Vol. 107: 99–111, 2013
doi: 10.3354/dao02671

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 12

# Virulence of viral hemorrhagic septicemia virus (VHSV) genotypes Ia, IVa, IVb, and IVc in five fish species

**Eveline J. Emmenegger[1,*], Chang Hoon Moon[2], Paul K. Hershberger[3], Gael Kurath[1]**

[1]US Geological Survey, Western Fisheries Research Center, 6505 NE 65th St. Seattle, Washington 98115, USA
[2]Biomedical Research Center, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan, Korea
[3]US Geological Survey, Western Fisheries Research Center, Marrowstone Marine Field Station, 616 Marrowstone Point Road, Nordland, Washington 98358, USA

ABSTRACT: The susceptibility of yellow perch *Perca flavescens*, rainbow trout *Oncorhynchus mykiss*, Chinook salmon *O. tshawytscha*, koi *Cyprinus carpio koi*, and Pacific herring *Clupea pallasii* to 4 strains of viral hemorrhagic septicemia virus (VHSV) was assessed. Fish were challenged via intraperitoneal injection with high ($1 \times 10^6$ plaque-forming units, PFU) and low ($1 \times 10^3$ PFU) doses of a European strain (genotype Ia), and North American strains from the West coast (genotype IVa), Great Lakes (genotype IVb), and the East coast (genotype IVc). Pacific herring were exposed to the same VHSV strains, but at a single dose of $5 \times 10^3$ PFU $ml^{-1}$ by immersion in static seawater. Overall, yellow perch were the most susceptible, with cumulative percent mortality (CPM) ranging from 84 to 100%, and 30 to 93% in fish injected with high or low doses of virus, respectively. Rainbow trout and Chinook salmon experienced higher mortalities (47 to 98% CPM) after exposure to strain Ia than to the other virus genotypes. Pacific herring were most susceptible to strain IVa with an average CPM of 80% and moderately susceptible (42 to 52% CPM) to the other genotypes. Koi had very low susceptibility (≤5.0% CPM) to all 4 VHSV strains. Fish tested at 7 d post challenge were positive for all virus strains, with yellow perch having the highest prevalence and concentrations of virus, and koi the lowest. While genotype Ia had higher virulence in salmonid species, there was little difference in virulence or host-specificity between isolates from subtypes IVa, IVb, and IVc.

KEY WORDS: VHSV · Virulence · Genotype · Perch · Salmon · Trout · Herring · Koi



## INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV), a rhabdovirus of freshwater and marine fishes, occurs as 4 unique genotypes (I, II, III, and IV) that demonstrate geographic differentiation (Einer-Jensen et al. 2004, Snow et al. 2004) and some host specificity (Skall et al. 2005a). The genotypes are distinguished phylogenetically by nucleotide sequence differences occurring in the genes coding for the viral nucleocapsid and glycoproteins. Genotype I likely originated in marine fishes from northern Europe, where isolations from sub-clinical fishes periodically occur (Skall et al. 2005a,b); however, it is also well-established in the European freshwater rainbow trout *Oncorhynchus mykiss* culture industry, where it causes serious economic loss from disease epizootics (reviewed by Wolf 1988 and Skall et al. 2005a). VHSV genotype I isolates sampled from rainbow trout at freshwater farms in continental Europe typi-

*Email: eemmenegger@usgs.gov

cally belong to subtype Ia and northern European marine isolates to subtype Ib (Einer-Jensen et al. 2004, Snow et al. 2004). Controlled laboratory challenge studies with marine isolates show low virulence for rainbow trout, while isolates from trout farms demonstrated greater pathogenicity in the same species, which suggested that the early marine isolates along with adapting to a new freshwater host may have also evolved higher virulence (Skall et al. 2005b). Genotype II occurs in sub-clinical Atlantic herring *Clupea harengus*, Atlantic cod *Gadus morhua*, and sprat *Sprattus sprattus* from the Baltic Sea, but has not been associated with any disease outbreaks. Genotype III also occurs exclusively in marine fishes, particularly in waters around the UK and Norway, where it has been isolated from both sub-clinical fishes and from epizootics involving sea-reared rainbow trout and turbot *Scophthalmus maximus* (Schlotfeldt et al. 1991, Ross et al. 1995, Dale et al. 2009). Genotype IV is delineated into 3 distinct phylogenetic subtypes (IVa, IVb, and IVc) which also demonstrate host and geographic distinction. Subtype IVa occurs among marine and anadromous fishes from western North America and Asia, where it can be highly virulent (Kocan et al. 1997) and occasionally causes disease epizootics in free-ranging and confined fishes including Pacific herring *Clupea pallasii*, Pacific hake *Theragra chalcogramma*, Pacific sardine *Sardinops sagax,* and Japanese flounder *Paralichthys olivaceus* (Meyers et al. 1999, Isshiki et al. 2001, reviewed by Hedrick et al. 2003). Among salmonids, genotype IVa periodically causes low-level mortality in cultured Atlantic salmon *Salmo salar* from marine netpens in British Columbia (Canada) and Washington (USA) and demonstrates low virulence to Pacific salmonids (*Oncorhynchus* spp.) where it is sporadically detected at low titers among sub-clinical adults (reviewed by Meyers & Winton 1995). Subtype IVc (Pierce & Stepien 2012) occurs among brackish fishes from the Atlantic coastal region of North America, where it has been isolated from fish in mortality events and epizootics in mummichog *Fundulus heteroclitus*, stickleback *Gasterosteus aculeatus aculeatus*, brown trout *Salmo trutta*, and striped bass *Morone saxatilis* (Gagné et al. 2007).

VHSV subtype IVb has invaded the North American Great Lakes and emerged since its first detection there in 2003, causing large-scale epizootics in muskellunge *Esox masquinongy*, freshwater drum *Aplodinotus grunniens*, round goby *Neogobius melanostomus*, gizzard shad *Dorosoma cepedianumand*, and yellow perch *Perca flavescens* (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Kane-Sutton et al. 2010, Faisal et al. 2012). Because of the rapid dissemination of highly virulent genotype IVb throughout the Great Lakes region during 2005 to 2010, the possibility of transport and establishment of this virus type in other hosts and geographical systems is of concern to regulatory agencies and resource managers. As a first step in examining the potential for further dissemination of VHSV genotypes, this study was performed to define the relative virulence of 4 VHSV isolates representing genotypes Ia, IVa, IVb, and IVc (hereafter referred to as Ia, IVa, IVb, and IVc). This study is the first direct comparison of these VHSV genotypes and an initial evaluation of the potential threat that VHSV-IVb infection could have in aquaculture-reared fish species of the western USA.

## MATERIALS AND METHODS

### Fish

All freshwater and anadromous fish species were transported to the Western Fisheries Research Center (WFRC) in Seattle, Washington (WA), and housed in circular tanks with single-pass, sand-filtered, and UV-treated fresh water from Lake Washington. The protocols for experimental use of live animals were approved by the Institutional Animal Care and Use Committee of the WFRC under the guidelines provided by the Guide for the Care and Use of Laboratory Animals (NRC 2011). The 5 fish species tested in this work were selected based on the following rationale: yellow perch is an important wild and cultured Great Lakes species known to be susceptible to mortality caused by genotype IVb; rainbow trout and Chinook salmon *Oncorhynchus tshawytscha* are important aquaculture species in the western US, and rainbow trout is known to be susceptible to mortality caused by genotype Ia; koi *Cyprinus carpio koi* are propagated and distributed world-wide, and with their long life span, they could serve as long-term reservoirs for various pathogens; and Pacific herring is an important Pacific coast forage species known to be susceptible to mortality caused by genotype IVa.

Two-month old yellow perch fry were obtained from the Chesapeake Sassafras River captive broodstock from the University of Wisconsin Great Lakes Water Institute (Milwaukee, Wisconsin), which has tested negative for VHSV during repeated fish health screenings. Yellow perch fry were held at a constant

temperature of 12°C and daily fed a 1.5 mm dry pellet feed (Life Stage Diet Food, Oregon Biodiet). Perch were 8 mo old with an average weight of 2.25 g at the start of the virus susceptibility challenge.

Fertilized eggs of fall Chinook salmon Portage Bay stock were transferred from the University of Washington hatchery (Seattle, WA) to the WFRC hatch room and placed in Heath tray stacks supplied with the same WFRC treated water as previously described, but at ambient lake water temperatures that ranged from 7.0 to 9.0°C. After 2 mo, hatching fry were transferred to tanks at a constant water temperature of 9.0°C and fed daily with Life Stage Diet Food. The Chinook fry were 6.5 mo old with an average weight of 1.85 g on the day of virus challenge.

Research grade rainbow trout fry, approximately 1.5 mo old and weighing 0.5 g, were received from Clear Springs Food Inc. (Buhl, Idaho) and transferred to WFRC tanks with water temperatures of 15°C. Fish were fed daily with a soft starter crumble diet (Oregon Biodiet) and then switched to the Oregon Biodiet BioClark fry 1.5 mm pellets at 2 mo of age. At the time of challenge, the 2.5 mo old rainbow trout weighed 1.5 g.

A domestic stock of koi fry approximately 2 mo old were transferred from the Pan Inter Corp breeding facility (Kenmore, WA) to the WFRC and reared at water temperatures ranging from 17 to 19°C. Koi were fed every other day with a mixed feed diet of moist and dry pellets consisting of Life Stage Diet Food and Hikari Gold (Kyorin Food Industries). At initiation of the challenge experiment, koi were 26 mo old with an average weight of 3.6 g.

Eight month old SPF (specific pathogen-free) Pacific herring weighing 1.5 g were obtained from the Cherry Point stock (Puget Sound, WA), reared in pathogen-free seawater at ambient seawater temperatures of 7 to 9°C at the US Geological Survey Marrowstone Marine Field Station (Nordland, WA), and transferred directly to the aquatic biosafety level 3 (BSL-3) laboratory at the WFRC with water temperatures ranging from 10 to 15°C. Herring were held in static seawater 1 d prior to challenge to cull any transfer mortalities and acclimate fish to BSL-3 tank conditions.

### Virus isolates

Four VHSV isolates of differing genotypes and biological attributes were used in the virus challenges (Table 1). The VHSV genotype I isolate, described as a VHSV F1 isolate (provided by Dr. Pierre De Kinkelin of the Institut National de Recherche Agronomique, Paris), was retrieved from the WFRC archival −80°C freezer. Jensen (1965) reported that the F1 strain was isolated during a rainbow trout epizootic at a Danish trout farm in which 270 000 fingerlings (30%) died. Our recent full G-gene sequencing and phylogenetic analyses identified our F1 isolate as genogroup subtype Ia (B. Batts, K. Einer-Jensen, and G. Kurath unpubl. data). Thus the isolate used in our challenges is referred to as an F1 variant with a Ia genotype to distinguish it from the presumed original DK-F1 isolate belonging to Genogroup I with no subtype designation. The pilchard *Sardinops sagax* genotype IVa isolate (received from Dr. Garth Traxler of the Department of Fisheries and Oceans [DFO] Pacific Biological Station in Nanaimo, British Columbia, Canada) came from a massive die-off of wild pilchards in northeast coastal waters of Vancouver Island, Canada. Many dead Pacific herring were also found along with a few dying blackcod *Anaplopoma fimbria*, ratfish *Hydrolagus colliei*, and shiner perch *Cytomaster aggregata* (Traxler et al. 1999). The genotype IVb isolate (sent by Dr. Mohamed Faisal of the Aquatic Animal Health Laboratory at Michigan State University, East Lansing, Michigan, USA) was sampled from a muskellunge collected during a fish health survey at Lake St. Clair, Michigan (Elsayed et al.

Table 1. Original source features of *Viral hemorrhagic septicemia virus* isolates used in virus challenge studies. NA: North America; BC: British Columbia, NB: New Brunswick, MI: Michigan

| Genotype | Isolate region (identifier) | Geographic origin | Isolation date | Fish species | Water environment | Host background/ Isolation setting | Reference |
|---|---|---|---|---|---|---|---|
| Ia | European (F1 variant) | Denmark | 1962 | Rainbow trout | Freshwater | Farm/Epidemic | Jensen (1965) |
| IVa | NA-Pacific Coast (99-001) | BC, Canada | 1999 | Pilchard | Marine | Wild/Epidemic | Traxler et al. (1999) |
| IVb | NA-Great Lakes (MI03GL) | MI, USA | 2003 | Muskellunge | Freshwater | Wild/Diagnostic | Elsayed et al. (2006) |
| IVc | NA-Atlantic Coast (149) | NB, Canada | 2000 | Mummichog | Brackish | Wild/Epidemic | Gagné et al. (2007) |

2006). The mummichog genotype IVc isolate (obtained from Dr. Gilles Olivier of DFO Fish Health Unit in Moncton, New Brunswick, Canada) was collected during a mortality event involving mummichog, three-spined stickleback *Gasterosteus aculeatus*, and striped bass *Morone saxatilis* (Walbaum) from the eastern Atlantic coast of New Brunswick in April and May of 2000. VHSV was also detected in a dead brown trout *Salmo trutta trutta* L (Gagné et al. 2007). Virus isolates were propagated at a multiplicity of infection of approximately 0.007 in an epithelioma papulosum cyprini (EPC) cell line (Fijan 1983) at a constant temperature of 15°C in minimum essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine (Invitrogen), and buffered to pH 7.5 with 7.5% sodium bicarbonate (SB) and Tris (hydroxymethyl) aminomethane (Fisher Scientific). Virus was harvested when the average cytopathic effect was at least 80% and stored as frozen aliquots at –80°C. Virus titers from a thawed aliquot were determined by plaque assay following the procedure outlined by Batts & Winton (1989). Because the European genotype Ia strain is considered an exotic virus and the genotype IVb freshwater isolate is a highly invasive strain in the Great Lakes, all *in vivo* challenge experiments were performed in an aquatic BSL-3 containment laboratory, which is designed for the testing and containment of fish pathogens that pose high risks to the environment. The aquatic BSL-3 laboratory has 1 dry laboratory and 2 wet laboratory rooms each containing 18 aquaria with a maximum 30 l volume. All tank water effluent is batch-treated with sodium hypochlorite, all outflowing air is HEPA-filtered, and all dry materials are autoclaved prior to disposal.

### Intraperitoneal (IP) injection challenges of freshwater and anadromous fish species

Each fish species was challenged independently with the various VHSV strains in the BSL-3 at separate times. On the day of the challenge, fish were transferred from rearing tanks in the main wet lab to the aquatic BSL-3 tanks with a starting water temperature of 10°C. Challenge water temperature set points were increased 0.5°C daily until the temperature reached 12°C, which was then maintained for the remainder of the experiment. The challenge temperature regime was selected to maximize the vulnerability of the fish species tested, since rapid cold water stress treatment and handling on the day of challenge has facilitated susceptibility testing of other rhabdoviruses (Emmenegger & Kurath 2008). Prior to injection, yellow perch, Chinook salmon, rainbow trout, and koi were anesthetized with a tricaine methanesulfonate (MS-222, 60 mg $l^{-1}$) and sodium bicarbonate (SB, 300 mg $l^{-1}$) solution. Fish in triplicate tanks (20 fish $tank^{-1}$), each containing a single species, were injected with either a high dose of $1 \times 10^6$ plaque-forming units (PFU) $fish^{-1}$ or a low dose of $1 \times 10^3$ PFU $fish^{-1}$ of each VHSV genotype (Ia, IVa, IVb, or IVc). Control fish, also in triplicate groups of 20 fish $tank^{-1}$, were injected with the same inoculum volume (100 µl) of diluent (MEM-0-TRIS; medium solution containing no fetal bovine serum). Fish were monitored daily and fed every other day the same feed type previously described for each species. All dead fish were collected on the day of death, and disease signs were recorded. Upon termination of the experiment, 28 d after injection, all surviving fish were euthanized by immersion in water containing 240 mg $l^{-1}$ of MS-222 and 1.2 g $l^{-1}$ SB. Individual fish carcasses were transferred directly into Whirl-Pak bags (Nasco) and frozen at –80°C until a subset was processed. Out of the dead fish sampled, a subset was selected that died early, midway, or later during the challenge, for virus quantitation. For virus titering, the whole fish was weighed, then diluted 1:8 with MEM-0 medium, and homogenized in a Stomacher (Seward) for 60 s at high speed. For each fish homogenized, 10 ml of homogenate were transferred into a 15 ml conical tube and centrifuged at 1000 × *g* (10 min). Supernatants were screened for the presence of virus by plaque assay as described previously (Batts & Winton 1989).

### Sampling of fish for virus replication at 7 d post-challenge

Concurrent with the susceptibility tests of yellow perch, Chinook salmon, rainbow trout, and koi, an additional 20 fish, each held in separate tanks, were injected with the high virus dose ($1 \times 10^6$ PFU $ml^{-1}$) of each VHSV genotype or with the mock (MEM-0) treatment to assess virus replication in the various host species 7 d after exposure. Seven days post-injection, all 20 fish were euthanized, and 10 fish were collected and frozen following the same procedures used for the survivors from the 28 d challenge. Viral load in a subset of sampled fish was assessed utilizing the same methodology as described above.

### Static seawater immersion challenge of Pacific herring

Previous laboratory immersion challenges of Pacific herring with VHSV genotype IVa strains have confirmed that $10^3$ PFU $ml^{-1}$ or greater consistently initiates acute disease (Kocan et al. 1997). Challenge conditions including a 14 d post-observation period and suspended feeding during VHSV exposure experiments were standardized by Hershberger et al. (2007). Our 2 pilot studies determined that larger tanks, lower fish densities, and immersion virus exposure instead of IP injection were needed in order to challenge herring in the aquatic BSL-3 containment laboratory under static seawater conditions (data not shown). Therefore in this study, duplicate tanks of 40 herring were exposed to each virus isolate at $5 \times 10^3$ PFU $ml^{-1}$ by immersion in a 20 l volume of sand-filtered and UV-irradiated seawater for 1 h with air saturation. Control herring in duplicate tanks received an identical mock inoculum volume of diluent (MEM-5-TRIS) using the same static challenge conditions. After a 1 h exposure, the treatment and mock tanks were refilled with seawater to 119 l. Seawater exchanges of approximately 20 l $tank^{-1}$ were performed daily in an effort to prevent tank fouling and maintain cooler water temperatures. Water temperatures fluctuated between 11.6 and 14.0°C during the 14 d challenge experiment. Dead fish were removed daily and stored individually at −80°C for virus titering via plaque assay. Herring survivors were sampled as previously described for the freshwater challenges.

### Analysis of challenge data

Mortality challenge data were statistically analyzed using GraphPad InStat Version 3.1a software. Cumulative percent mortality (CPM) values from duplicate treatment tanks for the herring virus challenges were statistically evaluated with a Fisher's exact test. CPM from the triplicate treatment groups of the freshwater and anadromous fish species challenges were arcsine transformed prior to statistical assessment by a 1-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparison post test if applicable to compare CPM between treatments. SPSS version 11.5 was only used to determine whether there was any variance among replicate tanks within each treatment. A significant relationship was designated for comparisons yielding p values ≤0.05. Mean day of death (MDD) values were calculated as the sum of days of death divided by the total number of dead fish in each tank, and then the average MDD was determined for the 3 tank replicates for each treatment.

## RESULTS

The time courses of mortality for each fish species challenged with the various VHSV genotypes are displayed in Figs. 1 & 2. Variance among replicate tanks for each treatment was not significant in any case, thus the mean CPM for the replicates of each challenged fish species treatment were used in the figures. Typical external clinical signs of VHS disease (Skall et al. 2005a), such as hemorrhages on the head, fins, body, and eyes, and ascites fluid swelling of the body cavity, were noted on at least some dead fish of each fish species challenged with all VHSV genotypes (photos not shown). Many virus-exposed fish also died with no evidence of disease. Mean CPM values and the MDD for each fish species after exposure to a VHSV isolate at a high or low dose are shown in Fig. 3. The results (i.e. significant or non-significant) from statistical comparisons of triplicate CPMs of each genotype for each fish species challenged are reported in Table 2.

### Susceptibility of freshwater and anadromous fish species to VHSV

Mortalities in mock treatments for all freshwater and anadromous fish species tested were ≤3.0% (data not shown). Yellow perch were overall the most susceptible species tested, with mortalities ranging from 84 to 100% and 30 to 93% in fish injected with high and low doses of virus, respectively (Fig. 1A,E). Total mortalities experienced by yellow perch challenged with any VHSV genotype were significantly higher than the mock exposed fish ($p < 0.0001$). Mortality levels between yellow perch exposed to various VHSV genotypes were all significantly different ($p < 0.0001$) except between genotypes IVa and IVb at both doses, and between genotypes IVc and Ia at the high dose (Table 2). Yellow perch were susceptible to all VHSV genotypes, but appeared to be the least susceptible to the European genotype Ia, especially when exposed at the lower viral dose. In addition, for all the fish species tested, the only significant difference in MDD was in yellow perch injected with the high virus dose, where genotype Ia had a significantly later MDD

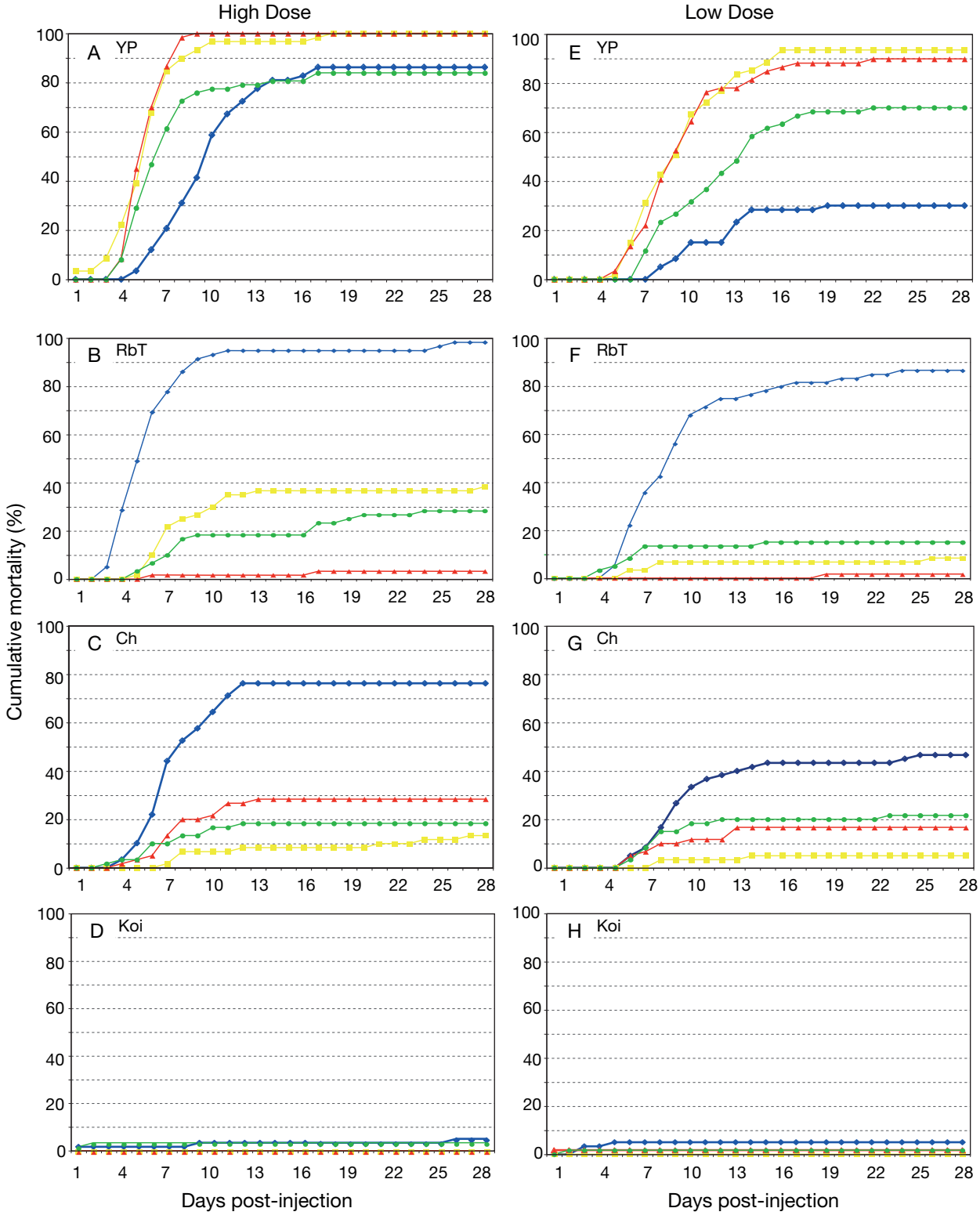


Fig. 1. Mean cumulative mortality of freshwater and anadromous fish species: yellow perch (YP), rainbow trout (RbT), Chinook (Ch), and koi, after intraperitoneal injection exposure to (A–D) a high dose ($1.0 \times 10^6$ plaque-forming units [PFU] fish$^{-1}$) or (E–H) low dose ($1.0 \times 10^3$ PFU fish$^{-1}$) of VHSV strains representing the following genotypes: European Ia (blue), North American West Coast IVa (yellow), Great Lakes IVb (red), and North American East Coast IVc (green). Mortalities of fish from all mock treatments were ≤3.0% and are not displayed

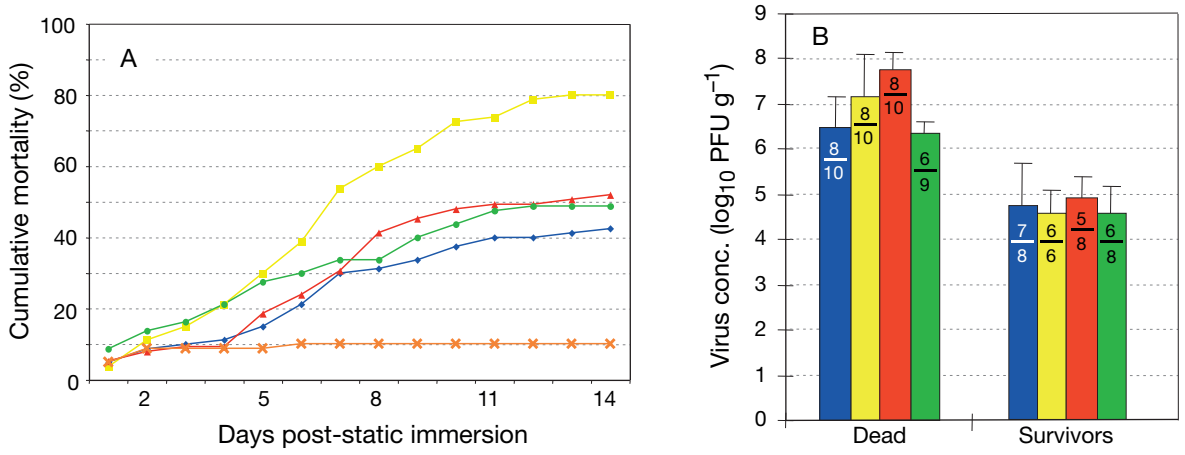


Fig. 2. *Clupea pallasii*. (A) Mean cumulative mortality of Pacific herring after immersion exposure to a dose of $5.0 \times 10^3$ plaque-forming units (PFU) $ml^{-1}$ of VHSV (blue: European Ia; yellow: North American West Coast IVa; red: Great Lakes IVb; green: North American East Coast IVc): or mock treatment (orange). (B) Virus concentrations (geometric mean of virus-positive fish) in dead and surviving fish (14 d post-challenge) with prevalence shown within the columns as the number of virus-positive fish per number tested. Error bars display the SD of virus titers

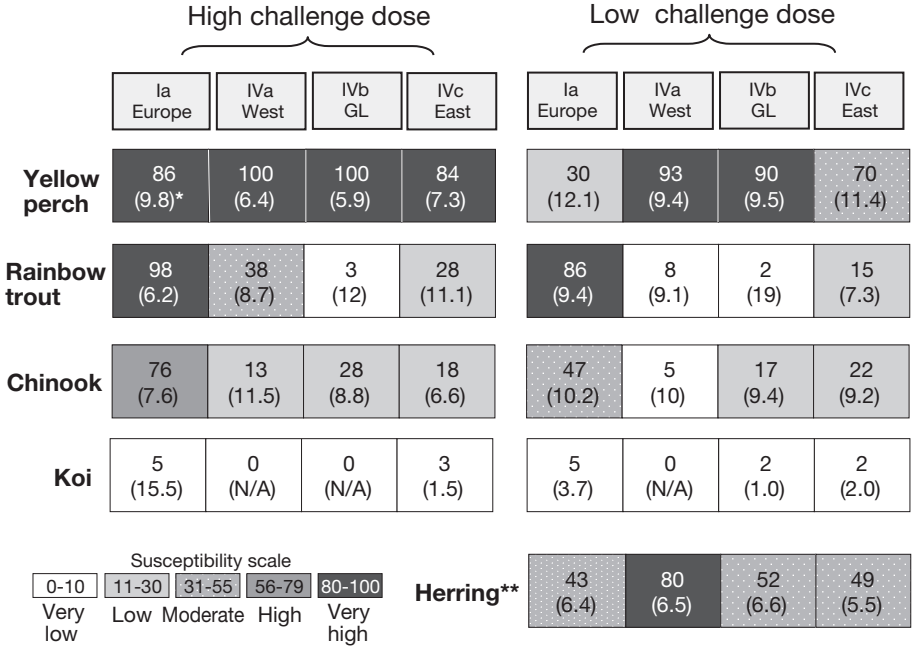


Fig. 3. Susceptibility categories (very low, low, moderate, high, very high) based on the mean cumulative percent mortality (CPM) of each fish species after exposure to a VHSV genotype at either a high or low dose ($1.0 \times 10^6$ or $1.0 \times 10^3$ plaque-forming units [PFU] $fish^{-1}$), displayed as background shading in each rectangle. CPM and mean day of death (MDD, in parentheses) for each species are listed. *Significantly different MDD between VHSV strains tested at the same dose. **Herring VHSV exposure differed from other hosts by being a 14 d static immersion challenge in seawater at $5.0 \times 10^3$ PFU $ml^{-1}$. N/A: MDD calculations not applicable to treatments where no mortalities occurred

(9.8; $p < 0.0001$) than perch exposed to the other VHSV genotypes at the same high dose (Fig. 3).

Both rainbow trout and Chinook salmon experienced higher mortalities, 47 to 98 % CPM, after exposure to the European genotype Ia strain than to any of the other virus genotypes (IVa, IVb, and IVc; Fig. 3). Rainbow trout were the most susceptible to genotype Ia and the least susceptible to IVb and IVc strains at both exposure doses ($p < 0.0001$). Cumulative mortality was significantly higher ($p < 0.0001$) in Chinook salmon challenged with genotype Ia than with the other genotypes (Table 2), with the exception that there was no significant difference between Chinook salmon exposed to genotype IVc (CPM 22 %) and genotype Ia at the lower dose (CPM 47 %). Although CPM was not compared statistically between different fish species, mortality induced by strain Ia was lower in Chinook salmon than in rainbow trout.

Koi were the least susceptible to VHSV infection of all the fish species tested (Fig. 1D,H). None of the mean cumulative mortalities exceeded 5 %,

Table 2. Summary of virulence comparisons between VHSV genotypes (European Ia, West coast IVa, Great Lakes IVb, East coast IVc) and mock treatments for yellow perch (YP), rainbow trout (RbT), Chinook salmon (Ch), and Pacific herring (PH). Statistical test outcomes for low or high challenge dose ($1.0 \times 10^3$ or $1.0 \times 10^6$ plaque-forming units $fish^{-1}$) cumulative percent mortality (CPM) replicates are displayed in the lower left (low dose) or upper right (high dose) matrix for each species. All CPM comparisons for koi were non-significant (data not shown). Comparisons between genotypes within each dose are shown relative to the genotypes in the first column (i.e. read genotypes and operator [< or >] from left to right), as significantly ($p < 0.0001$ for YP, RbT, and Ch, or $p < 0.0003$ for PH) higher (>) or lower (<), or non-significant (ns; $p > 0.05$)

| **YP** | Mock | Ia | IVa | IVb | IVc | |
|---|---|---|---|---|---|---|
| Mock | · | < | < | < | < | HIGH DOSE |
| Ia | > | · | < | < | ns | |
| IVa | > | > | · | ns | > | |
| IVb | > | > | ns | · | > | |
| IVc | > | > | < | < | · | |
| | | LOW DOSE | | | | |
| **RbT** | Mock | Ia | IVa | IVb | IVc | |
| Mock | · | < | < | ns | < | HIGH DOSE |
| Ia | > | · | > | > | > | |
| IVa | > | < | · | > | ns | |
| IVb | ns | < | ns | · | < | |
| IVc | > | < | ns | > | · | |
| | | LOW DOSE | | | | |
| **Ch** | Mock | Ia | IVa | IVb | IVc | |
| Mock | · | < | < | < | < | HIGH DOSE |
| Ia | > | · | > | > | > | |
| IVa | ns | < | · | ns | ns | |
| IVb | > | < | ns | · | ns | |
| IVc | > | ns | ns | ns | · | |
| | | LOW DOSE | | | | |
| **PH** | Mock | Ia | IVa | IVb | IVc | |
| Mock | · | | | | | HIGH DOSE |
| Ia | > | · | | | | |
| IVa | > | > | · | | | |
| IVb | > | ns | < | · | | |
| IVc | > | ns | < | ns | · | |
| | | LOW DOSE | | | | |

and there were no significant differences in mortality between the VHSV genotypes tested (Fig. 3, Table 2).

### Virus titers in freshwater and anadromous fish that died during challenge

Virus was detected in the majority of dead fish (Fig. 4A) for every species tested except koi. Yellow perch had the highest overall VHSV prevalence in dead fish of 95.7% (45/47) followed by Chinook at 90.9% (40/44) and rainbow trout 83.3% [30/36]. Dead koi had the lowest virus prevalence of 22.2% (2/9), and the European genotype Ia was the only genotype of VHSV recovered. No virus was found in the single rainbow trout that died 19 d after a low dose injection of genotype IVb. Overall, the mean virus titers found in dead fish for each species were comparable regardless of virus strain used in challenge. Titers in dead fish testing positive for virus ranged from $8.8 \times 10^5$ to $5.3 \times 10^7$ PFU $g^{-1}$ in yellow perch, $1.6 \times 10^5$ to $4.6 \times 10^6$ PFU $g^{-1}$ in rainbow trout, $8.3 \times 10^3$ to $6.6 \times 10^5$ PFU $g^{-1}$ in Chinook salmon, and $3.2 \times 10^3$ to $1.6 \times 10^4$ PFU $g^{-1}$ in koi (Fig. 4A).

### Detection of virus in freshwater/anadromous fish that survived the 28 d challenge

Among fish that survived the 28 d challenge, the overall prevalence of virus, regardless of genotype strain, was higher in yellow perch (27.5%, 11/40) and Chinook (26.0%, 13/50) than in rainbow trout (4.7%, 2/43) and koi (4.2%, 2/48; Fig. 4B). There was little difference in the geometric mean titer levels between the various VHSV strains detected in yellow perch survivors. The European genotype Ia virus mean titer was higher by 1.5 to 3 logs than North American genotypes IVb and IVc titers in Chinook salmon surviving a low-dose exposure. Overall, virus titers were generally higher in surviving yellow perch than in Chinook salmon except for genotype Ia, which were equivalent in concentration. Genotypes Ia and IVb were the only VHSV strains detected in rainbow trout and koi, respectively, both at a low prevalence of 33.3% (2/6), but with the highest virus titers found in all survivors tested.

### Detection of virus in freshwater and anadromous fish sampled 7 d post-challenge

Virus was detected in every fish species of each virus treatment group from the 7 d high-dose exposure challenge (Fig. 4C). Virus prevalence was the highest in yellow perch (100%, 20/20) followed by Chinook (79.4%, 27/34), rainbow trout (60.7%, 17/28), and koi (50%, 20/40) at 7 d post-challenge. In rainbow trout and Chinook salmon, VHSV genotypes Ia and IVc were detected in nearly all fish at 7 d post-challenge, while genotypes IVa and IVb were present in a smaller proportion of fish. All yellow perch tested with all VHSV challenge strains had titers

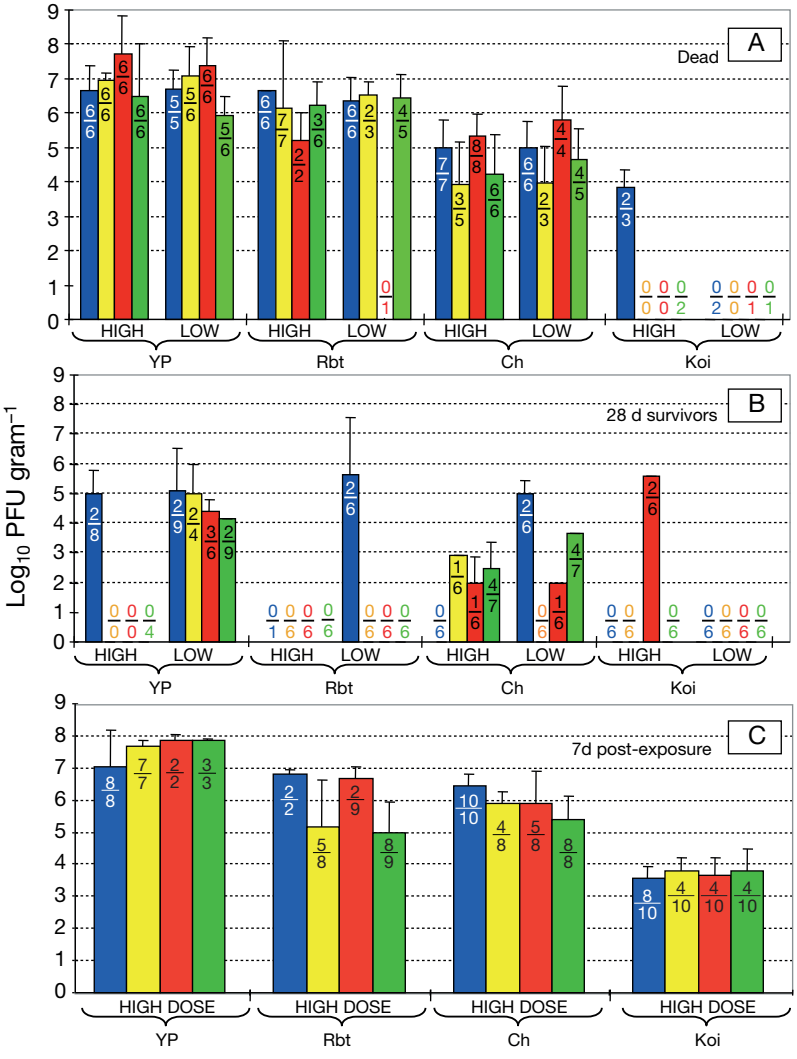


Fig. 4. Virus concentrations in (A) dead and (B) surviving fish from the 28 d challenge and in (C) fish euthanized at 7 d after exposure to different VHSV strains (blue: European genotype Ia; yellow: US West Coast IVa; red: Great Lakes IVb; green: US East Coast IVc) at both high and low dose or only a high dose (7 d challenge) in anadromous and freshwater fish species (YP: yellow perch; RbT: rainbow trout; Ch: Chinook). Titers reported as the log geometric mean of virus-positive fish ± SE. Prevalence is displayed as the number of virus positive fish per number tested within the columns or on the graph

higher than the original injection dose of $1 \times 10^6$ PFU. In rainbow trout genotypes Ia and IVb, and for Chinook salmon only the genotype Ia virus concentrations exceeded the initial inoculum dose. Virus concentrations in koi were approximately 2 logs lower at the 7 d time point. There was no consistent difference in virus concentrations between the VHSV challenge strains within each species tested, except for rainbow trout that had higher genotype Ia and IVb virus titers than the IVa and IVc virus concentrations (Fig. 4C).

### Pacific herring susceptibility to VHSV via immersion challenge

Mortality in the mock-exposed marine fish species (Pacific herring) was slightly higher, as compared to the mock-treated freshwater/anadromous species, with a total mortality of 10% (Fig. 2A). Pacific herring were most susceptible to the endemic West coast genotype IVa virus and moderately susceptible to other tested VHSV genotypes. Average cumulative mortality of herring after genotype IVa virus infection (80%) was significantly higher ($p < 0.0003$) than that of genotypes Ia, IVb, and IVc, which ranged from 42 to 52% CPM (Fig. 3, Table 2). Virus was detected in the majority of herring that died with titers on average higher than those found in surviving herring at 14 d post-challenge (Fig. 2B). No significant differences were noted between the MDD (Fig. 3).

## DISCUSSION

Due to the global importance of VHSV as a finfish pathogen, it has been extensively studied for many decades. In Europe, research has been conducted on freshwater trout farm genotype Ia isolates since the 1950s, and the virulence of marine genotypes Ib, II, and III has been investigated in many experimental challenge studies with various fish hosts since 2000 (Skall et al. 2005a). In these studies, the major finding regarding host specificity is that genotype Ia isolates from rainbow trout farms have high virulence for trout, while marine isolates in genotypes Ib, II, and III have low virulence for trout. This is consistent with the well supported hypothesis that VHS disease in trout farms is due to VHSV host jumps from marine reservoir fish into cultured rainbow trout, followed by adaptation to higher virulence in trout (Einer-Jensen et al. 2004). Our virulence results, showing that rainbow trout and Chinook salmon suffered higher mortalities (47 to 98% CPM) after exposure to genotype Ia than after challenge with all genotype IV isolates (2 to 38% CPM), indi-

cate that genotype Ia isolates from European trout farms pose a greater risk to salmonid species than the other tested genotypes. In western North America and Asia, VHSV isolates from Pacific genotype IVa have been examined in experimental challenges and found to vary in virulence for natural marine hosts, but they consistently have low virulence for rainbow trout or other salmonids (Meyers & Winton 1995, Hedrick et al. 2003). Since the emergence of VHSV genotype IVb in the Great Lakes region in 2003, it has also been actively studied using *in vivo* challenge experiments (Al-Hussinee et al. 2010, Kim & Faisal 2010a,b, Weeks et al. 2011, Cornwell et al. 2013). This has demonstrated that fish species vary in susceptibility to infection and mortality caused by genotype IVb, and the virus is of low virulence in most salmonids, including rainbow trout. To date, VHSV from genotype IVc has not been investigated in experimental challenge studies.

The research undertaken here is novel in simultaneously testing virulence of VHSV isolates from the European genotype Ia and North American genotype IV. A major goal of the study was to explore whether there were discernible differences in virulence between isolates from subtypes IVa, IVb, and IVc. This is of interest particularly in the western US, where VHSV genotype IVa is endemic in marine fish and is thought to pose minimal risk to aquaculture (Meyers & Winton 1995, Hedrick et al. 2003), but genotype IVb is perceived as a novel threat due to the recent emergence and potential for spread from the Great Lakes region (Faisal et al. 2012).

The mortality results from our challenge studies confirmed the expected high virulence of genotypes Ia, IVb, and IVa, in rainbow trout, yellow perch, and Pacific herring, respectively. Also as expected, genotype Ia was found to cause significantly higher mortality than the 3 genotype IV isolates in the 2 salmonid hosts (Table 2), but it also caused 30 to 86% mortality in yellow perch and 43% mortality in Pacific herring, indicating that it is not avirulent in these species. In yellow perch, genotype Ia was significantly less virulent than genotypes IVa and IVb, and in Pacific herring, it was equivalent to genotypes IVb and IVc, but less virulent than IVa. Thus, genotype Ia was either equal to, or less virulent than, the genotype IV isolates in non-salmonid hosts.

Comparison of mortality results for genotypes IVa and IVb revealed no significant difference for 7 of the 9 combinations of host species and challenge doses tested. In the other 2 experimental treatments, which were rainbow trout/high injection challenge dose and Pacific herring/low immersion challenge dose, genotype IVa caused significantly higher mortality than genotype IVb. Thus, in the 5 fish species tested here, the Great Lakes genotype IVb had equivalent or lower virulence than the West coast genotype IVa. Although this may not be universal for all fish species, these results have important implications for risk perception, suggesting that VHSV genotypes IVa and IVb should be viewed with equal caution. Comparisons of mortality results for the East coast genotype IVc revealed that it was not significantly different from IVa or IVb in Chinook or Pacific herring challenges, or from genotype IVa in rainbow trout. However, genotype IVc caused significantly higher mortality than genotype IVb in rainbow trout, and significantly lower mortality than IVa and IVb in yellow perch. Phylogenetic analyses and serological typing with monoclonal antibodies consistently indicate genotype IVc as antigentically distinct and the probable ancestor of the emergent genotype IVb in the Great Lakes (Elsayed et al. 2006, Thompson et al. 2011, Ito et al. 2012, Pierce & Stepien 2012). Therefore, the observation that genotype IVc caused significantly lower mortality than IVb in the Great Lakes yellow perch provides a preliminary indication that the emergence of genotype IVb may have been associated with an adaptation to higher virulence in yellow perch. Confirmation of this hypothesis would require additional challenge studies comparing genotypes IVb and IVc in yellow perch and other relevant Great Lakes fish species.

Although comparison of absolute mortality levels between experiments performed by different researchers must always be done with a great deal of caution, it is interesting to note that a previous study involving experimental challenges of Great Lakes yellow perch found this species to be only moderately susceptible to VHSV genotype IVb. Using the same genotype IVb isolate tested here (MI03), Kim & Faisal (2010b) challenged juvenile yellow perch by IP injection with doses ranging from $7 \times 10^2$ to $7 \times 10^7$ PFU fish$^{-1}$, and determined an IP lethal dose 50 ($LD_{50}$) of $2.5 \times 10^5$. Although we did not conduct a full $LD_{50}$ experiment, our yellow perch injected with $10^3$ or $10^6$ PFU fish$^{-1}$ experienced 90 to 100% mortality, indicating clearly that our $LD_{50}$ would be well below $10^3$ PFU. Relative to the yellow perch used in the previously reported study, our fish were younger, smaller, and likely from a different broodstock. Any of these variables might be responsible for the difference observed in susceptibility, providing a good example of variation sometimes observed in results of independent experimental challenges in fish, as noted previously by Snow et al. (2005). Although

absolute mortality levels may vary between different experiments due to the impact of numerous host, viral, and environmental factors, we would expect that the relative virulence of different virus isolates compared within each experiment should be consistent.

With regard to previous work with the other fish species tested here, our finding of low virulence for genotype IV isolates in salmonids is consistent with previously reported conclusions from several experimental challenge studies with VHSV genotypes IVa (Winton et al. 1991, Meyers & Winton 1995, Follett et al. 1997) and IVb (Kim & Faisal 2010a,b), and we extend that phenotype to IVc. Similarly, our finding of very low virulence in koi is consistent with results of challenge studies with genotype IVb (Cornwell et al. 2013), and we extend that phenotype to IVa and IVc. The high mortality of genotype IVa in Pacific herring confirms numerous publications (Meyers & Winton 1995, Hershberger et al. 2010), and the lower virulence of genotypes IVb, IVc, and Ia have not been reported previously to our knowledge. One publication has described high virulence of genotype IVb in lake herring *Coregonus artedi* from the Great Lakes region (Weeks et al. 2011), but this is a salmonid species not related to Pacific herring.

Beyond virulence, we also examined infection prevalence and persistence in the experimental challenges reported here. In the absence of disease or mortality, this is important as an indication of the potential of a fish species to serve as a vector or reservoir for virus, and also for assessing the risk of future host jumps. Our analyses of infectious virus titers in fish sampled at 7 to 14 d post-challenge clearly demonstrated that all genotypes of VHSV tested here were able to infect and replicate in at least some individual fish of each species. Prevalence of viral infection ranged from 40 to 100% for nearly all groups: yellow perch were notable for having 100% prevalence with all virus genotypes, and genotype Ia was notable as having 80 to 100% prevalence in all fish species at any timepoint tested. It was also interesting that all genotypes were detected in koi, a species thought to be resistant to VHSV infection, 7 d post-exposure, suggesting that there is potential of viral adaptation in this species. At 28 d post-challenge, all virus genotypes persisted in at least some individual yellow perch and Chinook salmon, but only genotype Ia was found in rainbow trout, and only genotype IVb in koi, with both species having the highest detected titer levels in the survivors. It was not unexpected that high virus titers for genotype Ia may occur in surviving rainbow trout, a well-established vulnerable host, but the high genotype IVb titers demonstrated in the surviving koi was unexpected, especially since mortality was so low. The potential persistence of genotype IVb in koi should be noted with caution given the ubiquitous distribution and propagation of ornamental koi.

The work presented here is subject to caveats that deserve mention. As in all experimental challenge studies, the results may be specific to the fish size and age, and to temperatures used for rearing and challenge. This may be relevant to our finding of extremely low susceptibility to disease and mortality in koi, which were larger and older than the other fish tested. However, a previous study challenging koi with genotype IVb using 2 mo old koi also demonstrated that they were highly refractory to VHS disease (Cornwell et al. 2013) at a younger age. Another caveat was that only 1 virus isolate from each genotype was tested, and it is possible that these isolates are not representative of the entire genotype or subtype. Further, the titration of the genotype Ia isolate (F1 variant) may have been skewed, since VHSV isolates from genogroup I may have lower plating efficiencies in EPC cells (Winton et al. 2007), which could have resulted in unequal challenge dose dilutions. If the Ia challenge dose was slightly greater than the other 3 IV isolate doses, it may have contributed to higher levels of mortality demonstrated in the highly susceptible species (e.g. rainbow trout and Chinook salmon). Finally, our methods constituted a 'worst-case scenario' by using IP injection challenge as the infection route. The observation that mortality was generally lower in the low-dose injection groups compared with the high-dose groups suggests the probability that there may have been even less infection and mortality if the exposures were done by immersion. Our intent in using injection challenge was to provide a most stringent test of the biological compatibility of these virus genotypes with these fish species. If any fish host had no detectable infection or mortality using injection challenge, we would have been able to conclude that host species was not at risk from exposure to VHSV. Instead, we found that all fish species could be infected, which is consistent with the known broad host range of VHSV.

In conclusion, simultaneous comparisons of VHSV European genotype Ia isolate with various North American genotype IV isolates confirmed the distinct host phenotypes previously reported in separate experimental challenge studies. We found no evidence that any genotype IV isolates are trout-adapted at this time, but they are all capable of infecting and

replicating in trout and Chinook, so there is potential for host jumps similar to those accomplished in the past by VHSV genotypes Ia (Schönherz et al. 2013), Ib (Nordblom 1998, Nordblom & Norell 2000), Id (Raja-Halli et al. 2006), and III (Dale et 2009). The most important finding here is that there was little difference in virulence or host-specificity between isolates from subtypes IVa, IVb, and IVc, suggesting that they should all be treated with appropriate caution.

*Acknowledgements*. This project was funded by Western Regional Aquaculture Center Grant 2004-38500-14698, grant 2005-38500-15812 from the US Department of Agriculture, National Institute of Food and Agriculture, and 'Exxon Valdez' Oil Spill Trustee Council, Project no. 10100132-I. We thank our colleagues who continue to support our research by donating test fish: S. LaPatra (Clear Springs Food) for rainbow trout, J. Burkard (Pan Intercorp) for koi, J. Wittouck (University of Washington) for Chinook salmon eggs, R. Goetz (now at NOAA) and F. Binkowski (University of Wisconsin Milwaukee) for yellow perch, and J. Gregg (Marrowstone Field Station) for Pacific herring. E.J.E. thanks M. Purcell for her statistics tutorial (and patience) in demonstrating the use of Graph Pad InStat; A. Wargo for help with SPSS analysis; and J. Winton for editorial assistance. This article has been peer reviewed and approved for publication consistent with USGS Fundamental Science Practices (http://pubs.usgs.gov/circ/1367/). Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government. This manuscript is submitted for publication with the understanding that the US Government is authorized to reproduce and distribute reprints for governmental purposes.

## LITERATURE CITED

Al-Hussinee L, Huber P, Russell S, LePage V and others (2010) Viral haemorrhagic septicaemia virus IVb experimental infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and fathead minnow, *Pimphales* [sic] *promelas* (Rafinesque). J Fish Dis 33:347–360

Batts WN, Winton JR (1989) Enhanced detection of infectious hematopoietic necrosis virus and other fish viruses by pretreatment of cell monolayers with polyethylene glycol. J Aquat Anim Health 1:284–290

Cornwell ER, LaBuda SL, Groocock GH, Getchell RG, Bowser PR (2013) Experimental infection of koi carp with viral hemorrhagic septicemia virus Type IVb. J Aquat Anim Health 25:36–41

Dale OB, Ørpetveit I, Lyngstad TM, Kahns S, Skall HF, Olesen NJ, Dannevig BH (2009) Outbreak of viral haemorrhagic septicaemia (VHS) in seawater-farmed rainbow trout in Norway caused by VHS virus genotype III. Dis Aquat Org 85:93–103

Einer-Jensen K, Ahrens P, Forsberg R, Lorenzen N (2004) Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. J Gen Virol 85:1167–1179

Elsayed E, Faisal M, Thomas M, Whelan G, Batts W, Winton J (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. J Fish Dis 29:611–619

Emmenegger EJ, Kurath G (2008) DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. Vaccine 26: 6415–6421

Faisal M, Shavalier M, Kim RK, Millard EV and others (2012) Spread of the emerging viral hemorrhagic septicemia virus strain, genotype IVb, in Michigan, USA. Viruses 4: 734–760

Fijan N, Sulimanovic E, Bearzotti M, Muzinic D and others (1983) Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Ann Inst Pasteur Virol 134:207–220

Follett JE, Meyers TR, Burton TO, Geesin JL (1997) Comparative susceptibilities of salmonid species in Alaska to infectious haematopoietic necrosis virus (IHNV) and North American viral haemorrhagic septicaemia virus (VHSV). J Aquat Anim Health 9:34–40

Gagné N, MacKinnon AM, Boston L, Souter B, Cook-Versloot M, Griffiths S, Olivier G (2007) Isolation of viral hemorrhagic septicemia virus from mummichog, stickleback, striped bass and brown trout in eastern Canada. J Fish Dis 30:213–223

Groocock GH, Getchell RG, Wooster GA, Britt KL and others (2007) Detection of viral hemorrhagic septicemia in round gobies in New York State (USA) waters of Lake Ontario and the St. Lawrence River. Dis Aquat Org 76: 187–192

Hedrick RP, Batts WN, Yun S, Traxler GS, Kaufman J, Winton JR (2003) Host and geographic range extensions of the North American strain of viral hemorrhagic septicemia virus. Dis Aquat Org 55:211–220

Hershberger PK, Gregg J, Pacheco C, Winton J, Richard J, Traxler G (2007) Larval Pacific herring, *Clupea pallasii* (Valenciennes), are highly susceptible to viral haemorrhagic septicaemia and survivors are partially protected after their metamorphosis to juveniles. J Fish Dis 30: 445–458

Hershberger PK, Gregg JL, Grady CA, Collins RM, Winton JR (2010) Susceptibility of three stocks of Pacific herring to viral hemorrhagic septicemia. J Aquat Anim Health 22:1–7

Isshiki T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T (2001) An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. Dis Aquat Org 47:87–99

Ito T, Kurita J, Sano M, Skall HF, Lorenzen N, Einer-Jensen K, Olesen NJ (2012) Typing of viral hemorrhagic septicemia virus by monoclonal antibodies. J Gen Virol 93: 2546–2557

Jensen MH (1965) Research on the virus of Egtved disease. Ann NY Acad Sci 126:422–426

Kane-Sutton M, Kinter B, Dennis PM, Koonce JF (2010) Viral hemorrhagic septicemia virus infection in yellow perch, *Perca flavescens*, in Lake Erie. J Gt Lakes Res 36:37–43

Kim R, Faisal M (2010a) Experimental studies confirm the wide host range of the Great Lakes viral haemorrhagic septicaemia virus genotype IVb. J Fish Dis 33:83–88

Kim R, Faisal M (2010b) Comparative susceptibility of representative Great Lakes fish species to the North American viral hemorrhagic septicemia virus Sublineage IVb. Dis Aquat Org 91:23–34

Kocan R, Bradley M, Elder N, Meyers T, Batts W, Winton J (1997) North American strain of viral hemorrhagic sep-

ticemia virus is highly pathogenic for laboratory-reared Pacific herring. J Aquat Anim Health 9:279–290

Lumsden JS, Morrison B, Yason C, Russell S and others (2007) Mortality event in freshwater drum *Aplodinotus grunniens* from Lake Ontario, Canada, associated with viral haemorrhagic septicemia virus, Type IV. Dis Aquat Org 76:99–111

Meyers TR, Winton JR (1995) Viral hemorrhagic septicemia virus in North America. Annu Rev Fish Dis 5:3–24

Meyers TR, Short S, Lipson K (1999) Isolation of the North American strain of viral hemorrhagic septicemia virus (VHSV) associated with epizootic mortality in two new host species of Alaskan marine fish. Dis Aquat Org 38: 81–86

National Research Council (NRC) (2011) Guide for the care and use of laboratory animals, 8th edn. Available at www.nap.edu/catalog/12910.html

Nordblom B (1998) Report on an outbreak of VHS in Sweden. Report 1998-05-21. Swedish Board of Agriculture, Jönköping, p 1–5

Nordblom B, Norell AW (2000) Report on an outbreak of VHS (viral haemorrhagic septicaemia) in farmed fish in Sweden. Report 2000-05-08. Swedish Board of Agriculture, Jönköping, p 1–3

Pierce LR, Stepien CA (2012) Evolution and biogeography of an emerging quasispecies: diversity patterns of the fish viral hemorrhagic septicemia virus (VHSv). Mol Phylogenet Evol 63:327–341

Raja-Halli M, Vehmas TK, Rimaila-Pärnänen E, Sainmaa S, Skall HF, Olesen NJ, Tapiovaara H (2006) Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. Dis Aquat Org 72:201–211

Ross K, McCarthy U, Huntly PJ, Wood BP and others (1995) A outbreak of viral haemorrhagic septicaemia (VHS) in turbot (*Scophthalmus maximus*) in Scotland. Bull Eur Assoc Fish Pathol 14:213–214

Schlotfeldt HJ, Ahne W, Jørgensen PEV, Glende W (1991) Occurrence of viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) — a natural outbreak. Bull Eur Assoc Fish Pathol 11:105–107

Schönherz AA, Lorenzen N, Einer-Jensen K (2013) Interspecies transmission of viral hemorrhagic septicemia virus (VHSV) from turbot (*Scophthalmus maximus*) to rainbow trout (*Onchorhynchus* [sic] *mykiss*). J Gen Virol 94:869–875

Skall HF, Olesen NJ, Mellergaard S (2005a) Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming: a review. J Fish Dis 28:509–529

Skall HF, Olesen NJ, Mellergaard S (2005b) Prevalence of viral haemorrhagic septicaemia virus in Danish marine fishes and its occurrence in new host species. Dis Aquat Org 66:145–151

Snow M, Bain N, Black J, Taupin V and others (2004) Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). Dis Aquat Org 61:11–21

Snow M, King JA, Garden A, Shanks AM, Raynard RS (2005) Comparative susceptibility of turbot *Scophthalmus maximus* to different genotypes of viral haemorrhagic septicaemia virus (VHSV). Dis Aquat Org 67:31–38

Thompson TM, Batts WN, Faisal M, Bowser P and others (2011) Emergence of *Viral hemorrhagic septicemia virus* in the North American Great Lakes region is associated with low viral genetic diversity. Dis Aquat Org 96:29–43

Traxler GS, Kieser D, Richard J (1999) Mass mortality of pilchard and herring associated with viral hemorrhagic septicemia virus in British Columbia, Canada. Am Fish Soc News 27:3–4

Weeks C, Kim R, Wolgamood M, Whelan G, Faisal M (2011) Experimental infection studies demonstrate the high susceptibility of the salmonid, lake herring, *Coregonus artedi* (Le Sueur), to the Great Lakes strain of viral haemorrhagic septicaemia virus (genotype IVb). J Fish Dis 34: 887–891

Winton JR, Batts WN, Deering RE, Brunson R, Hopper K, Nishizawa T, Stehr C (1991) Characteristics of the first North American isolates of viral hemorrhagic septicemia virus. In: Proc 2nd Int Symp Viruses of Lower Vertebrates. Oregon State University Press, Corvallis, OR, p 43–50

Winton J, Kurath G, Batts W (2007) Detection of viral hemorrhagic septicemia virus. Fact Sheet (FS 2007-3055). US Geological Survey, Western Fisheries Research Center, Seattle, WA

Wolf K (1988) Viral hemorrhagic septicemia. In: Wolf K (ed) Fish viruses and fish viral diseases. Comstock Publishing Associates, Cornell University Press, Ithaca, NY, p 217–249

*Editorial responsibility: Mark Crane, Geelong, Victoria, Australia*

*Submitted: June 17, 2013; Accepted: September 11, 2013*
*Proofs received from author(s): November 15, 2013*