**INTRODUCTION**

Viral hemorrhagic septicemia virus (VHSV) is the etiological agent of viral hemorrhagic septicemia (VHS), a devastating infectious disease of teleost fishes (Wolf 1988). The virus has a wide geographic distribution throughout the Northern Hemisphere and has been isolated from more than 80 marine and freshwater fish species (Faisal et al. 2012, OIE 2012). Nucleic acid sequencing has grouped VHSV into 4 major genotypic groups, where each displays a distinct geographic range (Skall et al. 2005, OIE 2012). Genotype I contains isolates from Europe, mainly inland freshwater and northern marine coastal areas, and is divided into 5 sublineages (‘a to e’). Genotype II consists of marine isolates from the Baltic Sea, while genotype III strains were collected from the North Sea, Skagerrak, and Kattegat. Genotype IV comprises primarily North American isolates but has also been reported from Japan and Korea (Snow et al. 2004, Nishizawa et al. 2002, Einer-Jensen et al. 2004, Elsayed et al. 2006). Molecular characterization of North American and Asian VHSV isolates has identified at least 2 sublineages (Skall et al. 2005, OIE 2012), genotype IVa, from the northern Pacific Ocean (Meyers & Winton 1995, Einer-Jensen et al. 2005) and genotype IVb from the freshwaters of the Laurentian Great Lakes (Elsayed et al. 2006). In addi-
tion, a new subgroup, IVc, consisting of estuarine and marine isolates from the Atlantic coast of North America, has recently been proposed (Gagné et al. 2007, Pierce & Stepien 2012).

The Laurentian Great Lakes represent the world's largest group of freshwater lakes and are an important source of both recreational and commercial fish species (Taylor et al. 2012). Since the first isolation from an adult muskellunge from Lake St. Clair, Michigan, in 2003 (ElSayed et al. 2006), VHSV genotype IVb emerged and spread throughout the entire Great Lakes basin as well as in several inland water bodies (Faisal et al. 2012). The virus has been reported from large die-off events of several fish species including the freshwater drum Aplodinotus grunniens (Lumsden et al. 2007), gizzard shad Dorosoma cepedianum (Kim & Faisal 2011), muskellunge Esox masquinongy (ElSayed et al. 2006), round goby Neogobius melanostomus (Grocock et al. 2007), and yellow perch Perca flavescens (Kane-Sutton et al. 2010). Outbreaks of the proposed VHSV genotype IVc have been more limited and include mummichog Fundulus heteroclitus, three-spined stickleback Gasterosteus aculeatus, striped bass Morone saxatilis, and brown trout Salmo trutta (Gagné et al. 2007). Experimental studies have identified other species susceptible to infection (Kim & Faisal 2010a,b, Grocock et al. 2012, Cornwell et al. 2013a,b, Grice et al. 2014), and previously, the US Department of Agriculture–Animal and Plant Health Inspection Service (USDA-APHIS) listed 28 VHSV-susceptible freshwater species that are typically found in the Great Lakes region (USDA-APHIS 2008).

Horizontal transmission from fish to fish through water, via excretion of virus in the urine, was traditionally suggested as one means of transmitting the VHSV virus (Kim & Faisal 2011), although viral shedding from the skin may also be a possibility for some species (Smail & Snow 2011). Recently, an oral transmission route was demonstrated for rainbow trout (Schönhrez et al. 2012) and tiger muskellunge (Getchell et al. 2013). A diverse number of animals, such as amphipod crustaceans (Faisal & Winters 2011), leeches (Faisal & Schulz 2009), freshwater turtles (Goodwin & Merry 2011), and piscivorous birds (Peters & Neukirch 1986), have been reported by several investigators as possible vectors, in addition to ballast water and boating (Bain et al. 2010). However, the movement of infected fish, for example baitfish (Goodwin et al. 2004, Getchell et al. 2013, Phelps et al. 2013), represents a risk factor for introducing VHSV to new areas (VHSV Expert Panel and Working Group 2010). Goldfish Carassius auratus are capable of adapting to a range of habitats worldwide and expanding to new environments where they may introduce exotic diseases. Viral infections in goldfish have been reported in Japan, Australia, Taiwan, and the USA (Humphrey & Ashburner 1993, Jung & Miyazaki 1995, Arthington & McKenzie 1997, Goodwin et al. 2006, Sadler et al. 2008). Goldfish are one of the major commercially farmed freshwater species in global aquaculture (FAO 2012), and nearly 150 million fish were produced and traded in the USA in 2005 (USDA 2006).

Other cyprinid species have displayed some level of susceptibility to VHSV infection (Al-Hussinee et al. 2010, Frattini et al. 2011, Cornwell et al. 2013a). Emmenegger et al. (2013) tested koi Cyprinus carpio koi susceptibility to 4 strains of VHSV. A report on the susceptibility of 2 new goldfish cell lines to 3 rhabdoviruses, infectious hematopoietic necrosis virus, snakehead rhabdovirus, and spring viremia of carp virus, suggested possible VHSV susceptibility in goldfish (Rougée et al. 2007). However, to our knowledge, there are no reports of VHSV being detected in goldfish, although there is some experimental evidence of susceptibility where an immersion trial using liver extract and cell culture supernatant was conducted; however, the data were insufficient to completely assess and verify goldfish as a VHSV-susceptible species (EFSA 2008). In this study, we examined different exposure routes of VHSV genotype IVb in goldfish to investigate whether this species is susceptible to infection under experimental conditions. The fish were challenged by injection, immersion, or feeding.

**MATERIALS AND METHODS**

**Fish**

Goldfish less than 1 yr old were purchased from a local pet store, a national pet store chain, and a commercial bait fish farm. All fish were initially held in 700 l fiberglass tanks (Frigid Units) at 15 ± 1°C with flow-through, chlorine-free, filtered water. Fish were inspected daily, maintained on a photoperiod of 12 h of daylight, and fed a commercial diet. Prior to the experiments, 10 fish were examined from each source, and several fish tested positive for monogenean parasites. All fish were treated 3 times with long-duration, low-dose formalin baths (3 to 24 h at 25 ppm) to remove the infestation prior to each experiment. Throughout the experiments, strict biosecurity protocols were maintained to ensure that
virus was not transferred between tanks of fish or outside of experimental rooms. Wastewater from the exposed tanks was disinfected with 12.5% sodium hypochlorite to maintain a free residual chlorine concentration of at least 1.0 mg l\(^{-1}\) for at least 10 min before discharge into the municipal sewage lines. The Cornell University Institutional Animal Care and Use Committee approved all use of animals.

**VHSV challenge isolate**

The MI03 isolate of VHSV (Elsayed et al. 2006) was propagated in epithelioma papulosum cyprini (EPC) cells (Winton et al. 2010), stored in Minimal Essential Medium with Hanks’ salts (HMEM) prepared with 10% fetal bovine serum (HMEM-10) at \(-80^\circ\text{C}\) (Grocock et al. 2007), and quantified using standard plaque assay protocols at the time of exposure to fish (Batts & Winton 1989).

**Expts 1 and 2: Intraperitoneal injection trials**

**Expt 1: Infection assessed by cell culture and PCR**

Fish were initially acclimated for 2 wk in a 700 l fiberglass tank at \(15 \pm 1^\circ\text{C}\). Then, 120 goldfish were randomized into 2 tanks using a randomizer program (Haahr 2011). One tank held 96 experimental fish, while the control tank held 24 fish. The water temperature in both tanks was lowered and kept at \(10 \pm 1^\circ\text{C}\). After 7 d, the 96 experimental fish were randomized into 12 plastic tanks (15 l), each containing 8 fish, while the 24 control fish were randomized into 3 flow-through plastic tanks, each containing 8 fish. Each fish received an intraperitoneal injection of either 0.1 ml of HMEM-5 containing VHSV type IVb MI03 at \(10^7\) plaque-forming units (pfu) ml\(^{-1}\) (experimental fish) or 0.1 ml of HMEM-5 (control fish) during the randomization process. The fish were monitored daily, mortalities were recorded, and moribund fish were euthanized with MS-222 as described above. Any fish removed for health reasons were counted as part of that week’s periodic 5-fish sample. To determine its infection status, the left pectoral fin was removed from each fish after euthanasia and analyzed as described by Cornwell et al. (2013c). Without damaging the internal organs of each sampled fish, an incision was made along the ventral midline to allow proper fixation in 10% neutral buffered formalin (v/v) for 48 h, and the fish was then placed in a decalcification solution for at least 1 wk prior to histological evaluation. These fish were routinely processed, sectioned at 5 µm, and stained with hematoxylin and eosin (Luna 1968).

**Expt 2: Infection assessed by histopathology**

An additional injection trial was set up to more accurately study the viral pathogenesis by obtaining samples for histology, using the same dose and challenge method as in Expt 1. The 35 goldfish were randomized into 4 (15 l) flow-through tanks, 3 experimental and 1 control. Two of the experimental tanks contained 8 fish each, while 9 fish were placed in the third. Ten fish were placed in the control tank. Five experimental fish (1 to 2 fish per virus treatment tank per week) as well as 2 control fish were randomly sampled each week for the 35 d trial. The fish were monitored daily, mortalities were recorded, and moribund fish were euthanized with MS-222 as described above. Any fish removed for health reasons were counted as part of that week’s periodic 5-fish sample. To determine its infection status, the left pectoral fin was removed from each fish after euthanasia and analyzed as described by Cornwell et al. (2013c). Without damaging the internal organs of each sampled fish, an incision was made along the ventral midline to allow proper fixation in 10% neutral buffered formalin (v/v) for 48 h, and the fish was then placed in a decalcification solution for at least 1 wk prior to histological evaluation. These fish were routinely processed, sectioned at 5 µm, and stained with hematoxylin and eosin (Luna 1968).

**Expt 3: Immersion trial**

Fifty goldfish acclimated for 4 wk in a 700 l fiberglass tank at \(15 \pm 1^\circ\text{C}\) were first randomly divided into experimental and control groups. Forty goldfish were then exposed to the virus in a single 6 l bath of VHSV type IVb MI03 at a final concentration of \(10^5\) pfu ml\(^{-1}\) for 24 h, while 10 goldfish were exposed in a control 6 l bath that contained equivalent concentrations of sterile HMEM-5 (1 ml l\(^{-1}\)) for 24 h. The water temperature in both tanks was lowered and kept at \(10 \pm 1^\circ\text{C}\) during the immersion challenge, and additional aeration was provided during this static condition (external water circulating around the static tanks). The virus-exposed fish were then randomized.
into 4 separate tanks (15 l), with 10 fish in each tank, with flow-through water maintained at 10 ± 1°C. The fish were monitored and processed during the 42 d trial following the same procedures as in Expt 1, except that all harvested tissues were stored in RNAlater at −80°C for qRT-PCR analysis.

**Expt 4: Feeding trial**

In this experiment, 120 goldfish were initially randomized into 2 groups, 1 experimental (96 fish) and 1 control (24 fish), and kept at 15 ± 1°C in separate 700 l fiberglass tanks. Two days later, the fish were randomized again: the experimental fish were placed into 12 plastic tanks (15 l) with 8 fish in each, while the control fish were placed in 3 tanks with 8 fish in each. The water temperature was lowered to 12 ± 1°C for the duration of the experiment. The feeding trial was initiated the next day; 0.4 g of a commercial fish feed was placed in a small weigh boat (46 × 46 × 8 mm) and soaked in either 1 ml of HMEM-5 containing VHSV type IVb at a concentration of 10^7 pfu ml⁻¹ (experimental fish) or 1 ml of HMEM-5 (control fish) for 5 min and then fed to each group of 8 fish. Day 0 water samples were collected from each tank along with soaked feed samples to confirm the quantity of VHSV the goldfish were exposed to. The fish were monitored and processed during the 42 d trial following the same procedures as in Expt 1, except that all harvested tissues were stored in RNAlater at −80°C for qRT-PCR analysis.

**RNA extraction and qRT-PCR testing**

Experimental and control samples were thawed and stored on ice at all times during processing. HMEM-10 (200 µl) was added to the tissues and feed samples in sterile 2 ml homogenizing tubes containing a single 1.3 mm chrome steel bead. Samples were then homogenized using a Minibeater-16 (BioSpec Products) for 1 min followed by centrifugation at 500 × g for 2 min prior to RNA extraction. Fifty microliters of the supernatant was used for the RNA extraction, which was performed using a MagMax magnetic bead extraction system and the MagMax-96 viral RNA isolation kit (Life Technologies) using the protocols described in the kit and the manufacturer-supplied extraction program AM1836_DW_50_V2. Eluted RNA was immediately placed in sterile microcentrifuge tubes following extraction and frozen at −80°C until use in the qRT-PCR assay.

The qRT-PCR assay used was slightly modified from that described by Hope et al. (2010) in that plates were run on an ABI 7500 detector, a linear regression was calculated from 5 RNA standards ranging from 1.5 × 10^2 to 1.5 × 10^6, and all plates were run for 42 cycles. Positive and negative controls were included in all runs. RNA quality and quantity were measured using a NanoVue spectrophotometer (GE Healthcare). All qRT-PCR copy numbers are reported per 50 ng total RNA.

**Virus isolation in cell culture**

VHSV infection was detected using isolation in cell culture for Expt 1 only (AFS-FHS 2010). The combined aliquots of spleen, heart, liver, and anterior and posterior kidney from each fish were pooled in groups of 3 or 4 and then homogenized, centrifuged for 2 min at 500 × g, diluted to 1:49 with HMEM-5 (Groocock et al. 2007), and filtered through a sterile 0.45 µm biological filter. A portion of the filtered homogenate was further diluted to 1:249, and both the 1:49 and 1:249 dilutions were plated in triplicate onto 48-well cell culture plates containing confluent monolayers of EPC cells less than 48 h old, for a final in-well dilution of 1:99 and 1:499, respectively. All plates were placed in a 15°C incubator and monitored at least twice a week for cytopathic effects (CPE). All plates were passaged at least twice to determine the presence of virus. Confirmation of VHSV isolation was performed by qRT-PCR assay. Individual brain samples also were pooled in groups of 3 or 4 and processed for virus isolation as described above.

**Statistical analysis**

Chi square tests on difference in prevalence (proportion of fish infected with VHSV) between virus-exposed and control groups or between the qRT-PCR positive pooled organs and qRT-PCR positive brain samples were performed using GraphPad Prism 6 statistical online software (GraphPad Software 2014).

**RESULTS**

**Expt 1: Intraperitoneal injection (cell culture and PCR)**

Eleven deaths in total occurred in the control (n = 1) and virus-exposed (n = 10) fish during the 42 d trial
The first 4 dead fish were recorded between Days 2 and 10. The last 7 dead fish were recorded between Days 21 and 40. Common clinical signs of VHS were found in 9 of the dead fish, including swollen abdomen, external and internal hemorrhage, and pale organs (see Table 1). These 9 virus-exposed fish also showed high levels of virus by qRT-PCR assay of their tissues (Fig. 1). One control and 1 virus-exposed fish were found dead on Day 2, and they both tested negative by qRT-PCR. The pooled organs from the 84 surviving virus-exposed fish showed high levels of VHSV RNA, with 13 of 35 positive fish $>1 \times 10^3$ N-gene copies per 50 ng of RNA (Fig. 1). The brain samples from the 84 survivors also showed high quantities of VHSV RNA, with 21 of 65 positive fish $>1 \times 10^3$ N-gene copies per 50 ng of RNA (Fig. 1). None of the control fish showed any clinical signs of VHSV or tested positive by qRT-PCR when their tissues were assayed. At the end of the trial, 2 fish were unaccounted for, and the cumulative percent mortality was assessed at 10% (10/96) in the virus-exposed fish, 42% (35/84) of the survivors were positive by qRT-PCR from pooled organ samples, and 77% (65/84) of the survivors were positive by qRT-PCR from brains after holding them for 42 d, which was significantly higher than the pooled organ samples and the control samples ($p < 0.0001$) (Fig. 1). Virus isolation in cell culture was successful for all of the 3- or 4-fish combined samples from the surviving virus-exposed fish, which included both qRT-PCR positive and qRT-PCR negative fish, as well as the 11 individual mortalities (with the exception of 1 virus-exposed fish and 1 control fish that both died on Day 2 post exposure). Virus isolation from the remaining control fish also tested negative. RNA from extracted cell culture supernatants was amplified by qRT-PCR to confirm the presence of VHSV in each sample that developed CPE.

**Expt 2: Intraperitoneal injection (histopathology)**

There was a 32% mortality (8/25) in the VHSV IVb injected fish (Fig. 2). Forty percent (10/25) of the left pectoral fins from the virus-exposed fish were positive by qRT-PCR versus none of the control fish ($p < 0.05$). Week 4 and 5 fins were negative by qRT-PCR, which coincided with when the mortalities stopped. The histological sections of infected fish exhibited classic hemorrhagic lesions (Fig. 3A–E). Eighteen of

<table>
<thead>
<tr>
<th>Day post infection</th>
<th>External</th>
<th>Clinical signs</th>
<th>Internal</th>
<th>Fish state</th>
<th>Tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Swollen abdomen</td>
<td>NSF; autolyzed</td>
<td>Dead</td>
<td>C3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Post-mortem hemorrhage of eye</td>
<td>NSF; autolyzed</td>
<td>Dead</td>
<td>E1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Distended abdomen with petechial hemorrhage</td>
<td>Red to clear ascites; swollen, fluid-filled intestines; pale gills and heart</td>
<td>Moribund</td>
<td>E4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Distended abdomen with petechial hemorrhage on ventrum</td>
<td>Serosanguinous ascites; Dead</td>
<td>E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>NSF</td>
<td>Serosanguinous fluid; peritoneal hemorrhage; pale heart; intestines empty; pale gills</td>
<td>Dead</td>
<td>E3</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>NSF</td>
<td>Swollen and pale spleen; hemorrhagic liver; pale intestines; hemorrhagic posterior kidney; pale gills</td>
<td>Dead</td>
<td>E7</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Swollen abdomen; small hemorrhage in left eye</td>
<td>Moderate intestinal hemorrhage; pale kidneys; moderate serous fluid in abdomen</td>
<td>Dead</td>
<td>E12</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Injury to vent from siphoning tanks</td>
<td>Pale heart</td>
<td>Moribund</td>
<td>E1</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Deformed head; otherwise NSF</td>
<td>Light serous fluid in abdomen; pale posterior kidney</td>
<td>Dead</td>
<td>E7</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Small hemorrhage in left eye</td>
<td>Pale heart; pale posterior kidney</td>
<td>Moribund</td>
<td>E8</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Swollen abdomen</td>
<td>Severe ascites; pale heart; pale kidneys</td>
<td>Dead</td>
<td>E4</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Signs of disease observed in all dead/moribund goldfish during Expt 1, viral hemorrhagic septicemia virus injection challenge. C: control; E: virus-exposed; NSF: no significant findings.
the 25 virus-exposed fish had hemorrhaging in the liver, kidneys, brain, intestines, or eyes. The severity of gross internal signs often coincided with the external symptoms the fishes displayed (Table 2). Most common signs observed included exophthalmia, petechial hemorrhage of the skin, ascites, internal hemorrhages, and enlarged spleen. Lepidorthosis also was noted in a few fish with severely swollen abdomens (Fig. 3F). Unidentified microsporidian cysts in the brains and spinal cords were observed in a few of both the virus-exposed and control fish (Fig. 3G). The presence of these parasites was considered a background finding that was of no significance to the present study.

Expt 3: Immersion trial

None of the 40 goldfish immersed for 24 h in $10^5$ pfu ml$^{-1}$ of VHSV showed any clinical signs, and no mortalities were recorded during the 42 d trial. Pooled tissues and brain from the virus-exposed fish as well as the 10 controls were negative when tested by qRT-PCR. Viral isolation was not conducted after qRT-PCR because past experience showed that the molecular assay was more sensitive than culture.

Expt 4: Feeding trial

In this modified feeding trial, the 8 fish in each experimental tank rapidly ate the commercial feed soaked in 1 ml of HMEM-5 containing $10^7$ pfu VHSV type IVb. Testing of Day 0 water samples and feed soaked in VHSV by qRT-PCR confirmed the quantity of virus consumed as well as a low level of VHSV in the water just subsequent to feeding (data not shown). Only a single moribund goldfish and a
Fig. 3. Expt 2 histopathology. (A) Day 5 goldfish mortality: spleen depleted of hemocytes (scale bar = 100 µm). (B) Day 7 moribund goldfish: liver hemorrhage (scale bar = 100 µm). (C) Day 15 goldfish mortality: posterior kidney necrosis (scale bar = 50 µm). (D) Day 5 goldfish mortality: hemorrhage in the meninges (scale bar = 500 µm). (E) Day 5 goldfish mortality: hemorrhage in the eye (scale bar = 500 µm). (F) Day 20 moribund goldfish: lepidorthosis (scale bar = 500 µm). (G) Day 28 control goldfish: unidentified microsporidian cysts in the brain (scale bar = 50 µm). Insets (A–G): Normal age-matched control tissue. (H) Day 5 goldfish mortality: intracranial hemorrhage and bilateral exophthalmia (scale bar = 5 mm)
Table 2. Signs of disease observed in goldfish during Days 5 to 21 of Expt 2, viral hemorrhagic septicemia virus injection challenge.

<table>
<thead>
<tr>
<th>Day post infection</th>
<th>External Clinical signs</th>
<th>Internal Clinical signs</th>
<th>Fish state</th>
<th>Tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Bilateral exophthalmia; intraocular hemorrhage in upper part of right eye; petechial hemorrhage at base of left eye; intracranial hemorrhage</td>
<td>Ascites present in peritoneal cavity</td>
<td>Dead</td>
<td>E1</td>
</tr>
<tr>
<td>6</td>
<td>NSF; autolyzed</td>
<td>NSF; autolyzed</td>
<td>Dead</td>
<td>E1</td>
</tr>
<tr>
<td>7</td>
<td>Swollen abdomen; moderate intestinal hemorrhage</td>
<td>Serosanguinous ascites;</td>
<td>Sampled</td>
<td>E2 W</td>
</tr>
<tr>
<td>7</td>
<td>NSF</td>
<td>Light intestinal hemorrhage</td>
<td>Dead</td>
<td>E3</td>
</tr>
<tr>
<td>7</td>
<td>Swollen abdomen; bilateral exophthalmia; light intestinal hemorrhage</td>
<td>Serosanguinous ascites;</td>
<td>Moribund</td>
<td>E3</td>
</tr>
<tr>
<td>14</td>
<td>NSF; light intestinal hemorrhage</td>
<td>Slightly enlarged spleen;</td>
<td>Sampled</td>
<td>E1 W</td>
</tr>
<tr>
<td>14</td>
<td>Petechial hemorrhage on left side of the body, just above the operculum</td>
<td>Ascites present in peritoneal cavity; moderate intestinal hemorrhage; enlarged spleen.</td>
<td>Sampled</td>
<td>E1 W</td>
</tr>
<tr>
<td>14</td>
<td>Bilateral exophthalmia; petechial hemorrhage on the caudal fin</td>
<td>Moderate intestinal hemorrhage</td>
<td>Sampled</td>
<td>E2 W</td>
</tr>
<tr>
<td>14</td>
<td>Slight bilateral exophthalmia; petechial hemorrhages on the left caudal part of the body and at the base of the right pectoral fin</td>
<td>Ascites in peritoneal cavity; moderate hemorrhage on intestines and body wall</td>
<td>Sampled</td>
<td>E3 W</td>
</tr>
<tr>
<td>14</td>
<td>Bilateral exophthalmia; swollen abdomen</td>
<td>Severe serosanguinous ascites; moderate hemorrhage on intestines and swim bladder</td>
<td>Sampled</td>
<td>E3 W</td>
</tr>
<tr>
<td>15</td>
<td>Bilateral exophthalmia; swollen abdomen; pale gills</td>
<td>Severe serosanguinous ascites; moderate hemorrhage on intestines and body wall</td>
<td>Dead</td>
<td>E1</td>
</tr>
<tr>
<td>15</td>
<td>Bilateral exophthalmia; swollen abdomen; pale gills</td>
<td>Severe serosanguinous ascites; moderate hemorrhage on intestines, body wall, caudal swim bladder, and posterior kidney; dark red, enlarged spleen</td>
<td>Dead</td>
<td>E3</td>
</tr>
<tr>
<td>17</td>
<td>Lethargic; problems maintaining buoyancy; loss of appetite; reduced respiration rate; pale gills; bilateral exophthalmia; severe swollen abdomen with protruding scales; petechial hemorrhages of skin caudal to the operculum</td>
<td>Ascites present in peritoneal cavity; moderate hemorrhage on intestines, body wall, caudal swim bladder, and posterior kidney; dark red, enlarged spleen</td>
<td>Moribund</td>
<td>E1</td>
</tr>
<tr>
<td>20</td>
<td>Lethargic; loss of appetite; protruding scales and reduced respiratory rate; bilateral exophthalmia, right eye larger than the left; swollen abdomen; pale gills; petechial hemorrhages of skin, caudal to the operculum</td>
<td>Red, hemorrhagic fluid in the abdominal cavity; intestinal hemorrhages; dark pigmentation spots along the interior wall of the body cavity and on the internal organs; dark red spleen</td>
<td>Moribund</td>
<td>E2</td>
</tr>
<tr>
<td>21</td>
<td>Bilateral exophthalmia</td>
<td>NSF</td>
<td>Moribund</td>
<td>E2 W</td>
</tr>
</tbody>
</table>

E: virus-exposed; NSF: no significant findings; W: weekly sample
single dead goldfish were observed, each with clinical signs characteristic of VHSV infection, at Days 5 and 11, respectively. Pooled tissues and brain from these 2 fish as well as the remaining goldfish assayed on Day 42 of Expt 1 were negative when tested by qRT-PCR. Viral isolation was not attempted.

**DISCUSSION**

We concluded that goldfish have limited susceptibility to VHSV genotype IVb. The mortality of infected goldfish was relatively low compared to other freshwater fish species (Groocock et al. 2012, Cornwell et al. 2013b,c), but high numbers of goldfish that survived infection were carriers of the virus in the brain at the end of Expt 1. Other investigators have reported chronic VHSV infections persistent in nervous tissue (Neukirch & Glass 1984, Neukirch 1986, Hershberger et al. 2010, Lovy et al. 2012). Brudeseth et al. (2002) found VHSV in brain specimens as early as Day 5 post infection and virus present in the neurons and neuroglia cells; all sections of the brain were infected, without any pattern of cell specificity. Olson (2013) observed a significant VHSV load in the brain of yellow perch, particularly in the later stages of infection, and suggested that latent virus might remain in the brain neurons undetected in asymptomatic carriers of the virus, classifying the VHS virus as neurotropic as well as hemorrhagic. Snow et al. (2000) proposed that the brain offers the potential for virus to persist and escape immunodetection, and this may have significant implications in the maintenance of viral reservoirs in wild fish.

Failure to detect VHSV by qRT-PCR from fin samples in Expt 2 at Days 28 and 35 was interesting, given that many positive samples were found in pooled organs and brain samples on Day 42 of Expt 1. The goldfish age in both experiments was similar and the dose of virus injected identical, but the fish were from different commercial sources. Failure to detect the virus in the fin samples after 3 wk may be linked to undetectably low levels of virus or perhaps related to the low susceptibility of goldfish. Future studies should examine the suitability of fin samples as a non-lethal option when chronic levels of VHSV are suspected.

The failure to observe morbidity and mortality in Expt 3 has been found in other susceptibility trials with VHSV IVb (Al-Hussinee et al. 2010, Groocock et al. 2012, Cornwell et al. 2013a,b, Grice et al. 2014). Grice et al.’s (2014) study with walleye exposed by waterborne challenge showed that both skin and gill were the most prominent tissues infected during early time points (0 to 8 d) and that only the skin was highly immunopositive during the later stages (9 to 26 d). Including earlier sampling time points and the addition of gill, skin, or intestine samples in future susceptibility trials may be warranted. The very limited evidence of disease (clinical signs only) in Expt 4 contrasted with the success in transmitting VHSV by feeding infected live baitfish to tiger muskellunge (Getchell et al. 2013). Clinical signs and histopathological changes associated with VHS are variable and cannot be used for definitive diagnosis. The possibility of low, undetectable levels of virus and false negative results cannot be ruled out. The Hope et al. (2010) qRT-PCR assay used in this study has similar limit of detection capabilities as 3 other published assays when testing for VHSV IVb isolates only (Warg et al. 2014). A similar feeding trial with VHSV-containing pellets fed to Atlantic cod came to the conclusion that they did not reach a high enough minimum dose to establish infection with their experiment, which may have been the case in our experiment (Snow et al. 2009). We observed goldfish eating the pellets immediately, and there was no feed remaining after 20 to 30 s. Feed assays and Day 0 water samples confirmed the quantity of the virus in this exposure method. VHSV is very sensitive to low pH (Mas et al. 2004); thus, the properties of the goldfish digestive tract may have prevented this route of virus entry, or goldfish are just not very susceptible to VHSV by the waterborne and feeding routes.

While these experiments showed that young goldfish had limited susceptibility to VHSV genotype IVb, if goldfish were to become infected in their natural environment with VHSV, they could become a major problem. Goldfish are used as bait, whether captured wild or from commercial sources, and are moved from place to place in many US states even though several states have made that practice illegal (Nathan et al. 2015). Baitfish will remain a disease hazard in North American waters and should be a significant part of the risk assessment process for VHSV (Phelps et al. 2014).

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