The Effect of Temperature Change on Viral Pathogenesis of Viral Hemorrhagic Septicemia Virus (VHSV) in Fathead Minnow (*Pimephales promelas)*

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Rebecca Fellman
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Supervised by Dr. Paul Bowser

Abstract

Viral hemorrhagic septicemia virus (VHSV) prevalence in Great Lakes fish populations varies seasonally, with greater prevalence during spawning seasons. Chronic infections appear to be associated with neural tissue, while acute forms target endothelial tissue. Given the potential immunosurpessive effect of water temperature variation in natural environments, I investigated the effect of a 5ºC temperature change on disease development due to VHSV infection in fathead minnow *Pimephales promelas*. Fish were exposed to VHSV genotype IVb and either a temperature increase from 10ºC to 15ºC, decrease from 20ºC to 15ºC, or a stable temperature of 15ºC. I evaluated prevalence of gross lesions and death, tested fish for VHSV via cell culture of pooled visceral tissue, and performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays on brain tissue and pooled visceral tissue extracts. Fish that experienced temperature decrease had greater VHSV prevalence than those that experienced temperature increase, and more tested positive by qRT-PCR of brain tissue than those that experienced no change, suggesting greater prevalence of infections. Visceral organ samples from fish that experienced either temperature change contained higher viral RNA N-gene copy numbers than those from fish that experienced stable temperature, while brain tissue samples contained similar viral RNA copy numbers among all groups. In fish that contained more viral RNA copies in viscera than brain, those fish that experienced either temperature change had a greater difference between the two quantities than those that experienced no temperature change. The severity of chronic infections was not affected by temperature change, but the severity of acute infections was increased in fish that experienced any temperature change. These results suggest that fish that encounter temperature changes, especially decreases, of 5ºC are at higher risk of contracting chronic VHSV infections and severe acute VHSV infections, helping to explain seasonal fish die-offs attributed to VHSV, especially in fish that encounter temperature change during the spawning season.

Introduction

Viral hemorrhagic septicemia virus (VHSV) is an aquatic rhabdovirus that affects a broad range of teleost fish species. The virus is classified in the order *Mononegavirales*, family *Rhabdoviridae*, and genus *Novirhabdovirus* (Kim and Faisal 2011). The single, negative-stranded RNA genome of VHSV is approximately 11,000 bases long and consists of six open reading frames (Schütze et al. 1999). In order from the 3' to the 5' end of the genome, the open reading frames encode a nucleoprotein, phosphoprotein, matrix protein, glycoprotein, nonstructural viral protein, and a polymerase (Schütze et al. 1999). The nonstructural protein contributes to viral replication and enables the virus to become more pathogenic (Ammayappan et al. 2011). Variations in the glycoprotein and nucleoprotein gene sequences contribute to the differentiation of VHSV isolates and their phylogeny, from which four general lineages emerge (Ammayappan and Vakaria 2009; Elsayed et al. 2006). Geographic tendencies, in addition to genetic variability, distinguish the different isolates of the virus. Viral hemorrhagic septicemia virus genotype IV is most relevant to North America and Japan, while the sub-lineage VHSV genotype IVb pervades the Great Lakes region, where it has been identified in a wide range of host species (Ammayappan and Vakaria 2009).

Viral hemorrhagic septicemia manifests as either an acute or chronic infection, and its ability to cause clinical signs in infected fish is extremely variable. Examples of such signs include pale gills and other signs of anemia, enlarged spleen, abnormal swimming behavior, exophthalmia, and hemorrhage of the skin, liver, kidney, and other internal organs (Kim and Faisal 2011, Ammayappan et al. 2010, Groocock et al. 2007). Though VHS in its acute form has been associated with high mortality and high rates of clinical symptom presentation, chronic, or sub-clinical, infections of VHSV have relatively low mortality and reduced symptom incidence (Hershberger et al. 2010, Oidtmann et al. 2011). In chronically infected fish, the virus has been detected in significantly higher quantities in the brain than in kidney, spleen or other internal tissues (Hershberger et al. 2010). Furthermore, rainbow trout

Oncorhynchus mykiss that have survived VHSV infection have been shown to have a higher viral load in the brain, while fish presenting VHS clinical symptoms have higher viral load in the internal viscera (Oidtmann et al. 2011). This may have implications for tissue selection during VHSV surveillance efforts. Whether the incidence of chronic versus acute infection are the result of differences in species resistance to VHSV, individual immune responses, or other factors has yet to be determined. However, fish with chronic infections of VHSV pose ecological risks as potential reservoirs of the virus, especially given the potential for the natural or human-induced movement of fish among bodies of water in the Great Lakes Basin (Bain et al. 2010).

Viral hemorrhagic septicemia (VHS) was first identified in 1938 as an infectious liver and kidney degenerative disease in Danish rainbow trout, and has since been identified across a wide geographic range, including Europe, eastern and western North America, and Japan (Kim and Faisal 2011, Ammayappan and Vakharia 2009). Viral hemorrhagic septicemia virus genotype IVb was first identified in the Great Lakes region from a large 2005 mortality event of freshwater drum *Aplodinotus grunniens* in the Bay of Quinte in Lake Ontario and in muskellunge *Esox masquinongy* collected in Lake St. Clair (Lumsden et al. 2007, Elsayed et al. 2006). Subsequently, the virus was detected in round gobies *Neogobius melanostomus* from a die-off in the St. Lawrence River, and in surveys and cases from Lakes Huron, Michigan, Erie, Ontario, Superior, and several other bodies of water from the Great Lakes Basin (Groocock et al. 2007, Bain et al. 2010, Thompson et al. 2011, Cornwell et al. 2011). Viral hemorrhagic septicemia virus was also isolated from frozen muskellunge kidney and spleen tissue collected in the northwest region of Lake St. Clair, Michigan, in 2003, indicating the presence of the virus in the Great Lakes region prior to the 2005 mortality event (Elsayed et al. 2006).

Viral hemorrhagic septicemia affects over 80 cool and cold water species of fresh and saltwater fish worldwide (Kim and Faisal 2009, Thompson et al. 2011, Ammayappan and Vakharia 2009). These species include the bluegill *Lepomis macrochirus*, walleye *Sander vitreus*, rainbow trout *Oncorhynchus*

mykiss, emerald shiner *Noropis atherinoides*, yellow perch *Perca flavescens,* and northern pike *Esox lucius,* among numerous others (Kim and Faisal 2009, Thompson et al. 2011). VHSV genotype IVb in particular has been shown to have an especially broad host range among fish species of the Great Lakes Basin (Kim and Faisal 2009). As previously mentioned, VHSV has been implicated in a number of large mortality events in some of these species, though it has not caused die-offs in every species that it has the potential to infect (Lumsden et al. 2007, Groocock et al. 2007, Al-Hussinee et al. 2011). Nonetheless, some of the affected species are of great importance to fish farming, bait fish, and sport fishing industries, and the virus has been associated with economic losses (Kim and Faisal 2011). Due to the threat that VHSV poses to both ecology and economy, the World Organization for Animal Health (OIE) recognizes the virus as a pathogen of interest, and outlines specific protocols to report and prevent the spread of the disease, and to track its already well documented presence worldwide and among the Great Lakes (OIE 2011).

Viral hemorrhagic septicemia virus is stable outside of the host, and remains viable longer in cold environments (Hawley and Garver 2008). The virus primarily infects new hosts via horizontal transmission, by which an infected fish sheds viral particles through the urine into the environment, allowing a new host individual to pick up the virus on its epithelium (Muroga et al. 2004, Kim and Faisal 2011). It is thought that the main sources of viral entry are the skin and gill epithelium (Yamamoto et al. 1992). However, though viral shedding is the most well-established route of infection, leeches and amphipods have also been shown to contain detectable levels of VHSV, suggesting other possible mechanisms of transmission (Faisal and Schulz 2009, Faisal and Winters 2011). Nonetheless, a number of recent experimental studies of VHSV simulate infection through injection or immersion challenges, the latter of which imitates a host's encounter with VHSV in the environment (Goodwin and Merry 2011, Echinas et al. 2010).

A number of techniques enable the detection of VHSV, with variable sensitivity and specificity.

The virus can be cultured on cell lineages, including *Epitheliosum papulosum cyprini* (EPC), fathead minnow *Pimephales promelas* (FHM), and bluegill fry *Lepomis gibbosus* (BF-2), among others (Winton et al. 2010, Olesen and Jørgensen 1992). Round, refractile cells and monolayer destruction characterize the presence of the virus (Jensen 1965). Gross pathology, histopathology, and clinical pathology also can contribute to viral detection (Groocock et al. 2007, Kim and Faisal 2011). However, quantitative reverse transcription quantitative PCR (qRT-PCR) provides one of the most sensitive techniques to detect the virus (Hope et al. 2010, Garver et al. 2011, Ammayappan et al 2011). This method amplifies the genomic negative strand and the mRNA positive strand of the nucleoprotein gene to identify a positive sample (Hope et al. 2010). Though cross reaction can take place between genotypes of VHSV, qRT-PCR is highly specific to only VHSV isolates, and discriminates against other related aquatic pathogens with great accuracy (Hope et al. 2010, Garver et al. 2011). Quantitative reverse transcription quantitative PCR is also approximately 1,000 times more sensitive in detection of the virus as cell culture, making it a highly useful tool in detecting viral loads at lower levels than previously possible (Hope et al. 2010). Furthermore, qRT-PCR can provide a measure of viral load, and the method is particularly useful in detecting positive samples in the absence of clinical signs (Hope et al. 2010). Since its development, qRT-PCR has been successfully used to perform surveys of VHSV genotype IVb in the Great Lakes region, in addition to its use in experimental settings (Cornwell et al. 2011).

Various environmental and demographic factors can affect the susceptibility of an individual or population of fish to VHSV. One such factor is the age of the fish. In some studies, young fish appear to have a much higher incidence of the virus, VHS symptoms, and mortality than their seniors (Eckerlin et al. 2011, Kim and Faisal 2011). However, there is no definite consensus on the relationship between age and susceptibility, as other studies have demonstrated older fish to have a greater probability of infection in wild yellow perch (Cornwell et al. 2012). Furthermore, biological stresses like spawning

also increase susceptibility to the disease. Smallmouth bass *Micropterus dolomieu*, for example, have a significantly higher prevalence of VHSV during the spring spawning season than other times throughout the year (Eckerlin et al. 2011). Water temperature also influences VHSV incidence and viability. An optimal temperature range for VHS incidence occurs between 9-12ºC, while viral viability decreases in temperatures greater than 18-20ºC (Kim and Faisal 2011, Goodwin and Merry 2011). On cell culture lines, viral replication is optimal at 14-15ºC (Kim and Faisal 2011).

In natural environments, fish may encounter temperature changes seasonally or as a result of differences within a body of water (Wells and Parker 2010, Finlay et al. 2001, Buchtíková et al. 2010). In addition to temperature variations due to depth, water temperatures fluctuate locally. In Lake Huron, for example, variations in thermocline position produces local temperature variations with rates of 5-10ºC per hour (Wells and Parker, 2010). Furthermore, littoral regions of Lake Opeongo in Ontario, Canada, demonstrated a mean variability of 3-4ºC with changes measured up to 7-15ºC at the same sites over the course of a few days (Finlay et al. 2001). These sites are used by the VHSV susceptible smallmouth bass *Micropterus dolomieu* during spawning in the spring, so the fish may encounter temperature change on this scale (Finlay et al. 2001). Being ectothermic, fish are prone to physiological changes, particularly in immune function, as a result of external temperature changes. Though each fish species will have an preferred temperature range, colder water temperature in general increases susceptibility to disease and suppresses immunity (Martin et al. 2011, Goodwin and Merry 2011, Xu et al. 2010). In a study of disease caused by *Ichthyopthirius multifiliis* in channel catfish, *Ictalurus punctatus*, fish held or cycled at lower than optimal temperatures showed higher mortality and a reduced the ability to form protective antibodies (Martin et al. 2011). Additionally, VHSV-infected bluegill *Lepomis macrochirus* are able to clear the virus at temperatures above 18ºC, while those at lower temperatures experienced higher mortality (Goodwin and Merry 2011). Fish can also experience fluctuations in immune responses seasonally, and some species are more likely to be immunosurpressed

in the spring (Buchtíková et al. 2010).

The well-documented effect of temperature on the fish immune system, the implication of VHSV in seasonal mortality events, and the differences between acute and chronic infections raise important questions about the role of water temperature on the course of VHSV infection. Through this experiment, I sought to explore the effect of temperature change on the incidence of chronic and acute infections of VHSV genotype IVb among fathead minnows *Pimephales promelas*. I evaluated the effects of a 5ºC temperature increase and 5ºC decrease surrounding a central temperature of 15ºC, based on the optimal temperature for VHSV replication in cell culture and the plausibility of such a change in the Great Lakes region (Kim and Faisal 2011, Wells and Parker 2010). I also evaluated brain tissue as a site of viral localization in chronically infected fish, and examined potential differences in the incidence of chronic infection based on temperature change. This experiment is part of an ongoing, larger effort currently being undertaken by the Aquatic Animal Health Program at the College of Veterinary Medicine of Cornell University (Ithaca, NY) to explore the pathogenesis of VHSV and the role of temperature change on the incidence of this virus.

Based on the increase in disease susceptibility that accompanies temperature stress, I hypothesized that a temperature change would result in a greater incidence of VHSV than temperature stability. More specifically, a decrease in temperature will likely produce a greater incidence of VHSV based on the immunosuppressive effect of temperature decrease (Martin et al. 2011, Goodwin and Merry 2011, Xu et al. 2010). This difference will manifest as a greater number of fish with clinical signs of VHS, greater mortality, higher VHSV prevalence, and infected fish with a higher viral load in those individuals exposed to a temperature decrease compared to those from other treatments. I expect these infection trends with relation to temperature change will exist in both tests of brain and visceral tissue. Furthermore, I expect that more infected fish will have a greater viral load in brain tissue than in pooled visceral tissue as chronic infections develop (Hershberger et al. 2010, Oidtmann et al. 2011).

Materials and Methods

Experimental structure

The subjects of this study consisted of 356 fathead minnows *Pimephales promelas* from Anderson Minnow Farms (Lonoke, Arkansas). These individuals were certified by the supplier to be free of common aquatic animal diseases, including viral hemorrhagic septicemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), spring viremia of carp virus (SVCV), *Aeromones salmonicidia, Yersinia ruckeri,* and Heterosporosis (Anderson Minnow Farms, Lonoke, Arkansas, 2011). Furthermore, complete necropsies were performed on a random sample of 10 fish upon their arrival at the Aquatic Animal Health Program at Cornell University (Ithaca, New York), to further testify to the subject's initial health. Fathead minnow were selected as the subject species due to their manageability in the laboratory setting and their susceptibility to VHSV infection (Al-Hussinee et al. 2010). The subjects were sorted randomly into 12 tanks of 30 (+/-2) fish. Of those 12 tanks, 6 tanks were designated as control tanks and 6 as treatment tanks. Within the control and treatment groups, two of the six tanks were assigned to one of three temperature shift groups. These temperature shift groups consisted of 10ºC to 15ºC and 20ºC to 15ºC, with a control group that remained at 15ºC (Fig. 1).

For two weeks, the water temperature of the respective tanks was maintained at a base temperature of 10ºC, 15ºC, or 20ºC. At the end of this acclimation period, the treatment groups of fish were exposed to 9 mL of Minimal Essential Medium with Hank's salts (Gibco, Invitrogen, Grand Island, New York) prepared with 10% fetal bovine serum (Gibco, Invitrogen), penicillin (200 IU/mL), streptomycin (200 µg/mL), glutamine (0.584 mg/mL) (Gibco, Invitrogen) and HEPES buffer (Gibco, Invitrogen), referred to hereafter as HMEM-10, containing 10^5 pfu/mL of VHSV IVb (isolate MI 03). The exposure was carried out by immersion in 9 L of water. Control groups were exposed to 9 mL of HMEM-10 in 9 L of water. The viral exposure lasted for 24 hours (Muroga et al. 2004). After this time, the fish were transferred to new static 9 L tanks (Sterilite Corporation, Townsend, Massachusetts) and

all tanks were held at a water temperature of 15ºC for the duration of the six week experiment. The treatment and control tanks were housed in separate rooms in the facilities of the Aquatic Animal Health Program in the Veterinary Medical Center of Cornell University. Each room contained three Living Streams (Frigid Units, Inc., Toledo, Ohio) of water acting as a 15ºC water bath to two static tanks and their alternates. All static tanks had their water changed twice each week. Each tank had a corresponding alternate that was filled the day before the fish were moved into it and left overnight in the Living Stream to ensure a temperature of 15ºC. The now vacant tank would then be rinsed with water and iodine solution (Providone-Iodine 7.5% Scrub; Butler Schein Animal Health, Dublin Ohio; contact time ≥ 60 seconds), wiped, and left to dry until its reuse. Subjects were fed approximately 0.5 g of Rangen Starter for Cultured Fish feed (Rangen Inc, Buhl, Idaho) per tank once daily and were checked twice daily, with control groups always being interacted with prior to treatment groups. During each check, the water temperature was read from a thermometer (Fisher Scientific, Pittsburg, Pennsylvania) in each of the living streams, and each tank was checked for deceased fish and any abnormal swimming behavior or obvious signs of disease.

Sampling

Day 0 of the experiment was designated as the day after the fish's 24 hour exposure to VHSV. Sampling occurred on days 0, 7, 14, 21, 28, and 35. On each sampling day, a water sample was taken from each tank. This was performed by dipping a 5 mL polypropylene round-bottom tube (Becton Dickinson, Franklin Lakes, New Jersey) into the static tank and filling it with at least 3mL of water. Sampling days were also days on which the water was changed, and the water sample was taken prior to the cleaning of the static tanks, so the fish had been living and potentially shedding viral particles in the sampled water for at least 3 days prior to water sampling.

Six fish- three per tank- were randomly selected from each temperature shift group from control and treatment tanks. The fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate,

Western Chemical, Ferndale, Washington) and immediately processed. The fish were weighed and measured longitudinally. Fish were then evaluated for any external abnormalities, such as hemorrhage and exophthalmia. Next, the fish were dissected and checked for internal abnormality and disease symptoms, including hemorrhage, abnormal organ appearance, and ascites. The viscera- including the anterior and posterior kidney, gastrointestinal tract, heart, liver, and spleen- were removed and pooled into a single sample in a 2-mL homogenizing tube (Biospec Products, Barletsville, Oklahoma) that also contained one 1.3mm chrome steel bead (Biospec). This tube was weighed before and after the addition of the sample. The brain was dissected out using a separate set of dissecting equipment, which was sterilized between individuals using a 20% household bleach solution (3-6% sodium hypochlorite; Chlorox Co., Oakland, California; final solution ~ 10,000 mg of active chlorine/L; contact time ≥ 60 seconds). The brain sample was placed in a 2-mL homogenizing tube (Biospec Products) containing 200 µL RNAlater (Ambion, Applied Biosystems, Inc. [ABI], Carlsbad, California). All equipment was disinfected using the same household bleach solution and dried with a clean paper towel between the processing of each fish. In the event of a moribund or dead fish, the fish was treated in the same manner as those processed on regular sampling days. If the fish could not be processed immediately, it was placed in a small, lidded plastic container with a moist paper towel and was processed no more than 30 hours following its discovery. On day 35 all remaining subjects were processed.

Viral Isolation in Cell Culture

Prior to sampling, new monolayer cultures of *epithelioma papulosum cyprini* (EPC) cells were prepared as described by Groocock et al. (2007). This cell line is known to be VHSV susceptible (Lorenzen et al. 1999). Though thought to have originated from the common carp *Cyprinus carpio,* it has been shown that the cell line has been contaminated with cells of the subject species, the fathead minnow (Winton et al. 2010). One day before sampling, cells were seeded at $5x10^5$ cells/mL with 250 µL/well in 48 well cell culture plates (Corning Inc., Corning, New York). These plates were then

incubated at room temperature until the next day. Based on the weight of the dissected pooled viscera samples, a volume of HMEM-10 (Gibco, Invitrogen), was added to the 2 mL homogenizing tube to produce a 1/10 dilution (weight/volume) of the sample. Brain samples were not used for cell culture. The viscera samples were homogenized for 60 s in a Minibead Beater (Biospec). The homogenized samples were then centrifuged at 8,000 x gravity (g) for 1.5 min to separate cell particles, debris, and the bead from the remaining components of the homogenate. Supernatant (500µL) was removed from the homogenizing tube and added to 2 mL of HMEM-5 (Gibco, Invitrogen) cell media in a 5 mL tube (Becton Dickinson) to generate a 1/100 dilution. The dilution was then drawn up into a 3 mL syringe (Becton Dickinson, Rutherford, New Jersey) and filtered with an Acrodisc 25mm Syringe Filter with 0.45 µm Supor Membrane (Pall Corporation, Ann Arbor, Michigan). The resulting filtered homogenate dilution was placed in a 1.7 mL microcentrifuge tube (VWR International, Radnor, Pennsylvania). 250 µL of this filtered dilution was then added to a second 1.7 mL microcentrifuge tube (VWR International) containing 1 mL of HMEM-5 (Gibco, Invitrogen) to produce a 1/500 dilution. Water samples were also drawn up into a 3 mL syringe (Becton Dickinson) and filtered with the same filters into 1.7 mL microcentrifuge tube (VWR International).

Cells were inoculated with 250 µL of each dilution of each filtered sample in triplicate and 250 µL of filtered water samples in triplicate. Cell plates were incubated at 15ºC until CPE was observed or for a maximum of 14 days. At least once every 4 days, the cells were checked for cytopathic effects (CPE) and cytotoxicity, as well as for fungal contamination and other abnormalities. After inoculation, if the cells began to exhibit signs of CPE or cytotoxicity, the cells and media were removed from the plate and the triplicates of each dilution were consolidated into two 2 mL microcentrifuge tubes (VWR International). These were centrifuged at 8,000 x g for 1.5 minutes and 250 µL of the supernatant was redistributed in triplicate onto a fresh plate, prepared as previously described. Apparently negative

samples were observed for two weeks, then replated in the same fashion as those with observed CPE or cytotoxicity. If the cells were observed to have contracted fungal contamination, or other abnormalities, they were filtered with an Acrodisc 25mm Syringe Filter with 0.45 µm Supor Membrane (Pall Corporation) before replating. Each replating constituted one passage of the sample. After two weeks, if the second passage also failed to demonstrate CPE or cytotoxicity, the sample was ended. Only samples that were positive for CPE after 2 or more passages were considered positive for VHSV viral isolation through cell culture. If CPE was observed, the supernatant from the 1:00 and 1:500 dilutions of samples were saved in 1.7 mL microcentrifuge tubes (VWR International) and stored in a -80ºC freezer.

Quantitative reverse transcription PCR

RNA extraction was carried out via a MagMax magnetic bead extraction system and the MagMax-96 viral RNA isolation kit (Life Technologies, Grand Island, New York) as per the protocols described by the manufacturer and the extraction program AM1836_DW_50_V2. Each well of the deep well sample plate contained 130 µL of prepared lysis binding solution, 20 µL of a 1:1 solution of magnetic beads and binding enhancer, and 50 µL of homogenized sample of visceral organs or brain samples, prepared as described above for cell culture. Two deep well plates containing 150 µL of Wash Solution 1 per well and two deep well plates containing 150 µL of Wash Solution 2 per well were prepared. Elution was performed in 75 µL of elution buffer. The extraction program was optimized for viral RNA extraction from large volumes and involves agitated lysis binding for 10 min, 3 min 30 sec in each Wash 1 plate, 2 min 40 sec in each Wash 2 plate, 1 min of drying, 3 min of heated elution, and 1 min of bead collection. Eluted RNA was placed in a sterile, nucleic acid free 1.7 mL microcentrifuge tube (VWR International) and frozen immediately following extraction at -80ºC until qRT-PCR testing.

Quantitative reverse transcription PCR was carried out as described by Hope et al. (2010). This technique recognizes the presence and quantifies the amount of VHSV present in a sample based on RNA gene copies of the viral nucleoprotein (N). Each 96-well PCR microplate (Axygen, Union City, California) was loaded with five standards with N gene copies of 10^2, 10^3, 10^4, 10^5, and 10^6 VHSV N gene copies/mL. Each plate also contained a negative control consisting of sterile water. The remainder of the plate was loaded with extracted sample RNA. Each well was loaded in duplicate with 5 µL of the sample, standard, or control. The assay was carried out by an ABI PRISM Model 7500 sequence detector (Applied Biosystems, Inc.), with settings based on the manufacturer's instruction and the methods described by Hope et al. (2010). A NanoVue spectrophotometer (GE Healthcare, Piscataway, New Jersey) was used to evaluate the quantity and quality of each extracted RNA sample. Based on linear regression derived from qRT-PCR standard results and a normalization of RNA concentrations, the quantity of N gene copies, and thus viral load, was determined in all positive samples.

Evaluation of Data

The determination of a positive sample was evaluated independently in each of the three tests-cell culture, viscera qRT-PCR, and brain qRT-PCR. In cell culture, a positive sample was considered as any sample that demonstrated CPE in all three replicates of either or both of the two dilutions beyond the second passage of the sample. In qRT-PCR of viscera or brain samples, only those samples that tested positive in both duplications of the sample were considered positive. Furthermore, a sample was only considered a true positive if the pattern and slope of signal amplification and detection by the ABI PRISM Model 7500 sequence detector (Applied Biosystems, Inc.) was consistent with that of RNA amplification as described by Hope et al. 2010. Statistical analysis of the data was performed in R version 2.13.1 (R Development Core Team 2011). A Fisher's exact test was used to evaluate differences in incidence of clinical signs and abnormalities through pairwise comparisons of proportions of affected fish in each of the six groups ($\alpha = 0.05$). The incidence of death was also evaluated through a

Fisher's exact test in R, as were the differences in proportions of positive fish over time and the relative distribution of positive results among the three diagnostic tests between the three temperature treatment groups ($\alpha = 0.05$). The mean quantities of VHSV viral RNA detected in brain and visceral tissue extracts by qRT-PCR were analyzed via ANOVA tests in R ($\alpha = 0.05$). This technique was also applied to analysis of the difference in viral RNA quantity between visceral and brain tissues, and to viral RNA quantity and sample viral RNA differences over time. Pairwise relationships in ANOVA tests were further illustrated by TukeyHSD operations in R. Specificity, sensitivity, positive and negative predictive values, and diagnostic accuracy were determined via a Diagnostic or Screening Test in OpenEpi version 2.3.1 (Dean et al. 2011).

Over the course of this experiment, it was found to be necessary to retest certain samples, both in cell culture and qRT-PCR. Cell culture was repeated in samples that demonstrated cytotoxicity or were inconclusive for more than four passages. The cell culture was performed with the same methodology as previously described. In qRT-PCR of brain and viscera samples, any sample that tested positive for only one of the two duplicates was repeated through qRT-PCR, as was the single control sample, brain sample number CC-41B, that tested positive through qRT-PCR.

Fifty-three samples initially tested positive in cell culture, but tested negative in viscera qRT-PCR. For these, the saved cell culture fluids were put through RNA extraction and qRT-PCR in duplicate as previously described. For those that tested positive, confirming the cell culture result, the original sample was thawed and put through RNA extraction, qRT-PCR, and cell culture for a second time, all as previously described.

Results

Water Samples

Water samples from all six VHSV exposed treatment tanks tested positive for VHSV by qRT-

PCR and cell culture on Day 0 of the experiment. Quantities of viral RNA in the six samples ranged from 9.32 x 10^5 to 1.53 x 10^6 viral N gene copies/50 ng RNA. The mean of the viral RNA quantities of experimental samples from Day 0 was 1.19 x 10^6 viral N gene copies/50 ng RNA (SD = 2.7 x 10^5 viral N gene copies/50 ng RNA). The only other water sample to test positive for VHSV was taken on Day 7 of the experiment, and came from an experimental tank that experienced a temperature decrease. This sample tested positive in both cell culture and qRT-PCR and contained a viral RNA quantity of 3.07 x 10^3 viral N gene copies/50 ng RNA. All other water samples from experimental and control groups tested negative for VHSV in cell culture and qRT-PCR.

Four of the water samples, all from experimental tanks taken on Day 21 of the experiment, initially tested positive by cell culture but not by qRT-PCR. This may have been due to contamination of that particular cell culture plate, but all either tested negative upon qRT-PCR testing of cell culture fluids, or tested negative when reevaluated in cell culture and qRT-PCR of the initial water sample, as described in Materials and Methods.

Incidence of Clinical Abnormality and Death

Gross examination of all 356 fish showed a broad range of internal and external abnormalities, in addition to gross lesions associated with VHS (Table 1). Among the most common conditions were the presence of internal parasites (n=95), internal and external hemorrhagic lesions (n=21), spleen abnormalities (n=14), liver abnormalities (n=7), and the presence of ascites (n=4) (1 incidence consisted of an abnormality occurring in a single anatomical structure or region within a fish, as quantified in Table 1).

Because each fish could potentially present multiple incidences of abnormality, the distribution of clinical signs was not directly used as a point of comparison between temperature treatments. Instead, I compared groups based on the proportion of individual fish that presented abnormalities (Table 2). The presence of internal parasites in adult or cystic forms was not considered to be directly

related to VHS incidence, and fish whose only abnormalities were parasites were not included in analysis. Among experimental groups, clinical signs of VHSV infection occurred with greater incidence than in control groups, with 33 (18.8%) VHSV exposed fish experiences abnormalities, compared to 6 (3.3%) unexposed fish. Between experimental groups, abnormality occurred in 13 (22.0%) fish that experienced a 5ºC increase, 8 (13.8%) fish that experienced no temperature change, and 12 (20.3%) of fish that experienced a 5ºC decrease. Among control groups, abnormalities occurred in 0 (0.0%) fish that experienced a 5ºC increase, 1 (1.7%) fish that experienced no temperature change, and 5 (8.3%) of fish that experienced a 5ºC decrease (Table 2). The frequency of abnormalities was significantly different between experimental and control fish ($p < 0.001$). However, there were no significant differences in the frequency of abnormalities between temperature change groups among VHSV exposed fish, nor were there significant differences between temperature change groups among control fish ($p > 0.05$) (Table 2). Abnormality frequency between experimental fish that experienced no temperature change was not significantly different than that of control fish that experienced a 5ºC temperature decrease ($p = 0.071$). This was the only case of a experimental group being statistically similar to a control group. Otherwise, all experimental groups were exclusively statistically similar to one another, and likewise for all control groups ($p > 0.05$).

Over the course of the experiment, two control fish and five VHSV exposed fish died. Both control fish died on Day 7 of the experiment and tested negative for VHSV by cell culture and qRT-PCR of both brain and visceral tissue. One experimental fish from the temperature increase group died on Day 35 of the experiment, and tested positive for VHSV by cell culture and qRT-PCR of brain and visceral tissue. Brain tissue was found to contain more viral RNA at 5.81×10^9 viral N gene copies/50 ng RNA, while viscera tissue contained 1.14×10^6 viral N gene copies/50 ng RNA. One experimental fish from the stable temperature group died on Day 7 of the experiment and tested positive for VHSV only in cell culture. The cell culture fluids tested positive for VHSV by qRT-PCR, and visceral tissue

samples did not test positive upon repeat qRT-PCR testing. Three fish from the temperature decrease group died, one on Day 10 and two on Day 32. The fish from Day 10 tested positive for VHSV in cell culture and in qRT-PCR of brain tissue, but not in qRT-PCR of visceral tissue. In the brain, the viral RNA quantity was found to be 4.84×10^9 viral N gene copies/50 ng RNA. Again, cell culture fluids tested positive by qRT-PCR, but visceral tissue retested as negative by the same method. Of the two fish that died on Day 32, one tested positive in qRT-PCR of brain and visceral tissue, but negative in cell culture, while the other tested positive in all three methods. Respectively, the visceral samples contained 9.74×10^6 viral N gene copies/50 ng RNA and 4.42×10^8 viral N gene copies/50 ng RNA, while the brain tissue contained 4.30×10^8 viral N gene copies/50 ng RNA and 3.00×10^8 viral N gene copies/50 ng RNA. A Fisher's exact test comparing the temperature decrease group, with the highest incidence of death (5.1%), and the stable and temperature decrease control groups, with the lowest incidence of death (0.0%), did not reveal any significant difference in death prevalence between them ($\alpha = 0.05$, $p = 0.119$). Therefore, incidence of death among all groups was not significantly different.

Viral Hemorrhagic Septicemia Virus (VHSV) Prevalence and Quantity as Related to Temperature Change and Time

In total, 59 out of 176 VHSV exposed fish tested positive for VHSV in one or more of the three diagnostic tests. No control fish tested positive for VHSV. Of those VHSV exposed fish that experienced a temperature increase, 14 of 59 fish tested positive, while 26 of 58 that experienced a temperature decrease tested positive. Of the VHSV exposed fish that experienced no temperature change, 19 of 58 tested positive (Fig. 2). Fish that experienced a temperature decrease had a significantly higher incidence of VHSV than fish that experienced a temperature increase ($\alpha=0.05$, $p = 0.032$). Neither temperature change group showed a significant difference in proportion of positive fish from the stable temperature group ($p > 0.05$). The proportions of fish that tested positive for VHSV were separated by the week at which the sample was taken (Fig. 3). There were no significant

differences in the proportion of positive fish within each temperature group over time ($p > 0.05$).

In total, eight fish tested positive in cell culture of visceral tissue extracts but not in qRT-PCR of visceral tissue. One of these was from the temperature increase group, five from the stable temperature group, and two from the temperature decrease group. The diagnostic tests were found to have a sensitivity of 77.1%, specificity of 98.75%, positive predictive value of 87.1%, negative predictive value of 97.5%, and diagnostic accuracy of 96.63% (Table 4).

Of the fish that tested positive for VHSV in one or more of the three diagnostic tests, 35 of 59 tested positive by cell culture, 31 tested positive by qRT-PCR of viscera, and 42 tested positive by qRT-PCR of brain. In the temperature increase group, 10 of 14 tested positive by cell culture, 9 by qRT-PCR of viscera, and 12 by qRT-PCR of brain. In the stable temperature group, 11 of 19, 8 of 19, and 9 of 19 fish tested positive, and in the temperature decrease group, 14 of 26, 14 of 26, and 21of 26 fish tested positive by cell culture and qRT-PCR of pooled visceral tissue and brain tissue respectively (Fig. 4). There was no significant difference in proportion of fish in each temperature group that tested positive for VHSV by cell culture or qRT-PCR of visceral tissue extracts ($p > 0.05$). However, significantly more fish from the temperature decrease group tested positive by qRT-PCR of brain tissue than those from the stable temperature group (p-value = 0.019).

The mean quantity of VHSV viral RNA detected in visceral tissue of fish exposed to VHSV was 1.30×10^8 viral N gene copies/50 ng RNA. In those that experienced a temperature increase, the mean viral RNA quantity was 1.59×10^8 ($\log_{10}$ transformed: mean = 16.33; SD = 3.31) viral N gene copies/50 ng RNA. In fish that experienced a temperature decrease, this value was 1.83×10^8 ($\log_{10}$ transformed: mean = 14.64; SD = 3.15) viral N gene copies/50 ng RNA, while fish that experienced no temperature change had a mean value of 5.42×10^6 ($\log_{10}$ transformed: mean = 11.12; SD = 4.42) viral N gene copies/50 ng RNA (Fig. 5). The mean VHSV viral RNA quantities of the three temperature change groups (10ºC to 15ºC, 15ºC to 15ºC, and 20ºC to 15ºC) were significantly different from one another

(F-value = 5.53, df = 2, p = 0.0095). Specifically, there were significant differences between both temperature change groups and the stable temperature group (Temperature increase: p = 0.0112: temperature decrease: p = 0.0275). There was no significant difference between the temperature change groups (p-value = 0.7624).

The mean quantity of VHSV viral RNA detected in the brain tissue was 5.34 x 10^8 viral N gene copies/50 ng RNA. In fish that experienced a temperature increase , the mean viral RNA quantity was 5.9 x 10^8 ($\log_{10}$ transformed: mean = 16.30; SD = 4.08) viral N gene copies/50 ng RNA. Fish that experienced a temperature decrease had a mean quantity of 5.42 x 10^8 ($\log_{10}$ transformed: mean = 15.77; SD = 4.12). Fish that did not experience temperature change had a mean quantity of 4.46 x10^8 ($\log_{10}$ transformed: mean = 15.77; SD = 4.12) (Fig.6). Viral RNA quantities between the three groups were not significantly different (F-value = 0.139, df = 2, p = 0.871).

The quantities of viral RNA detected in brain and pooled visceral tissue samples was also evaluated over the course of the six weeks as a function of time. In the temperature increase group, fish that tested positive in weeks 4 and 5 did have significantly higher viral RNA quantities in viscera than earlier weeks (F value = 6.000, df = 2, p = 0.026) (Fig. 5). Quantities of viral RNA in visceral tissue from stable temperature and temperature decrease groups did not differ significantly between weeks (stable temperature : F value = 5.674, df = 2, p-value = 0.0517; temperature decrease: F value = 1.276, df = 2, p = 0.317). The quantity of viral RNA in brain tissue also did not differ significantly over the course of the experiment in all three temperature treatment groups (temperature increase: F value = 0.472, df = 2, p = 0.641; decrease: F value = 0.195, df = 2, p = 0.825; stable: F value = 3.689, df = 2, p = 0.081).

Relative Viral Hemorrhagic Septicemia Virus Quantity in Brain vs Visceral Tissue as Related to Temperature Change and Time

Relative levels of VHSV viral RNA in the brain and viscera were evaluated by taking the

absolute value of the difference between the two quantities. In total, 20 fish had a higher viral RNA quantity in the visceral tissue, while 31 fish had a higher viral RNA quantity in the brain. Those fish that had a higher quantity in the viscera had a mean difference between the two of 1.18×10^8 ($\log_{10}$ transformed: mean = 16.46; SD = 4.13) viral N gene copies/50 ng RNA, while fish with a higher quantity in the brain had a mean difference of 6.87×10^8 ($\log_{10}$ transformed: mean = 14.34; SD = 3.95) viral N gene copies/50 ng RNA. There was no significant difference between these mean quantities (t-value = 1.8391, df = 42.062, p = 0.073).

Of the 20 cases in which viscera contained higher VHSV viral RNA quantities than brain, 5 were found in the temperature increase group (35.7% of positive fish), 4 in the stable temperature group (21.1% of positive fish), and 11 in the temperature decrease group (42.3% of positive fish) (Fig. 7). There was statistical difference between these proportions ($p > 0.05$). In the temperature increase group, the mean quantity by which viscera was higher was 3.72×10^7 ($\log_{10}$ transformed: mean = 16.22; SD = 2.87) viral N gene copies/50 ng RNA. In the stable temperature group, the mean was 4.07×10^4 ($\log_{10}$ transformed: mean = 9.56; SD = 2.13) viral N gene copies/50 ng RNA, and in the temperature decrease group, the mean was 1.98×10^8 ($\log_{10}$ transformed: mean = 15.22; SD = 3.63) viral N gene copies/50 ng RNA. The differences between the three groups were statistically significant (F-value = 5.613, df = 2, p = 0.013). Both temperature change groups differed significantly from the stable temperature group, but not from one another (temperature increase to stable p = 0.018; temp. decrease to stable p = 0.021; temp. increase to decrease p = 0.837).

Of the 31 cases in which brain contained higher VHSV viral RNA quantities than viscera, 8 were from the temperature increase group, 10 from the stable temperature group, and 13 from the temperature decrease group (Fig. 8). There was no statistical difference between these proportions ($p > 0.05$). In the temperature increase group, the mean quantity by which brain samples were higher than viscera was 7.36×10^8 ($\log_{10}$ transformed: mean = 15.59; SD = 4.92) viral N gene copies/50 ng RNA. In

the stable temperature group, the mean was 4.42 x 10^8 (log_{10} transformed: mean = 16.51; SD = 3.82) viral N gene copies/50 ng RNA, and in the temperature decrease group, the mean was 8.45 x 10^8 (log_{10} transformed: mean = 16.95; SD = 4.11) viral N gene copies/50 ng RNA. There was no significant difference between these measurements (F-value =0.258, df = 2, p = 0.775).

Most temperature groups had no significant relationship between the difference in brain and visceral viral RNA quantity and time. This was true for all cases of greater viral RNA quantity in the brain, and in cases of greater viral RNA in the viscera for stable temperature and temperature increase groups (greater brain temperature increase: F value = 0.538, df = 2, p = 0.614; decrease: F value = 0.556, df = 2, p = 0.591; stable: F value = 3.673, df = 2, p = 0.081); greater viscera temperature increase: F value = 1.478, df = 1, p = 0.311). The stable temperature group with greater viral RNA quantities in the viscera did not contain enough samples to provide meaningful analysis. In the temperature decrease group, visceral tissue that contained greater viral RNA quantities was greater by a significantly larger quantity in the later four weeks than in the first two weeks (F value = 41.116, df = 2, $p < 0.01$).

Discussion

The results of this experiment expose dynamics between temperature change and VHSV prevalence, severity, and the presence of chronic vs. acute infection that have important implications for wild fish in VHSV affected environments. The experiment raises questions about the distinction between the fish's ability to prevent VHSV infection and its ability to cope with established VHSV infection, and the results demonstrate a difference between the responses of acute and chronic infections to temperature change. On a larger scale, the impact of temperature change on VHSV susceptibility and disease manifestation provide insight to seasonal differences in VHSV prevalence and fish die-off events that have occurred in the Great Lakes Basin and other regions.

Due to the regular water changes, and the highly infrequent events of viral shedding beyond the

first week of the experiment, the incidence of VHSV infection can be confidently attributed to experimental exposure to VHSV that occurred simultaneously with temperature change as opposed to viral shedding from other fish. It is likely that the difference in prevalence is due to an effect on the immune system of the fish rather than an effect on viral replication within the fish, since 15ºC is within the optimal range for VHSV replication and this temperature was central to all three temperature treatment groups (Kim and Faisal 2011, Goodwin and Merry 2011). Thus, the increase in VHSV prevalence probably reflects a detrimental effect of temperature decrease on the function of the fish immune system, as other studies have also suggested (Martin et al. 2011, Goodwin and Merry 2011, Xu et al. 2010). However, neither temperature change group experienced a significantly different VHSV prevalence than the stable temperature group.

Eight fish in total tested positive only in cell culture. Though cell culture is a widely used and relied upon test for VHSV, qRT-PCR has been shown to be a more sensitive test and it is generally expected that a positive visceral sample in cell culture would test positive by qRT-PCR as well (Hope et al. 2010). However, analysis showed qRT-PCR of pooled visceral tissue to be highly sensitive and very highly specific compared to cell culture, and to have a high positive predictive value and very high negative predictive value, as well as a very high diagnostic accuracy (Table 4). Due to this information and the positive qRT-PCR results of the cell culture fluids of the eight samples, I am confident in the accuracy of the measurements of VHSV prevalence, both in cell culture and qRT-PCR, even if a sample were only to test positive in cell culture. Therefore, if fish that experienced a temperature decrease had a higher prevalence of VHSV due to reduced immune system function, it is possible that temperature increase improved immune function in some way so as to reduce VHSV infection, while fish that experienced no temperature shift did not experience a positive or negative effect on the immune system. However, as this and other aspects of this experiment demonstrate, immune function that prevents viral infection should perhaps be considered separately from immune function that mediates

an already established viral presence in the body.

Fish that experienced a temperature decrease also had a higher prevalence of fish that tested positive by qRT-PCR of brain tissue than those that experienced no temperature change (Fig. 4). Though there is not much available information on the mechanisms of chronic vs acute VHSV infection, viral presence in the brain or neural tissue is associated with chronic infection (Hershberger et al. 2010). However, based on viral localization, temperature decrease resulted in more brain samples that tested positive for VHSV than temperature increase or stability. It is not known how VHSV enters the brain or localizes there as opposed to other tissues, but given the immune depression associated with temperature decrease, perhaps immunological responses are involved in preventing chronic infections from developing (Martin et al 2011., Goodwin and Merry 2011, Xu et al. 2010). Thus, temperature decrease and the associated reduced immune responses would have allowed more chronic infections to develop.

Despite the increase in VHSV prevalence among fish that experienced a temperature decrease, the prevalence of death was comparably small among control and treatment groups, and did not reflect the relative prevalence of VHSV (Table 1, Table 2, Table 3). Also, clinical signs of VHSV may not be representative of VHSV prevalence. Chronic infections are associated with reduced incidence of clinical signs, so therefore not all infected fish would be represented by a measure of physical abnormalities (Hershberger et al. 2010, Oidtmann et al. 2011). Furthermore, clinical signs associated with VHSV are not exclusive to VHSV. Though the experimental fish were determined to be healthy upon arrival, this attests to the idea that clinical signs are not a definitive measure of VHSV prevalence. Therefore, these lack of differences between the temperature groups does not detract from the credibility of the differences by the other, more definitive tests for VHSV.

The quantity of viral RNA in viscera is more indicative of acute infections, and therefore temperature change resulted in more severe acute infections (Hershberger et al. 2010). This brings up

important questions regarding immune system function, and how different aspects of immune function may be variably affected by the same influencing factor. For example, though temperature decrease increases the likelihood of VHSV infection as opposed to temperature increase, once an acute infection is established, it seems that the stress of either temperature change results in an increase in the degree of viral replication in the body. The increasing quantity of viral RNA in the temperature increase group further highlights the possible variation in the effect of temperature change on different aspects of the fish immune system. While this group experienced relatively low VHSV prevalence, the degree of acute infections was comparable to that of the temperature decrease group, and rose over time. These findings suggest a distinction between the mechanisms of VHSV infiltration of the body and the management of an acute infection. Ultimately, the severity of acute infection increased with temperature change, regardless of how that same temperature treatment affected VHSV prevalence.

In contrast, the quantity of VHSV viral RNA in brain tissue samples was comparably high in all temperature treatment groups (Fig. 6). Though the number of fish with apparently chronic infections was greater with temperature decrease, it seems that once VHSV established itself in brain tissue, the degree of the infection was less variable. This trend may be due to the less accessible nature of neural tissue to immune actors, or different types of interactions between neural tissue and the immune system. Alternatively, this difference may reflect the different mechanisms by which VHSV establishes a chronic neural infection as opposed to an acute epithelial infection, which may be affected differently by various temperature treatments. Viral replication is optimum at lower temperatures, which would potentially increase the demand on the immune system already compromised by the temperature change, possibly contributing to this trend (Kim and Faisal 2011). Unfortunately, there is a shortage of work on these topics, and a clearer understanding of the these processes is outside the scope of this experiment. Nonetheless, it is apparent that the degree of chronic infection is less dependent on variations in immune function or other factors as produced by temperature change.

Acute and chronic VHSV infection are not necessarily exclusive to one another, and the degrees of infection in both areas of the body can be compared. In cases where the pooled visceral sample contained a greater viral RNA quantity than the brain sample, the margin by which viscera contained more RNA was significantly greater in fish that experienced either temperature change (Fig. 7). In cases where the brain sample contained a greater viral RNA quantity than the pooled visceral sample, the margins were similar between all temperature treatment groups (Fig. 8). These trends correspond well to those regarding the severity of acute and chronic infections in response to temperature treatment.

There are several overall trends that emerge from these results. First, a temperature decrease increased the prevalence of VHSV, and temperature decrease regularly resulted in greater numbers of fish testing positive for VHSV in high quantities. The consistency of this trend is worth noting and contributes to the conclusion that temperature decrease has the greatest influence on prevalence of VHSV infection. Furthermore, of those fish that contracted VHSV, temperature change increased the severity of acute infections. Though temperature change had no influence on the intensity of chronic infections, temperature decrease did generate more chronic infections. With regard to my initial hypotheses, temperature decrease did increase VHSV susceptibility, but only in comparison to temperature increase, and not by every method of evaluation that I applied. I did not anticipate the trends in brain and visceral tissue viral RNA quantities to be so revealing about the differences between chronic and acute infection. I expected a stronger relationship between chronic vs acute infections and time, but instead found a relationship between acute infection severity and temperature change, and between chronic infection prevalence and temperature decrease.

These findings and the possible physiological meanings they imply have ramifications for fish in natural environments. Thermocline turnover, change in depth, and other regular variations within a body of water produce 5ºC and greater temperature changes in the Great Lakes and other bodies of

water where VHSV is present (Wells and Parker 2010, Finlay et al. 2001). Fish that experience 5ºC temperature decreases or greater will be at higher risk of acquiring VHSV infections, and at higher risk of developing chronic neural infections. It is not known whether temperature change affects VHSV shedding behavior in fish, but if so, an increase in shedding combined with a increased susceptibility in the case of temperature decrease may also contribute to higher infection risk. Even without an effect on shedding, temperature decrease would increase susceptibility to environmental VHSV. Furthermore, previously established acute infections may be exacerbated by any temperature change, and fish carrying VHSV in both brain and neural tissue may experience an increase in acute visceral infection severity relative to their chronic infection.

The results also provide potential insight into mortality events and periods of high viral prevalence. A study on temporal variation of VHSV in smallmouth bass in the Great Lakes Basin suggested that VHSV prevalence was highest during the spawning period (Eckerlin et al. 2011). During the spawning season, these fish are known to migrate to sites at which have been recorded to have temperature variations of 5ºC or greater (Finlay et al. 2001). Smallmouth bass are VHSV susceptible, and therefore it is reasonable to suggest that, based on these results, exposure to temperature changes might play an instrumental role in increasing VHSV susceptibility or exacerbating existing VHSV infections during this time. Likewise, these principles may apply to a number of large VHSV mortality events or outbreaks, many of which have occurred during spawning periods or with seasonal regularity that might relate to behavioral differences in the affected fish (Lumsden et al. 2007, Groocock et al. 2007, Al-Hussinee et al. 2011). Various seasonal activity changes may bring fish into contact with temperature changes, influencing the population dynamics of VHSV infection.

This experiment leads into a wide variety of future work that can be performed to further illuminate the pathogenesis of VHSV. Very little is known about the difference between acute and chronic infections. The variable responses of VHSV infection in the brain and viscera to temperature

change suggest that there are different mechanisms taking place to establish and maintain the virus in these two locations. Once a fish contracts VHSV, the mechanisms of its localization to different areas of the body are unknown. Furthermore, though this experiment has suggested that temperature change can play a role in influencing these mechanisms, whether it does so through immune responses alone or also by affecting viral mechanisms is cause for further study. Even the characterization of and the relative demographic susceptibility to chronic vs. acute infections requires further investigation.

The implications of the relative degree of acute vs. chronic infections on the spread of VHSV can also be expanded upon. For example, it is unknown whether an established chronic infection can ever recur as an acute infection, or whether this type of response can be provoked by manipulation of environmental factors like temperature change. The dynamics of VHSV viral shedding may also be relevant to chronic vs. acute infections. This experiment evaluated the effect of temperature change with a simultaneous VHSV exposure, but one could investigate the effect of temperature change on established VHSV infections to examine viral shedding or other aspects of disease progression. Since some species are more susceptible to death due to VHSV while others can be long-term carriers of the virus, the effect of temperature change and other factors on viral shedding may provide insight to the risk some species pose as reservoirs of the disease, and as possible sources of VHSV spread (Eckerlin et al. 2011, Groocock et al. 2007, Al-Hussinee et al. 2011). Finally, this work lends itself to further investigation of the correlations between spawning behavior or seasonal activity changes and VHSV. Surveys could focus on seasons, geographic areas, or species with behavior that would be more likely to bring fish into contact with areas of temperature change, in order to further establish if these relationships between temperature and VHSV infection hold true in natural environments as laboratory investigations such as this suggest.

In summary, this experiment found that temperature decrease increases VHSV susceptibility and the prevalence of chronic infection. Temperature change in either direction increases the severity of

acute infections, while the severity of chronic infections remains more constant. These results also suggest that the mechanisms that govern acute vs. chronic infections, and the immune functions that prevent infection vs. those that manage established infections differ in ways that are variably affected by temperature changes in the environment. Ultimately, this experiment provides insight into the increase in VHSV prevalence during seasons in which fish are more likely to experience temperature change, and justifies a broad spectrum of future work involving the intricacies of acute and chronic infections of VHSV and their responses to variation in aquatic environments.

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Works Cited

Al-Hussinee, L., P. Huber, S. Russell, V. LePage, A. Reid, K. M. Young, E. Nagy, R. M. W. Stevenson, and J. S. Lumsden. 2010. Viral haemorhagic septicaemia virus IVb experimental infection of rainbow trout, Oncorhynchus mykiss (Walbaum), and fathead minnow Pimephales promelas (Rafinesque). Journal of Fish Diseases. 33:347-360.

Al-Hussinee, L., S. Lord, R. M. W. Stevenson, R. N. Casey, G. H. Groocock, K. L. Britt, K. H. Kohler, G. A. Wooster, R.G. Getchell, P. R. Bowser, and J. S. Lumsden. 2011. Immunohistochemistry and pathology of multiple Great Lakes fish from mortality events associated with viral hemorrhagic septicemia virus type IVb. Diseases of Aquatic Organisms. 93:117-127.

Ammayappan, A. G. Kurath, T. M. Thompson, and V. N. Vakharia. 2011. A reverse genetic system for the Great Lakes strain of viral hemorrhagic septicemia virus: the NV gene is required for pathogenicity. Mar Biotechol. 13:672-683.

Ammayappan, A., and V. N. Vakaria. 2009. Molecular characterization of the Great Lakes viral hemorrhagic septicemia virus (VHSV) isolate from USA. Virology Journal. 6:171.

Bain, M. B., E. R. Cornwell, K. M. Hope, G. E. Eckerlin, R. N. Casey, G. H. Groocock, R. G. Getchell, P. R. Bowser, J. R. Winton, W. N. Batts, A. Cangelosi, and J. W. Casey. 2010. Distribution of an invasive aquatic pathogen (Viral hemorrhagic septicemia virus) in the Great Lakes and its relationship to shipping. PloS ONE. 5(4): e10156.doi:10.1371/journal.pone.0010156.

Buchtíková, S., A. Šimková, K. Rohlenová, M. Flajšhans, A. Lojek, E. Lilius, and P. Hyršl. 2011. The seasonal changes in innate immunity of the common carp (*Cyprinus carpio*). Aquaculture. 318:169-175.

Cornwell, E. R., G. E. Eckerlin, R. G. Getchell, G. H. Groocock, T. M. Thompson, W. N. Batts, R. N. Casey, G. Kurath, J. R. Winton, P. R. Bowser, M. B. Bain, and J. W. Casey. 2011. Detection of viral hemorrhagic septicemia virus by quantitative reverse transcription polymerase chain reaction from two fish species at two sites in Lake Superior. Journal of Aquatic Animal Health. 23:4, 207-217.

Cornwell, E. R., G. E. Eckerlin, T. M. Thompson, W. N. Batts, R. G. Getchell, G. H. Groocock, G. Kurath, J. R. Winton, R. N. Casey, J. W. Casey, M. B. Bain, and P. R. Bowser. 2012. Predictive factors and viral genetic diversity for viral hemorrhagic septicemia virus infection in Lake Ontario and the St. Lawrence River. Journal of Great Lakes Research. Article in press. Available: http://dx.doi.org/10.1016/j.jglr.2012.01.006.

Dean, A. G., K. M. Sullivan, M. M. Soe. 2011. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1. Available: www.OpenEpi.com.

Echinas, P., M. A. Rodriguez-Milla, B. Novoa, A. Estrepa, A. Figueras, and J. Coll. 2010. Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicemia rhabdovirus. A proteomic and transcriptomic approach. BMC Genomics. 11:518.

Eckerlin, G. E., J. M. Farrell, R. N. Casey, K. M. Hope, G. H. Groocock, P. R. Bowser, and J. Casey. 2011. Temporal variation in prevalence of viral hemorrhagic septicemia virus type IVb among upper St. Lawrence River smallmouth bass. Transactions of the American Fisheries Society. 140:529-536.

Elsayed, E., M. Faisel, M. Thomas, G. Whelan, W. Batts, and J. Winton. 2006. Isolation of viral haemorrhagic septicaemia virus from muskellunge, *Esox masquinongy* (Mitchill), in Lake St. Clair, Michigan, USA reveals a new sublineage of the North American genotype. Journal of Fish Disease. 29:611-619.

Faisal, M., and C. A. Schulz. 2009. Detection of viral hemorrhagic septicemia virus (VHSV) from the leech *Myzobdella lugubris* Leidy, 1851. Parasites and Vectors. 4:25.

Faisal, M., and A. D. Winters. 2011. Detection of viral hemorrhagic septicemia virus (VHSV) from diporeia spp. (Pontoporeiidae, Amphipoda) in the Laurentian Great Lakes, USA. Parasites and Vectors. 4:2.

Finlay, K. P., H. Cyr, and B. J. Shuter. 2001. Spatial and temporal variability in water temperatures in the littoral zone of a lake. Canadian Journal of Fisheries and Aquatic Sciences. 58: 609-619.

Garver, K. A., L. M. Hawley, C. A. McClure, T. Schroeder, S. Aldous. F. Doig, M. Snow, S. Edes, C. Baynes, and J. Richard. 2011. Development and validation of a reverse transcription quantitative PCR for universal detection of viral hemorrhagic septicemia virus. Diseases of Aquatic Organisms. 95:97-112.

Goodwin, A. E., and G. E. Merry. 2011. Mortality and carrier status of bluegills exposed to viral hemorrhagic septicemia virus genotype IVb at different temperatures. Journal of Aquatic Animal Health. 23:85-91.

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

Hawley, L. M., and K. A. Garver. 2008. Stability of viral hemorrhagic septicemia virus (VHSV) in freshwater and seawater at various temperatures. Diseases of Aquatic Organisms. 82: 171-178.

Hershberger, P. K., J. L. Gragg, C. A. Grady, L. Taylor, and J. R. Winton. 2010. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. Diseases of Aquatic Organisms. 93: 43-49.

Hope, K. M., R. N. Casey, G. H. Groocock, R. G. Getchell, P. R. Bowser, and J. W. Casey. 2010. Comparison of quantitative RT-PCR with cell culture to detect viral hemorrhagic septicemia virus (VHSV) IVb infections in the Great Lakes. Journal of Aquatic Animal Health. 22:50-61.

Jensen, Mogens H. 1965. Research on the virus of Egtved disease. Annals of the New York Academy of Sciences. 126: 422-26.

Kim, R., and M. Faisal. 2009. Experimental studies confirm the wide host range of the Great Lakes viral haemorrhagic septicaemia virus genotype IVb. Journal of Fish Diseases. 33:83-88.

Kim, R. and M. Faisal. 2011. Emergence and resurgence of the viral hemorrhagic septicemia virus (*Norvirhabdovirus, Rhabdoviridae, Mononegavirales*). Journal of Advanced Research. 2:9-23

Lumsden, J. S., B. Morrison, C. Yason, S. Russell, K. Young, A. Yazdanpanah, P. Huber, L. Al-Hussinee, D. Stone, and K. Way. 2007. Mortality event in freshwater drum *Aplondinotus grunniens* from Lake Ontario, Canada, associated with viral haemorrhagic septicemia virus, type IV. Diseases of Aquatic Organisms. 76:99-111.

Lorenzen, E., B. Carstensen, and N. J. Olesen. 1999. Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV, and IPNV. Diseases of Aquatic Organisms. 37:81-88.

Martins, M. L., D. H. Xu, C. A. Shoemaker, and P. H. Klesius. 2011. Temperature effects on immune response and hematological parameters of channel catfish *Ictalurus punctatus* vaccinated with live theronts of *Ichthyophthirius multifiliis*. 31: 774-780.

Muroga, K. H. Ilda, K. Mori, T. Nishizawa, and M. Arimoto. 2004. Experimental horizontal transmission of viral hemorrhagic septicemia virus (VHSV) in Japanese flounder Paralichthys olivaceus. Diseases of Aquatic Organisms. 58:111-115.

Oidtmann, B., C. Joiner, D. Stone, M. Dodge, R. A. Reese, and P. Dixon. 2011. Viral load of various tissues of rainbow trout challenged with viral haemorrhagic septicaemia virus at various stages of disease. Diseases of Aquatic Organisms. 93:93-104.

OIE (World Animal Health Organisation). 2011. Viral haemorrhagic septicaemia. Chapter 10.9 in Aquatic animal health code, 2011 edition. OIE, Paris. Available: http://www.oie.int/fileadmin/Home /eng/Health_standards/aahc/2010/en_chapitre_1.10.9.pdf. (March 2012).

Olesen, N. J., and P. E. V. Jørgensen. 1992. Comparative susceptibility of 3 fish cell-lines to egtved virus, the virus of viral hemorrhagic septicemia (VHS). Diseases of Aquatic Organisms. 12: 235-237.

Schütze, H., E. Mundt, and T. C. Mettenleiter. 1999. Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. Virus Genes. 19: 59-65.

Thompson, T. M., W. N. Batts, M. Faisal, P. Bowser, J. W. Casey, K. Phillips, K. A. Garver, J. Winton, and G. Kurath. 2011. Emergence of viral hemorrhagic septicemia virus in the North American Great Lakes region is associated with low viral genetic diversity. Diseases of Aquatic Organisms. 96:29-43.

Wells, M. and S. Parker. 2010. The thermal variability of the waters of Fathom Five National Marine Park, Lake Huron. Journal of Great Lakes Research. 36:570-576.

Winton, J., W. Batts, P. deKinkelin, M. LeBarre, M. Bremont, and N. Fijan. 2010. Current lineages of the *epithelioma papulosum cyprini* (EPC) line are contaminated with fathead minnow, *Pimephales promelas*, cells. Journal of Fish Diseases. 33:701-704.

Xu, G., X. Sheng, J. Xing, and W. Zhan. 2010. Effect of temperature on immune response of Japanese flounder (*Paralichthys olivaceaus*) to inactivated lymphocystis disease virus (LCDV). Fish and Shellfish Immunology. 30: 525-531.

Yamamoto, T., W. N. Batts and J. R. Winton. 1992. Vitro infection of salmonid epidermal tissues by infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus. Animal Health. 4:231-239.

Figures and Tables

Table 1. Distribution of internal abnormalities, external abnormalities, and gross lesions associated with viral hemorrhagic septicemia virus (VHSV) infection among fathead minnows *Pimephales promelas* subjected to temperature changes surrounding 15ºC. Temperature change groups include 10ºC to 15ºC, 15ºC to 15ºC, and 20ºC to 15ºC. Simultaneously with the temperature change, experimental fish were exposed to 10^5 pfu/mL viral hemorrhagic septicemia virus (VHSV) genotype IVb (isolate MI 03) while control fish were exposed to the same concentration of HMEM-10. See Materials and Methods for definition of HMEM-10. Data collection occurred over the course of six weeks.

Temperature Group			Experimental			Control		
			10°C to 15°C	15°C to 15°C	20°C to 15°C	10°C to 15°C	15°C to 15°C	20°C to 15°C
External Abnormalities	Hemorrhage	All	10	3	0	0	1	4
		At Base of Anal Fin	3	1	0	0	1	1
		At Base of Caudal Fin/ On Caudal Peduncle	1	2	0	0	0	3
		At Base of Pelvic Fin	2	0	0	0	0	0
		At Base of Pectoral Fin	2	0	0	0	0	0
		Surrounding Eyes	1	0	0	0	0	0
		At Head	1	0	0	0	0	0
	Bilateral Exophthalmia		2	0	1	0	0	0
	Pale Gills		1	0	0	0	0	0
	Pink Gills		0	0	0	0	0	2
	Emaciated		1	1	1	0	0	0
	Swollen Abdomen		0	0	1	0	0	0
	Eroded Caudal Fin		0	0	0	0	0	1
	Necrosis on Ventrum (cranial to pelvic fin)		0	0	1	0	0	0
	Subdermal Congestion		0	0	1	0	0	0
	Cut Nose		0	0	0	0	0	1
Internal Abnormalities								
	Hemorrhage	All	2	0	6	0	0	0
		In Spleen	0	0	1	0	0	0
		In Liver	0	0	1	0	0	0
		In Mesentary	1	0	1	0	0	0
		In Brain	0	0	1	0	0	0
		In Swim Bladder	1	0	0	0	0	0
		Severe Ceolomic Hemorrhagic Effusion	0	0	1	0	0	0
		Hemorrhagic Exudative Effusion	0	0	1	0	0	0
	Digeneans	Heavy	4	4	6	7	5	4
		Moderate	6	8	6	7	14	5
		Light	1	0	1	2	2	1
		Cysts	0	0	0	0	1	0
	1-3 Nematodes in Ceolomic Cavity		1	2	2	2	2	1
	Monogenean Cysts in Liver		0	0	0	0	1	0
	Full Gall Bladder		0	0	0	0	0	1
	Fluid in GI Tract		0	0	0	0	0	1
	Mottled Swim Bladder		0	1	0	0	0	0
	Small Liver		1	0	0	0	0	0
	Pale Liver		2	0	1	0	0	0

Dark Liver	0	1	0	0	0	0
Soft, Friable Liver	1	1	0	0	0	0
Enlarged Spleen	3	1	6	0	0	0
Dark Spleen	0	1	1	0	0	0
Soft, Friable Spleen	1	1	0	0	0	0
Pale Heart	0	0	1	0	0	0
All Ascites	3	0	1	0	0	0
Milky Ascites	1	0	0	0	0	0
Excess Visceral Fat	1	0	0	0	0	0
Diffuse Visceral Pallor	0	1	0	0	0	0
Autolysis	0	0	1	0	0	0

Table 2. Total number and proportion of fathead minnows *Pimephales promelas* presenting gross lesions associated with of viral hemorrhagic septicemia virus (VHSV) infection over the course of six weeks following experimental exposure to temperature changes surrounding 15ºC. Temperature change groups include 10ºC to 15ºC, 15ºC to 15ºC, and 20ºC to 15ºC. Simultaneously with the temperature change, experimental fish were exposed to 10^5 pfu/mL viral hemorrhagic septicemia virus (VHSV) genotype IVb (isolate MI 03) while control fish were exposed to the same concentration of HMEM-10. See Materials and Methods for definition of HMEM-10. Groups were compared pairwise by Fisher's exact tests ($\alpha = 0.05$).

Temperature Group	Experimental			Control		
	10ºC to 15ºC	15ºC to 15ºC	20ºC to 15ºC	10ºC to 15ºC	15ºC to 15ºC	20ºC to 15ºC
Number of Fish with Internal Abnormalities	17	3	21	16	24	12
Number of Fish with External Abnormalities	7	17	3	0	1	3
Total Number of Fish with Abnormalities	22	19	21	16	25	14
Total Proportion of Fish with Abnormalities	37.3%	32.8%	35.6%	26.7%	41.7%	23.3%
Total Number of Fish with Abnormalities Excluding Internal Parasites	13	8	12	0	1	5
Total Proportion of Fish with Abnormalities Excluding Internal Parasites	22.0%	13.8%	20.3%	0%	1.7%	8.3%
Significantly Different Groups with p-Values	Control 10ºC to 15ºC ($p < 0.001$)	Control 10ºC to 15ºC ($p = 0.003$)	Control 10ºC to 15ºC ($p < 0.001$)	Experimental 10ºC to 15ºC ($p < 0.001$)	Experimental 10ºC to 15ºC ($p < 0.001$)	Experimental 10ºC to 15ºC ($p = 0.043$)
	Control 15ºC to 15ºC ($p < 0.001$)	Control 15ºC to 15ºC ($p = 0.016$)	Control 15ºC to 15ºC ($p < 0.001$)	Experimental 15ºC to 15ºC ($p = 0.003$)	Experimental 15ºC to 15ºC ($p = 0.016$)	
	Control 20ºC to 15ºC ($p = 0.043$)			Experimental 20ºC to 15ºC ($p < 0.001$)	Experimental 20ºC to 15ºC ($p < 0.001$)	

Table 3. Number of deaths among fathead minnows *Pimephales promelas* over the course of six weeks following experimental exposure to temperature changes surrounding 15ºC. Deaths are presented with results of testing each fish for viral hemorrhagic septicemia virus (VHSV) in cell culture of pooled visceral tissue and quantitative reverse transcription PCR (qRT-PCR) of pooled visceral and brain tissue. Temperature change groups include 10ºC to 15ºC, 15ºC to 15ºC, and 20ºC to 15ºC. Simultaneously with the temperature change, experimental fish were exposed to 10^5 pfu/mL viral hemorrhagic septicemia virus (VHSV) genotype IVb (isolate MI 03) while control fish were exposed to the same concentration of HMEM-10. See Materials and Methods for definition of HMEM-10. A Fisher's exact test comparing the groups of greatest mortality incidence difference showed no significant difference between groups ($\alpha = 0.05$, $p = 0.119$). * = cell culture fluids were tested by qRT-PCR and visceral tissue was retested by qRT-PCR

Temperature Group	Total Number of Deaths	Number of Deaths by Temperature Group	Date of Death	Cell Culture Results	Viscera qRT-PCR Results	Brain qRT-PCR Results
Control	2	10ºC to 15ºC 0				
		15ºC to 15ºC 0				
		20ºC to 15ºC 2	11/1/2011	Neg (-)	Neg (-)	Neg (-)
			11/1/2011	Neg (-)	Neg (-)	Neg (-)
Experimental	5	10ºC to 15ºC 1	11/29/2011	Pos (+)	Pos (+)	Pos (+)
		15ºC to 15ºC 1	11/1/2011	Pos (+)*	Neg (-)	Neg (-)
		20ºC to 15ºC 3	11/4/2011	Pos (+)*	Neg (-)	Pos (+)
			11/26/2011	Neg (-)	Pos (+)	Pos (+)
			11/26/2011	Pos (+)	Pos (+)	Pos (+)

Table 4. Frequency of fathead minnows *Pimephales promelas* that tested positive for viral hemorrhagic septicemia virus (VHSV) by cell culture and quantitative reverse transcription PCR (qRT-PCR) of pooled visceral tissues. Sample size consisted of 356 fish, 176 of which were exposed to 10^5 pfu/mL viral particles for twenty-four hours and 180 of which were exposed to HMEM-10 (defined in Materials and Methods) in the same concentration and duration.

		Pooled Visceral Tissue Extract Cell Culture Results	
		Pos (+)	Neg (-)
Pooled Visceral Tissue Extract qRT-PCR Results	Pos (+)	27	4
	Neg (-)	8	317

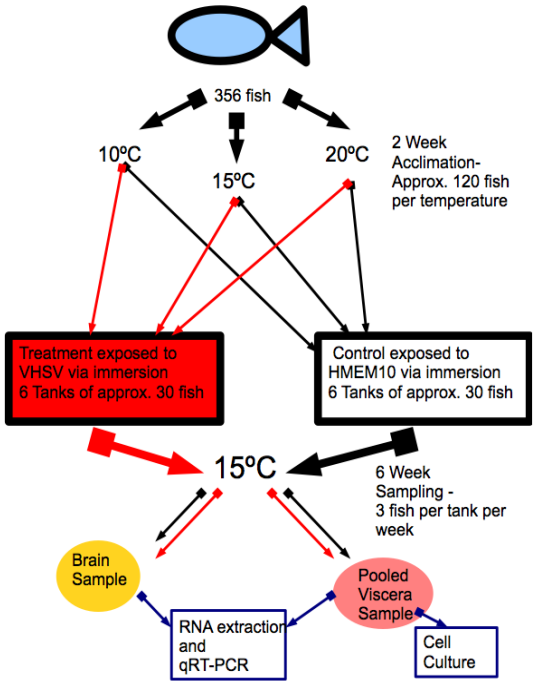

Fig. 1. A schematic plan of the experiment to investigate the effect of 5ºC temperature increase to, decrease to, and stability at 15ºC on the incidence of viral hemorrhagic septicemia virus (VHSV) among fathead minnow *Pimephales promelas.* This experiment was carried out in the facilities of the Aquatic Animal Health Program at Cornell University (Ithaca, New York).

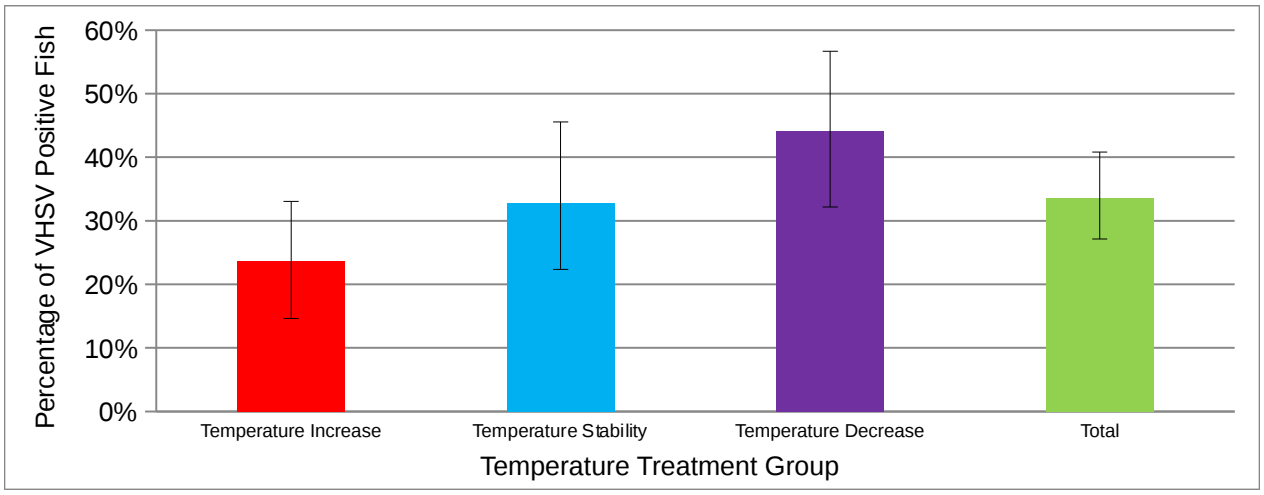

Fig. 2. Total proportion of fathead minnows *Pimephales promelas* that tested positive for viral hemorrhagic septicemia virus (VHSV) following experimental exposure to temperature changes surrounding 15ºC. Temperature change groups included 10ºC to 15ºC, 15ºC to 15ºC, and 20ºC to 15ºC. Simultaneously with the temperature change, experimental fish were exposed to 10^5 pfu/mL VHSV genotype IVb (isolate MI 03). No control fish (0.0%) of any temperature group tested positive for VHSV. Fish were considered positive if VHSV was detected by cell culture, quantitative reverse transcription PCR (qRT-PCR) of pooled visceral tissue, and/or qRT-PCR of brain tissue. Eight fish tested positive in cell culture but not in qRT-PCR of viscera. One was from the temperature increase group, five from the temperature stability group, and two from the temperature decrease group.

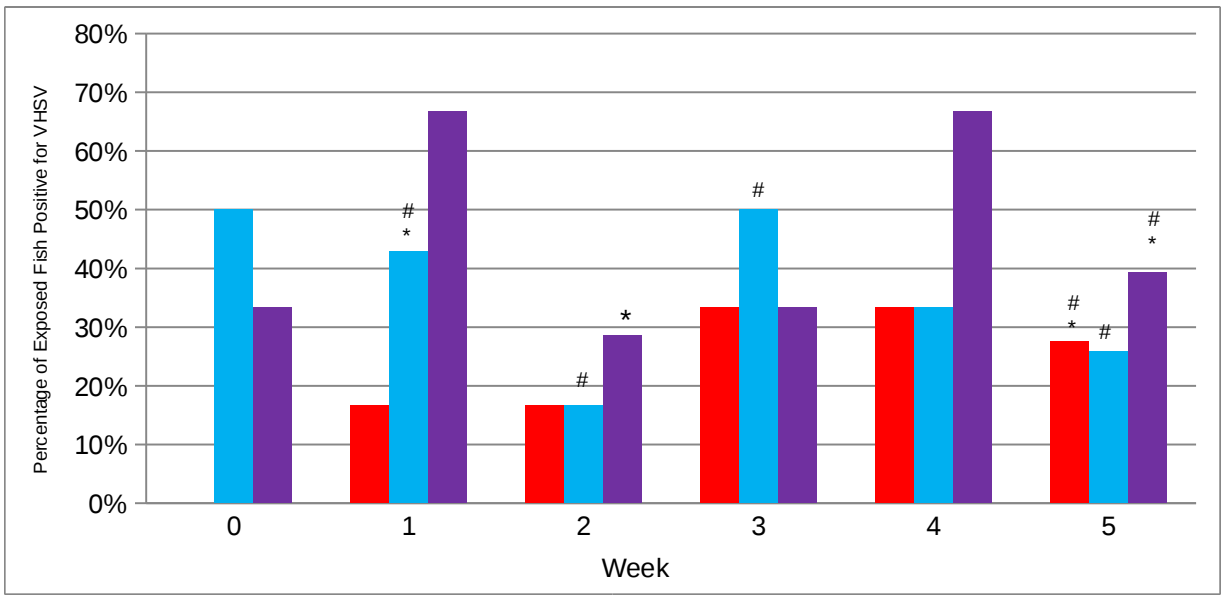

Fig. 3. Proportion of fathead minnows *Pimephales promelas* that tested positive for viral hemorrhagic septicemia virus (VHSV) over the course of six weeks after experimental exposure to temperature changes surrounding 15ºC. Temperature change groups include increase from 10ºC to 15ºC (red), stability at 15ºC to 15ºC (blue), and decrease from 20ºC to 15ºC (purple). Simultaneously with the temperature change, experimental fish were exposed to 10^5 pfu/mL VHSV genotype IVb (isolate MI 03). No control fish (0.0%) of any temperature group tested positive for VHSV, so this figure represents only VHSV exposed fish. Fish were considered positive if they tested positive for VHSV by one or more of the following: cell culture, quantitative reverse transcription PCR (qRT-PCR) of pooled visceral tissue, or qRT-PCR of brain tissue.
* = 1 to 2 fish died during this week, altering the total number of tested fish accordingly.
# = 1 to 2 fish that tested positive from this week tested positive in cell culture but not in qRT-PCR of pooled visceral tissue. Cell culture fluids tested positive by qRT-PCR, and visceral tissue was retested in qRT-PCR.

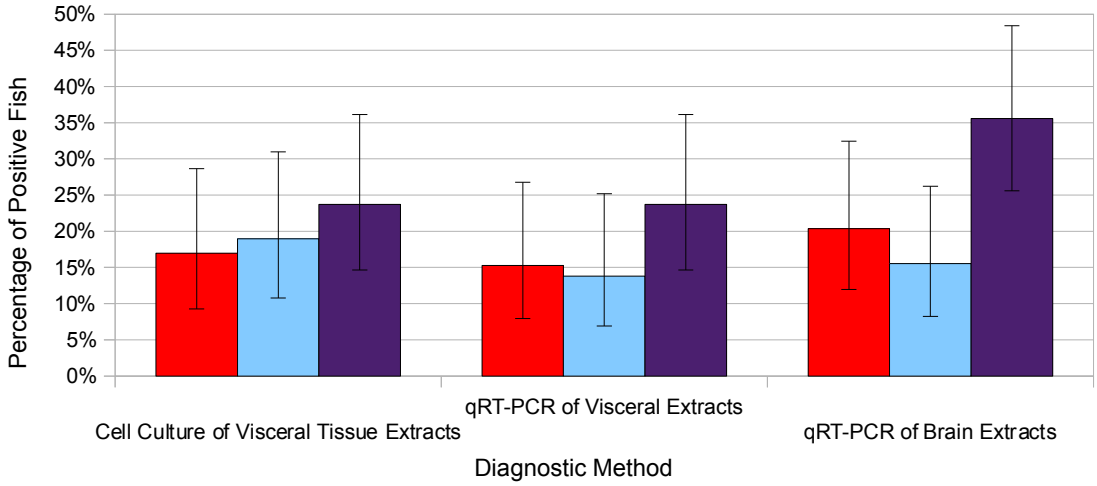

Fig. 4. Proportions of viral hemorrhagic septicemia virus (VHSV) exposed fathead minnows *Pimephales promelas* that tested positive for the virus by cell culture or quantitative reverse transcription PCR (qRT-PCR) of pooled visceral and brain tissue extracts. Red represents fish that experienced a 5ºC increase from 10ºC to 15ºC; blue represents fish that experienced temperature stability at 15ºC; purple represents fish that experiences a 5ºC decrease from 20ºC to 15ºC. Of 176 fish that were exposed to 10^5 pfu/mL viral particles for twenty-four hours at the same time as the temperature change, these percentages reflect the proportion of the 59 fish among all groups that tested positive for VHSV in one or more diagnostic methods during the six week experiment.

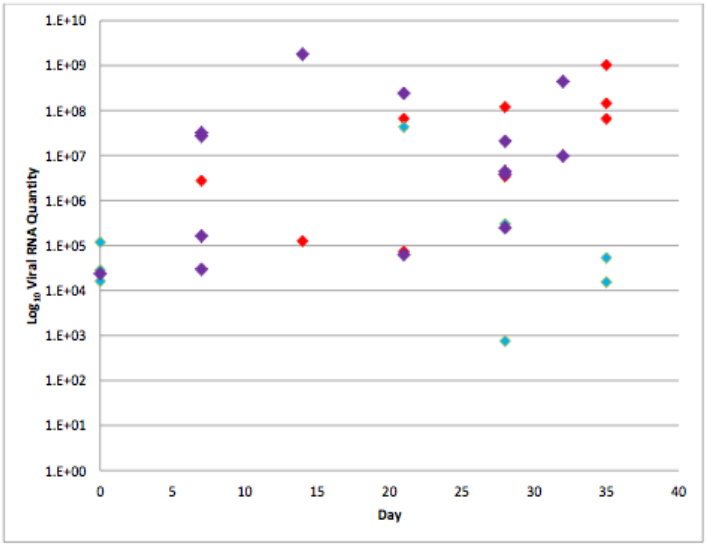

Fig. 5. Logarithmically scaled quantity of viral hemorrhagic septicemia virus (VHSV) N gene RNA copies per 50 µL sample of pooled visceral tissue from fathead minnows *Pimephales promelas.* A total of 176 experimental fish were exposed to 10^5 pfu/mL viral particles for twenty-four hours at the same time as a temperature change event. Red represents fish that experienced a 5ºC increase from 10ºC to 15ºC; blue represents fish that experienced temperature stability at 15ºC; purple represents fish that experiences a 5ºC decrease from 20ºC to 15ºC. Data were collected over the course of six weeks.

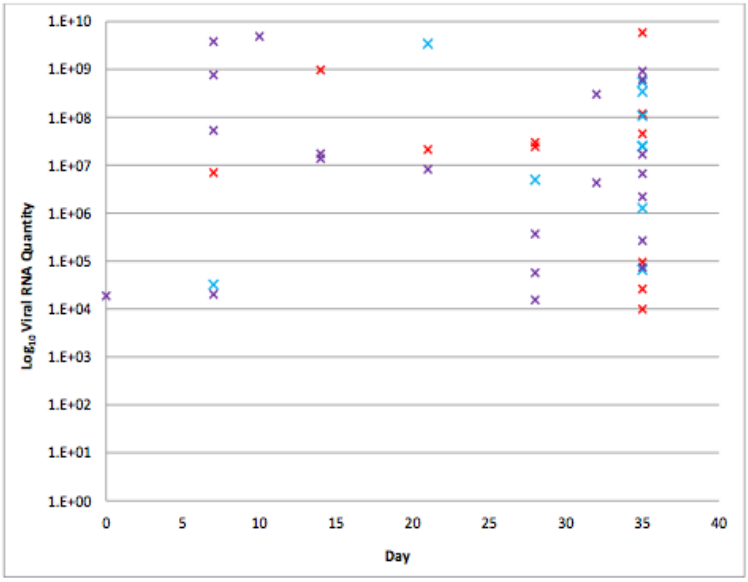

Fig. 6. Logarithmically scaled quantity of viral hemorrhagic septicemia virus (VHSV) N gene RNA copies per 50 µL sample of brain tissue from fathead minnows *Pimephales promelas.* Fish were exposed to 10^5 pfu/mL viral particles for twenty-four hours at the same time as a temperature change event. Red represents fish that experienced a 5ºC increase from 10ºC to 15ºC; blue represents fish that experienced temperature stability at 15ºC; purple represents fish that experiences a 5ºC decrease from 20ºC to 15ºC. Data were collected over the course of six weeks.

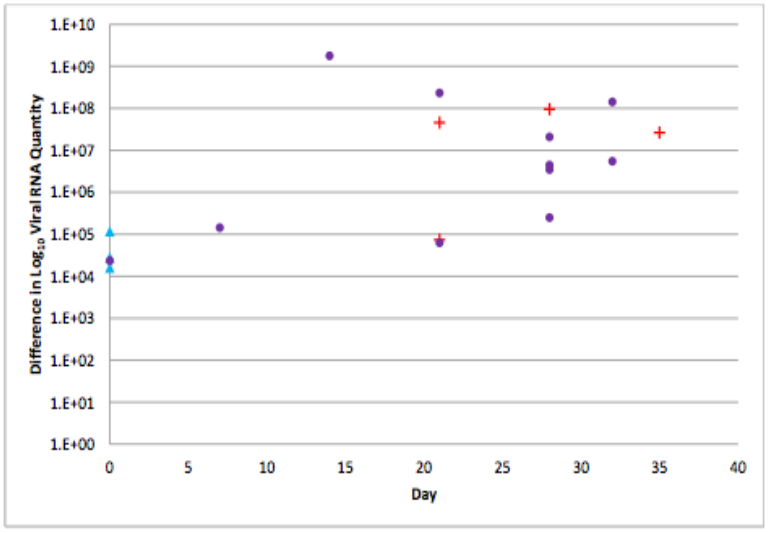

Fig. 7. Logarithmically scaled differences between quantities of viral hemorrhagic septicemia virus (VHSV) N gene RNA copies per 50 µL sample of pooled visceral and brain tissues from fathead minnows *Pimephales promelas* in which visceral tissues contain greater quantities of viral RNA. Fish were exposed to 10^5 pfu/mL viral particles for twenty-four hours at the same time as a temperature change event. Red crosses represent fish that experienced a 5ºC increase from 10ºC to 15ºC; blue triangles represent fish that experienced temperature stability at 15ºC; purple circles represent fish that experiences a 5ºC decrease from 20ºC to 15ºC. Data were collected over the course of six weeks.

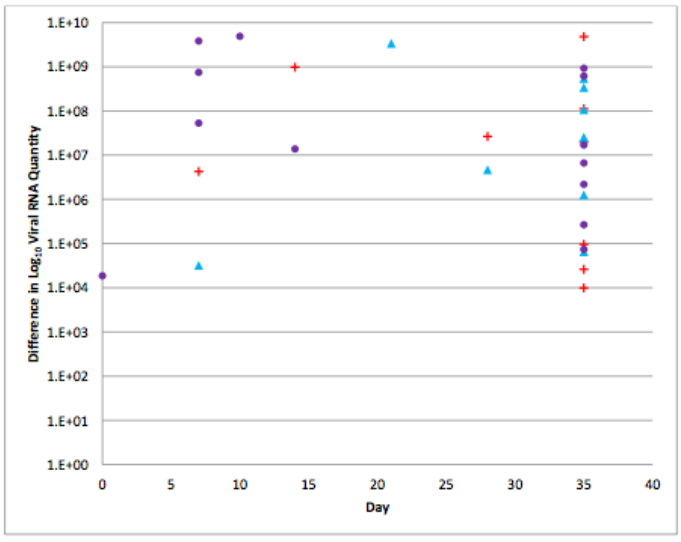

Fig. 8. Logarithmically scaled differences between quantities of viral hemorrhagic septicemia virus (VHSV) N gene RNA copies per 50 µL sample of pooled visceral and brain tissues from fathead minnows *Pimephales promelas* in which brain tissues contain greater quantities of viral RNA. Fish were exposed to 10^5 pfu/mL viral particles for twenty-four hours at the same time as a temperature change event. Red crosses represent fish that experienced a 5ºC increase from 10ºC to 15ºC; blue triangles represent fish that experienced temperature stability at 15ºC; purple circles represent fish that experiences a 5ºC decrease from 20ºC to 15ºC. Data were collected over the course of six weeks.