHSV. Because the assay does not distinguish between virion RNA and viral mRNA, N-gene copy number does not directly correlate with the amount of infectious VHSV in fish tissue. However, the qRT-PCR-derived N-gene copy number is closely comparable, only 0.4 to 1.3 logs higher than the VHSV titer determined by cell-culture-based plaque assays (Figure 2.6). This further emphasizes that the increased qRT-PCR signal in total RNA isolated from infected fish tissue as compared to that identified using plaque assays is due to the presence and detection of viral mRNA. To directly measure the viral-genome copy number during replication of VHSV and at different disease stages, as has been done with IHNV (79), a two-step qRT-PCR strategy selectively amplifying either the positive or negative strand will be required.

Analytical sensitivity.

The high sensitivity and reproducibility of this qRT-PCR assay is apparent when comparing 23 different standard curves run at different times. A correlation coefficient of 0.997 indicates both a good linear fit and high reproducibility of the standard samples. Furthermore, the slope of – 4.02 is within an acceptable range of the

theoretical yield of –3.3, indicating an accurate reading of the dilution series and near optimal primer and probe concentration. The standard error associated with these averages ranged from 0.1 to 0.4, with the higher variations occurring with the more dilute samples, which is a range consistent with other qRT-PCR assays for other fish pathogens (64, 79, 110).

This qRT-PCR assay is approximately linear with RNA input from 50 to 1,000 ng per reaction (Figure 2.2). A 2-fold observed reduction in the expected signal (20-fold) was observed at the highest input sample (1000 ng); however, from assays where higher amounts of total RNA (4–8 μg) were used, a further reduction in expected signal was observed. With this lack of linearity at high concentrations of total RNA in a reaction, quantifiable value of the viral copy number would have to be derived from an established concentration dependent standard curve. Thus, in order to keep the quantifiable aspects of this assay, we recommend not adding more that 1000ng of total RNA under the present conditions.

Analytical specificity.

Although the qRT-PCR approach presented here was focused on targeting VHSV IVb, the primers and probe also cross-react with at least one other isolate of VHSV (Table 2.1; Figure 2.4). The primers and probe designed for genotype IVb amplified both genotypes IVa and IVb since the target sequences were well conserved with only a one-base substitution in the probe. Amplification was not observed with the fish rhabdovirus SVCV RNA due to the extensive sequence divergence between these viruses. To differentiate between different genotypes of VHSV, a different set of primers and probe would need to be designed and tested. It appears that other

genotypes of VHSV are less of a concern at the present time in this region since there are very few sequence differences between Great Lakes isolates, all of which are still classified as genotype IVb (104).

Comparison of qRT-PCR with cell culture.

This qRT-PCR assay is more sensitive than a cell culture based method of detecting VHSV in fish tissues. Approximately 10^4 copies of the N gene (detection limit as predicted by qRT-PCR) must be present in a tissue sample to isolate VHSV in cell culture. Using the qRT-PCR method, the limit that reproducibly detects VHSV is approximately 10^2 copies of viral RNA, or 100 times more sensitive than cell culture identification. If the qRT-PCR assay is carried out with 1 μg of input RNA instead of 50 ng, the sensitivity becomes 10-fold greater and is now 1,000 more sensitive than cell culture.

As well as higher sensitivity, qRT-PCR provides a quantitative analysis that conventional RT-PCR cannot, while also eliminating post amplification steps, thereby reducing the risk of cross contamination events in subsequent testing (42). Although qRT-PCR cannot replace cell culture, since it does not evaluate the infectivity of the virus nor detect sequence variants, it will serve as a valuable screening tool in detecting a defined pathogen like VHSV IVb, especially where high-throughput sampling is necessary (27, 56, 62). Furthermore, in endemic regions, RT-PCR based methods are often sufficient (107).

Real-world applications.

RNA quality is an important issue in this assay. Besides the obvious concerns for RNA degradation during processing of tissues, there could be selective degradation

of mRNA compared to virion RNA that may be somewhat protected by its capsid. Similarly, some RNA-preparation methods may offer advantages in limiting RNAse activity during thawing of frozen fish. We have compared the efficacy of two RNA-isolation procedures, RNAbee and RNeasy. The two methods were essentially indistinguishable with regard to RNA suitability in this qRT-PCR assay (Figure 2.5). Slightly higher amounts of RNA were achievable with RNAbee, but precautions in handling toxic chemicals and residual waste are major concerns. RNeasy is convenient and presented in a well-defined kit format, and the yield of RNA (100 μg) is more than sufficient for these applications. In both methods, we saw no loss of RNA during the homogenization step when we would have predicted tissue RNAses to be most active. Surprisingly, it appears that tissue homogenized in PBS versus guanidine thiocyanate-based lysis buffers had no effect on preserving RNA integrity as measured by qRT-PCR. However, this qRT-PCR assay targets a very short 100-bp region of the N gene and some RNA hydrolysis could have occurred without compromising the assay.

Comparing this qRT-PCR assay and cell culture protocols using wild fish samples taken from the Great Lakes during 2006 and 2007, qRT-PCR was found to be as accurate as cell culture identification, but far more sensitive to detect VHSV infection. For example, of 1,428 samples evaluated, all 69 VHSV-cell culture positive samples were also positive by qRT-PCR. However, of 1,359 samples negative according to cell culture, 325 (24%) were positive according to qRT-PCR. Four of these 326 cases (1%) had copy-number values of 10^4 or more, which we would have predicted to be positive by cell culture. Although it's unclear why this small fraction

was not identified in cell culture, we suspect it may be due to limitations such as the amount of available sample, toxicity of the sample, or the age of the sample.

Although often used to first identify the potential presence of VHSV, clinical signs of disease, including external hemorrhaging, are not sufficient to determine whether a fish is positive or negative. Of the 1,359 cell culture negative samples, 32 showed clinical signs and only two of these 32 were qRT-PCR positive. On the other hand, of the 69 fish that were both cell culture and qRT-PCR positive, 37 of these fish showed no signs of disease. These data ultimately indicate that the clinical signs of disease, although helpful when seen, are not indicative of VHSV infection. Furthermore, clinical signs for VHSV are also shared with many other pathogens, including SVCV.

In summary, the qRT-PCR assay described here, with its increased sensitivity and specificity and decreased time requirements is beneficial both as a diagnostic aid to rapidly and accurately identify VHSV in fish and as a research tool to follow the fate of the virus in an experimental setting. This assay will be particularly useful in survey work to detect fish with sub-clinical infections and ultimately determine the threat that VHSV poses in both endemic and non-endemic regions. The ability to accurately detect the presence of the VHSV is critical, especially for an agent with such compelling ecological and financial impacts on the Great Lakes region (104).

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CHAPTER 3

THE DEVELOPMENT OF A ZEBRAFISH, *DANIO RERIO*, INFECTION MODEL FOR THE GREAT LAKES ISOLATE OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IVB

Kristine M. Hope, Paul R. Bowser, and James W. Casey

ABSTRACT

Viral hemorrhagic septicemia is a severe fish disease caused by the fish rhabdovirus, viral hemorrhagic septicemia virus, VHSV. Of the four described genotypes, the North American isolate VHSV IVa is most closely related to a recent fresh-water isolate VHSV IVb that invaded the Great Lakes in 2003. From the onset, VHSV IVb was associated with numerous mortality events but since 2008 now persists in fish in the absence of clinical disease. The stress of temperature change and spawning are thought to play a major role in disease susceptibility since disease and viral expression are highest at this time. We have employed a zebrafish infection model to define VHSV IVb infection conditions that favor mortality versus subclinical disease. Zebrafish are susceptible to VHSV IVb infection at 15°C, and susceptibility decreases based on both the length of time of acclimation to 15°C prior to infection and the virus dose. The dose exposure threshold for hemorrhagic disease induction and mortality occurs at a threshold between 10^5 and 10^6 pfu/mL regardless of the length of time of 15°C acclimation. At an infectious dose of 10^6 pfu/mL, 100% mortality results for fish acclimated to 15°C for 24-hours or for 2-weeks, however the mortality profiles are significantly different. The 24-hour acclimated fish reach 50% mortality at 6 dpi

while mortality is delayed to 13 dpi in fish acclimated for 2-weeks suggesting recovery from the initial short-term stress of rapid temperature change. In contrast, at exposure doses between 10^2 and 10^4 pfu/mL, many fish show levels of 10^2 to 10^6 copies of VHSV N gene RNA in their tissues and harbor infectious VHSV IVb measured by cell culture virus isolation. Maximal VHSV IVb shedding occurs in zebrafish that are infected at 10^4 pfu/mL, conditions where subclinical infections (70%) are favored. In summary, zebrafish offer a highly reproducible and accurate model that parallels environmental conditions and the pathogenic outcome of fish infected in the wild. Extension of these studies will help further define mechanisms of viral transmission and host immune responses that govern the long-term effects of invasive pathogens, like VHSV IVb, on the Great Lakes ecosystem.

*Hope wrote and performed all experiments in this chapter. Bowser contributed intellectually. Casey is the primary investigator of this work and contributed significantly in an intellectual manner.

INTRODUCTION

A new isolate of Viral hemorrhagic septicemia virus (VHSV IVb) has emerged in the Great Lakes and affects at least 28 fish species (11). VHSV is divided into four genotypes that generally correlate with geographic location (104). Interestingly, numerous isolates of VHSV IVb from different geographic sites, show little genetic diversity (104) suggesting that the invasion was one genotype. VHSV IVb was first identified in the Great Lakes region in 2005 associated with fish mortalities in Lake St. Clair and the Bay of Quinte, Lake Ontario, Canada (61). In the three years following the initial identification of VHSV IVb, additional mortality events at new locations occurred. However, from 2008 to the present, no significant mortality events were observed in the Great Lakes but VHSV IVb was found to persist in fish sampled from four of the Great Lakes tested in the absence of clinical disease(6).

Recent surveillance of the Great Lakes has focused on trying to better understand VHSV IVb persistence. Several possibilities exist for the observed persistence of VHSV in Great Lakes and most likely more than one contribute to this aspect of viral survival. The 2008 survey work found many species of fish to be likely "asympotomic carriers" of VHSV IVb, as they were clinically normal but had varying levels of viral RNA in their tissues. Surprisingly, the highest amounts of N gene copies detected were on the order of 10^7, which usually result in mortality. Additionally infectious VHSV IVb was rescued by cell culture in several cases and suggests that additional abiotic and biotic factors maybe necessary for overt disease(6). Interestingly, the persistence of VHSV IVb in the Great Lakes parallels earlier studies of clinically normal migrating salmon to the west coast of the United

States that were positive for VHSV IVa but also subclinically infected(13, 45). Further, VHSV IVb can persist free in freshwater and retain its infectivity at 4°C for more than a year (40). And, Faisal et al. found that *Diporeia* sampled from Lakes Huron, Ontario, and Michigan were positive for infectious VHSV IVb, indicating other species may also serve reservoirs for and contribute to viral persistence (28).

To better understand if this virus will persist in the future requires an understanding of the mechanisms the virus uses to replicate, shed and transmit in the environment. With respect to transmission, fish challenged in laboratory settings are primarily infected via intraperitoneal (IP) injection or immersion, which is most relevant to transmission in the natural environment. Although less relevant to natural transmission, the most prominent outcomes of IP injection experiments are the vast differences in species susceptibility, severity of disease, and infectivity (47). VHSV has also been isolated from reproductive fluids, suggesting the possibility of vertical transmission. However, due to the difficulty in experimentally testing this possibility, direct evidence of this has yet to be shown (16). Several organs have been identified as targets for VHSV infection, particularly the kidney, spleen, liver, and heart. Interestingly, however, VHSV IVb has not been isolated from blood, even though it is known to cause hemorrhaging by destruction of endothelial cells lining blood vessels (47). All genotypes have the unique ability to persist in the environment for years. This is supported by the fact that European fish farms have been dealing with reoccurring outbreaks of VHSV since the 1930s (47). And although research is being done to investigate VHSV IVb persistence, a laboratory model to help address questions of persistence and transmission would be particularly useful for aquaculture

and the environment.

In this manuscript, we use zebrafish via immersion infection as a model for VHSV IVb pathogenesis. Zebrafish have been successfully used as an infection model for IHNV, SVCV, Snakehead rhabdovirus, and even VHSV genotype I (26, 60, 76, 78, 84). Although zebrafish are normally maintained at 26°C to allow for viral replication the fish must adapted to 15°C. Zebrafish, naturally live between 18°C and 25°C, and are readily adapted to 15°C. Our work shows that zebrafish infected with high doses of VHSV show similar signs of clinical disease, namely external hemorrhaging, lethargy, occasional exophthalmia, and death, similar time frame of the onset of mortality, and similar mortality to both natural VHSV infected fish and natural fish hosts that have been challenged in a laboratory setting. We investigate the mortality profile and the infectivity of VHSV IVb in zebrafish as a function of the acclimation time to 15°C prior to infection and by altering the dose in which the fish are exposed. We have further investigated shedding of VHSV IVb from infected fish both with and without clinical signs of disease. Finally, we have investigated viral expression in the population that is in a "carrier" state.

METHODS

Reagents.

The Cornell strain of zebrafish was generously provided by Dr. Kate Whitlock and maintained in an Aquatic Habitats (AHAB) System. The fish are maintained at 26°C, with a 14 hour light and 10 hour dark cycle had an average mass of 116.2 ± 2.5mg, for the 689 fish used. The fish were fed a combination of brine shrimp and ground TetraMin Tropical Flakes daily (Tetra).

The VHSV IVb isolate (MI03) was used for these experiments and propagated in epithelioma papulosum cyprini (EPC) cells.

Acclimation to 15°C prior to infection.

To allow for viral infection, zebrafish were adapted to 15°C prior to infection. Zebrafish were moved into a static container in a mixture of reverse osmosis (RO) water and Instant Ocean at 26°C at a average density of 2.7 ± 0.4 mg/mL, and then moved into a 15°C incubator. For the first 3 hours, the rate of temperature decline was 0.05 °C/min, followed by a slower decrease for the next 10 hours averaging at 0.008 °C/min, reaching 15°C by 16 hours. The zebrafish were then kept at 15°C prior to infection for various amounts of time depending on the experiment. Water parameters were checked every 3 days for ammonia, pH, chlorine, nitrates, nitrites, hardness, and alkalinity; and full water changes were also done every 3 days. Fish were fed ground TetraMin Tropical Flakes daily (Tetra) during all phases of the experiments.

Infection of zebrafish with VHSV MI03.

After the zebrafish were acclimated to 15°C, typically either 24 hours or 2 weeks, the fish were separated into two groups: an uninfected group, exposed to media only, and an infected group, exposed to VHSV MI03 in media. Both groups were treated via bath exposure in 500mL of water at an average density of 0.3 ± 0.01 mg/mL. Twenty-four hours post infection the zebrafish were netted into 1000 mL of 15°C clean RO water with instant ocean at an average density of 0.1 ± 0.007 mg/mL. The fish were then observed for signs of viral infection including external signs of hemorrhaging on the base of the fins and abdomen, exophthalmia, erratic swimming

behavior including lethargy, and death. Zebrafish were removed from the containers post-mortem and immediately frozen at -20°C until further processing. To evaluate virus shedding, one-milliliter daily water samples were taken and frozen at -80°C. An aliquot of the viral stock used as inoculums for each experiment was also stored at -80°C for qRT-PCR and cell culture confirmations.

Post infection processing.

Post-mortem, whole frozen zebrafish were processed both for qRT-PCR and cell culture. For lysis and homogenization, a whole zebrafish was weighed, mixed with 1 mL of HMEM-5FPSH (media with 5% FBS, penicillin, streptomycin, HEPES) and 100 to 150 mg of 0.1 mm zirconia/silica beads in the designated sterile 2 mL screw-cap tubes (Bio-Spec Products), and homogenized for 10 s using a Bead-Beater (Bio-Spec Products). Following homogenization, the sample was centrifuged at 15.7 rcf for 5 min at room temperature in an Eppendorf Model 54-15 centrifuge. The supernatant was transferred to a sterile 1.5 mL microcentrifuge tube and kept on ice until further processing. After the cells were lysed, homogenized, and centrifuged, a portion of the supernatant, from 5 to 30 mg of tissue depending on the sample size was used to isolate total RNA for qRT-PCR. The RNA was stored at -80°C. The remaining supernatant was further diluted when possible at a tissue to volume ratio of 1:30 using HMEM-5FPSH for immediate application in cell culture.

Cell culture application of whole zebrafish homogenate.

EPC cells used for virus isolation were obtained from ATCC (30). These cells were routinely grown in 75 cm^2 or 25 cm^2 tissue culture flasks at 23°C (Corning) using Minimum Essential Medium Eagle, With Hanks′ salts, L-glutamine and sodium

bicarbonate, liquid, sterile-filtered, cell culture tested (Sigma) with added 10% Fetal Bovine Serum (Invitrogen/Gibco), penicillin/streptomycin (Invitrogen/Gibco), and HEPES (Invitrogen/Gibco) (HMEM-10FPSH). Cells were sub-cultivated through the use of a 0.05% trypsin (Invitrogen/ Gibco) solution to dislodge the cells from the surface of a flask, followed by re-suspending the cells in HMEM-5FPSH prior to seeding in new culture vessels. Virus isolations were performed in 48-well tissue culture plates (Corning). One confluent 25-cm^2 tissue culture flask was used to seed each 48 well tissue culture plate, for an approximate 1:2 split. Cells were harvested as described above for cell passage, but were then re-suspended in HMEM-5FPSH. To each well of the 48 well plate, 500 µL of cell suspension was seeded. Inoculation of cells with the tissue homogenate was performed on sub-confluent cultures. The diluted zebrafish homogenate was filtered using a 0.22 µm syringe filter and 250 µL of each sample was added to each of three wells of a 48-well plate of EPC cells. To control both cross contamination between wells and cell degeneration not associated with the zebrafish homogenate, 250 µl of HMEM-5FPSHFPSH was added to 3 wells on each 48 well plate.

After inoculation, the 48 well plates were incubated at 15°C and examined for cytopathic effects (CPE) daily. Once 90-100% CPE was observed, the remaining cells and media were removed and combined from each of the three wells, filtered using 0.22 µm syringe filter, and re-plated in 3 wells of a fresh 48 well plate of EPC cells, passage one (p1). This was repeated one more time (p2). From the p2 plate, the remaining presumed positive cells and media were collected at 90-100% CPE, all three wells for each sample combined, and stored at -20°C. If the diluted homogenate

did not show CPE, it was left at 15°C for 14 days, passage as described above (p1), and then with continued lack of CPE, the cells and media from the p1 plate were collected and frozen at -20°C as described above and were presumed cell culture negative. All samples were processed for confirmation of VHSV using qRT-PCR as described below.

To ensure detection of VHSV IVb, from cell culture experiments and define limits of detection versus tissue toxicity control experiments were performed with the following dilutions of tissue mass to media volume: 1:5, 1:10, 1:30, 1:50, 1:100, 1:250, and 1:500, and with the following amounts of VHSV: 0, 10^2, 10^3, 10^4, 10^5, and 10^6 pfu/mL (data not shown). However, wells were ranked both for degree of CPE as well as extent of tissue toxicity, compared to mass to volume dilutions that had no VHSV present. An aliquot of the final cell passage was evaluated for levels of VHSV N gene using qRT-PCR. Tissue toxicity occurred at a mass to volume ratio at or above 1:10, with minimal tissue toxicity observed at a ratio of 1:30. No tissue toxicity was observed at any of the ratios of 1:50 or below. When infected with VHSV, 2.5 pfu showed CPE in 4 days. Thus, VHSV is highly infectious in cell culture and is only inhibited when tissue toxicity overcomes the infection. For subsequent experiments we used a 1:30 mass to media ratio as our highest value and 1:300 as our lowest ratio. However, qRT-PCR detects both virion RNA and mRNA and higher qRT-PCR levels are predicted than in cell culture positive detection. Our threshold of qRT-PCR copies detected that are consistently positive in all three wells is 10^3 N gene copies.

Isolation of total RNA for detection using qRT-PCR.

Total RNA was isolated from the aliquot of homogenized fish, 150 µl of water,

or 150 µl of cell culture cell/media mixture (CC), using a modified version of the manufacture's protocols for an RNeasy mini kit (Qiagen). All centrifugation steps were performed at 15.7 rcf. For fish samples, 600 µl of both Buffer RLT and 70% ethanol was added; for the water and CC samples, 150 µl of Buffer RLT and 300 µl of 70% ethanol was added, prior to vortexing and application to the RNeasy columns. The columns were centrifuged for 30 s once, for the water and CC samples, and twice for the fish samples to allow for the full volume to be absorbed to the column. The protocol was then the manufacturer's directions. To fully remove the wash buffers, an additional 2 min centrifugation step into a clean sterile collection tube was included following the final RPE centrifugation step. Elution of total RNA was accomplished with 50 µL of RNase-free water followed by centrifugation for 1 min at 15.7 rcf. The following additional steps were carried out for fish samples. To allow for a more concentrated sample, the elution step was repeated by eluting the same 50 µL back through the column. The concentration of RNA was determined by an OD_{260} reading and diluted to achieve a final stock concentration of 50 ng/ 6 µl in preparation for qRT-PCR.

qRT-PCR analysis of VHSV IVb.

The qRT-PCR assay for the MI03 isolate of VHSV is outlined in Hope et al. 2010 (Chapter 2), with the following changes for water and CC samples. These samples were evaluated on a 384-well format (Applied Biosystems, MicroAmp® Optical 384-Well Reaction Plate with Barcode, MicroAmp® Optical Adhesive Film) using a ViiA 7 Real-Time PCR System (Applied Biosystems). The total reaction volume was decreased to 15 µL using the same ratios as in Hope et al. 2010, with 6 µl

of total RNA to be loaded per well and run in duplicate (44). This volume was tested in comparison to the previous reaction volume and found acceptable for the detection of VHSV from these sources. The standards were also adjusted accordingly.

To insure detection of samples with less than 50ng of total RNA, serial dilutions of zebrafish total RNA samples known to have a given N gene copy number were run in qRT-PCR (data not shown). As the relationship between total RNA loaded per well and N gene copies detected is not linear above 500 ng, we found that at least 0.2 N gene copies / ng of total RNA is required to reduce the chance of false negatives using lower amounts of starting material.

Results

Zebrafish Susceptibility to the MI03 Isolate of VHSV.

Prior to infection, 4 to 6 month old zebrafish were acclimated to 15°C for either 24 hours or 2 weeks (Figure 3.1A). Following this acclimation, fish were either VHSV infected with 10^6 pfu/mL of the MI03 isolate or mock infected with the

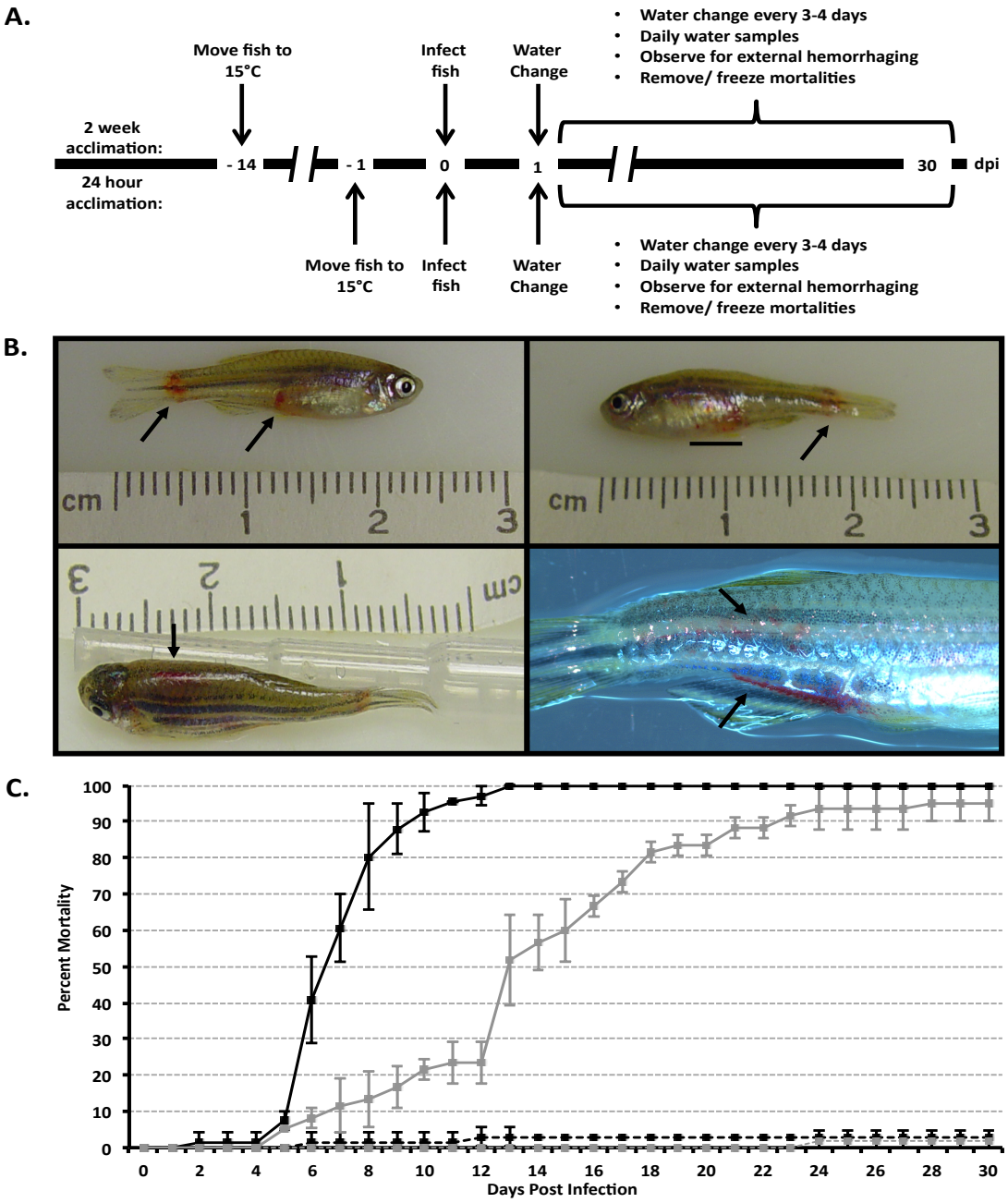

Figure 3.1 High mortality and external hemorrhaging are observed in zebrafish infected with VHSV and can be altered based on duration of 15°C acclimation. (A) Experimental design for zebrafish exposed to 10^6 pfu/ml of VHSV. (B) Severe external hemorrhaging is observed largely at the base of fins and abdomen, as is shown here in fish that have been acclimated to 15°C for 24 hours prior to infection. (C) Percent mortality of fish acclimated to 15°C for either 24 hours (solid black) or 2 weeks (solid gray). Uninfected controls are also shown for each condition (dashed black and dashed gray respectively). Error bars represent biological triplicates of 19 to 22 fish each.

comparable volume of media via immersion for 24 hours. Fish were observed over the course of 30 days for clinical signs of disease, in particular external hemorrhaging, lethargy, and mortality.

Both 24 hour and 2 week acclimated fish showed varying levels of clinical disease. External hemorrhaging ranged from slight to severe and was most visible at the base of the fins (Figure 3.1B). Abdominal petechial hemorrhaging, hemorrhaging of the eye, and exophthalmia were also observed. Fish with signs of external hemorrhaging were also lethargic, increasingly so as the severity of the hemorrhaging increased. Once hemorrhaging was visible, the fish would die within 1-2 days or 2-4 days for 24 hour or 2 week acclimated fish respectively, and these fish were not able to be rescued by moving the fish back to 26°C to halt viral replication. In fish acclimated for 24 hours, external hemorrhaging was observed on one fish in one group as early as 2 dpi, however, the other two groups consistently had fish showing external hemorrhaging around 5 dpi and external hemorrhaging preceded death in most cases. For the 2 week acclimated fish, external hemorrhaging was observed consistently in all three groups around 5 to 6 dpi and external hemorrhaging preceded death in all cases.

Fish acclimated to 15°C for both 24 hours and 2 weeks eventually showed near 100% mortality (Figure 3.1C). However, the mortality kinetics are very different. The 24 hour acclimated fish had a steep mortality curve, with mortalities beginning as early as 2 days post infection (dpi), with 50% mortality occurring at 6-7 dpi, and 100% by 13 dpi. In contrast, the 2 week acclimated fish did not start dying until 5 dpi, with 50% mortality occurring around 12-14 days, and near 95% by 24 dpi. One fish from

each control group died, but showed no clinical signs of disease and were negative for VHSV by cell culture and qRT-PCR.

Histological analysis showed lesions consistent with viral infection (Figure 3.2). The most common internal lesions included mild to moderate multifocal acute necrosis of the epithelium of the skin over the whole fish, mild to moderate multifocal acute necrosis of the epithelium of the oral cavity, pharynx, filaments, and lamellae of the gill, mild to moderate multifocal acute necrosis of the epithelium and lymphatic tissue of the thymus, mild to moderate acute diffuse, global degeneration and necrosis of the capillary epithelium of the glomeruli of the kidney. In severely affected fish, severe hemopoietic depletion from the kidney interstitium, moderate multifocal intramuscular hemorrhage in both white and red skeletal muscle, and degeneration of spermatids in the testis with spermatid giant cell formation was observed. In fish sampled later post infection, multifocal hyperplasia of the epithelium and mucous cells in the skin, oropharynx, and gills, regenerative hyperplasia of the kidney hemopoietic tissue with a profound left shift, in that most of the cells were blasts or immature, were observed. Glomeruli in the kidney also showed crescent formation in Bowman's capsule and in a few cases cuboidal metaplasia of the epithelium lining of Bowman's capsule, indicating sub-chronic inflammation of the glomeruli, was observed. Other lesions observed include locally extensive necrosis of the epithelium of the pneumatic duct, necrosis of the epithelium lining of the lateral line canal, locally extensive necrosis of the nasal epithelium, and mild necrosis of the pseudobranch epithelium.

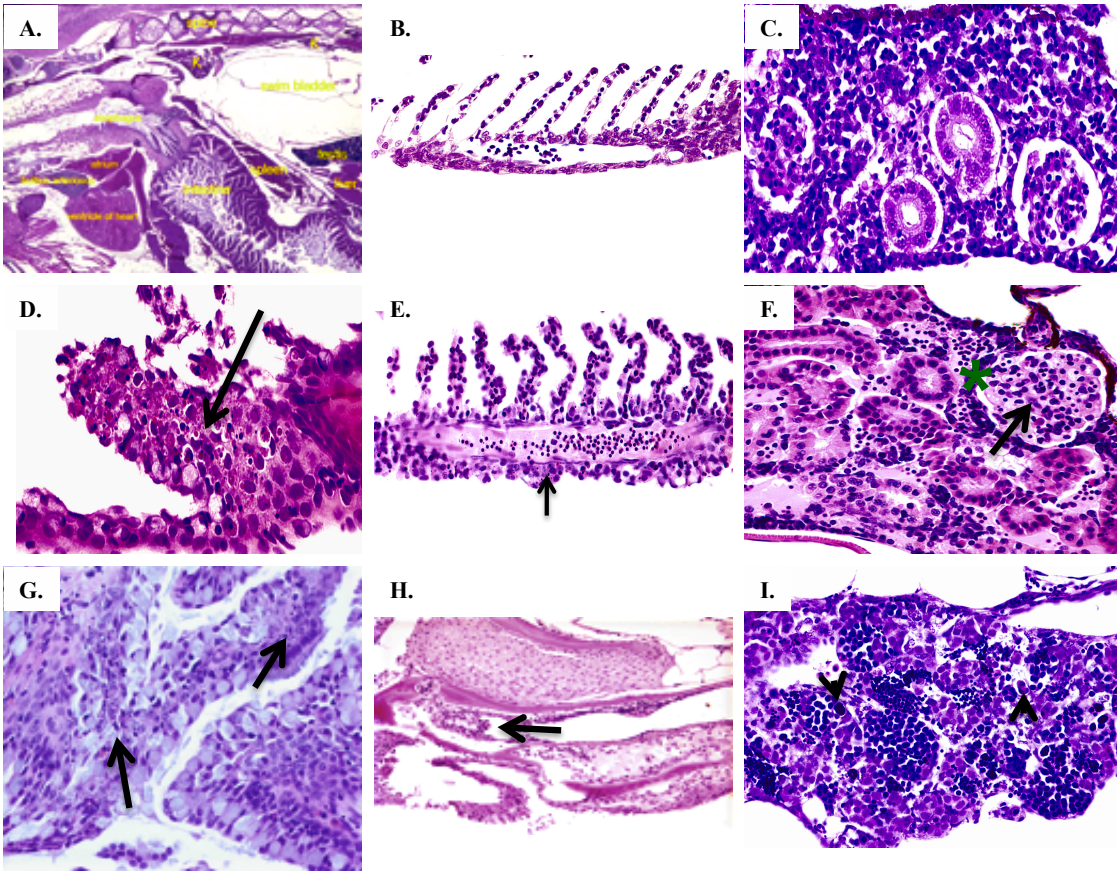

Figure 3.2 Zebrafish exposed to VHSV display varying internal clinical signs of infection. Histological analysis with hematoxylin and eosin staining for the following uninfected fish sections: (A) Whole section of an uninfected zebrafish at 1x, (B) mock infected gill lamellae at 20x, and (C) mock infected kidney at 40x, or infected conditions: (D) moderate locally extensive necrosis of the epithelium of the skin at 100x, (E) mild multifocal necrosis of the epithelium of the gill filaments and lamellae at 20x, (F) depletion of the hemopoietic tissue from the kidney (the asterisk indicates sinusoids that contain degenerating red blood cells and the arrow indicates a glomerular capillary that is intact but contains necrotic red blood cells) 40x, (G) locally extensive necrosis of the epithelium of the pneumatic duct 40x, (H) necrosis of the epithelium lining of the lateral line canal 10x, and (I) degeneration of spermatids in the testis 20x.

To evaluate the levels of VHSV in each fish, both qRT-PCR and cell culture were performed on whole tissue homogenates (Figure 3.3, Table 3.1). For both the 24 hour and 2 week acclimated mock infected fish, all were negative for VHSV in qRT-PCR and cell culture as expected (data not shown). All VHSV infected fish had high

levels of N gene copies (Figure 3.3) and were positive for infectious VHSV in cell culture (Table 3.1). For the 24 hour acclimated VHSV infected fish, one fish each on the order of 10^5 copies, while the rest had 10^6 or more copies. For the 2 week acclimated VHSV infected fish, one fish had 10^3 copies, while the remaining had 10^6 or more copies. Although no fish survived the infection after being acclimated for only 24 hours, the fish that survived the infection after a 2 week acclimation had 10^3, 10^6, and 10^6 N gene copies, representing the lowest concentrations of VHSV of all of the 2 week acclimated fish.

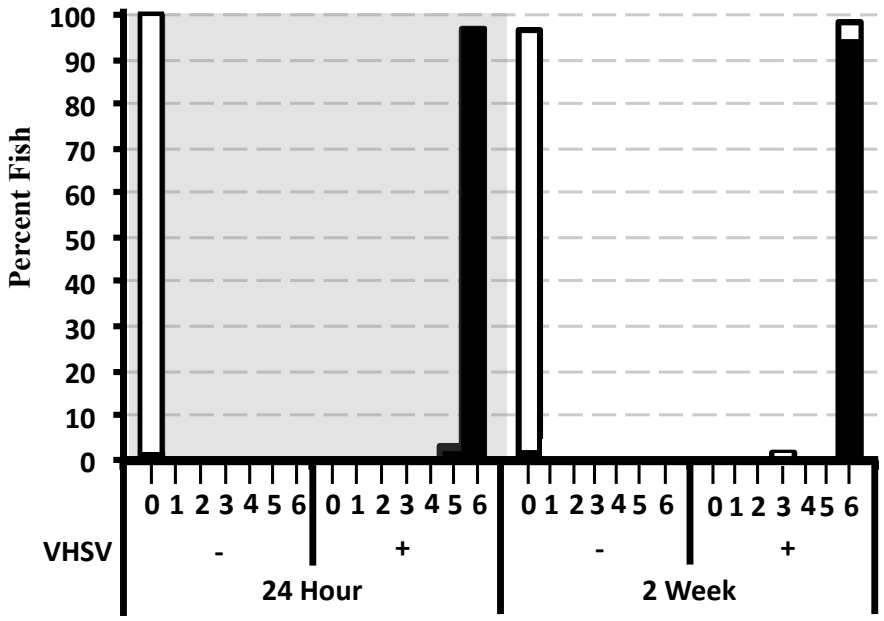

Figure 3.3 VHSV confirmed in zebrafish tissue using qRT-PCR targeting the N-gene. Total RNA from zebrafish acclimated to 15C for 24 hours (left) or 2 weeks (right) prior to mock infection (VHSV -) or infection (VHSV +) were quantified based on copies of the N-gene per 50ng of total RNA. The percent of fish with a given N-gene copy number is plotted. Copy number categories are as follows: 0 = not detected; 1 = less than or equal to 10^1 N-gene copies; 2,3,4,5 = on the order of 10^2, 10^3, 10^4, or 10^5 N-gene copies respectively; 6 = greater than or equal to 10^6 N-gene copies. Black bars indicate death; white bars indicate survival.

Table 3.1 Cell culture results confirm infectious virus in zebrafish.

Acclimation to 15°C	Exposure to VHSV	qRT-PCR		Cell Culture	
		Positive	Negative	Positive	Negative
24 Hour	-	0 (0/65)	100 (65/65)	0 (0/65)	100 (65/65)
	+	100 (64/64)	0 (0/64)	100 (64/64)	0 (0/64)
2 Week	-	0 (0/59)	100 (59/59)	0 (0/59)	100 (59/59)
	+	100 (60/60)	0 (0/60)	100 (60/60)	0 (0/60)

Top number is percent of total. In paratheses is ratio to total fish number.

Zebrafish susceptibility to VHSV is dose dependent and affected by 15°C acclimation.

To investigate the effects of the dose of VHSV on zebrafish susceptibility, fish were acclimated to 15°C for either 24 hours (Figure 3.4A) or 2 weeks (Figure 3.4B) prior to exposure to VHSV via immersion at 0 (mock infection), 10^2, 10^3, 10^4, 10^5, or 10^6 pfu/mL for 24 hours. Fish were observed over the course of 30 days for clinical signs of disease, including external hemorrhaging, lethargy, and mortality.

Monitoring external hemorrhaging over the course of infection was the first distinguishing metric (data not shown). For both the 24 hour and 2 week acclimated fish, no hemorrhaging was seen in the uninfected group or the group infected with 10^2 pfu/mL of VHSV. However, as the dose increased for both acclimated groups of fish, the appearance of clinical signs both increased and began to be seen earlier post infection. On average, the first signs of hemorrhaging were seen on 13 ± 3.5 dpi, 9.7 ± 1.5 dpi, 4.7 ± 1.2 dpi, and 3.7 ± 1.5 dpi for the 24 acclimated fish and on 13 dpi,

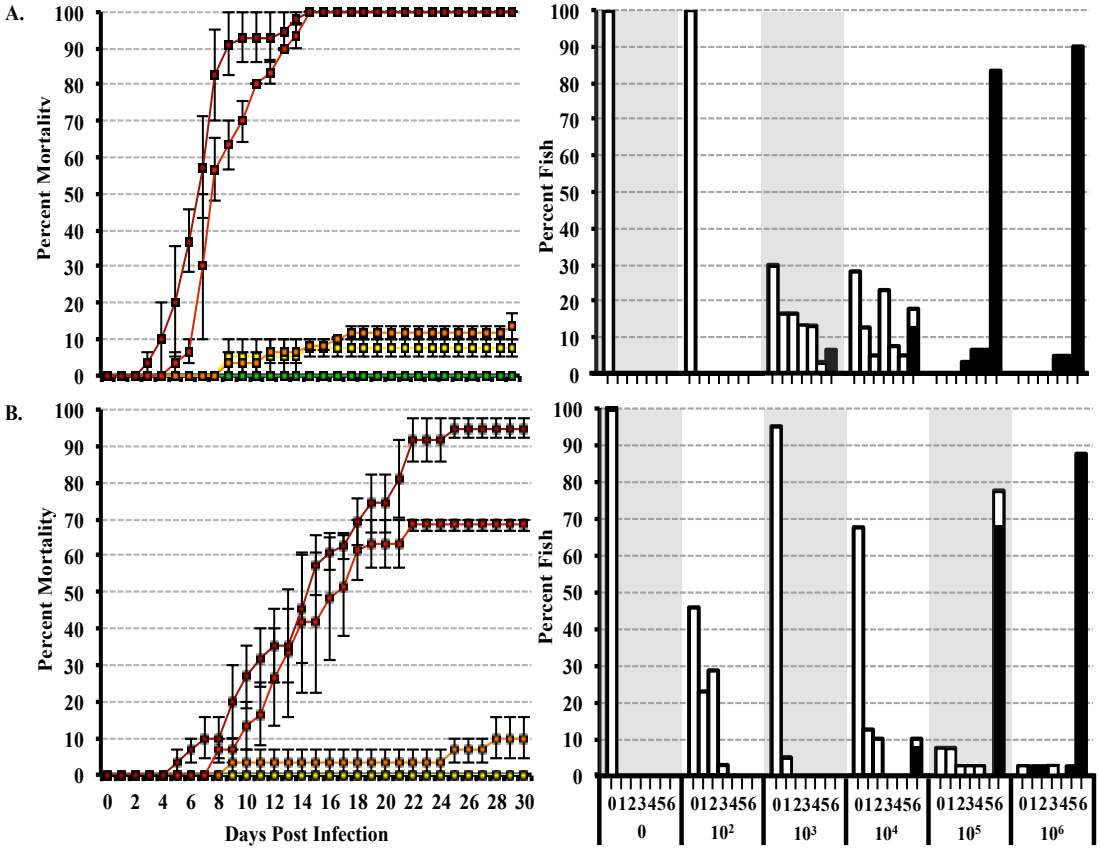

Figure 3.4 Mortality and susceptibility of zebrafish to VHSV are dependent on dose. Fish were acclimated to 15°C for 24 hours (A) or 2 weeks (B) prior to infection with either 0 (blue), 10^2 (green), 10^3 (yellow), 10^4 (orange), 10^5 (light red), or 10^6 (dark red) pfu/mL VHSV for 24 hours. Mortality curves are shown on the left and copies of N gene in each fish for each dose are shown on the right for each acclimated group. The N gene copies are plotted as a distribution as follows: 0 = below the limit of detection, 1 – 5 = on the order of 10^1, 10^2, 10^3, 10^4, 10^5 respectively and 6 = on the order of 10^6 or greater. Error bars represent biological triplicates of 10 to 15 fish each.

18 ± 11 dpi, 9.7 ± 3.8 dpi, and 7.0 ± 2.6 dpi for the 2 week acclimated fish exposed to 10^3, 10^4, 10^5, and 10^6 respectively.

Mortality during the course of infection was our second distinguishing metric (Figure 3.4). For the 24-hour acclimated fish (Figure 3.4A), the mortality profiles at 10^5 and 10^6 pfu/mL were comparable with the same steep curve and 100% mortality by 15 dpi. The first mortalities were on 5 dpi and 3 dpi, reaching 50% around 7 to 8

dpi and 6 to 7 dpi for the 10^5 and 10^6 doses, respectively. The 10^4 pfu/mL dose reached a maximum mortality of 14%, with the initial mortality on 9 dpi, remaining at 12% from 18 to 29 dpi, only reaching 14% on 30 dpi. The 10^3 pfu/mL dose had a maximum mortality of 8% by 15 dpi, with the initial mortality on 9 dpi. The 10^2 pfu/mL dose and the mock infected fish had no mortalities. For the 2 week acclimated fish (Figure 3.4B), the 10^6 and 10^5 pfu/mL doses also had similar mortality profiles to each other through 21dpi, with a dramatically shifted mortality curve than what was observed for the 24 hour acclimated fish. Initial mortalities for these two doses were observed at 5 and 8 dpi and reached 50% by 14 to 15 and 16 to 17 dpi, respectively. However, whereas the 10^6 pfu/mL dose reached about 95% mortality by 25 dpi, the 10^5 pfu/mL dose only reached its maximum mortality of 68% by 22 dpi, indicating substantial differences between these two doses after a 2 week acclimation. As the dose decreases further, the mortality profiles become more suppressed. The 10^4 pfu/mL dose had its initial mortalities on 9 dpi, remaining at about 6% from 9 dpi through 27 dpi, only to achieve the maximum 10% mortality at 28 dpi. No mortalities were observed in the 10^3, 10^2, and mock infected doses.

The third distinguishing metric involves the quantification of VHSV using qRT-PCR (Figure 3.4) and the presence of infectious VSHV using cell culture (Table 3.2). For the 24 hour acclimated mock and 10^2 pfu/mL dose, none of the fish had detectable levels of N gene and all were cell culture negative, corresponding to the lack of mortalities seen in these groups. For the 10^3 and 10^4 pfu/mL doses, 70% and 72%, respectively, were positive for VHSV N gene, with 40% and 56% positive in cell culture, even with the low rate of fish mortality at these doses. Interestingly, the

distribution of N gene copies in each of these doses varied quite dramatically with fish containing anywhere from undetectable levels to 10^6 or greater. All of the fish that died in these doses had 10^6 or more N gene copies. For the 10^5 and 10^6 doses, all of the fish were 100% positive in both qRT-PCR and cell culture. Unlike the 10^3 and 10^4 doses, however, the fish had N gene copies from 10^4 through 10^6 or more. For the 2 week acclimated fish, the mock infected were negative for both qRT-PCR and cell culture as expected. For the 10^2 pfu/mL dose, unlike the 24 hour acclimated fish at this dose, 46% of the fish had levels of N gene at 10^3 or below, even though all were cell culture negative. For the 10^3 dose, only 5% had levels of N gene equal to or less than 10^1 N gene copies and all were cell culture negative. For the 10^4 pfu/mL dose, 33% of the fish had N gene copies, with 13% less than or equal to 10^1, 10% on the order of 10^2, and 10% equal to or higher than 10^6 N gene copies, while 10% of the fish were cell culture positive. For the 10^5 pfu/mL dose, 93% had N gene copies, with 7% less than or equal to 10^1, ~3% on the order of 10^2, 10^3, and 10^4, and 77% greater than or equal to 10^6, with 80% cell culture positive. For the 10^6 pfu/mL dose, 98% had N gene copies, 2.5% were less than or equal to 10^1, 2.5% were on the order of 10^2, 10^3 and 10^5, and 87.5% were greater than or equal to 10^6 N gene copies, with 93% being cell culture positive. Unlike the 24 hour acclimated fish at the 10^5 and 10^6 doses, the distribution of N gene copy numbers in the 2 week acclimated fish was more diverse.

Table 3.2 Cell culture results confirm infectious virus in zebrafish in a dose dependent manner.

Acclimation to 15°C	Exposure to VHSV	qRT-PCR		Cell Culture	
		Positive	Negative	Positive	Negative
24 Hour	0	0 (0/39)	100 (39/39)	0 (0/39)	100 (39/39)
	2	0 (0/39)	100 (39/39)	0 (0/39)	100 (39/39)
	3	70 (21/30)	30 (9/30)	40 (12/30)	60 (18/30)
	4	72 (28/39)	28 (11/39)	56 (22/39)	44 (17/39)
	5	100 (30/30)	0 (0/30)	93 (28/30)	7 (2/30)
	6	100 (40/40)	0 (40/40)	100 (40/40)	0 (0/40)
2 Week	0	0 (0/32)	100 (32/32)	0 (0/32)	100 (32/32)
	2	54 (19/35)	46 (16/35)	0 (0/25)	100 (25/25)
	3	5 (2/40)	95 (38/40)	0 (0/40)	100 (40/40)
	4	33 (13/40)	67 (27/40)	10 (4/40)	90 (36/40)
	5	93 (37/40)	7 (3/40)	80 (32/40)	20 (8/40)
	6	98 (39/40)	2 (1/40)	93 (37/40)	7 (3/40)

Top number is percent of total. In parentheses is the ratio to total fish number.

Viral release of VHSV from zebrafish is dose dependent.

To evaluate the viral shedding of the fish, an aliquot of water was taken both before and after infection and before and after every water change (Figures 3.5 and 3.6). In both the 24 hour and 2 week acclimated fish, from 10^2 though 10^4 as the dose increases, more VHSV N gene can be detected in the water, with significant amounts

detected starting at 10^3 and 10^4 for the 24 hour and 2 week acclimated fish respectively. Interestingly, this trend does not continue at the higher doses. For the 24 hour acclimated fish, little VHSV N gene was detected in the water at a dose of 10^5, even though the mortality curve was very steep with all fish having high levels of infectious virus in their tissues. The fish exposed to 10^6 pfu/mL of VHSV had high VHSV N gene copies in the water throughout the experiment. For the 2 week acclimated fish, the levels of VHSV N gene in both the 10^5 and 10^6 pfu/mL doses were highly detectable, however very similar to each other. Overall, there does appear to be a spike in VHSV N gene in the water when mortality occurred; however, more data would need to be collected to better understand this correlation. Furthermore, variation between the replicate tanks was also apparent, particularly in the lower doses.

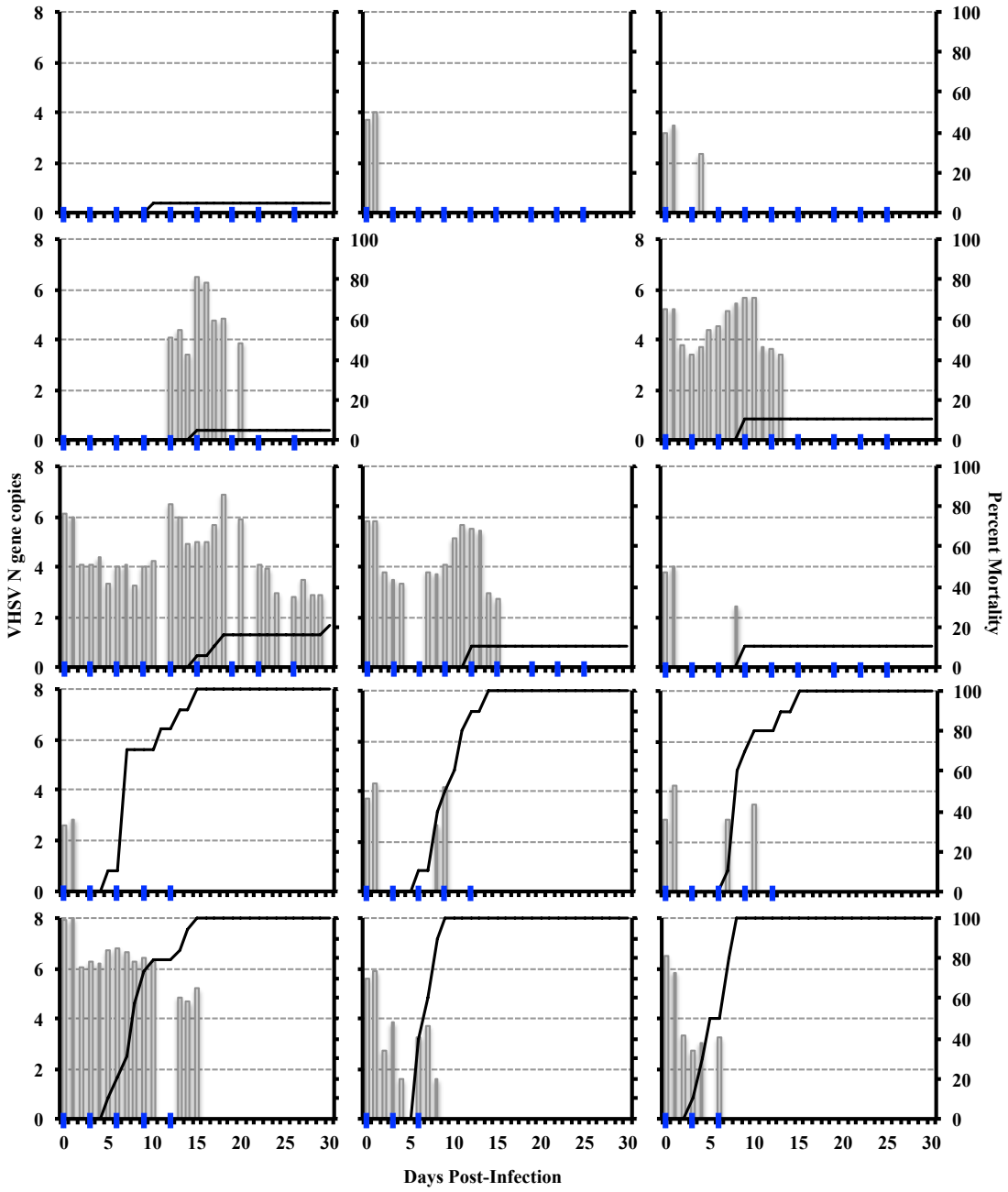

Figure 3.5 VHSV released into the environment of fish acclimated to 15°C for 24 hours is dose dependent. Fish were acclimated to 15°C for 24 hours prior to infection with either 10^2 (1^{st} row), 10^3 (2^{nd} row), 10^4 (3^{rd} row), 10^5 (4^{th} row), or 10^6 (5^{th} row) pfu/mL VHSV for 24 hours. VHSV N gene copies/mL (grey bars), corresponding to the left axis and reported as log transformed values, are superimposed onto the mortality curves (black lines), corresponding to the right axis. Each column represents a biological replicate of 10 to 15 fish each for each dose, trial A (left), trial B (center), and trial C (left). Blue marks indicate water change days.

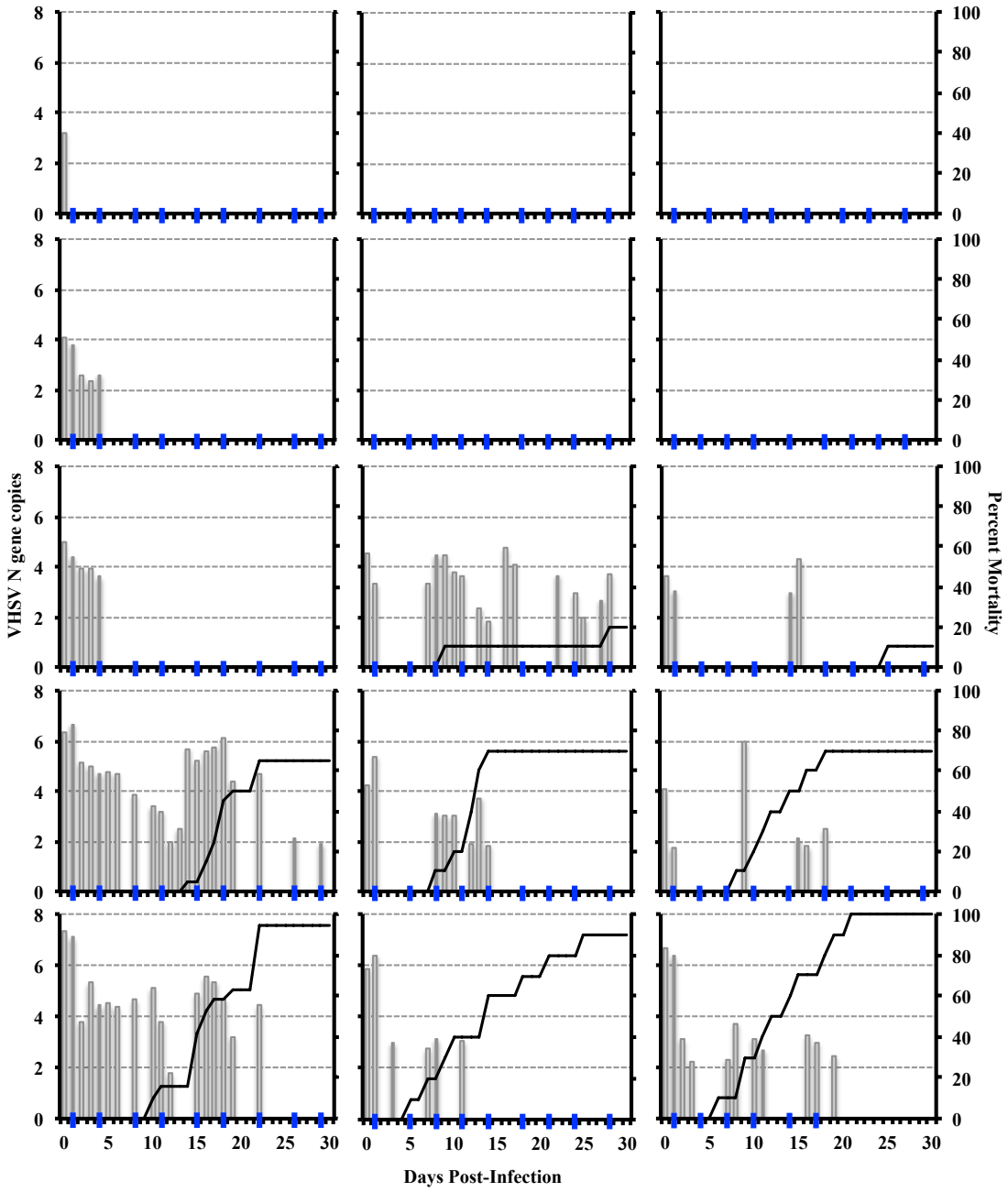

Figure 3.6 VHSV released into the environment of fish acclimated to 15°C for 2 weeks is dose dependent . Fish were acclimated to 15°C for 2 weeks prior to infection with either 10^2 (1^{st} row), 10^3 (2^{nd} row), 10^4 (3^{rd} row), 10^5 (4^{th} row), or 10^6 (5^{th} row) pfu/mL VHSV for 24 hours. VHSV N gene copies/mL (grey bars), corresponding to the left axis and reported as log transformed values, are superimposed onto the mortality curves (black lines), corresponding to the right axis. Each column represents a biological replicate of 10 to 15 fish each for each dose, trial A (left), trial B (center), and trial C (left). Blue marks indicate water change days.

Effect of age on the susceptibility of zebrafish to VHSV.

In the development of our model for translation to natural hosts of VHSV, preliminary work tested the VHSV susceptibility of different age groups of zebrafish, including 8 week old juveniles (data not shown), 4 to 6 month adults (as described here), and greater than 2 year old adults (data not shown).

For the juvenile experiments, the fish were acclimated at 15°C for 2 weeks, infected with either 10^4, 10^5, or 10^6 pfu/mL or mock infected for 2 hours, and then observed for 30 days with water changes every 3 to 4 days. The juveniles did not show increased sensitivity to VHSV, with the highest mortality at 70%, whereas adults exposed to 10^6 pfu/mL for 2 hours reached 100% mortality. The total mortality for the 10^4 dose was lower, at 40%, than the 10^5 and 10^6 doses, which were similar to each other at 70%. N gene copies increased in a dose dependent manner, with 60%, 83%, and 100% positive for VHSV N gene, on the order of 10^1 to 10^2, 10^2 to 10^3, and 10^4 to 10^7 copies for the 10^4, 10^5, and 10^6 doses respectively. Juveniles were also used to test sensitivity to temperature change and an increased susceptibility was observed when the fish were exposed to either a rapid higher or lower temperature shift (data not shown).

The older adult fish were used to further understand the effect of acclimation time on VHSV susceptibility. The fish were acclimated to 15°C for 16 hours, 2 weeks, 4 weeks, 8 weeks, and 16 weeks prior to a 2 hour infection with 10^6 pfu/mL (Table 3.3). For the 16 hour acclimated fish, the first mortality occurred on 1 dpi, 50% on 5dpi, and 100% at 10 dpi. All of the fish had detectable levels of VHSV N gene. Those that died on 1 dpi and 2 dpi had copies on the order of 10^2 to 10^3 and the rest on

Table 3.3 Long term threshold of the effects of 15°C acclimation on VHSV susceptibility in zebrafish older than 2 years.

Acclimation Time[a]			Infection		Day of Initial Mortality[c]	Day of 50% Mortality[c]	Day of Final Mortality[c, d]
			Dose[b]	Time			
16 weeks			10^6	2 hours	10	13-14	35 (90)
8 weeks			10^6	2 hours	8	17	34 (100)
4 weeks			10^6	2 hours	11	28	35 (60)
2 weeks			10^6	2 hours	10	11-12	23 (100)
16 hours			10^6	2 hours	1	5	10 (100)
0 hours	Trial 1	Mock	0	2 hours	2	1-2	3 (100)
		VHSV	10^6	2 hours	1	4-5	5 (60)
	Trial 2	Mock	0	2 hours	1	3-4	6 (100)
		VHSV	10^6	2 hours	2	3	6 (100)

[a] Acclimation refers to 15°C prior to infection.
[b] Dose is pfu/mL.
[c] Day refers to day post infection.
[d] Number in paraentheses refers to percent total mortality.

the order of 10^4 to 10^7. For the 2 week acclimated fish, the fist mortality was on 10 dpi, 50% between 11 and 12 dpi, and 100% on 23 dpi, with VHSV N gene copies on the order of 10^6 to 10^7 for all of the fish. Interestingly, for acclimation times greater than 2 weeks, the mortality profiles are very similar to the 2 week profile. For the 4 week acclimated fish, the first mortality was on 11 dpi, 50% on 28 dpi, and 60% on 35 dpi, with VHSV N gene copies on the order of 10^6 to 10^7 for the mortalities, and undetectable, 10^2, and 10^6 for the survivors. For the 8 week acclimated fish, the first mortality was on 8 dpi, 50% on 17 dpi, and 100% on 34 dpi, with VHSV N gene

copies on the order of 10^6 to 10^7 for all of the fish. For the 16 week acclimated fish, the fist mortality was on 10 dpi, 50% between 13 and 14 dpi, and 90% on 35 dpi, with VHSV N gene copies on the order of 10^6 to 10^7 for the mortalities, and undetectable for the survivors. Although there is variation as to total mortality, the profiles themselves clump together from 2 weeks through 16 weeks, indicating a threshold for acclimation affecting VHSV susceptibility at 2 weeks, after which the mortality profile is not significantly affected by longer acclimation periods.

To determine whether there is an early threshold of acclimation, adult fish were also moved directly to 15°C without acclimation. As suspected, this immediate temperature shock resulted in high mortality in both the mock infected and the VHSV infected fish and this mortality occurred earlier than the 16 hour or 24 hour acclimated fish. For two trials, the total mortality was 100% and 60% for the mock infected fish, and 100% and 100% for the VHSV infected fish. Strikingly, the mock infected mortalities were more sporadic, whereas the VHSV infected mortalities were almost identical between the two trials. For the mock infected fish, the first mortalities were on 2 dpi and 1 dpi, 50% on 1 to 2 dpi and 4 to 5 dpi, and the maximum mortality of 100% on 3 dpi and 60% on 5 dpi for trial one and trial two respectively. For the VHSV infected fish, the first mortalities were on 1 dpi and 2 dpi, 50% on 3 to 4 dpi and 3 dpi, and 100% on 6 dpi and 6 dpi for trial one and trial two respectively.

DISCUSSION

Zebrafish infection model is similar to natural outbreaks and laboratory challenge experiments in natural host species.

We have focused on using zebrafish as a relevant model for VHSV IVb

infection of fish in the Great Lakes. Although zebrafish are tropical, they can be acclimated to 15°C and productively infected with a number of fish rhabdoviruses including VHSV genotype 1 (55, 76, 78, 84). Characteristic clinical signs of disease appear on infected zebrafish, which include external hemorrhaging and internal lesions that are consistent with both natural infections of VHSV as well as laboratory challenge experiments on fish that are natural hosts (49). Similarly, the time scale of when mortality begins and the profile of disease are also consistent with these VHSV IVb challenge experiments using natural hosts (49).

Zebrafish model insights into the age of fish on the susceptibility to VHSV.

Juvenile, adult, and older adult all show similar mortality profiles when exposed to the same dose for a similar amount of time. However, in both juveniles and adults, rapid temperature shift is a key component in increasing the susceptibility to VHSV. Furthermore, with both the adults and older adults, temperature acclimation is also a major contributor to increased viral susceptibility. The length of time for the fish to reach an equilibrium with respect to acclimation to 15°C is as early as 2 weeks, since the mortality profiles after 2 weeks are similar to the 2 week profile for both groups of adults. Time points between 24 hours and 2 weeks have not been investigated, but would help to fully determine this long-term threshold.

In natural hosts of VHSV, increasing age has often been correlated with decreasing susceptibility to VHSV. The preliminary results for the zebrafish VHSV infection model shown here do not indicate an age preference; but replicates of these experiments would increase the strength of these conclusions. However, even with the typical trend of increasing age and decreasing susceptibility, this has not been tested in

all natural host species of VHSV and may be partially species specific. Interestingly, there is evidence in Pacific herring that fish exposed to VHSV in the larval stage are more protected after their metamorphosis to juveniles than juveniles that had never been exposed to VHSV, indicating that even at early stages of development, there are differences in the susceptibility to VHSV (43). Evaluating the effects of the age of the fish on VHSV susceptibility warrants further study.

Zebrafish model insights into the entry of VHSV into the host fish.

The primary site of VHSV entry and its movement through the host has been under debate for some time. Recent evidence indicates entry through the gills, orally, and through the fin base (39, 47). Harmache et al. have used bioluminescence to follow IHNV in live fish and have found movement through the tail and other fins into the fish. Further more, the Encinas et al. have found that at one time point post VHSV infection, there is a strong immune response in the fins and not in the internal organs, implying that the virus encounters the fins and then hematologically spreads, becoming a systemic infection (26). The data obtained here from the zebrafish infection model also support this mode of entrance based on the following: (1) hemorrhaging at the base of the fins is observed on the majority of all fish showing signs of disease, and (2) hemorrhaging at the base of the fins are usually the first clinical signs of an infected fish. However, current data cannot exclude the possibility that the virus is entering through additional routes and then spreads early in the course of the infection to the fin bases.

Zebrafish model insights into the replication of VHSV in the host fish.

The persistence of VHSV in the Great Lakes, in spite of the lack of mass

mortality from 2008-2011 (6), emphasizes both the extreme hardiness of this virus, as well as the lack of understanding as to how this virus persists and is rescued as an infectious agent. Previous work in our lab examined over 1000 different fish collected from four of the Great Lakes; and, through the use of a highly sensitive qRT-PCR approach to detect VHSV IVb coupled with cell culture, both fish that show clinical signs of disease and fish that are sub-clinically infected with VHSV were found (44). Furthermore, recent survey work found that, not only can infectious VHSV be isolated from clinically normal fish in various locations throughout the Great Lakes, but that VHSV IVB can also be detected in water samples in some of these locations (6), indicating the shedding activity and persistence of VHSV IVB in the absence of disease.

To better understand the separation between subclinical infection and disease associated infection, we exposed zebrafish to different doses of VHSV IVb mimicking what we observe in fish surveyed in the Great Lakes, including all three categories of fish: VHSV infected fish with clinical signs of disease, infected fish not showing clinical signs of disease, and VHSV exposed fish that do not have VHSV in their tissues. Observing these groups of fish for 1) external hemorrhaging, 2) degree of mortality, and 3) presence and infectivity of VHSV in both higher (24 hour acclimation prior to infection) and lower (2 week acclimation prior to infection) temperature associated stress conditions, allows us to hypothesize the existence of additional stressors, such as spawning that may have an effect on susceptibility to VHSV IVb in natural hosts.

Temperature acclimation stress appears to be a major contributor to the

susceptibility of zebrafish to VHSV IVb. Although the degree that the immune system is compromised at 15°C has not been thoroughly evaluated for zebrafish, recent work which has assessed the immune response after a one week acclimation to 15°C prior to exposure to VHSV I and found that fish were able to mount an immune response and that the response varied depending on the tissue analyzed (26). In our study, zebrafish infected after 24 hours acclimation to 15°C, appear to be partially immunocompromised as is evidenced by increased disease susceptibility. Recovery from this temperature shift occurs within 2 weeks and perhaps sooner, but we have not examined shorter time periods. Using this temperature acclimation variable as an indicator of a stress (like the temperature changes that fish in the Great Lakes encounter in spring), we evaluated the effects of different exposure doses to VHSV at two acclimation time intervals. As expected, external hemorrhaging increased with increased exposure dose in both temperature-acclimated groups. For the 24 hour acclimated fish, significant signs were observed at 10^4 pfu/mL, whereas for the 2 week acclimated fish hemorrhaging was not observed until 10^5 pfu/mL. Interestingly, fish acclimated for 24 hours died within one to two days of exhibiting hemorrhaging, however, fish acclimated for 2 weeks, died significantly later, on average of 4 to 5 days after hemorrhaging was visible. Both this increased threshold for clinical signs of disease and this longer ability of the fish to maintain severe states of disease, indicate the decreased susceptibility to VHSV after having been acclimated for 2 weeks.

Predictably, mortality also increased with dose, however, for the 24 acclimated fish mortalities were observed at a dose of 10^3 pfu/mL, whereas for the 2 week acclimated fish, mortalities were not observed until 10^4 pfu/mL, again indicating

about a log difference in the viral threshold between a 24 hour acclimation and a 2 week acclimation. For both time acclimations, 10^5 to 10^6 pfu/mL seems to be a plateau of mortality. Interestingly, however, the profiles of mortality are very different between the two time points when comparing the 10^5 and 10^6 doses, with 50% mortality reached by 7 dpi for the 24 hour acclimated fish and by 16 dpi for the 2 week acclimated fish. The total mortality is also different between these two acclimation times, with a 30% increase in mortality when decreasing the acclimation time. In short, this data reconfirms that zebrafish are far more susceptible to VHSV IVb pathogenesis when encountering virus shortly after a 24 hour temperature shift.

Overall, the threshold of VHSV IVb infectious disease is 10^4 pfu/mL. A log decrease in virus exposure is observed when temperature acclimation time is reduced from 2 weeks to 24 hours. The threshold for mortality for zebrafish is around 10^5 pfu/mL with both a 30% increase in total mortality and a 9 day decrease in time to 50% mortality when acclimation time is shifted from 2 weeks to 24 hours. The threshold for detection of VHSV IVb shed in the water using either qRT-PCR or cell culture, is around 10^4 pfu/mL regardless of the acclimation time. Interestingly, the delayed onset of disease and mortality seems to be a consistent trait with respect to VHSV infection, as different species exhibit this type of profile even when exposed to the same dose of VHSV and the same temperature conditions (49). Since this trend is mirrored in zebrafish using differences in temperature acclimation, evaluating fish for host factors that influence susceptibility may help to further elucidate those that cause these types of profile differences in natural host species.

Zebrafish model insights into the exit of VHSV from the host fish and its transmission from host to host.

At low doses of VHSV exposure, 10^2 and 10^3 pfu/mL, VHSV can be detected in the water for a few days post exposure, but it becomes undetectable past 4 dpi. This could be due to residual carry over of virus initially from water changes, either through the net or the fish. However, at these lower exposure doses, VHSV IVb cannot be detected again in water. At 10^4 pfu/mL, VHSV is shed into the water throughout the experimental time frame even in the absence of external hemorrhaging and death. At higher doses, VHSV in the water corresponds to mortalities and fish with external hemorrhaging. Interestingly, this pattern indicates that a carrier state for zebrafish would be around 10^4 pfu/mL, where few clinical signs and mortalities are observed, but a significant amount of VHSV is still being shed into the water, posing a subclinical threat to uninfected fish, a log lower than the dose required for extensive mortality. Future extension of viral shedding studies from these different categories of infected or exposed fish, whether showing clinical signs or not will be informative to better understand the transmission of VHSV.

The future use of the zebrafish VHSV IVb infection model.

In conclusion, the sustained persistence of VHSV in the Great Lakes region and its wide host range, allow VHSV to be a continued threat to other naïve bodies of water and fish. To better understand the role this virus plays in a rapidly changing environment, the mechanisms of virus replication and transmission need to be delineated. Using zebrafish, a species that models disease in natural populations, as well as laboratory challenge infections of wild fish, will allow detailed investigations

of VHSV pathogenesis. Taking advantage of zebrafish genomics to define host immune response to rhabdovirus infection coupled with analysis of viral replication and transmission promises to provide new insights into the mechanisms employed by VHSV IVb to invade and persist in environments like the Great Lakes.

CHAPTER 4

INVESTIGATING THE PERSISTENCE AND STABILITY OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IVB IN POST-MORTEM FISH

Kristine M. Hope, Mrinalini Modak, Steven E. Zhang, Randall A. Meyer, and James W. Casey

Abstract

The earliest detection of viral hemorrhagic septicemia virus IVb (VHSV IVb) in the Great Lakes was in 2003 in Lake Saint Clair. Since then VHSV IVb has spread throughout the Great Lakes including inland bodies within or near the Great Lakes watershed. The long-term stability of VHSV IVb at low temperatures is remarkable (up to a year) and likely a major factor in allowing the virus to persist both in the environment and in aquaculture. We have similarly investigated the stability of VHSV IVb in post mortem infected zebrafish to model the fate of infected natural mortalities in the environment. We find that detection of VHSV IVb N gene using qRT-PCR is readily accomplished through 30 days at 22°C and 100 days at 4°C although the copy number drops off by 2-3 logs at the later time points. We are able to isolate infectious VHSV IVb by cell culture on EPC cells for 1 day post-mortem (dpm) and occasionally from dry carcasses up to 14 days at 22°C. Remarkably, infectious VHSV IVb can be consistently recovered for up to 100 dpm at 4°C from carcasses kept dry in air or in water. These findings substantiate that VHSV IVb can be detected at vastly longer time intervals than previously assumed and that infected dead fish might serve as

reservoirs, possibly enhancing transmission through consumption by susceptible hosts.

*Hope wrote this chapter, helped with various steps for the experiment, and contributed significantly in an intellectual manner. Modak and Zhang performed all experiments in this chapter. Meyer assisted in the 22°C experiments. Casey is the primary investigator and contributed intellectually.

Introduction

A member of the Rhabdovirus family within the genus Novirhabdovirus, viral hemorrhagic septicemia virus (VHSV) is the etiological agent of viral hemorrhagic septicemia (VHS), a highly fatal and persistent fish disease. Upon infection, fish may exhibit symptoms of lethargy and petechial hemorrhaging, specifically under the dorsal fins, near the gills, or surrounding the mouth (47). Multiple marine and freshwater fish from the coasts of Japan, Europe, the United States, and more recently, the Great Lakes, have been found to be susceptible to VHSV (47, 107). Four distinct genotypes of VHSV have been discovered and generally correlate with location (9). Genotype IVb include isolates from the Great Lakes and the Atlantic coast of Canada.

The route of VHSV movement into the Great Lakes watershed, although initially thought to involve shipping routes, has yet to be clearly understood. However, genotype IVa, the closest phylogentic relation to IVb, has been found primarily on the Pacific Northwest of the United States. Furthermore, although the major outbreaks of disease in the Great Lakes occurred between 2005 to 2007, with massive mortalities in the tens of thousands, a dead muskellunge (*Esox masquinongy*) captured from the Great Lakes and stored frozen in 2003 and then tested for VHSV in 2006 was found to be positive for VHSV, indicating the presence of VHSV in the region prior to outbreaks of disease (25, 37, 61). Although few mortality events have been reported, infectious VHSV has been found throughout the region, substantiating that VHSV has the ability to persist even in the absence of clinical disease (6).

Due both to the broad host range of VHSV IVb, which encompasses over 28 fish species in the Great Lakes, and to the persistence of VHSV in the environment,

understanding how the virus is transmitted and the behavioral interactions within susceptible fish species is central to being able to predict the future role of VHSV IVb in both this region and its spread to neighboring regions (32, 37, 61).

Although susceptibility to VHSV varies greatly depending on the fish species, with respect to both infection and disease, the mechanisms of transmission are still unclear. Fish exposed to VHSV via immersion become infected and have been shown to shed virus into the water. Direct evidence of how virus enters the host, whether through the skin, gills, mouth, or some combination of these, is being studied, with different preferences being observed depending on the experimental design and technology employed. VHSV IVb has been found to be shed in both urine and ovarian fluids, suggesting another source for spread and a potential for vertical transmission (29, 52). Interestingly, species other than fish, including *Diporiea ssp*, leeches, lampreys, and turtles have also been found to be susceptible to VHSV, indicating the possibility that these additional reservoirs could contribute to VHSV IVb persistence (28, 29, 31, 34). Hawley et al. has convincingly shown that the infectivity of VHSV is maintained for up to a year at 4°C (40). Based on the surprising stability of VHSV IVb in water, especially for an RNA virus, the potential for this virus to remain stable in post-mortem hosts is intriguing.

To test the stability of infectious VHSV in post-mortem hosts, our study has utilized a zebrafish VHSV IVb infection model. Zebrafish have several advantages, including a short generation time, access to large numbers for population studies, and a well- developed model for VHSV IVb pathogenesis. In this paper, we investigate the stability and infectivity of VHSV IVb in post-mortem zebrafish at two temperatures,

4°C and 22°C, temperatures that mimic those in the environment, and two conditions of post-mortem fish, 1) washed up on sand or 2) floating or sinking in water.

Materials and Methods

Zebrafish care and maintenance.

The zebrafish used in this study are wild-type of the Cornell line (K.E. Whitlock; Cornell University), AB fish crossed to commercial available zebrafish. They were maintained in a closed recirculation system (Aquatic Habitats) with a 12 h light-dark cycle at 26ºC. The fish were fed crushed Tetramin Tropical Fish Flake and brine shrimp.

Zebrafish acclimation and VHSV infections

Before infection, the zebrafish, at an average density of 1.8 mg/mL, were acclimated to 15ºC over the course of 12 hours (Chapter 3) in a static container containing a solution of reverse-osmosis (RO) water and Instant Ocean at a concentration of 60 mg/L. The fish were then kept at 15ºC for an additional 12 hours prior to infection.

After acclimation, the fish, at an average density of 7.2 mg/mL, were either infected with 10^6 PFU/mL of the MI03 isolate of VHSV or mock infected with the corresponding volume of HMEM-5FPSH via immersion for 24 hours. Following the infection, the fish were transferred into clean, pre-chilled to 15ºC water, at an average density of 1.8 mg/mL. Water parameters, including pH, ammonia, nitrate, nitrite, chlorine, hardness, and alkalinity were measured every 3 days to ensure the quality of the water during this period. Fish were fed daily with crushed Tetramin Tropical Fish

Flake and complete water changes were performed every 3 to 4 days with a one-milliliter water sample collected before and after water change for each condition.

Clinical signs of VHSV infection, including lethargic movement and hemorrhaging near the fins, mouth, and abdomen, began to show around 6 to 8 days post infection (dpi). Infected fish were collected as indicated, either 1) after death occurred or 2) after euthanization when external hemorrhaging was visible using an overdose of tricaine mesylate at a concentration of 0.02 g/mL. Mock infected fish were euthanized using an overdose of MS-222 to match the death or euthanization of each infected fish.

Post-mortem plating and collection.

Once euthanized or collected, each dead fish was placed in a 10 cm petri dish in one of three conditions, either 1) dry air, 2) water, or 3) wet sand, at either 4°C or 21°C. The pH of the sand was tested to be 7 and mixed with 15 mL of RO water. The water environment consisted of a volume of 25 mL RO water. An 800 or 1000 µl sample was taken from the water conditions daily. For each infected fish plated, a corresponding mock-infected fish was also plated for the same condition and time point. Three fish per condition per temperature were plated to achieve biological triplicates.

Upon sample collection, the fish were placed in 1.6 mL microcetrifuge tubes and frozen at -20°C until sample processing could occur. Three fish were collected at each of the following time points, for 21°C: 0, 0.25, 0.5, 1, 2, 3, 4, 5, 7, 14, and 30 dpm, and for 4°C: 0, 0.25, 1, 2, 3, 4, 5, 7, 15, 30, 60, and 100 dpm.

Zebrafish tissue preparation for qRT-PCR and cell culture.

Fish tissue for cell culture and qRT-PCR were prepared according to Chapter 3. Briefly, whole fish were homogenized for 10 seconds using a Mini Bead Beater (Bio-Spec) in 1 mL of HMEM-5FPSH with 100 mg of silica beads and then centrifuged at 9.3 x gravity (g) for 4 minutes. The supernatant was collected into sterile tubes. Thirty-milligrams of homogenized tissue was aliquoted for qRT-PCR and the remaining tissue was diluted in a 1:30 mass:volume ratio for use in cell culture analysis.

Detection of VHSV using qRT-PCR and cell culture.

For qRT-PCR detection of VHSV, total RNA was extracted from the 30 mg centrifuged tissue homogenate using Qiagen's RNeasy kit following the manufacturer's protocol, including the optional spin prior to elution, and the concentrations were determined using a spectrophotometer (General Electric). qRT-PCR was performed using a Taqman one-step qRT-PCR kit (ABI), with 50 ng of total RNA per reaction. The primers and probes were constructed to amplify approximately a 100 bp region of the nucleoprotein gene of VHSV IVb. The sequence of the forward primer is 5'-ACCTCATGGACATCGTCAAGG-3'. The sequence of the reverse primer is 5' CTCCCCAAGCTTCTTGGTGA-3'. The probe sequence is 50-56FAM/CCCTGATGACGTGTTCCCTTCTGACC/36-TAMSp/-30. The thermal cycling sequence was the following: 48ºC for 30 minutes, followed by 10 min at 95 ºC, and finally 1 minutes at 95 ºC 60 ºC for 15 seconds for 42 cycles using an Applied Biosystems ViiA 7 instrument.

Cell culture virus isolation was performed on epithelioma papulosum cyprini cells (EPC) obtained from the American Type Culture Collection (30) using the protocol of Winton et al. modified by Hope et al. with the following variation (44, 105). After the final passage, the etiological agent for CPE was confirmed to be VHSV using the previously described qRT-PCR assay instead of endpoint RT-PCR.

Results

To investigate the potential of post-mortem hosts as reservoirs of VHSV, either mock infected or VHSV infected zebrafish presenting with clinical signs of disease, were euthanized and then exposed to different conditions mimicking the environment that a naturally infected post-mortem host might encounter. The fish were exposed to either air, water, or wet sand at 22°C or 4°C for 0.25, 0.5, 1, 2, 3, 4, 5, 7, 14, 30, 60, or 100 dpm. Each condition and time point was plated with three different fish to achieve biological triplicates and to observe the effects of individual variation on the ability of VHSV to remain in post-mortem hosts. Three fish were also collected post-infection prior to plating at time zero to give measure the initial infection status of the fish. The fish were analyzed for VHSV using both qRT-PCR and cell culture. For the water conditions, water samples were taken frequently to evaluate viral release into the surrounding environment.

At 22°C, VHSV N gene copies can be detected through 30 dpm in air, water, and wet sand, with copies ranging from 10^1 to 10^6 (Figure 4.1, 4.4). However, by 14 dpm, a decrease by approximately 2 orders of magnitude was observed. In cell culture, infectious VHSV IVb was not detected past 1 dpm in the water and wet sand conditions; however, in air, after a lack of infectious VHSV IVb detection between 2

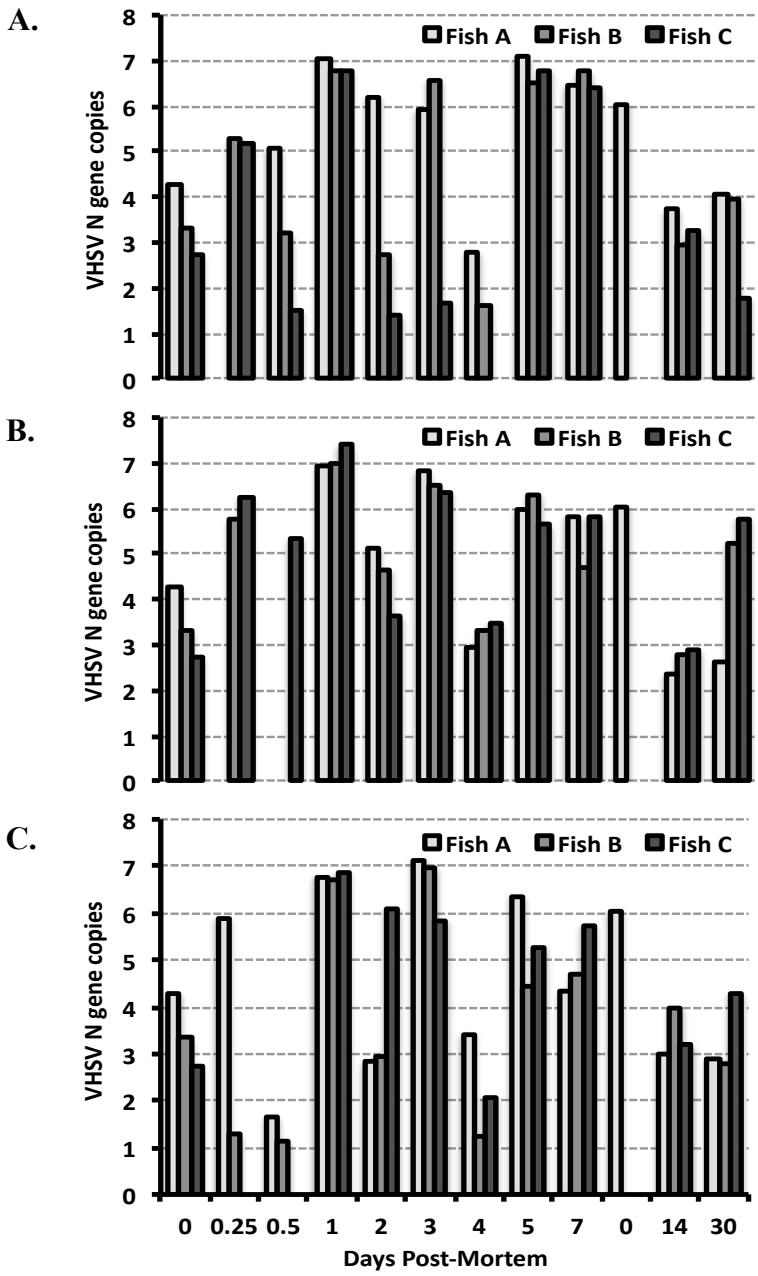

Figure 4.1. VHSV N gene can be detected through 30 dpm at 22°C under all conditions. Fish were plated on dry petri dishes representing air (A), on petri dishes with water (B), or on petri dishes with wet sand (C). Each fish was then collected at the designated day post-mortem. Fish homogenate was then analyzed for VSHV using qRT-PCR. White, light grey, and dark grey bars represent each fish from a biological triplicate, as indicated by Fish A, B, or C. VHSV N gene copies are reported as log transformed values.

and 5 dpm, infectious virus was detected at both 7 and 14 dpm (Table 4.1). Interestingly, a spike of virus is detected in the water environment at 1 dpm and then drops off slowly after this initial peak (Figure 4.2).

Table 4.1 Infectious VHSV stability in post-mortem hosts at 22°C.

Days Post-Mortem	Pre-Plating		Air		Sand		Water	
	qRT-PCR[a,b]	CC[c]	qRT-PCR[a,b]	CC[c]	qRT-PCR[a,b]	CC[c]	qRT-PCR[a,b]	CC[c]
0	3.8	+						
0.25			5.1	+	5.5	+	5.9	+
0.5			4.3	+	0.9	-	4.9	+
1			6.9	+	6.9	+	7.0	+
2			5.3	-	5.5	-	4.7	-
3			6.2	-	6.7	-	6.7	-
4			1.2	-	2.9	-	3.4	-
5			6.7	-	5.8	-	6.1	-
7			6.5	+	5.3	-	6.0	-
0	2.4	+						
14			3.4	+	3.6	-	2.7	-
30			3.8	-	3.8	-	5.4	-

[a] Each value is the log transformation of the N gene copies of VHSV per 50ng of total RNA.
[b] Each value is the corresponding qRT-PCR value for the pool of fish used to get the CC data.
[c] CC stands for cell culture and represents the presence of CPE on EPC cells.

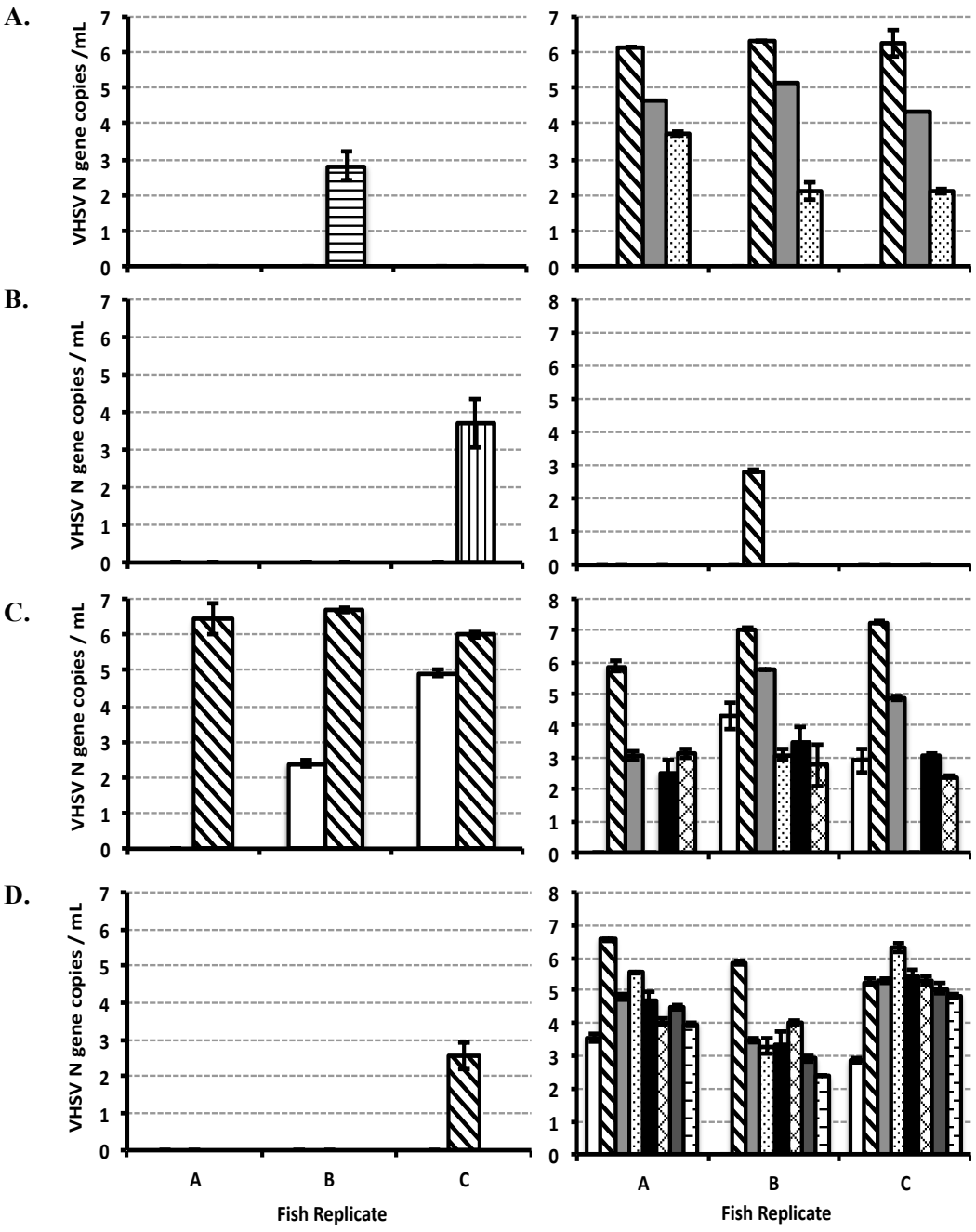

Figure 4.2. VHSV N gene copies in the water condition peak at 1dpm at 22°C. Eight hundred microliter samples were taken upon plating (t = 0), upon collection, and daily when possible from each petri dish with water. Each panel represents the fish that were collected on the following days: Left side: 0.25 (A), 0.5 (B), 1 (C), 2 (D) and Right side: 3 (A), 4 (B), 5 (C), 7 (D) days post-mortem. Bars represent the day the water sample was collected: 0 (white), 0.25 (horizontal stripes), 0.5 (vertical stripes), 1 (diagonal stripes), 2 (light grey), 3 (dots), 4 (black), 5 (exes), 6 (dark grey), and 7 (dashes).

At 4°C, VHSV N gene copies can also be detected throughout the course of the experiment, in this case 100 dpm, with copies ranging from 10^2 to 10^7 (Figure 4.3, 4.4). By 100 dpm. the signal is reduced about three orders of magnitude in both air

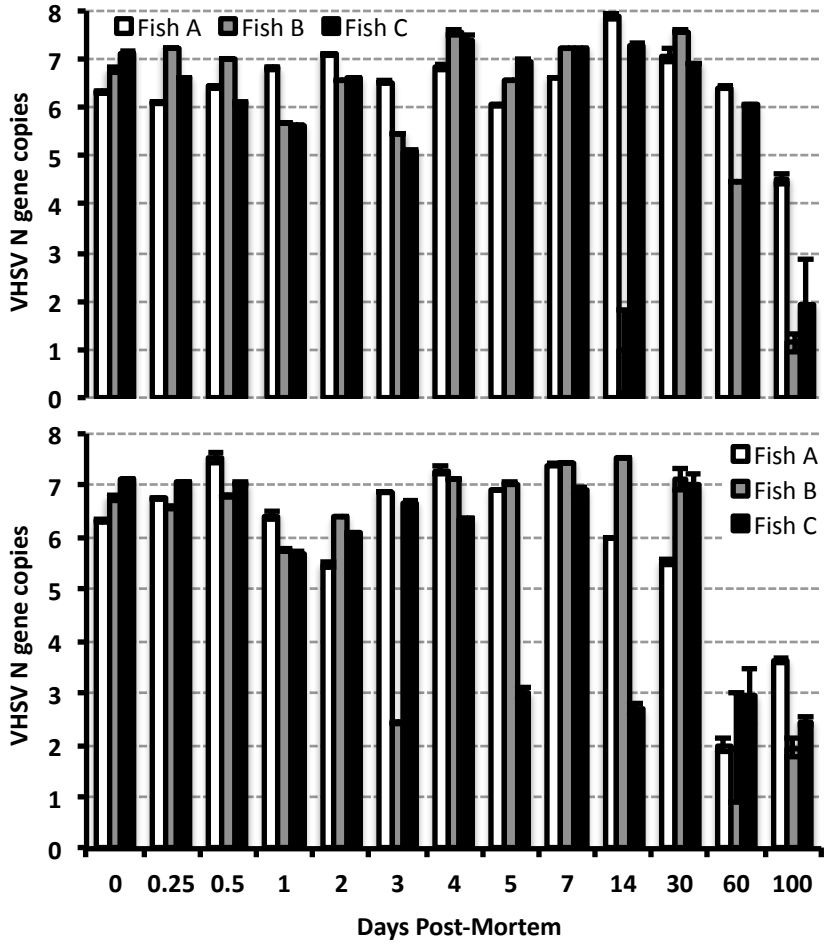

Figure 4.3. VHSV N gene can be detected through 100 dpm at 4°C under all conditions. Fish were plated on either dry petri dishes representing air (A) or on petri dishes with water (B). Different colored bars represent each fish from a biological triplicate, as indicated by Fish A, B, or C. VHSV N gene copies are reported as log transformed values.

and water. This decrease occurs by 60 dpm in water, but not until 100 dpm in air. By cell culture, infectious VHSV could also be detected to 100 dpm in air and water (Table 4.2). In air, infectious VHSV was not detected between 4 and 14 dpm, but

detectable at 30 dpm. At least one water sample at each time point through 100 dpm is positive for infectious VHSV.

Table 4.2 Infectious VHSV stability in post-mortem hosts at 4°C.

Days Post-Mortem	Pre-Plating		Air		Water	
	qRT-PCR[a,b]	CC[c]	qRT-PCR[a,b]	CC[c]	qRT-PCR[a,b]	CC[c]
0	6.3, 6.8, 7.1	+, -, +				
0.25			6.1, 7.2, 6.6	-, +, +,	6.8, 6.6, 7.1	-, +, +
0.5			6.4, 7.0, 6.1	+, +, +	7.6, 6.8, 7.0	+, +, -
1			6.8, 5.7, 5.6	-, -, +	6.4, 5.8, 5.7	+, +, +
2			7.1, 6.6, 6.6	-, +, +	5.5, 6.4, 6.1	-, -, +
3			6.5, 5.5, 5.1	-, -, +	6.9, 2.4, 6.7	-, -, +
4			6.8, 7.6, 7.4	-, -, -	7.3, 7.1, 6.4	-, -, +
5			6.1, 6.5, 7.0	-, -, -	6.9, 7.0, 3.0	+, +, +
7			6.6, 7.2, 7.2	-, -, -	7.4, 7.4, 6.9	-, -, +
14			7.9, 1.5, 7.3	-, -, -	6.0, 7.5, 2.7	-, +, +
30			7.1, 7.6, 6.9	-, -, +	5.5, 7.2, 7.1	+, -, -
60			6.4, 4.5, 6.0	+, +, +	2.0, 2.7, 3.2	+, -, +
100			4.5, 1.2, 2.6	+, +, -	3.6, 2.0, 2.5	+, +, +

[a] Each value is the log transformation of the N gene copies of VHSV per 50ng of total RNA.
[b] Each value is the corresponding qRT-PCR value for the pool of fish used to get the CC data.
[c] CC stands for cell culture and represents the presence of CPE on EPC cells.

Discussion

Our study examined the stability of VHSV genotype IVb in post-mortem fish in simulated lake environments. Previous studies have suggested that VHSV may be infective for up to one year in water at 4°C (40). However, the effects of VHSV in post-mortem hosts have not been investigated. Our results have expanded upon this

idea by suggesting that post-mortem fish infected by VHSV may act as reservoirs of the virus, especially at 4°C.

VHSV N gene can be detected through the latest time point in each temperature, 30 dpm at 22°C and 100dpm at 4°C, in all simulated environments. It is possible that N gene could be detected later than 30 dpm at 22°C if the trial was extended, however, as both late time points for each temperature show a decrease by about 3 orders of magnitude, this is likely close to the detection limit of VHSV in post-mortem fish at both temperatures. The increased stability of VHSV N gene RNA at 4°C is further emphasized by looking at the distribution of copies based on orders of magnitude, where almost 70% of the fish in the 4°C trial had N gene copy numbers around 10^6 or greater, but only about 30% were around this copy number for the 22°C trial (Figure 4.4). We cannot rule out the possibility that a portion of this discrepancy is due to the fish at 22°C replicating less virus into their tissues during infection. However, fish were examined for clinical signs of disease upon plating with an attempt to minimize these differences. Interestingly, by examining different fish at each time point in each condition at each temperature, we do see individual variation; however, this is likely a result of the infection status of the fish upon plating. For example, in the 22°C trial, the 0.5 and 4 dpm fish were plated toward the end of the experiment with fish showing very slight clinical signs of disease. Regardless, the levels of VHSV N gene RNA are consistently detected out to 30 dpm and 100 dpm at 22°C and 4°C respectively.

Significant differences are seen when evaluating the cell culture results both between different conditions and between temperatures. At 22°C, infectious VHSV

can still be detected up to 1 dpm in all three environmental conditions. However, in wet sand infectious VHSV is not detected at 0.5 dpm, but due to the low levels of N gene in these fish and the lower sensitivity of cell culture, we are not surprised by this result. Even with the low levels of RNA at this time point, the detection at 1dpm is consistent with the sand condition. Interestingly, in air, where the fish dry out as they remain in the petri dish provides a very different infectious VHSV IVb profile. VHSV IVb is detected up to 1dpm, like the water and wet sand, but then is detected again at both 7 and 14 dpm and finally dropping off to negative at 30 dpm. This may be a result of the different ways that tissue degrades depending on the environment and how this effects the degradation of VHSV IVb, since both water and wet sand are moist environments as compared to dry air. At 4°C, infectious VHSV is recovered up to 100 dpm in both air and water. We suggest that wet sand represents a fish washing up on shore and since this does not often happen at 4°C, particularly during the spring when VHSV outbreaks have previously been seen, we did not use this condition at this temperature. Interestingly, in air there is a gap of detection of infectious virus, similarly to what is observed at 22°C. Infectious VHSV is detected up to 3 dpm, then negative from 4 to 14 dpm. Infectious virus is recoverable from 30 to 100 dpm. The lack of detection of infectious VHSV during 4 to 14 dpm may reflect the time frame of tissue degradation in air, where some virions survive the complete degradation of tissues of the level of encapsulated virus RNA compared to free RNA. This discrepancy may also reflect where and how the virus is sequestered in individual fish. Interestingly, in water at 4°C, infectious VHSV can be detected in at least one fish at all time points through 100 dpm, with all three fish having detectable infectious virus

at 1, 5, and 100 dpm. When examining daily water samples taken from zebrafish placed in water, infectious VHSV peaks at 1 dpm in the water and then decreases slowly over time at both 22°C and 4°C. One day post mortem appears to be a significant time point where infectious virus is both released into the environment and is detectable in tissues at both 22°C and 4°C and would be a concern of transmission into the environment and into hosts feeding on the degrading tissues.

Combined, our data give compelling insight into VHSV's mode of transmission and identifies another possible reservoir of infectious VHSV IVb that may contribute to the persistence of VHSV in the Great Lakes. At 22°C, infectious VHSV is present in tissues up to 1dpm, but this time increases through 100 dpm at 4°C, which is particularly relevant to fish that have died and washed ashore or are floating on the surface or even those fish such as Round Gobies that sink when they die. These post-mortem hosts may either be consumed by other fish and possibly transmit the virus through the oral route, or release infectious virus into their surrounding environment and transmit infectious virus in a waterborne manner. Furthermore, once this infectious virus is released into the water, the stability VHSV IVb in freshwater has been shown to be detected through 60 and 10 days at 4°C and 20°C, thus increasing the impact of infectious VHSV released from post-mortem hosts. Ultimately, post-mortem hosts are reservoirs of infectious VHSV and likely contribute to the persistence of VHSV in the Great Lakes.

CHAPTER 5

DETERMINATION OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IVB VERTICAL TRANSMISSION USING A ZEBRAFISH INFECTION MODEL

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*Hope wrote this chapter based off of Monroe's thesis, helped with various steps for the experiment, and contributed significantly in an intellectual manner. Monroe performed all experiments in this chapter. Casey is the primary investigator and contributed intellectually.

INTRODUCTION

Viral Hemorrhagic Septicemia (VHS) is a deadly marine and freshwater fish disease that affects over 48 species of fish worldwide (85). Acute clinical signs include skin darkening, anemia, exophthalmia, and epidermal hemorrhages sometimes with ulceration (24, 51). Internal lesions consist of petechial and/or ecchymotic hemorrhages located in most visceral organs and serosanguinous ascites (85). Discovered in the 1930's in European Rainbow Trout farms, VHS has now caused massive damage to commercial as well as recreational fish populations worldwide. The viral etiology of VHS was determined in the 1960s and the associated *Novirhabdovirus* was called Viral Hemorrhagic Septicemia Virus (VHSV). Today, it is one of the most studied fish pathogens and has expanded its geographic range and habitat occupation in the last two decades (68). VHSV is usually classified into sub-

genotypes, ranging from Type I to IVb, and these genotypes are usually associated with geographic locations. Type I to Type IVa have been known and studied for decades, but more recently, a novel genotype of VHSV, genotype IVb, has been isolated from various fish across various species in the North American Great Lakes region and is expanding to other freshwater bodies in the United States (37).

The virus was first isolated in the Great Lakes in 2005 from freshwater drum, *Aplodinotus grunniens,* and round goby, *Neogobius melanostomus*, after large die-off events in Lake Ontario (15). Afterward, VHSV was isolated from archived samples of muskellunge, *Esox masquinongy*, captured in Lake St. Clair, Michigan in 2003, which were clinically normal, although little is known about VHSV in this region between 2003 and 2005 (25). Additional fish mortality episodes appeared in 2006 through 2007 at several locations in Lakes Michigan, Erie, St. Clair, and connected waters (15, 37, 61, 98). Between 2005 and 2007, the major fish mortality events, observed only in the spring, presented immediate concerns about the effects, spread and virulence of VHSV, resulting in a continued survey effort from 2008 through 2011 in the Great Lakes region. Interestingly, during this period very few mass mortality events were reported; however, infectious VHSV was found in all lakes surveyed (6). The extreme persistence of VHSV in this region is not well understood, although several theories are being researched, including horizontal transmission, other species aside from fish as potential reservoirs for VHSV, and vertical transmission. Fish exposed to VHSV through immersion studies have been found to be infectable in this manner; however, there is a noted difference from different species of fish as to their susceptibility to VHSV via immersion and this is not well understood. Furthermore, VHSV has been

isolated from other species, such as lamprey, leeches, and *Diporeia ssp*, although what roles these species play in the persistence of VHSV in the Great Lakes remains unknown (6, 28, 29, 31). And although VHSV has been identified in ovarian and seminal fluids from infected fish, due to the difficulty of studying these mechanisms in natural host fish, direct evidence to support or deny vertical transmission, defined by either direct transmission within germ cells or via exposure to reproduction fluids contaminated pre or post fertilization, as a key component of persistence in the Great Lakes region, has yet to be provided (21, 52, 71).

However, due to the newly developed zebrafish model for VHSV IVb, vertical transmission can now be studied in earnest (Chapter 3). This model shows that zebrafish are susceptible to VHSV IVb, exhibit many similar symptoms of VHS as native species both naturally infected and laboratory infected, are easy to manipulate in terms of environmental parameters, are easily maintained due to their small size, and are able to be bred year round allowing for testing the potential for vertical transmission of VHSV Ib.

The main goal of this project was to develop and implement a zebrafish model to test vertical transmission of VHSV IVb. Since a breeding infectivity model for zebrafish has not been reported, several parameters were tested in order to elucidate the most optimal conditions for vertical transmission of VHSV IVb, including 1) the effects of temperature on embryo viability and development, 2) the effects of temperature on breeding viability, using both traditional single temperature environments, as well as shifting temperature environments, 3) the efficacy of a disinfection protocol for the removal of external VHSV from the embryos, and 4) the

ability of qRT-PCR and cell culture to detect virus and confirm viral infectivity, respectively. After determining the appropriate parameters to test vertical transmission, fish were selected for this protocol after 2 days post-infection (dpi), 4 dpi, or 11 dpi to determine the effects of infectivity on the ability of VHSV IVb to be vertically transmitted. Using our model, vertical transmission of VHSV IVb is not a significant contributor to the persistence of the virus in the Great Lakes.

MATERIALS AND METHODS

Zebrafish maintenance and care.

Zebrafish, *Danio rerio*, used were wild-type Cornell strain, between the ages of 6 months and 2 years. Males and females were maintained separately for as short as 3 weeks prior to use, as determined by visualization of outer features typical to male and females, including underbelly shape and form (males have a more streamlined underside while females have a protruding belly due to the ovaries), size (females are usually larger than males), and color (male zebrafish often have a pink-yellow undertone while females have more of a white-blue undertone) (102). Before use in experimental procedures, zebrafish were maintained between 26°C to 30°C in a temperature and humidity controlled animal care facility, using a typical closed filtered recirculation system (103).

During all experiments, unless otherwise stated, the following conditions were used. The water was reverse osmosis (RO) filtered water with Instant Ocean (9.0 g per 40 L). The static container's included 4 L, 2 L and 1 L containers filled with 3 L, 1.5 L and 0.5L of water respectively, depending on the number of fish to be grouped. The density of housed fish ranged from 3.3 to 6.0 mg/mL. Breeding was conducted using

green translucent breeding containers and traditional breeding protocol (103).

To ensure the overall health of the fish in each experiment, periodic water samples and water condition assays were collected and recorded respectively. Parameters measured included nitrate levels, nitrite levels, ammonia levels, pH, free chlorine levels, and alkalinity, and appropriate water changes, every third to fourth day, when not otherwise stated, was performed to maintain healthy water conditions.

Effects of temperature on embryo viability and development.

To determine the temperature allowance of zebrafish embryos, three sets of uninfected female and male fish were bred at 26°C, 3 males and 3 females per breeding, to achieve between 200 to 250 viable embryos. The embryos from all breedings were collected 4 hours post fertilization (hpf), counted, dead embryos and unfertilized eggs removed, and the remaining 200 to 250 embryos were placed in a 10 cm petri dish with a 50% embryo media to RO water mix, and moved to either 15°C, 22°C, or kept at 26°C. They embryos were then observed until hatching. Each trial was repeated with different fish at different times to reduce individual breeding pair variation and to provide biological triplicates.

Embryos used in the temperature dependence of zebrafish embryos experiment were photographed every 8 hours until the embryos hatched and became juveniles. The Wild Makroshop M420 was the dissecting microscope used (Wild Heerbrugg Scope Instrument Company) at a magnification of 20X using a 10X eyepiece, for a total magnification of 200X. The camera used was 11.2 Color Mosaic (Diagnostic Instruments Inc.) with associated software: Insight Firewire Spot.

Determination of viral RNA extracted from zebrafish embryos.

To determine the number of embryos required for viral detection in cell culture and for extracted viral RNA in qRT-PCR, three sets of uninfected female and male fish were bred at 26°C, 3 males and 3 females per breeding, and the resulting embryos were divided as follows: 50, 100, 200, 300, 400, and 500 embryos. The embryos were collected in pre-weighed 2ml sterile microcentrifuge tubes, and all excess water was removed by decantation. The embryos were weighed using a Mettler AE163 Model analytical scale (Mettler Toledo International Inc,). This was repeated three times to achieve biological triplicates.

Determination of the efficiency and efficacy of the embryo disinfection protocol.

To determine whether the disinfection protocol would be useful for the determination of vertical transmission in zebrafish, three sets of 3 males and 3 females, maintained separately prior to breeding, were bred at 26°C (103). The embryos were collected, separated into groups of 60 embryos each, or 70 mg. Two groups of 60 embryos were infected with 10^6 pfu/mL of VHSV, while the other two groups of 60 embryos were exposed to an equal volume of HMEM-5FPSH only, via immersion for 4 hours.

Post-infection, one of the infected and one of the uninfected groups of embryos were exposed to a 0.003% hypochlorite solution in RO water for approximately 1 minute, with a continuous mixing motion, followed by two RO washes for 1 minute each, again with a continuous mixing motion (103). To determine whether VHSV can be detected from washed embryos, the other infected and uninfected groups of embryos were not exposed to the hypochlorite, but were washed three times as

described above with RO water only.

The embryos were then collected using a transfer pipette in 2 mL microcentrifuge tubes, followed by decantation of excess water, and stored in a -20°C freezer until sample processing. Hypochlorite solution was made fresh for each group of embryos being disinfected. This protocol was repeated three times in order to achieve biological triplicates.

Effects of temperature on zebrafish breeding.

To determine the acceptable temperature conditions for breeding zebrafish under the temperature constraints of VHSV, three pairs of 3 uninfected males and 3 females were maintained separately and then bred at one of the following four temperature conditions: 26°C, 15°C, after a 2 week acclimation, and two temperature shifting conditions, whereby after a 2 week acclimation to 15°C, the fish were moved back to 26°C for either 24 or 48 hours prior to breeding. The fertilized embryo numbers were recorded for each temperature condition. This protocol was repeated three times to achieve biological triplicates.

Determination of VHSV in fish tissues in a post-infection time course.

To determine the amount of VHSV in fish tissues at different times during infection, males and females were maintained together, acclimated to 15°C, infected and terminated at given times post infection. Twenty-five fish were moved into static containers for 2 weeks at 15°C for acclimation purposes, as previously described, with 7 fish in one container and 18 in another, density controlled. Post-acclimation, the group of 18 fish was infected at a dose of 10^6 pfu/mL, while the group of 7 fish was exposed to HMEM-5FPSH only, for 24 hours via immersion. A complete water

change was performed after this 24 hour infection period for both infected and uninfected fish. Three infected fish were collected on 0, 1, 3, 5, 7, 9, and 11 dpi, with corresponding single control fish collected each day. The collected fish were euthanized using an overdose of MS-222 (tricaine methanesulfonate; Western Chemical, Ferndale, Washington), approximately 8 mg/mL of MS-222 diluted in RO Water. The protocol for ensuring the fish were properly euthanized included observing the fish in the MS-222 solution until movement of the gills ceased, followed by an additional one minute in the solution to ensure death. After confirmed death, fish were collected in 2 mL microcentrifuge tubes and stored in a -20°C freezer until sample processing could commence. This protocol was repeated three times to achieve biological triplicates.

Protocol for the determination of vertical transmission of VHSV in zebrafish.

To test for vertical transmission using the pre-determined conditions above, male and female fish were maintained and kept separated, acclimated to 15°C, infected with VHSV, moved back to 26°C for 48 hours, combined for 24 hours, and then the embryos were collected. Briefly, approximately 10 males and 10 females were used per experiment, housed separately in 2 L containers acclimated to 15°C for 2 weeks, infected or mock infected with either 10^6 pfu/mL VHSV or HMEM-5FPSHFPSH, respectively, via immersion for 24 hours, followed by a complete water change. Fish were moved back to 26°C for 48 hours prior to breeding at 11, 4, or 2 dpi. Breeding pairs consisted of three males and three females each, three separate breeding groups per experiment. This protocol was repeated three times for each time point post infection to achieve biological triplicates.

Protocol for the tissue lysis and homogenization prior to VHSV detection protocols.

Whole fish were partially thawed, weighed, and mixed with 1ml of Hank's Minimal Essential Medium (HMEM) (Gibco®, Invitrogen) supplemented with 5% fetal bovine serum (FBS), Streptomycin (200 IU/ml) (Gibco®, Invitrogen), Penicillin (200 µg/mL) (Gibco®, Invitrogen), and HEPES Buffer (1M 0.015 mL/mL) (Gibco®, Invitrogen), and with 100 to 150 mg of 0.1-mm zirconia–silica beads in designated sterile 2 mL screw-cap tubes (Bio-Spec Products), and homogenized for 10 to 20 s depending on the size of the fish sample in a Bead Beater (Bio-Spec Products,). Samples were kept on ice when not being homogenized. After homogenization, the samples were centrifuged at 9,300xg for 5 min at room temperature in an Eppendorf Model 54–15 centrifuge.

The supernatant was then transferred to a sterile 2 mL microcentrifuge tube and a portion of the supernatant (15-30 mg tissue weight) was used to isolate total RNA for qRT-PCR and the remaining portion (100–500 mg) was further diluted, approximately 1:30, for cell culture.

Protocol for the isolation of total zebrafish RNA.

Total RNA was isolated using the QIAGEN RNeasy kits, with a slightly modified protocol. Seven hundred microliters of both buffer RLT and sterile 70% ethanol were added to the 10 -30 mg tissue weight cleared lysate from the Bead Beater procedure, vortexed and then applied to the RNeasy column. Two centrifuge steps were required to allow the full volume to be added to the column. A new collection tube was used for each centrifugation step to avoid cross contamination. To elute the total RNA from the column, 50 µL of RNase-free water was added and centrifuged for

1 min at 13,700 g. To provide a more concentrated sample, the final elution step was repeated, eluting the same 50 µL of water back through the column. Total RNA was measured for purity and concentration using a NanoVue spectrophotometer. In preparation for qRTPCR, dilutions were prepared to achieve 50 ng of total RNA per well of either a 96- or 384-well plate.

Determination of quantitative VHSV RNA using qRT-PCR.

The following primers and probe were designed to target the N gene of the MI03 isolate of VHSV IVb, as previously described (8, 18, 23, 32, 37, 44, 83).

Forward- 5'- ACCTCATGGACATCGTCAAGG- 3',

Reverse- 5' – TCCCCAAGCTTCTTGGTGA – 3',

Probe- 5' -/56-FAM CCCTGATGACGTGTTCCCTTCTGACC/36-TAMSp/- 3'.

The assay was run on an Applied Biosystems-Prism model 7700 and Applied Biosystems ViaII model sequence detector (Applied Biosystems, Inc.) for 96 and 384 well plates, respectively, and performed according to ABI using their TaqMan One-Step RT-PCR Master Mix reagents. VHSV N gene copy number standards were prepared and run exactly according to published methods (44).

The unknowns, standards, and no-template controls (which contained RNase-free water in place of template) were run in triplicate on a MicroAmp Optical 96- or 384 well reaction plate from ABI. For the 96 well plates, each 25 µL-per-well reaction was comprised of 15 µL from the master mix solution (final amounts per reaction: 1X Multiscribe, 1X TaqMan Universal PCR Master Mix, 200 nM forward primer, 200 nM reverse primer, and 200 nM probe) and 10 µL of sample at a concentration of 50 ng total RNA/10 µL unless stated otherwise. For the 384 well plates, each 15 µL-per-

well reaction was comprised of 9 μL from the master mix solution (final amounts per reaction: 1X Multiscribe, 1X TaqMan Universal PCR Master Mix, 120 nM forward primer, 120 nM reverse primer, and 120 nM probe) and 6 μL of sample at a concentration of 50 ng total RNA/6 μL unless stated otherwise. The PCR conditions were as follows: 30 min at 48°C for reverse transcription; 10 min at 95°C for AmpliTaq activation; 15 s at 95°C for denaturing, followed by 1 min at 60°C for annealing and extension (repeated for 42 cycles). Absolute copy numbers in unknown samples were determined from a standard regression fit using the supplier's software (SDS; ABI). This standard regression fit used the applicable standard curve for VHSV IVb, which was constructed previously by Hope et al. (44).

Determination of infectious VHSV using cell culture.

Epithelioma papillosum cyprini (EPC) cells were obtained from the American Type Culture Collection for virus isolation (30). Cells were routinely grown in T75- or T150- cm^2 tissue culture flasks (Corning, Inc.) in Hank's Minimum Essential Medium (HMEM) with HEPES buffer containing 10% fetal bovine serum (FBS) with penicillin and streptomycin (HMEM-10FPSH). The cells were sub-cultivated using a 0.05% trypsin wash to dislodge them from the surface of the flasks and then re-suspended in an appropriate volume of HMEM-10FPSH before being seeded in new culture flasks. Virus isolations were performed in 48-well tissue culture plates (Corning, Inc.). The equivalent of one confluent 25-cm^2 tissue culture flask was used to seed each 48-well tissue culture plate, for an approximately 1:2 split.

The cells were harvested as described above for cell passage but then re-suspended in HMEM-5FPSH. To each well of the 48-well plate 250 μL of cell

suspension was seeded. The sample homogenate was then used (or thawed in cases of homogenate having been frozen) and used to inoculate triplicate wells of the 48-well plate for virus isolation. During the inoculation process, 250 µL of filtered inoculum was carefully placed in each well. After inoculation, EPC cells were incubated at 15°C for no more than 14 days. The cells were examined for cytopathic effects (CPE) at 1, 5, 7, and 14 days post inoculation. If at any time CPE was noted, the cells and media were removed and passaged to fresh EPC cell monolayers after being filtered through a 0.2 µm porosity filter. All samples were passaged at 14 days even if there were no signs of CPE, for a total passage count of three. If there was no CPE observed after three passages, the cells were scored as VHSV negative. If the sampled showed CPE for three consecutive passages (or rather throughout the entire cell culture passaging process) it would be scored as VHSV positive, pending confirmation of the passage filtrate using qRT-PCR.

RESULTS

Effects of temperature on embryo viability and development.

To determine the acceptable temperature for zebrafish embryo growth and development under the temperature constraints of VHSV as a low temperature replicating virus, embryos were obtained via optimal breeding conditions at 26°C, separated, placed into one of three temperatures, either 15°C, 22°C, or 26°C, and then observed for mortality and developmental stages.

Low mortality events were observed with the 22°C and 26°C embryos, 19% and 12% respectively, while those embryos held at 15°C experienced 100% mortality (Figure 5.1).

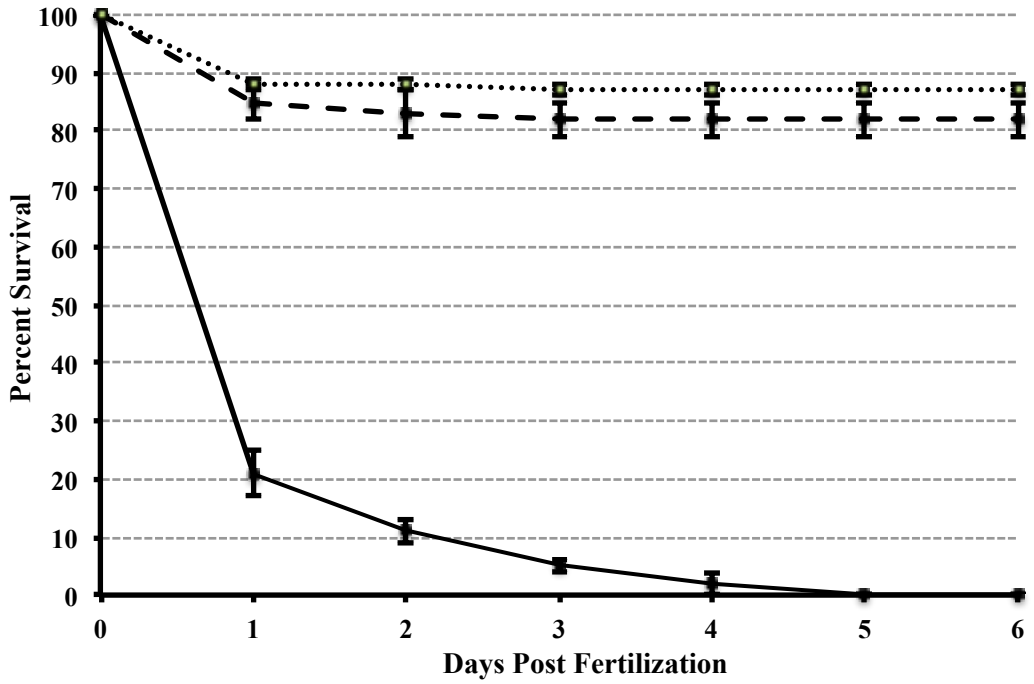

Figure 5.1. Zebrafish embryo survival is temperature dependent. Zebrafish embryos, collected 4 hours post fertilization at 15°C (solid), 22°C (dashed), or 26°C (dotted), were observed for embryo death. Bars represent biological triplicates at each temperature condition.

To assess the developmental differences of the embryos at these different temperatures, the embryos were observed in each condition at 2.5, 15, 37, 61, 77, 97, and 121 hours post fertilization (hpf) and representative images are shown in Figure 5.2. Although the mortality of the embryos was not significantly different between the 22°C and the 26°C groups, differences in the development were very apparent (103). Between the embryos kept at 26°C and 22°C there is an approximate 1:2 developmental delay throughout the time course. At 2.5 hpf, the embryos at 26°C were at the 16-cell stage, or the 1.5 hour stage, whereas the 22C embryos were at the 8-stage, or what would be expected for a 1.25 hour stage. At 15 hpf, the 26°C embryos were at the 3-somite stage, or the 11 hour stage, whereas the 22C embryos were in the

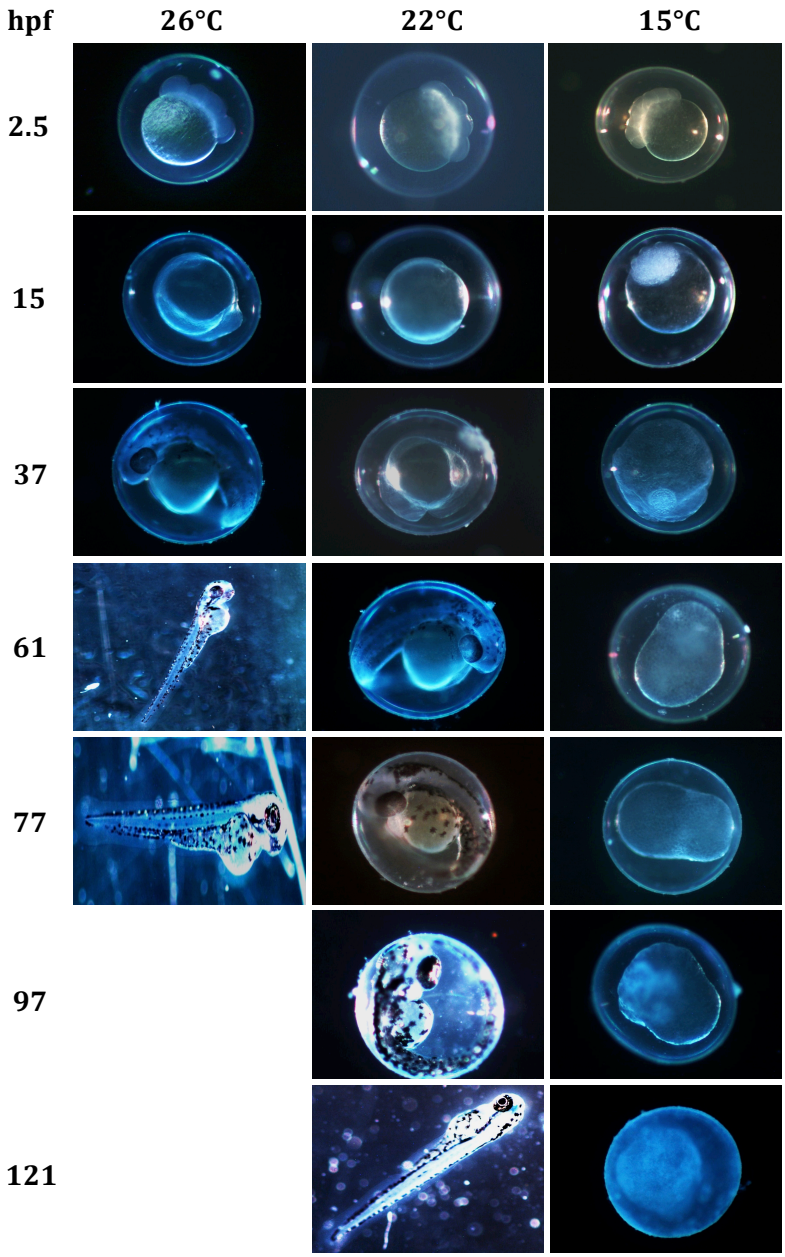

Figure 5.2 Embryo developmental defects are observed at 22°C and 15°C. Photographs represent the developmental stage of more than 70% of the embryos taken at 2.5, 15, 37, 61, 77, 97 or 121 hpf. Magnified at 20X using a 10X eyepiece.

shield, 6 hour stage. By 37 hpf, the 26°C embryos were at the prim 22, or 35 hour, stage, and the 22°C embryos were at the 18-somite, or 18 hour stage. At 61hpf, the 26°C embryos were now hatched at the pec fin, or 60 hour stage, while the 22°C embryos were still only at the prim-16, or 31 hour stage. At 77 hpf, the 26°C embryos were now at the protruding mouth, or 72 hour, stage, while the 22°C embryos were just reaching the high pec, or 42 hour, stage. By 97 hours, the 26°C fish are now considered juveniles, whereas the 22°C embryos had just reached the pec fin, or 60 hour stage, not reaching the protruding mouth, or 72 hour, stage until 121 hpf.

Although the embryos kept at 22°C fully developed albeit taking twice as long, the embryos at 15°C never fully developed. Interestingly, the 15°C embryos at 2 hpf reached the same stage as the 22°C embryos, the 8 cell, or 1.25 hour, stage. However, at 15 hpf, the 15°C embryos had only reached the 256-cell, or 2.5 hour stage, with a highly irregular shape with a bi-lobe appearance. This irregular shape was maintained until 121 hpf, when the embryos died.

The effects of embryo mass on the detection of VHSV using qRT-PCR and cell culture.

Typical yields necessary for our cell culture and qRT-PCR assays using whole zebrafish tissue range from 50 to 100 mg of tissue for cell culture and 5 to 30 mg of tissue for qRT-PCR; however, the amount of material in an embryo had not been determined using either cell culture or qRT-PCR in our lab. Thus, to determine the amount of embryonic material for detection using cell culture and qRT-PCR, uninfected male and female fish were bred at 26°C and the embryos collected. The embryos were then counted into groups of 50, 100, 200, 300, 400, or 500 embryos and

the mass was determined for each group. As expected, embryo weight increased linearly with embryo number (Figure 5.3). As 100 embryos provide about 120 mg of tissue and 200 embryos provide 240 mg of tissue, 100 to 200 embryos would provide enough tissue for both of our assays.

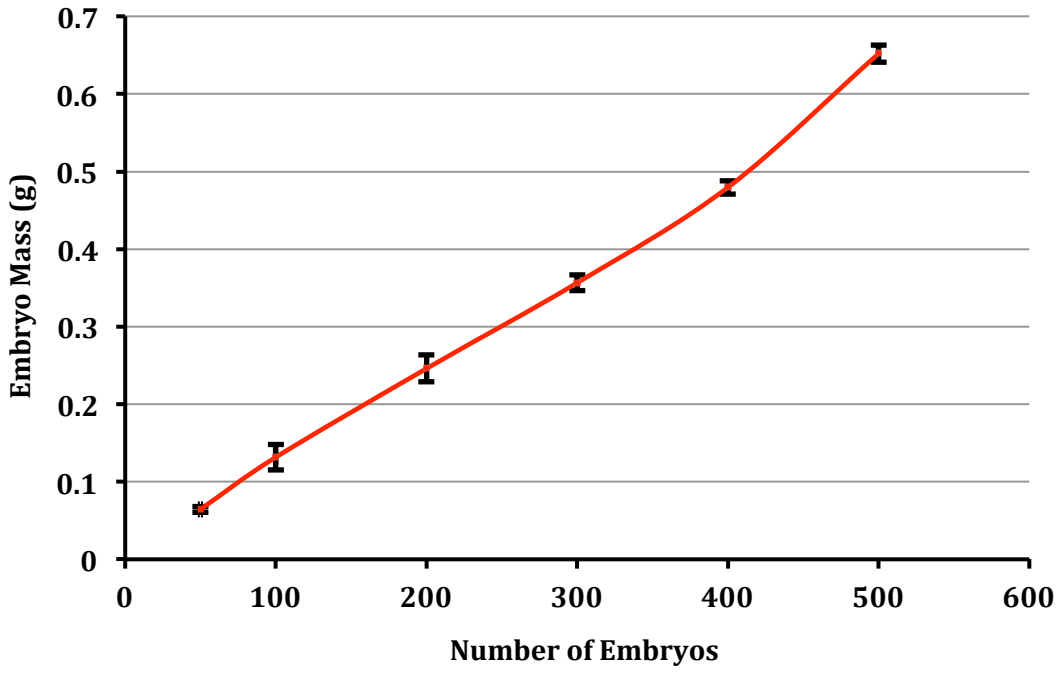

Figure 5.3: Embryo mass increases linearly with embryo number.
Uninfected embryos were collected, counted, separated, and weighed using an analytical scale. Error bars represent biological triplicates.

Determination of the efficiency and efficacy of the embryo disinfection protocol.

In order to differentiate whether VHSV is inside the embryo, indicating vertical transmission, or attached to the exterior of the embryo, a disinfection protocol using a dilute hypoclorite solution of bleach was tested to remove external VHSV (Figure 5.4). Uninfected embryos, collected 2.5 to 4 hpf, were either infected with VHSV or mock infected. Following this, the different groups were either disinfected

using dilute bleach or mock disinfected with RO water. These groups were then washed twice with RO water to remove any lingering VHSV and bleach, and excess water removed.

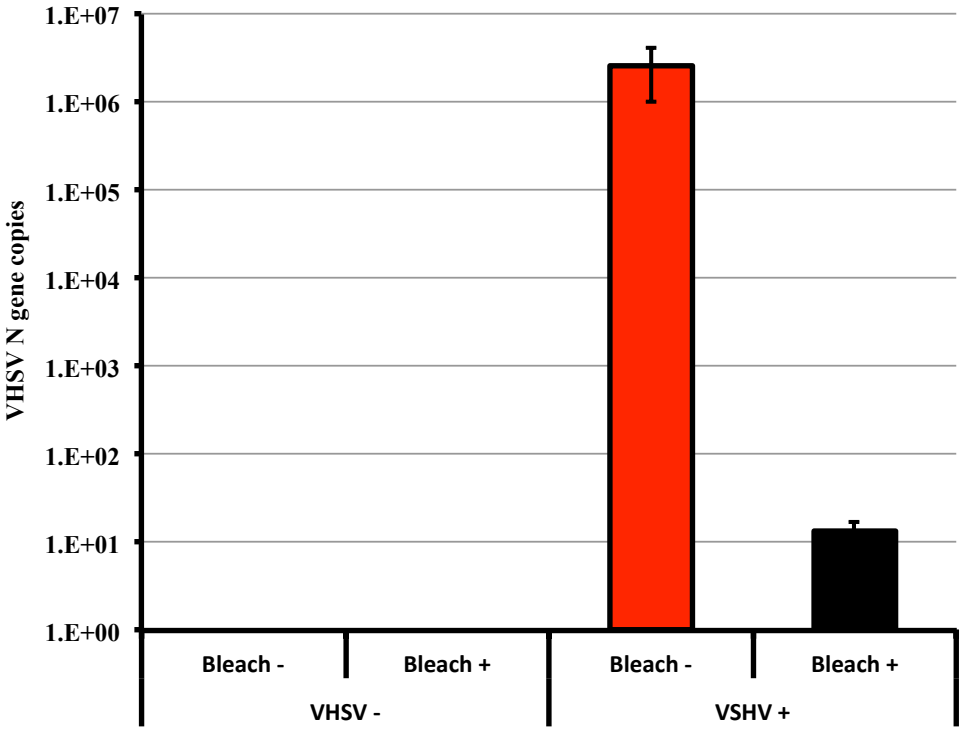

Figure 5.4. External VHSV is significantly reduced using dilute bleach. Hours post-fertilization that previously uninfected embryos were exposed to 10^6 pfu/mL of VHSV or an equal volume of HMEM-5FPSHFPSH for 4 hours via immersion. After infection, the embryos were disinfected with 0.005% hypoclorite solution or mock disinfected with RO water. Following two washes with RO water, the embryos were evaluated for VHSV using both qRT-PCR (values of bars) and cell culture (bar color; red indicates cell culture positive and black indicates cell culture negative). Error bars represent biological triplicates.

All uninfected embryos survived the disinfection protocol and were negative for VHSV. For the infected embryos that were not disinfected, the embryos were positive for VHSV both in cell culture and qRT-PCR, with N gene copies on the order of 10^6 copies per 50ng of total RNA. By exposing infected embryos to bleach, VHSV

was significantly reduced, on the order of 10^1 N gene copies per 50 ng of total RNA, and were now cell culture negative. These results show that a significant amount of external VHSV can be removed using this bleaching protocol. Furthermore, these results also corroborate previous work with snakehead rhabdovirus, which requires embryos to be de-chorionated for external viral entry (78).

Effects of temperature on zebrafish breeding.

Along with the effects on the outcome of embryo development, temperature also plays a crucial role in breeding. To determine the acceptable temperature for zebrafish breeding under the temperature constraints of VHSV as a low temperature replicating virus, zebrafish were bred at one of the following four temperature conditions: 26°C, 15°C, and two temperature shifting conditions, whereby after a two week acclimation to 15°C, mimicking conditions for infection, the fish were moved back to 26°C for either 24 or 48 hours prior to breeding.

The average embryo yield at 26°C was approximately 377 embryos, whereas at 15°C no embryos were observed, which is consistent with embryo viability at 15°C (Figure 5.5). To adjust for the lack of embryo production or release at 15°C while still allowing for a VHSV infection at 15°C, zebrafish, after a 2 week acclimation to 15°C, were shifted back to 26°C for either 24 or 48 hours prior to breeding, yielding on average 45±11 embryos and 197±91 embryos respectively.

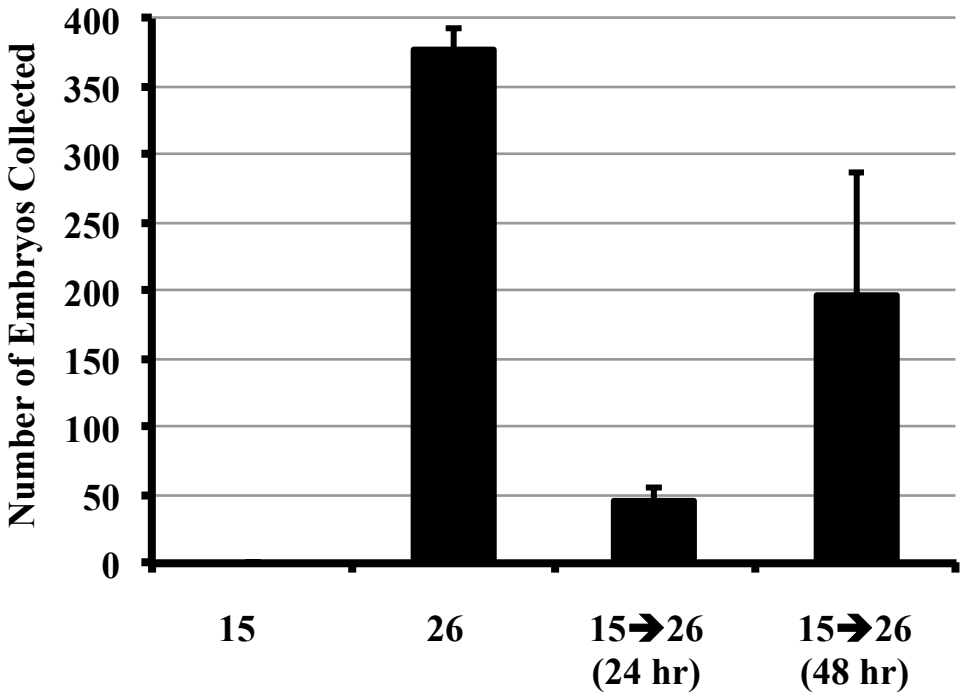

Figure 5.5 Zebrafish breeding success decreases with decreasing temperature. Zebrafish were acclimated to either 15°C or 26°C for 14 days prior to breeding. Two groups were moved up to 26°C for 24 or 48 hours after this acclimation period prior to breeding. Breeding success was determined by the number of embryos collected from each temperature condition. Error bars represent biological triplicates from three different breeding pairs.

Determination of vertical transmission of VHSV using a zebrafish model.

Acknowledging the significant effects that temperature has on zebrafish breeding, the effects of infection on zebrafish breeding also needs to be elucidated. To determine when the fish should be shifted to 26°C prior to breeding to test for vertical transmission, zebrafish were acclimated to 15°C for 2 weeks prior to infection with 10^6 pfu/mL of VHSV or mock infected with HMEM-5FPSH for 24 hours. At each time point, 0, 1, 3, 5, 7, 9, and 11 dpi, 3 infected fish and one control fish were euthanized and tested for VHSV in qRT-PCR and cell culture (Figure 5.6). VHSV N gene copies increased linearly through 5 dpi, reaching approximately 10^5 copies, with

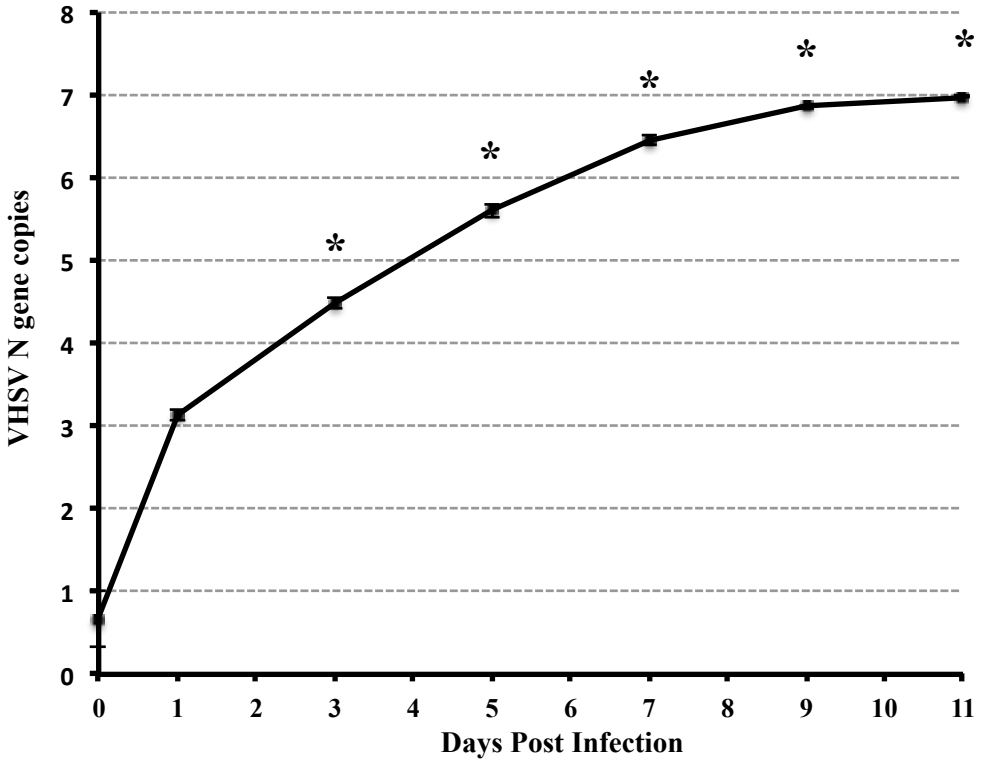

Figure 5.6 Infectious VHSV increases during infection. Zebrafish were acclimated to 15°C for 2 weeks prior to an infection with 10^6 pfu/mL VHSV. Three fish were collected on each dpi. Bar quantities indicate VHSV N gene copies; bar color indicated cell culture positive (red) or negative (black). Error bars represent biological triplicates of three separate infections.

slight increases through 11 dpi, reaching a maximum of approximately 10^6 copies. Infectious VHSV could be detected at 3 through 11 dpi. External signs of hemorrhaging were visible beginning 8 dpi, where VHSV N gene copies were already significantly high.

Using this data and the conditions previously determined, vertical transmission was tested in zebrafish using three time points: early, as defined by pre-onset of clinical signs with borderline cell culture positivity, middle, as defined by pre-onset of clinical sings with definite cell culture positivity, and late, as defined by the presence

of clinical signs with cell culture positivity for VHSV. Gender separated fish were acclimated at 15°C for 2 weeks prior to infection with 10^6 pfu/mL of VHSV or mock infection with HMEM-5FPSH for 24 hours. Following this infection period, fish were moved back to 26°C for 48 hours either at 2, 4, or 11 dpi. After this 26°C acclimation period, the male and female zebrafish were bred and breeding success was evaluated by embryo number (Table 5.1).

For the fish temperature shifted 11 dpi, where clinical signs were observed, breeding was not successful, no embryos or eggs were found, regardless of the breeding sets and no sperm was visible. For the fish temperature shifted 4 dpi, where the fish showed no clinical signs, again no embryos or eggs were found; however, sperm release was visible. For the fish temperature shifted 2 dpi, where clinical signs were not observed and the cell culture was negative, again no embryos or eggs were present; however, sperm release was visible. All mock infected breeding sets were successful at each temperature shift post exposure to HMEM-5FPSH with similar clutch sizes of that shown in Figure 5.5, implying the viral infection as the cause of the lack of embryo release.

Temp. Shift [a]	Breeding Set [b]	Gender	Cell Culture	VHSV N gene Copies [c]	Average VHSV N gene Copies [c, d]	Eggs Visible	Sperm Visible	Embryos Collected
2	1	F	+, +, +	6.0, 6.6, 7.0	6.5 ± 0.5	No		0
		M	+, +, +	4.6*, 5.7, 6.8	6.3 ± 0.6		Yes	
	2	F	-, +, +	3.3, 7.7, 7.9	6.3 ± 2.6	No		0
		M	-, +, +	3.0, 4.6*, 6.9	5.0 ± 2.0		Yes	
	3	F	-, +, +	3.6, 7.0*, 7.0	5.3 ± 1.7	No		0
		M	+, +, +	5.8*, 6.9, 7.5	7.2 ± 0.3		Yes	
4	1	F	-, +, +	2.6, 6.9, 7.6	5.7 ± 2.7	No		0
		M	+, +, +	5.1, 7.9, 8.0	7.0 ± 1.6		Yes	
	2	F	+, +, +	5.4, 6.5, 6.7	6.2 ± 0.7	No		0
		M	+, +, +	5.8, 6.0 7.5	6.4 ± 0.9		Yes	
	3	F	-, +, +	2.7, 5.8, 7.7	5.4 ± 2.5	No		0
		M	+, +, +	4.7*, 5.3, 7.9	6.6 ± 1.3		Yes	
11	1	F	+, +, +	4.5*, 5.5, 6.7	6.1 ± 0.6	No		0
		M	-, +, +	3.7, 6.3, 6.8	5.6 ± 1.7		No	
	2	F	-, +, +	3.1, 6.8, 7.3	5.7 ± 2.3	No		0
		M	+, +, +	5.5*, 6.9, 7.1	7.0 ± 0.1		No	
	3	F	+, +, +	3.6*, 4.6*, 6.7	6.7	No		0
		M	-, +, +	2.9, 7.0, 7.7	5.9 ± 2.6		No	

Table 5.1 Zebrafish trials of vertical transmission of VHSV

[a] Temperature shift is defined as days post infection that the fish were moved back to 26°C prior to breeding.

[b] Each breeding set consisted of three males and three females where possible. Each breeding set counted as a single trial and three independent trials were done for each temperature shift condition.

[c] N gene copies are reported as a log value.

[d] Error reported is the standard deviation or range of the fish actually bred.

*Died prior to breeding.

DISCUSSION

Better understanding the modes of transmission of such a deadly virus as VHSV with such a broad host range is critical to understanding how it can be controlled, how to predict its movements into new environments, and how it needs to be handled in a hatchery environment. Although vertical transmission has been targeted as potential mode of transmission for VHSV, direct evidence of this has yet to

be reported. In this study, we directly tested for vertical transmission of VHSV utilizing a zebrafish model, that has been previously developed for VHSV IVb (Chapter 3), allowing an in depth study into the practicality of VHSV vertical transmission.

Effects of temperature on embryo development and zebrafish breeding.

In order to determine a compromise for the optimal higher temperature for zebrafish breeding and the optimal lower temperature for VHSV replication, several temperature parameters and schemes for both embryo development and zebrafish breeding were evaluated. At 15°C, embryos did not develop normally and died by 121 hpf even in uninfected zebrafish. Interestingly, however, the embryos developed at the same delayed rate as the 22°C embryos that ultimately survived and hatched at 2 hpf. By 15 hpf, the 15°C embryos were now delayed even from the 22°C embryos and began to display a highly irregular shape with a bi-lobe appearance. The embryos remained in this state until 121 hpf when they all died, displaying the characteristic white haziness inside the chorion. These are some of the first observations of zebrafish embryos at 15°C and imply that some development does occur even at such a low temperature but at a distinct stage of development, the embryos are halted and do not survive. At 22°C, embryo development took consistently twice as long as what was observed for the embryos at 26°C, but fully developed and hatched. Further temperature data would be interesting to fully understand these trends in developmental delay for zebrafish embryos.

Breeding at 15°C was also not successful, since embryos were not observed, though whether this is a result of the females not producing eggs or just not releasing

the eggs is unclear, but sperm was also not observed in these breeding sets. However, by applying a temperature shift from 15°C to 26°C for either 24 or 48 hours, breeding was successful, however, both embryo clutch sizes were much lower than the embryos kept at 26°C, about 8-fold and 2-fold lower respectively. Thus, allowing the zebrafish to acclimate to 26°C for an additional 24 hours significantly affected the ability of the zebrafish to breed more normally. Although there was concern about VHSV being cleared at 26°C, a preliminary test of the vertical transmission infection model (data not shown) proved that even after 72 hours at 26°C, zebrafish infected with VHSV still showed high levels of virus when screened, and thus, acclimating the zebrafish at 26°C for longer periods of time to increase the clutch size is another option. Furthermore, lower temperature acclimation has already been show to be crucial on the susceptibility of zebrafish to VHSV, where significant differences can be seen between 24 hours and 2 weeks, with little change even after acclimating the fish at 15°C prior to infection up to 16 weeks (Chapter 3). The ability of zebrafish to acclimate to new temperature environments appears to be a very rapid response and most likely is even quicker when the temperature shift is upwards, affecting not only viral susceptibility but now breeding is observed as such as well. This is consistent with data indicating that zebrafish are not completely immune-compromised at 15°C, and likely other species of fish will show similarly variable responses to temperature change (26).

Effects of bleach on disinfecting VHSV exposed embryos.

To ensure that vertical transmission was occurring and to exclude the possibility of detecting VHSV that was attached to the outside of the chorion,

zebrafish embryos were exposed to dilute bleach using a protocol previously used on zebrafish embryos (103). The protocol was both highly efficient and efficacious in removing external VHSV, reducing the external VHSV levels by five orders of magnitude, while not harming the embryos themselves. Interestingly, however, the embryos that were exposed to VHSV without being exposed to bleach, with N gene copies on the order of 10^6, still underwent three washes with RO water and were then completely removed from any supernatant prior to processing them for qRT-PCR, emphasizing the ability of VHSV to adhere strongly to the surfaces of the embryos and is crucial in considering how VHSV is primarily spread from fish to fish. Further exploring the levels of external versus internal VHSV may shed some light into species specificity to this virus or at least into the possibility of transmission via physical contact with the exterior of the embryo post hatching.

Determination of vertical transmission of VHSV using a zebrafish model.

With the parameters determined to test the vertical transmission of VHSV utilizing a zebrafish model, males and females were kept separate, acclimated and infected at 15°C, followed by acclimation and breeding at 26°C. Fish were moved back to 26°C prior to breeding at 3 different stages post-infection: on 2 dpi, prior to clinical signs of disease and prior to the detection of infectious VHSV, on 4 dpi, prior to clinical signs but after the detection of infectious VHSV, or on 11 dpi, after clinical signs of disease with the detection of infectious VHSV. Regardless of the stage post-infection, none of the breeding sets resulted in the development of embryos. Interestingly, although the presence of sperm was observed in the two earlier stages, eggs were never released from the females, even at low exposure doses, indicating a

potential gender specificity of the susceptibility to VHSV. Furthermore, infected female fish presented clinical signs of disease before the males with more severe signs of disease (data not shown). Overall, the female zebrafish appear to be unable to produce and/or release eggs when exposed to VHSV, even at small doses, even though their uninfected counterparts did not exhibit this problem, whereas the male zebrafish seem to have a highly tolerance to VHSV and produce and release sperm when bred using the model after being moved as late as 4 dpi.

Interestingly, even at 1 dpi, VHSV N gene copies were as high as 10^3, even though infectious VHSV was not detected. This could be a result of the increased sensitivity of the qRT-PCR assay compared with the cell culture assay, where the limit in cell culture is around 10^3 pfu/mL from fish samples. However, this could also indicate that, as is consistent with the ability of VHSV to adhere to surfaces, the initial amount of virus is located on the exterior of the fish, prior to moving into the fish and being able to replicate in the host cells, thus creating a highly concentrated exposure to VHSV that stays with the fish even as it moves through its environment. This would also help explain survey work from the Great Lakes and Canada, where fish have been found to have high levels of VHSV, without high levels in nearby waters, though the limitations of detecting VHSV in water samples cannot be ruled out.

The main goal of this manuscript was to test for the vertical transmission of VHSV by analyzing embryos produced from infected fish for the presence of infectious VHSV. However, embryo production was not observed even using fish that had yet to show clinical signs of disease. There are several possibilities to explain these observations. First, since there were no eggs released, an early effect of VHSV

exposure could be to halt egg production, egg release, or both. Exposing zebrafish to lower initial doses could also be done to further test whether any exposure to VHSV will result in this phenotype. Second, the lack of egg release may be a result of too many insults to the female host and maybe able to be reproduced with other insults, such as chemicals or bacteria, and is not a specific effect of VHSV exposure. Third, as there does appear to be a difference in susceptibility between male and female fish to VHSV, females may require more time acclimated to 26°C prior to being bred after being exposed to VHSV. Furthermore, if infected females are not releasing eggs, then exposing just males to VHSV and testing for vertical transmission may be a better model for what occurs in the natural environment, which could be controlled in our system by breeding the fish at 26°C and thus preventing the infection of females to any VHSV from the males. To further address the potential of vertical transmission using this model in the absence of infected embryo production, eggs and sperm from uninfected males and females can be squeezed out of the fish. The eggs can be separated from ovarian fluids, either bleached or not, and all fractions can be tested for VHSV, further directly addressing whether VHSV is even in the eggs to begin with. One piece of evidence indicates that VHSV is not in the eggs, whereas another virus, INPV, is located in the eggs (17). Furthermore, using uninfected eggs and sperm in the presence of varying amounts of VHSV in *in vitro* fertilization would determine that, if egg release did occur, would VHSV present during the fertilization process enter the embryo. Ruling out these further possibilities would go even farther to negate vertical transmission as being a likely source for transmission of VHSV.

However, although vertical transmission may not be a key factor since infected

females did not release eggs, but due to the ability of VHSV to strongly adhere to surfaces, the potential for horizontal transmission early in development is highly likely. In natural hosts of VHSV, juveniles display an increased susceptibility to VHSV (50). Furthermore, newly hatched zebrafish feed on the chorions as part of their first meal until further developments occur for algae digestion. Although we have shown that VHSV is unable to enter the embryo during development when exposed post fertilization, which is consistent with work done with snakehead rhabovirus, where zebrafish embryos required de-chorination to be infected, the effects of VHSV exposure post-hatching has yet to be evaluated. High levels of VHSV attached to the juvenile fish during development may explain some of the increased susceptibility to VHSV at this early age. However, with SHRV, even upon de-chorionating the embryos and then infecting, the embryos were not able to survive this viral exposure, and likely VHSV would be similar (78).

Based on the fact that 1) the female fish would not release eggs even with lower levels of VHSV in their tissues and 2) VHSV exposure to post-fertilized embryos, even though VHSV strongly adhered to the surfaces of embryos, does not enter embryos, it is unlikely that vertical transmission is a significant contributor to the persistence of VHSV in the Great Lakes region.

CHAPTER 6

VIRAL SHEDDING AND HORIZONTAL TRANSMISSION OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IVB UTILIZING A ZEBRAFISH INFECTION MODEL

Kristine M. Hope, Randall A. Meyer, and James W. Casey

*Hope wrote this chapter, helped with various steps for the experiment, and contributed significantly in an intellectual manner. Meyer performed all experiments in this chapter. Casey is the primary investigator and contributed intellectually.

Introduction

Viral hemorrhagic septicemia virus (VHSV), a rhabdovirus of fish, is the causative agent of viral hemorrhagic septicemia, one of the most serious fish diseases that is notifiable under the OIE. VHSV has affected various fish populations since the early 1930s and has continued to both spread and persist worldwide. Four distinct genotypes have been identified, which generally correlate with their location. VHSV IVb includes all of the isolates from the Great Lakes, having been only recently identified as of 2005 (47). From 2005 through 2007, massive mortality events occurred each spring in the Great Lakes region as a result of VHSV infection, initiating survey efforts for all of the Great Lakes. Interestingly, although mortality events dwindled after this time, with few noted outbreaks of disease, VHSV has continued to persist in the environment (6). This raises the question of how VHSV has maintained itself in these environments. In addition subclinical persistence of VHSV

IVb poses a threat to naïve bodies of water, raising concerns of future epidemics.

Understanding VHSV persistence requires investigating several aspects of the virus life cycle, including viral entry, replication area and status in the host, viral exit, transmission between hosts, and possible secondary host reservoirs. VHSV entrance into its host has been studied using various methods of infection, including oral, via the gills, or through the fin bases (47), which of these routes is dominant in natural virus acquisition is unknown. Once in the host, VHSV has been identified in most of the major organs including spleen, kidney, liver, and heart. VHSV can exit/shed from the host in number of ways. VHSV has been identified in ovarian fluid, feces and urine, but it is unclear as to whether these are the major sites of exit or if additional shedding occurs in tissues like skin. Once shed, VHSV appears to be stable in the environment for extended periods at low temperatures. VHSV can spread from host to host via water, but there is also evidence that species other than fish, such as leeches, *Diporeia ssp*, potentially contribute to spread and persistence (28, 29, 31). Interestingly, physical contact is another potential mechanism for spread of VHSV, as European fish farms were the first to identify the disease caused by VHSV where population density is high.

One particularly interesting element of VHSV transmission includes not only how VHSV is shed into the environment, but also at what rate shedding occurs and how this relates to persistence and disease outbreaks. Recent studies have been conducted with fish rhabdoviruses to address some of these questions using various fish models. VHSV shedding during a challenge infection of a Pacific Herring was demonstrated to have peaks corresponding to increases in cumulative percent

mortality during the course of the infection indicating a probable point during the infection for a water-borne transmission. Furthermore, survivors did not shed detectable levels of the virus indicating either the virus was cleared or persisted sub-clinically in the fish (43). Similar trends were noted with a trial infection of Japanese flounder utilizing VHSV (72) and Atlantic salmon with infectious salmon anemia virus (36). Although these studies confirm that VHSV and SVC are shed at an appreciable rate during infection they do not confirm how VHSV persists in fish and is transmitted from host to host.

To investigate the mechanisms of VHSV horizontal transmission, we have utilized a zebrafish model for VHSV IVb, focusing on two main questions. First, we have compared viral shedding profiles in group housed and individualized fish to evaluate both the fate of fish after an initial infection and the role of viral spread on this process. Second, we evaluated the ability of VHSV to infect both with and without physical contact from host to host, to determine the importance of physical contact in the transmission of VHSV.

Materials and Methods

Fish maintenance and 15°C acclimation.

Zebrafish from two sources were utilized for the following experiments. The first were a wild type Cornell strain about 5 to 6 months old, with an average mass of 187 mg. These fish were maintained in a flow through system at an average temperature of 26°C in a 12 hour light-dark cycle. The second were a wild type fish raised in Florida about 6 to 12 months old, with an average mass of 341 mg. These fish were kept in filtered aquaria until use at an average temperature of 27°C on a 12

hour light-dark cycle. All fish were fed ground flakes (Tetra) and brine shrimp daily.

Prior to each experiment, fish were acclimated for either 24 hours or 2 weeks, as indicated in each experiment, to 15°C in reverse osmosis filtered water with 60 mg/L of Instant Ocean (RO-IO) salts added. These fish were moved in static containers, as was done in Chapter 3, achieving 15°C by about 12 hours post move. Water changes occurred daily and all fish were fed granulated fish flakes for the duration of acclimation and experimental periods. Water was tested for pH, ammonia levels, and chlorine every 7 days. Fish were maintained at a density of 1.86 mg/mL for Cornell strain and 3.41 mg/mL for Florida strain in a stagnant system of RO water with 60 mg/L of IO for all acclimation, infection, and experimental periods and all exceptions are noted.

VHSV stock and infection.

Viral hemorrhagic septicemia virus (VHSV) isolate M103 of Genotype IVb was employed through out these experiments. All virus stock was stored at -80°C. Virus was quickly thawed in a warm water bath prior to use in all experiments.

Zebrafish were infected with 10^6 pfu/mL of viral stock or mock infected with an equivalent volume of HMEM-5FPSH media via immersion for 24 hours. Immediately after initial exposure and before fish were sequestered into individual experimental groups, 800 µL water samples were taken and stored at -20°C until future processing.

Evaluation of viral shedding.

For this experiment, 10 Florida fish were mock infected and 30 fish (20 Florida and 10 Cornell) were VHSV infected with 10^6 pfu/mL for 24

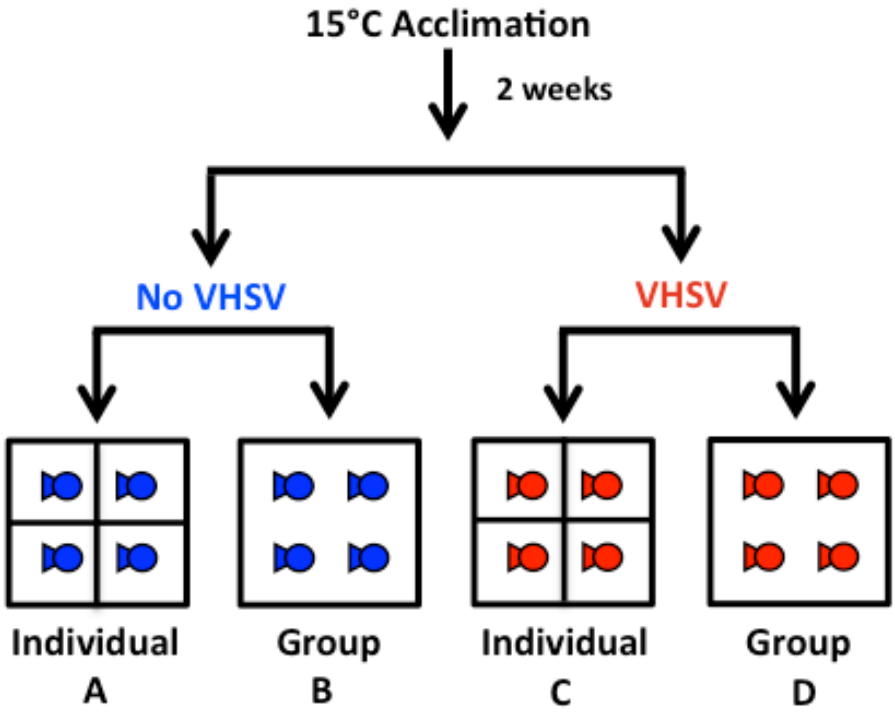

Figure 6.1. Schematic for VHVS shedding protocol. After 2 weeks of acclimation to 15°C, 15 fish were infected and 15 fish mock infected. Following a 24 hour exposure, 5 fish from the mock infected group were kept as a group while the 5 others were separated into individual containers, while 10 infected fish were kept as group and the 10 others were separated into individual containers. This was repeated three times to produce biological triplicates.

hours as described above after a 15°C, and then separated as described in figure 6.1. Fish from each group were either separated into individual containers in 300 mL of RO-IO, 5 fish for the mock infected group and 10 fish for the infected group, at an average density of 1.46 mg/mL, or kept as groups in RO-IO, 5 fish for the mock infected and 10 fish for the infected, at an average density of 1.74 mg/mL for Cornell strain fish and 3.71 mg/mL for Florida stain fish. The fish were observed for external hemorrhaging and mortality for 27 days. Complete water changes were done daily and 800 µl water samples were taken before and after each water change. As fish died,

they were removed from the containers and at 27 days post infection (dpi), survivors were terminated in an overdose of MS 222 (20 mg/mL). All specimens were stored at -20°C upon collection until processed for qRT-PCR or cell culture.

Evaluation of physical contact in VHSV infections.

To evaluate the importance of physical contact in VHSV infections, 36 Florida fish were acclimated to 15°C for 2 weeks. Following this acclimation period, two fish were tagged by clipping the caudal fin prior to infection. Twenty-two fish, including one tagged fish, was mock infected and 2 fish, including the other tagged fish, were VHSV infected with 10^6 pfu/mL for 24 hours. After infection, the fish were combined in the following ways: 1) one clipped mock infected fish with 5 mock infected fish, 2) one clipped VHSV infected fish with 5 mock infected fish, 3) one mock infected fish separated from 5 mock infected fish by a fish barrier, and 4) one VHSV infected fish separated from 5 mock infected fish by a fish barrier (Figure 6.2). The fish barrier consisted of two layers of a 2 mm plastic mesh barrier (Plastic Canvas) installed using hot glue and placed in the same containers used for all other zebrafish experiments. This apparatus was tested with uninfected fish for health and durability prior to using in the experiments.

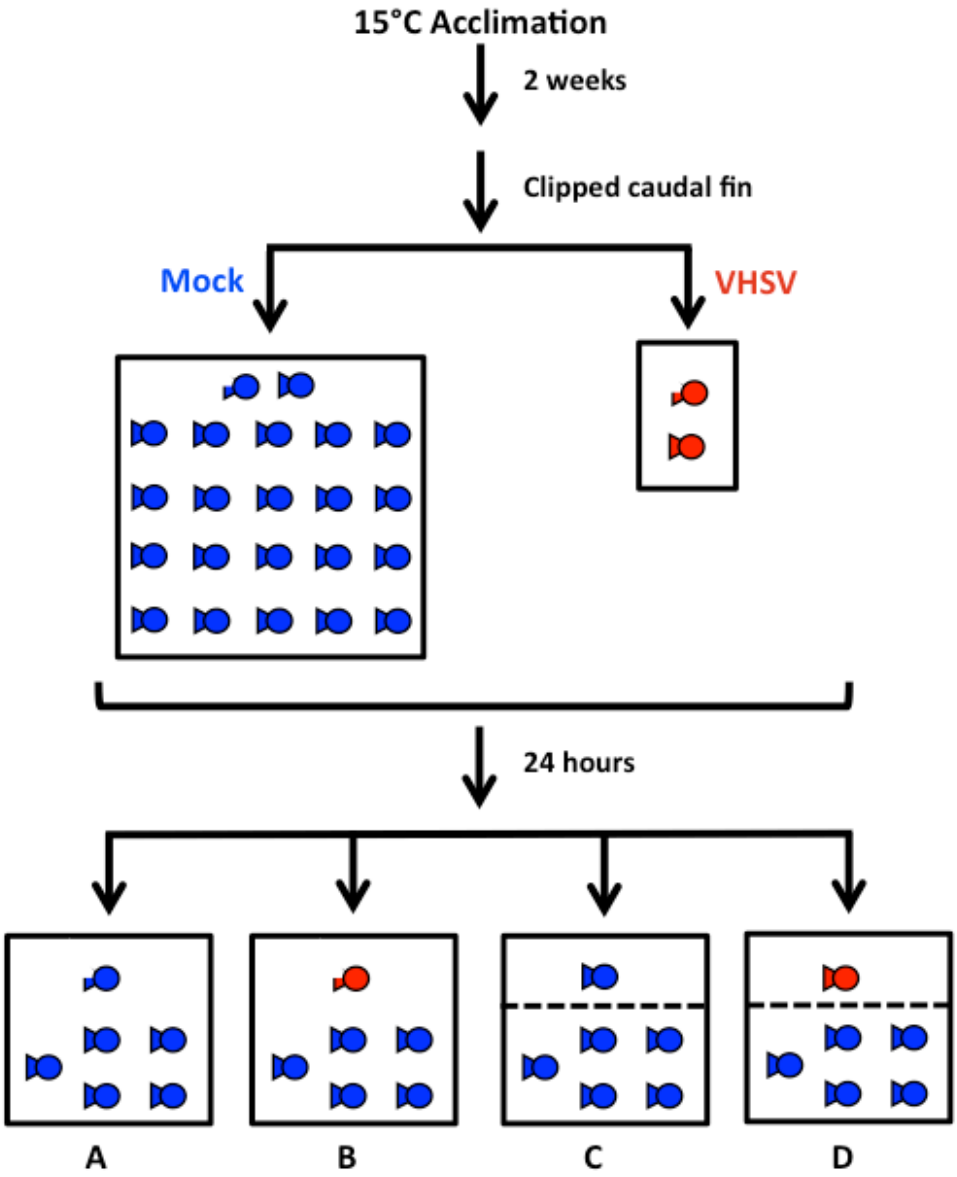

Figure 6.2. Schematic for VHVS co-housing protocol. After 2 weeks of acclimation to 15°C followed by two fish being tagged, 22 fish were mock infected and 2 fish VHSV infected for 24 hours. Following infection, for the physical contact groups, (A) one mock infected tagged fish or (B) one VHSV infected tagged fish were combined with 5 mock infected fish. For the non-physical contact groups, (C) one mock infected or (D) one VHSV infected fish were placed on the opposite side of a semi water permeable barrier from 5 mock infected fish. This was repeated three times to produce biological triplicates.

Following separation into groups, fish were observed for external hemorrhaging and mortality over 27 days. Complete water changes were performed every 3 days and 800 µL water samples were taken daily, both before and after water changes. As fish died, they were removed from the container and at the end of the experiment, all survivors were terminated utilizing an overdose of MS-222 at 20 mg/mL. All water and fish samples were stored at -20°C until processed for qRT-PCR and cell culture. This protocol was repeated 3 times to produce biological triplicates.

Detection of VHSV using cell culture and qRT-PCR.

Fish samples were tested for viral infectivity utilizing epithelioma papillosum cyprini (EPC) cells from American Type Culture Collection (ATCC) following the protocol outlined in Chapter 3 for both maintenance and infectivity testing. Briefly, fish samples were thawed, weighed, and homogenized in a Bio-Spec Mini Bead Beater. The homogenate was centrifuged at 2700xg for 5 min using a Eppendorf Centrifuge model 5430. Liquefied fish homogenate, or supernatant, was then withdrawn and the equivalent volume of 30 mg of tissue was withdrawn from each tube for qRT-PCR. The remainder of the supernatant was diluted to achieve a 1:30 mass (mg) to volume (µL) ratio, syringe filtered, and 250 µL was added to each well of a 48 well plate. Each sample was analyzed in triplicate wells and labeled as passage 0. Inoculated cell monolayers were stored at 15°C and were observed regularly for cytopathic effects (CPE). Upon 100% CPE in 2 of the 3 replicates or after 2 weeks, samples were passaged onto fresh EPC cell monolayers (P1). Cells showing no CPE after 2 weeks at P1 were considered VHSV negative, pending qRT-PCR confirmation. Cells showing CPE at P1 were then passaged onto fresh EPC cells to achieve P2.

Resulting CPE on P2 cells were collected and considered VHSV positive pending qRT-PCR confirmation.

Fish samples were also tested directly by qRT-PCR using the 30 mg volume supernatant described above as outlined in Chapter 2. Briefly, total RNA was prepared using the Qiagen RNeasy kit using the manufacturer's protocol with the following modifications. For tissue homogenates collected during cell culture preparation, 600 µL of lysis buffer was added to the sample followed by one volume of 70% ethanol. The solution was centrifuged through the RNeasy column 3 times to collect all RNA from each sample. For water samples, 150 µL of sample was mixed with 200 µL of lysis buffer and then 1 volume of 70% ethanol was added. Wash step centrifugations were performed for 30s at 18,000xg. Post washing, samples were centrifuged for 1 min at 18,000xg to completely remove all liquid from the column. Following total RNA preparation, for tissue homogenates only, total RNA concentrations were evaluated utilizing a NanoVue spectrophotometer. Samples were diluted to 8.3 ng/µL for analysis on a 384 well plate or 5 ng/µL for analysis on a 96 well plate achieve 50 ng of total RNA per well. Water samples were not diluted due to undetectable levels of RNA and are reported as copies/volume.

To assess VHSV N gene copy number, one step TaqMan qRT-PCR assays were performed as described in Hope et. al. (47) utilizing primers and probes as described. Assays were performed on a 96 well plate on an Applied Biosystes 7500 Fast Real Time PCR System) and on a 384 well plate utilizing a VIIa 7 Real-Time PCR system. reaction comprised of 1 X TaqMan One Step RT-PCR Master Mix, 1 X Multiscribe Reverse Transcriptase, 200nM of the forward primer, the reverse primer,

and the probe. Standards were prepared via serial dilutions of samples of known VHSV RNA copy number. Thermal cycler method was as follows: 30 min at 48°C for reverse transcription, 10 min at 95°C for Taq polymerase activation, and then 42 cycles of 15s at 95°C for denaturation, followed by 1 min at 60°C for annealing and extension. Data was analyzed utilizing a linear regression of standard samples to quantify VHSV N-gene RNA copy number for each sample utilizing manufacturer's software.

Analysis

All VHSV N-gene levels in fish are presented as copies per 50 ng total RNA. For VHSV N-gene levels in water samples, each qRT-PCR value was presented on a per mL of tank water basis.

For the shedding experiment, further data analysis was required to compute the daily shedding rate. Each shedding data point was computed as follows:
(N-gene copies/mL before water change) – (N gene copies/mL after water change the day before).
This simple formula corrected for any carry over of VHSV during a water change and allowed for a daily shedding rate to be derived. VHSV N-gene water level data for the transmission experiment was not processed in this manner.

Results

Investigation of VHSV release into their water environment.

To evaluate viral shedding from VHSV infected fish into their water environment, zebrafish were infected for 24 hours after a 2 week 15°C acclimation and then 100% water change was performed daily. Water samples were collected

before and after 1) infection and 2) each daily water change and evaluated for VHSV N gene levels using qRT-PCR. Fish were either infected and maintained as a group or individualized.

The grouped Cornell strain reached a total percent mortality of 60%, reaching 50% mortality between 14 and 15 dpi (Table 6.1 and Figure 6.3). The average N gene

Table 6.1. Individual fish are more susceptible to VHSV.

		Fish Strain [a]	Percent of fish [b]	Average N gene copies [c]	Percent Positive in Cell culture [b]
Group		**C**			
	Mortalities		**60 (6)**	**6.6 ± 0.1**	**100 (6)**
	Survivors		**40 (4)**	**3.9 ± 0.3**	**100 (4)**
Group		**F**			
	Mortalities		**30 (3)**	**5.5 ± 0.5**	**100 (3)**
	Survivors		**70 (7)**	**1.3 ± 0.4**	**14 (1)**
Individual		**F**			
	Mortalities		**90 (9)**	**5.4 ± 0.5**	**89 (8)**
	Survivors		**10 (1)**	**0.8**	**0 (0)**

[a] Fish strain represents either Cornell (C) or Florida (F).
[b] Number in paraenthesis is the number of fish in that category.
[c] Copies are per 50 ng of total RNA. Values are given as log transformations. Error reflects standard error of the mean.

copies for these mortalities approximated 10^6, whereas the survivors averaged of 10^3. Both mortalities and survivors were positive in cell culture. Interestingly, the grouped Florida line had only 30% total mortality, reaching 50% mortality between 13 and 14 dpi. The average N gene copies were approximately 10^5 for the mortalities and 10^1 for the survivors. Although all of the mortalities were positive in cell culture, only 14% of the survivors were positive. The individualized Florida line fish had a cumulative mortality of 90%, reaching 50% mortality between 10 and 11 dpi. For the mortalities,

the cumulative N gene copies were on the order of 10^5, with 89% positive in cell culture. For the survivor, the N gene copies were below 10^1 and the fish was negative in cell culture.

The progression of VHSV infection was also evaluated by qualitative examination of external hemorrhaging throughout the course of infection as well as upon mortality. The grouped Cornell line fish first exhibited first signs of slight hemorrhaging at 8 dpi. As each fish in this group died, severe external hemorrhaging was noted on the abdomen, tail fin, and dorsal fin. The survivors exhibited no discernible signs of hemorrhaging. The grouped Florida line fish exhibited very slight signs of external hemorrhaging 9 dpi corresponding to the first mortality in that group. Further mortalities were accompanied by only very slight signs of external hemorrhaging. For the individual Florida line fish external hemorrhaging was first noted 11 dpi corresponding to the fifth mortality in that group. Prior mortalities were not accompanied with clinical signs of VHSV infection. Fish after 11 dpi exhibited moderate hemorrhaging upon mortality on the abdomen, dorsal fin, and pelvic fin.

The VHSV levels in each group or with individual fish are shown in Figure 6.3 for each day post infection. The zero time point is a sample taken during infection representing the initial infection to which all of the fish were exposed. For both groups and the individual fish, the average copies during infection were on the order of 10^6 to 10^7, as expected for a target dose of 10^6 pfu/mL. A peak occurs 4 to 5 dpi and no VHSV is detected in the water past the last mortality in all three conditions.

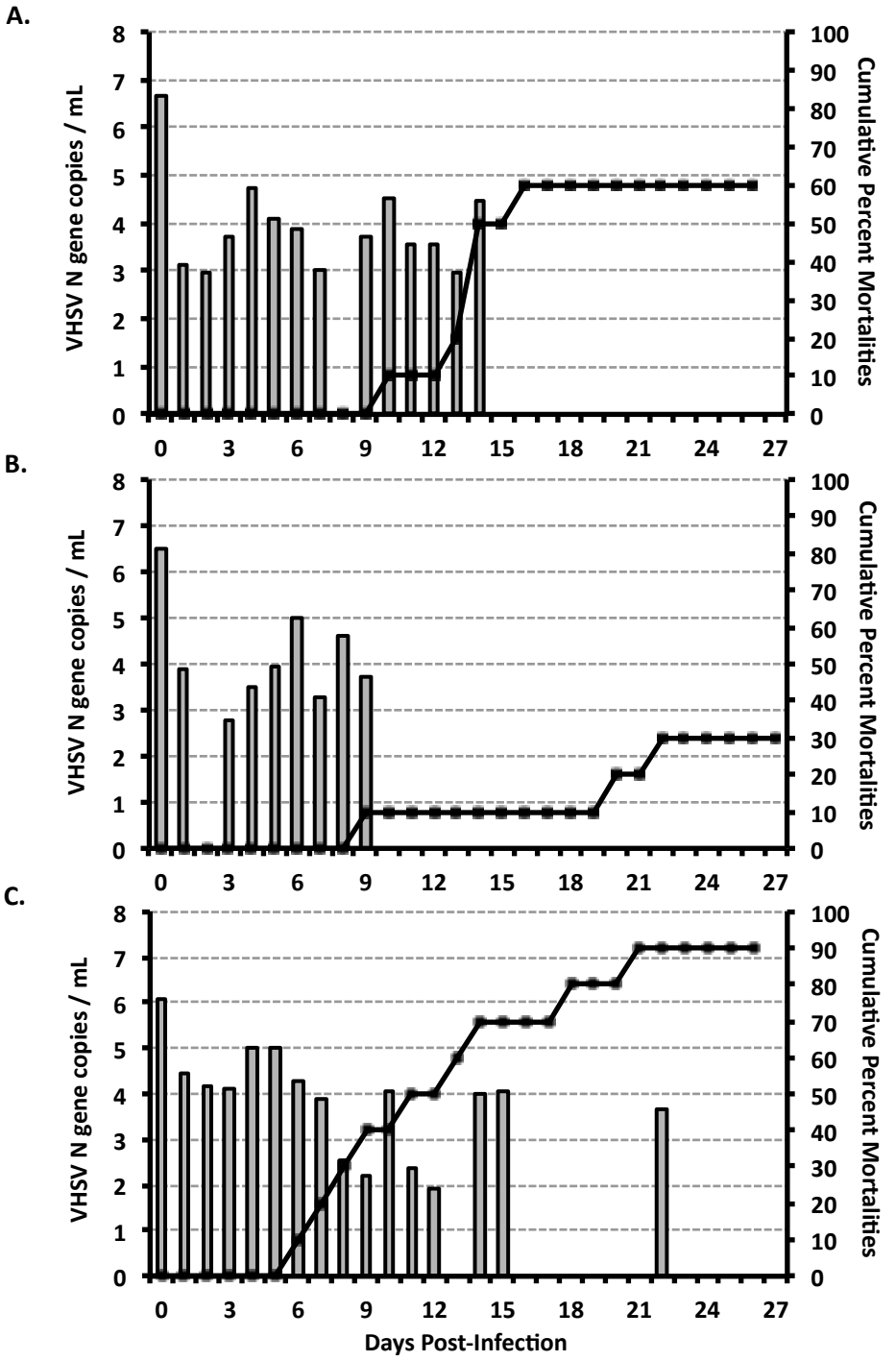

Figure 6.3 VHSV N gene in water is not detected past the final mortality. The cumulative percent mortalities (line graph) are overlaid on the corresponding VHSV N gene in water samples on each day (bar graph) for fish either grouped, the Cornell line (A) or the Florida Strain (B), or individualized using the Florida line (C).

Figure 6.4 shows the individual fish shedding profile for VHSV. Only one fish survived the experiment and VHSV was only detected in the water through 2dpi. The rest of the fish died between 7 and 22 dpi, with VHSV detected through the day of death in 7 of the 9 containers (Figure 6.4, Fish # 1, 2, 3, 5, 6, 7, and 9). Fish # 4 and fish # 8 died 3 and 6 days after VHSV was detected in the water, respectively. Six of the 9 mortalities had a gap in the levels of VHSV prior to death (Figure 6.4, Fish # 1 and 5 through 9).

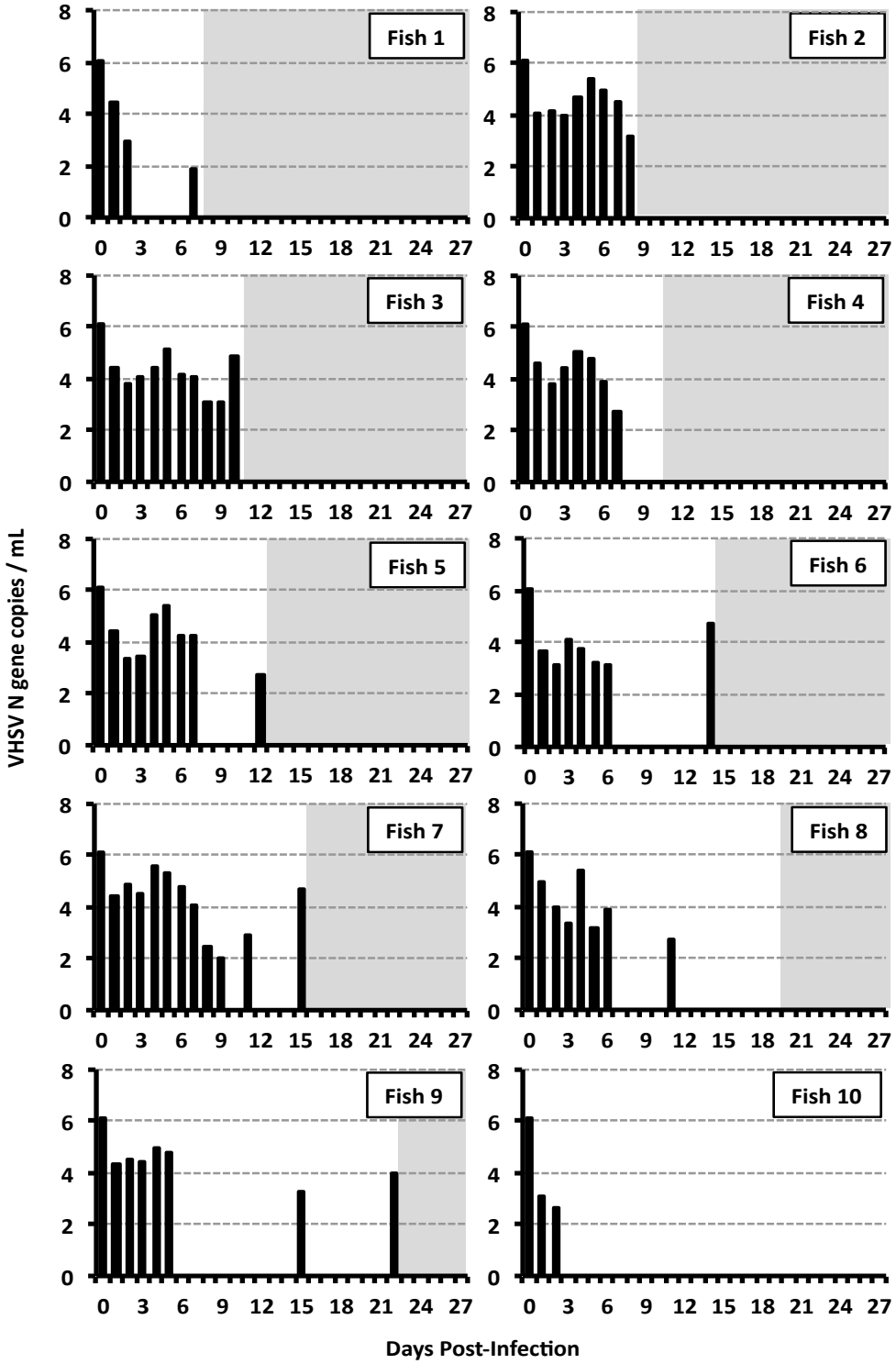

Figure 6.4 VHSV N gene varies depending on the infection status of an individual fish. Levels of VHSV N gene copies in the water environment of each of the 10 individual fish are plotted above as log transformed values. The fish were infected together and thus day 0 is the same for all 10 fish. After the death of each fish, the rest of the time line is greyed out.

Evaluation of physical contact in VHSV infections.

To evaluate the role of physical contact in VHSV susceptibility, zebrafish were infected for 24 hours after a 2 week 15°C acclimation and then one infected fish was placed either in a barrier free tank or in a fish barrier adjacent to 5 mock infected fish, also referred to as target fish. These groups were then observed over a 27 day period for external hemorrhaging and mortality. VHSV N gene levels using qRT-PCR and cell culture detection methods were measured on each fish.

For the group in physical contact, in all three trials, the qRT-PCR levels for each of the infected fish were in the range of 10^4 to 10^7 VHSV N gene copies (Figure 6.5A). In trials A and C, two of the target fish died with high levels of VHSV N gene copies, where one and three other target fish had low levels of VHSV respectively. For the group separated by the water permeable barrier, only the infected fish in trial A had high levels (10^7) of VHSV N gene. In trial A, no mortalities in the target fish were observed but all five fish had measurable low levels of VHSV N gene copies, even though none were cell culture positive. Interestingly, in trial A, although the infected fish was negative for VHSV in both cell culture and qRT-PCR, four of the five target fish had measurable levels of VHSV N gene with no cell culture positives.

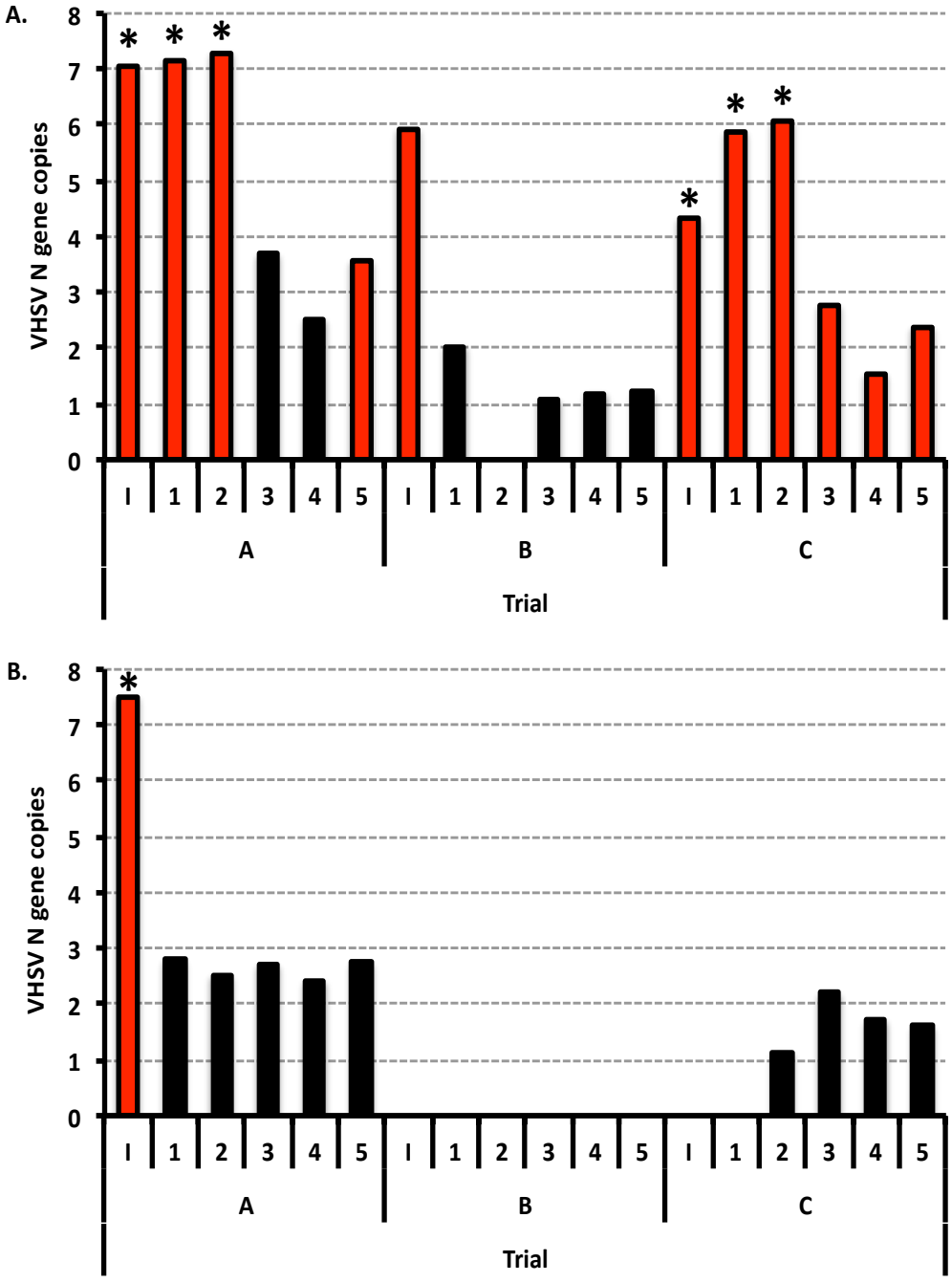

Figure 6.5 Direct contact increases the transmission of VHSV. VHSV N gene copies per 50 ng total RNA are graphed as log transformed values (bars) for fish in direct contact (A) or separated (B). Bar color represents either cell culture positive (red) or negative (black). Each bar represents a different fish for one of 3 trials. The "I" identifies the fish that was initially infected and the numbers 1 to 5 represent the 5 mock infected target fish. * indicates a fish mortality.

Figures 6.6 and 6.7 show the levels of VHSV N gene in the water of the fish in physical contact and fish separated by a barrier respectively. For the group in physical contact, significant levels of VHSV were detected in all three trials. For trial B, where the infected fish died on 8 dpi and none of the target fish died, levels of VHSV drop off dramatically by 9 dpi. For trials A and C, VHSV N gene copies can be detected throughout the course of the experiment, with an observable lack of VHSV N gene for 2 or 3 days prior to the deaths of the target fish. For the fish separated by a barrier, for trials B and C, the infected fish did not die, did not have detectable levels of infectious VHSV, and N gene copies were only detectable through either 1 or 6 dpi, respectively. For trial A, the infected fish had high levels of infectious VHSV, died on 11 dpi, and the target fish all had detectable levels of VHSV N gene, although none of the target fish died, VHSV N gene can be detected in the water through 14 dpi.

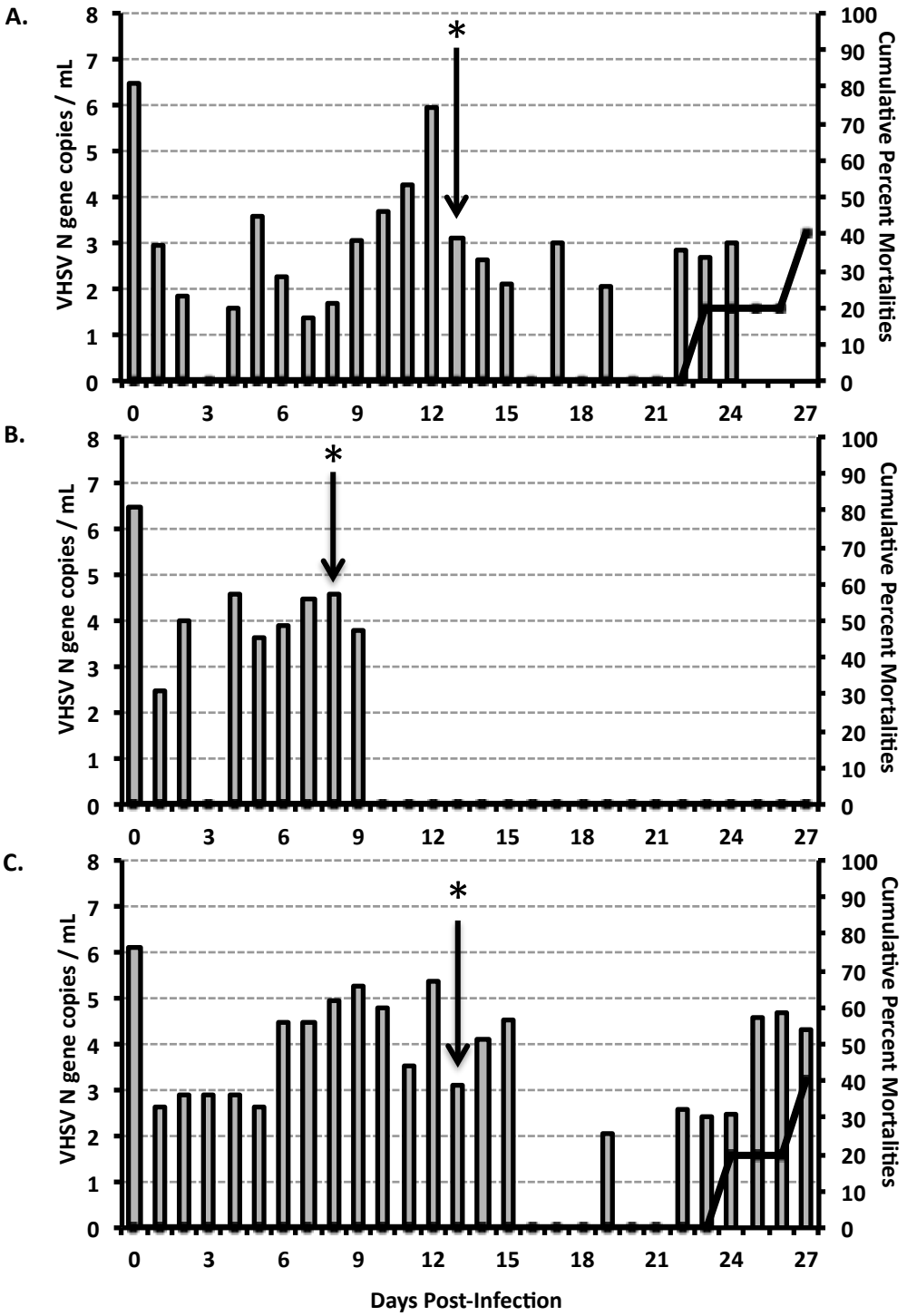

Figure 6.6 Transmission of VHSV in fish with direct contact is dependent on the exposure time to the infected fish. VHSV N gene copies per 50 ng total RNA are graphed as log transformed values (bars) overlaid by the cumulative percent mortality for the mock infected target fish for fish in direct contact for each of three trials (A, B, C). The * indicates the day that the initial infected fish died and was removed from the system.

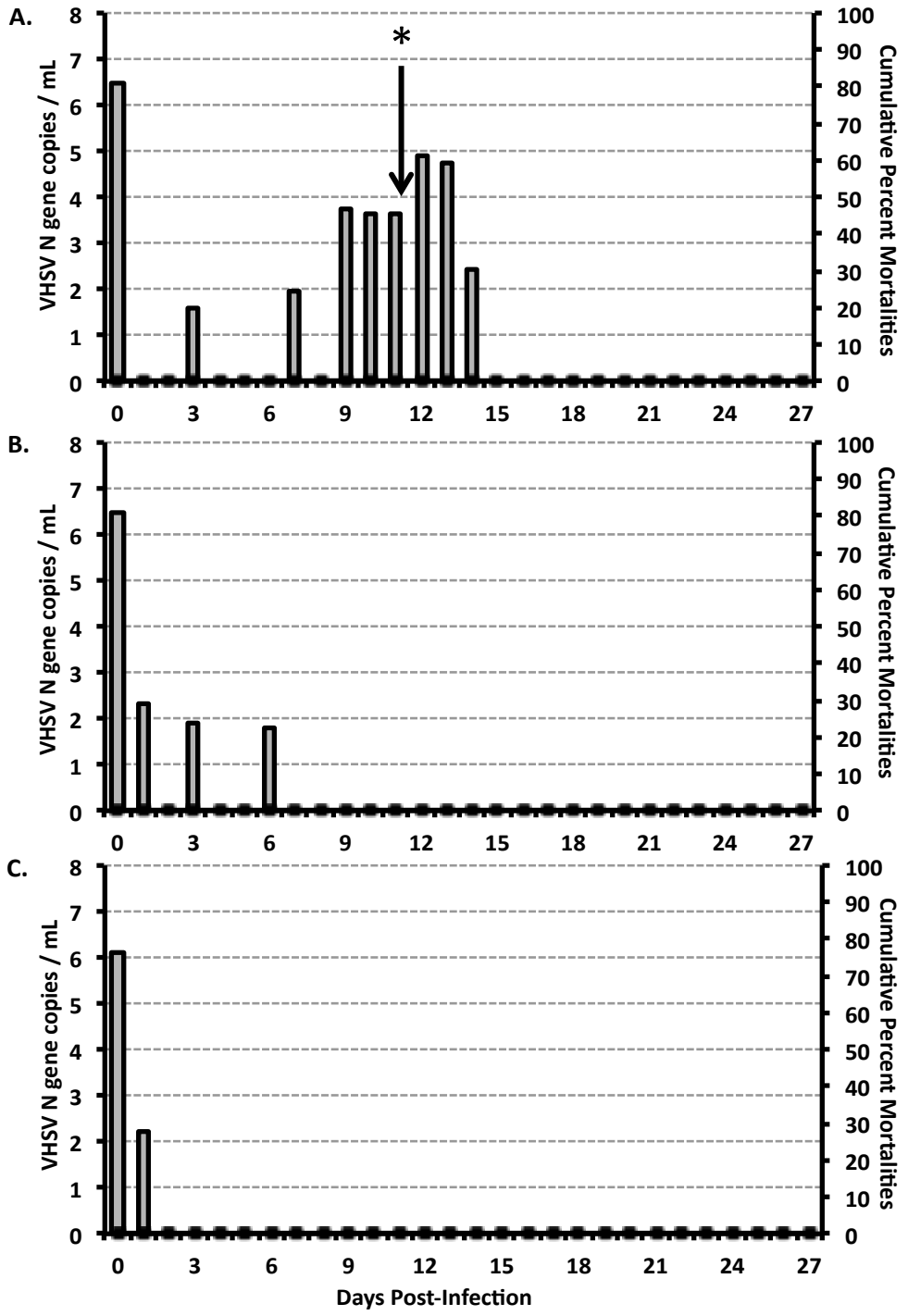

Figure 6.7 Transmission of VHSV is decreased in fish that are separated. VHSV N gene copies per 50 ng total RNA are graphed as log transformed values (bars) overlaid by the cumulative percent mortality for the mock infected target fish for fish in direct contact for each of three trials (A, B, C). The * indicates the day that the initial infected fish died and was removed from the system. No * indicates that the fish did not die during the experimental time period.

Conclusions

Using the zebrafish model for VHSV IVb infection, we evaluated 1) the levels of VHSV N gene in water over the course of infection, 2) the fate of an individually affected fish and the role of secondary infection during the course of infection, 3) and the importance of physical contact in VHSV susceptibility. Based on the water profiles of VHSV N gene, we observed that the release of VSHV into the water environment occurs prior to the fish presenting external hemorrhaging and long before the first mortality (Figure 6.4 and 6.5). The fate of each infected fish differs significantly from fish kept in a group, emphasizing the importance of re-infection of VHSV. Individualized fish died from 7 dpi to 22 dpi with one survivor, a range much larger than the group fish where one fish died on 9 dpi and the rest died from 19 to 22 dpi for the Florida fish. The Cornell strain died from 10 to 16 dpi. Interestingly, the Florida line showed a decreased susceptibility to VHSV, while the individualized Florida fish show an increased susceptibility to VHSV than both the Cornell and Florida groups, indicating the added stress of individualizing these fish. The Cornell group had 60% mortality with VHSV N gene levels on the order of 10^6, while the Florida group had only 30% mortality with VHSV N gene levels on the order of 10^5. The individualized Florida fish, however, had 90% mortality with VHSV N gene levels on the order of 10^5. The decreased susceptibility by the Florida strain compared with the Cornell strain are similar to what has been observed for different strains of some natural VHSV hosts and utilizing these differences might help explain the mechanisms and factors involved in the dramatic species differences to VHSV IVb.

Furthermore, the dramatically decreased susceptibility of the Florida fish when individualized, from 30% to 90% total mortality, may also represents the increased stress that these fish experience when laboratory adapted. Since this trend is not observed when the fish are separated by a barrier, direct contact is not required; however, water contact is a requirement to decrease the individualization stress. To further determine if the stress is a factor evaluation of a stress indicator, like cortisol levels, in the individual fish compared to the group fish could be attempted.

By comparing fish either in direct contact with a single infected fish or separated from an infected fish (via a barrier), the importance of physical contact in VHSV susceptibility was determined. Interestingly, the fish in physical contact were more susceptible to VHSV, showing more severe signs of disease, including mortalities (Figure 6.6, 6.7, and 6.8). Furthermore, high levels of infectious VHSV can be isolated from fins and skin of infected animals alluding to the importance of VHSV on the exterior of the fish (27, 50, 73). However, until this aspect is investigated, the importance of surface virus in transmission remains untested.

In conclusion, we have evaluated the horizontal transmission of VHSV IVb from several different approaches. We have shown that viral release into the water environment occurs prior to the appearance of clinical signs of disease. Additionally, survivors carrying infectious VHSV do not always release VHSV into the environment, indicating a potential difference in how the virus is compartmentalized internally or externally. We have also shown that individualization increases the susceptibility of zebrafish to infection by VHSV and different strains of zebrafish exhibit different responses in susceptibility to VHSV. Lastly, we have shown that

physical contact increases the susceptibility to VHSV. This latter transmission mechanism relies on fish species behavior, which ultimately may impact our understanding of species susceptibility.

CHAPTER 7

FUTURE DIRECTIONS FOR THE ZEBRAFISH INFECTION MODEL FOR VHSV IVB

Kristine M. Hope

- Evaluate the host immune response during the course of infection, with a focus on the different categories of fish, including survivors and mortalities.
- Compare and expand the histology analysis alongside immunohistochemistry during the course of infection time course, with a focus of making a correlation of both viral and disease movement through the host.
- Evaluate the effects of external VHSV on the host susceptibility by testing a dilute bleaching protocol post-mortem to remove external VHSV and compare this to internal VHSV without the necessity of cutting into the fish.
- Evaluate the transmission of VHSV from a post-mortem host to naïve live hosts, both orally and through waterborne exposure.
- Evaluate the different zebrafish lines for differences in VHSV susceptibility by utilizing the sequenced genome of the host.
- Evaluate the effects of VHSV on the exterior of the embryos on post-hatching fish.
- Evaluate the effects of other biological pathogens/chemical toxins on VHSV susceptibility.
- Evaluate long-term immunity to VHSV through re-challenging survivors.
- Evaluate VHSV isolate differences in host susceptibility, focusing on both survivors and mortalities using a dose response model.

- Evaluate the potential of VHSV to adapt to higher replication temperatures using an infection model, while taking advantage of the broad temperature range of zebrafish.

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