Retail Baitfish in Michigan Harbor Serious Fish Viral Pathogens

Trainat Boonthai, Thomas P. Loch, Qingli Zhang, and Michelle Gunn Van Deuren
Aquatic Animal Health Laboratory, Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, 1129 Farm Lane, Room 177K, East Lansing, Michigan 48824, USA

Mohamed Faisal*
Aquatic Animal Health Laboratory, Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, 1129 Farm Lane, Room 177K, East Lansing, Michigan 48824, USA; and Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan 48824, USA

Gary E. Whelan and Seth J. Herbst
Michigan Department of Natural Resources, Fisheries Division, Post Office Box 30466, Lansing, Michigan 48909, USA

Abstract
Indigenous small cyprinid fish species play an important role in Great Lakes ecosystems and also comprise the backbone of a multimillion-dollar baitfish industry. Due to their widespread use in sport fisheries of the Laurentian Great Lakes, there are increasing concerns that baitfish may introduce or disseminate fish pathogens. In this study, we evaluated whether baitfish purchased from 78 randomly selected retail bait dealers in Michigan harbored fish viruses. Between September 2015 and June 2016, 5,400 baitfish divided into 90 lots of 60 fish were purchased. Fish were tested for the presence of viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), golden shiner reovirus (GSRV), fathead minnow nidovirus (FHMNV), fathead minnow picornavirus (FHMPV), and white sucker bunyavirus (WSBV). Using the epithelioma papulosum cyprini cell line and molecular confirmation, we demonstrated the presence of viruses in 18 of the 90 fish lots (20.0%) analyzed. The most prevalent virus was FHMNV, being detected in 6 of 30 lots of Fathead Minnow Pimephales promelas and 3 of 42 lots of Emerald Shiners Notropis atherinoides. We also confirmed GSRV in two fish species: the Golden Shiner Notemigonus crysoleucas (5 of 11 lots) and Fathead Minnow (3 of 30 lots). Two VHSV (genotype IVb) isolates were recovered from a single lot of Emerald Shiners. No SVCV, FHMPV, or WSBV was detected in any of the fish examined. Some of the infected fish exhibited clinical signs and histopathological alterations. This study demonstrates that live baitfish are a potential vector for the spread of viral pathogens and underscores the importance of fish health certifications for the Great Lakes baitfish industry.

Invasions of aquatic species have inundated the Laurentian Great Lakes (Mills et al. 1993), and invasive pathogens continue to threaten native aquatic communities. Unfortunately, little is known about the nature of these multiple invasions, ranging from microscopic pathogens to vertebrates, and factors that contribute to their establishment.

*Corresponding author: faisal@cvm.msu.edu
1Present address: Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong 266071, China.
Received October 26, 2017; accepted June 20, 2018
in the new environment. Mandrak and Cudmore (2013) incriminated commercial shipping, live baitfish trade, migration, and releasing hatchery-raised fish into public waters as potential routes of invasive species introduction into the semi-closed Great Lakes ecosystem. Although it has been recognized that the live baitfish trade contains inherent risks for the introduction of aquatic invasive species (Kilian et al. 2012; Drake and Mandrak 2014), the documented evidence of its potential to introduce and spread harmful invasive pathogens is only beginning to be exposed (Phelps et al. 2014a; Brabec et al. 2016; Boonthai et al. 2017a). It is essential that research illuminating these risks become available to provide managers with adequate tools for handling potential threats to the aquatic ecosystem resulting from future introductions.

Native cyprinids, particularly those that serve as a forage base, are integral to the stability of the aquatic ecosystem and food web of the Great Lakes. These fish are often collected or farmed to be sold as baitfish for sportfishing, an industry that contributes more than $20 million in annual revenues for Michigan’s economy (Michigan Department of Natural Resources [MDNR], unpublished data) and likely contributes revenues in the tens of millions of dollars across North America (USDA National Agriculture Statistics Service 2006). Baitfish are also widely used as forage for piscivorous fish species that are raised for stock enhancement programs at state and federal hatcheries (e.g., Walleye Sander vitreus and Muskelunge Esox masquinongy). Since baitfish can harbor pathogens, they may be a source for the introduction and dissemination of fish pathogenic viruses, bacteria, and parasites to susceptible fish species, including farmed and free-ranging fish (Faisal 2007), and can then result in significant epizootic events with high mortalities (Faisal 2007).

Baitfish in Michigan are not only collected from within the Great Lakes basin but also from several out-of-state sources. Using a variety of supply chains, minnows are transported from source waters to holding facilities and then to retailers. This network can serve as a potential vector for efficiently transferring invasive pathogens to widely distributed bodies of water. Baitfish that are harvested and sold within Michigan are largely composed of Emerald Shiners Notropis atherinoides, Spottail Shiners Notropis hudsonius, White Suckers Catostomus commersonii, and Sand Shiners Notropis stramineus. Primary collection for these wild minnows within Michigan occurs along the shores of Lake Huron, including Saginaw Bay and the St. Clair River, and supports recreational ice fishing activities in the winter and popular Yellow Perch Perca flavescens and Walleye fisheries in the spring. In addition, retail baitfish shops import minnows from other states, mainly from aquaculture operations in Arkansas, Minnesota, South Dakota, and North Dakota, as well as wild-harvested fish from the Wisconsin River in Wisconsin. The majority of minnow species imported from aquaculture facilities into Michigan are Fathead Minnow Pimephales promelas, Golden Shiners Notemigonus crysoleucas, and White Suckers, along with a smaller number of wild-caught Emerald Shiners and White Suckers.

Michigan regulations currently prohibit the export of any wild-caught baitfish. Imported minnows from aquaculture facilities can be found nearly anywhere in the state. All in-state Emerald Shiners, Spottail Shiners, and White Suckers require certification for the viral hemorrhagic septicemia virus (VHSV), while all imported minnows require testing for both VHSV and Heterosporis sp. Although fish health inspections for some pathogens in Michigan are conducted for specific baitfish species, current inspections are inadequate to fully assess the loads of harmful pathogens in the baitfish trade. There is a dire need to determine the prevalence of viruses in the baitfish trade and the risk of nonnative viruses spreading throughout Michigan.

In the past 16 years, a number of viruses has been isolated from several cyprinid species commonly used in the baitfish industry. For example, a novel coronavirus, the fathead minnow nidovirus (FHNV), was isolated from farmed Fathead Minnow during a mortality episode (Iwanowicz and Goodwin 2002). Subsequent studies revealed that FHNV could infect Muskellunge naturally (Faisal et al. 2016) and experimentally (Baird 2015), causing high morbidity and mortality. In the same context, Plumb et al. (1979) isolated an Aquareovirus C, known as the golden shiner reovirus (GSRV), from moribund Golden Shiners during the summer of 1977. Additional viruses were also recently detected in baitfish, including the fathead minnow picornavirus (FHMPV; Phelps et al. 2014b) and the white sucker bunyavirus (WSBV; Hutchings 2015). The pathogenicity of these viruses to piscivorous fish remains to be elucidated.

During recent VHSV outbreaks in the Laurentian Great Lakes, VHSV genotype IVb was isolated from a number of native fish species, including Emerald Shiners and Spottail Shiners, from 2006 to 2017 (Faisal et al. 2012). This led to concerns regarding the potential dissemination of VHSV along with infected baitfish into other waterbodies by sport anglers. Similar concerns arose regarding another World Organization for Animal Health (OIE)-reportable virus, the spring viremia of carp virus (SVCV), which has a wide host range that includes most cyprinid species (OIE-2016) and was isolated from Common Carp Cyprinus carpio in Hamilton Harbor, Lake Ontario, within Great Lakes waters (Garver et al. 2007).

The purpose of this study was to assess the presence of two OIE-notifiable fish viruses (i.e., VHSV and SVCV), as well as four other baitfish-associated viruses, in the baitfish supply chain in Michigan. Because baitfish can carry
Viruses capable of infecting economically and ecologically important fish species, there is a critical need to examine the role that retail baitfish dealers may play in disseminating baitfish infected with viruses. Given the potential public trust resource damage these pathogens could cause from the indiscriminate movement across the state due to baitfish, this information will be used to develop and improve management and disease prevention tools.

METHODS

Fish and sample collections.—One or two lots of baitfish, each lot constituting 60 fish of a single species, were anonymously purchased from retail stores (n = 78) throughout Michigan by personnel (Figure 1). The retail stores sampled were randomly selected from a list of all licensed baitfish shops in Michigan. In addition, the baitfish lots purchased during each sampling event were later categorized as originating from an in-state or out-of-state collection source based on post hoc personal communications with individual baitfish shop owners. The source categories were used to determine the effect that importing or exporting baitfish would have on the potential introduction or spread of viral pathogens.

During the course of this study (from September 2015 to June 2016), we examined a total of 5,400 baitfish (90 lots total), including 42 Emerald Shiner lots, 30 Fathead Minnow lots, 11 Golden Shiner lots, 3 Sand Shiner lots, and 1 lot each of Spottail Shiner, Northern Redbelly Dace Chrosomus eos, and Blacknose Dace Rhinichthys atratulus. In one case, 60 fish of a single species were not available; thus, one lot consisted of 30 Weed Shiners Notropis texanus and 30 Sand Shiners.

The fish were transported alive to the Aquatic Animal Health Laboratory at Michigan State University (MSU), East Lansing, where they were examined for the presence of behavioral changes as well as external clinical disease signs within 24 h of collection from the baitfish stores. Fish were then euthanized using an overdose (250 mg/L) of sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Western Chemical Laboratories, Ferndale, Washington). Fish were individually dissected using sterile scissors and forceps and were examined for the presence of internal gross lesions. All fish handling, clinical examination, anesthesia, euthanasia, and sample collection were approved by the MSU Institutional Animal Care and Use Committee (AUF 09/15-138-00).

Tissue processing and virus testing.—Kidney, spleen, and heart tissues were aseptically collected from each fish. Samples from five fish were pooled in sterile Whirl-Pak bags (Nasco Whirl-Pak, Modesto, California) and kept frozen at −80°C until processed. For virus isolation, pooled tissue samples were diluted (1:10, weight/volume) in Eagle’s minimum essential medium (EMEM; Gibco Life Technologies, Grand Island, New York) supplemented with 0.3% tryptose phosphate broth (TPB; BD Biosciences, Sparks, New York), 2.0-mM L-glutamine (Invitrogen, Carlsbad, California), penicillin (100 units/mL)/streptomycin (100 μg/mL; Gibco Life Technologies), and amphotericin B (2.5 μg/mL; Lonza BioWhittaker, Walkersville, Maryland) and then homogenized twice using a Biomaster Stomacher (Wolf Laboratories, Pocklington, UK) at high speed for 2 min. The homogenates were centrifuged at 5,000 revolutions/min (4°C) for 30 min, and the supernatant was used to inoculate cell cultures.

The epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) was used throughout this study. The EPC cells were grown in a 150-cm² tissue culture flask (Corning Life Sciences, Corning, New York) containing EMEM supplemented with 0.3% TPB, 2.0-mM L-glutamine, penicillin (100 units/mL)/streptomycin (100 μg/mL), and amphotericin B (2.5 μg/mL). The medium was supplemented with 10% fetal bovine serum (FBS; GemCell, West Sacramento, California) and sodium bicarbonate (12 mM; Sigma, St. Louis, Missouri) to propagate cell lines or 5% FBS and Tris buffer (14 mM) during virus isolation.

Virus testing was performed according to the protocols outlined in the American Fisheries Society Fish Health Section’s Blue Book (USFWS and AFS–FHS 2016). Homogenized tissue sample supernatants (25 μL) were
inoculated in triplicate onto 96-well tissue culture plates containing EPC cells, and the plates were incubated at 15 ± 1°C and 20 ± 1°C and monitored over two passages of 14 d for signs of cytopathic effects (CPE). Samples that exhibited CPE at any time during incubation were collected, centrifuged, and used for RNA extraction.

**Identification of viruses.**—Extraction of RNA was conducted from cell culture supernatants showing CPE using a QIAamp Viral RNA Minikit (Qiagen Sciences, Gaithersburg, Maryland) according to the manufacturer’s protocol. Extracted RNA was quantified using a Qubit fluorometer (Invitrogen, Eugene, Oregon) and used as template (~10 ng/μL). This study focused on six viruses: VHSV, SVCV, GSRV, FHMNV, FHMPV, and WSBV. Reverse-transcription (RT) loop-mediated isothermal amplification (LAMP) reactions were carried out to confirm the identity of FHMNV according to the method developed by Zhang et al. (2014) using three pairs of RT-LAMP primers (Table 1). For GSRV confirmation, we used a set of primers targeting the S10 segment of GSRV developed in our laboratory (Table 1). For VHSV identification, RT polymerase chain reaction (PCR) was performed as described in the OIE Aquatic Code (OIE 2006) using primers that target the nucleocapsid gene (Table 2). The target amplicon of 811 base pairs was purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) and then was sequenced bi-directionally at the MSU Research Technology Support Facility. Sequences were assembled into contigs using the BioEdit Sequence Alignment Editor version 7.1.3.0. Sequence data were initially compared to reference sequences within GenBank and were subsequently compared to VHSV reference sequences belonging to the five primary genotypes (Ib, III, IVa, IVb, and IVc) that were previously deposited in the National Center for Biotechnology Information database (Altschul et al. 1990). For phylogenetic analysis, sequences from this study were aligned with one another and with VHSV reference sequences using ClustalW in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software (Tamura et al. 2013). Model selection for phylogenetic reconstruction was then performed in MEGA 6.0, whereby the model with the lowest Bayesian information criterion (K2+G) was selected. Neighbor-joining analysis was conducted in MEGA 6.0, and topology robustness was assessed via bootstrap analysis (n = 1,000 re-samplings). In addition, isolates were sent to a reference laboratory for confirmation and additional sequence analysis (National Veterinary Services Laboratories, Ames, Iowa).

A semi-nested RT-PCR protocol was used to test for the presence of SVCV (USFWS and AFS-FHS 2016), whereas the RT-PCR assays of Mor et al. (2015) and Hutchings (2015) were utilized to test for the presence of FHMPV and WSBV, respectively (Table 2).

**Histopathology.**—Samples from external or internal lesions were taken from representative fish, fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μm), and then stained with hematoxylin and eosin as described by Prophet et al. (1992). Stained tissues were visually examined under a light microscope (Model BX41TF; Olympus, Kyoto, Japan), and photographs were taken using image software (Olympus DP25-BSW version 2.2) connected to a camera (Olympus DP25).

**Statistical analysis.**—In this study, we used the definitions of Bush et al. (1997) for prevalence (number of infected lots of one species divided by the total number of lots examined of that same species) and mean intensity (the average titers of a virus in a single infected fish lot).

We used ANOVA to determine whether response variables differed among baitfish species collected from baitfish shops throughout Michigan. Our response variable

| Virus | Primer | Sequence (5′ to 3′) |
|-------|--------|---------------------|
| FHMNV | FIP    | CGTCTGCTGTTTGTGTTTGTGACAAACTTTTGTGCAGCAAACCTTTCAAGGC |
|       | BIP    | TTGCAGCAGTAGAATGAAGTGATTGATTTATGCGGATATCCTTTTAAGGG |
|       | LB     | CAAAGGATTCACAATACACATGC |
|       | LF     | GCGTGTGCTTTTCTTTCGATG |
|       | F3     | ACGTAAAGATCTCTGAGATG |
|       | B3     | TGAACCTTTTGAAGGGTACT |
| GSRV  | FIP    | ACGGTACGGCTACCGAGTAGTTTTAAACGAAGCCATTCGCTCT |
|       | BIP    | TGTGAAAGCTGGATTCGTCGTCGCTTTTGACGATGTCTTGGAGG |
|       | LB     | CCAACACAGATGAGACARTCR |
|       | LF     | GCGCGTTGTCACCATCTTTCGA |
|       | F3     | AGACTCCCAACGCTTGCTC |
|       | B3     | CAGGTGCAGAAACGGAAGY |
was prevalence by species for each virus but also included intensity values for VHSV-positive fish samples. We used the same statistical analysis to determine whether our response variable differed by bait fish source or origin (i.e., where the baitfish were collected). For this analysis, we had two predictor variables, which included in-state and out-of-state sources. When significant differences were detected, we used a post hoc Tukey’s honestly significant difference test (Zar 1999) to determine pairwise differences in prevalence and infection intensity among the various baitfish species collected.

RESULTS

Internal and External Gross Observations

Among the 90 lots examined, 35 lots (38.8%) contained fish that displayed behavioral changes, including loss of equilibrium and erratic swimming, as well as external and/or internal disease signs. External signs of disease included raised scales, exophthalmia, and/or hemorrhages of variable sizes on the mandible, maxilla, ventrum, isthmus, and/or caudal peduncle. Notable internal lesions included accumulation of fluid in the abdominal cavity; hemorrhages on visceral fat and intestine; friable, pale, edematous, and swollen kidneys and liver; distended gall bladder; and friable, hemorrhagic, darkened, and enlarged spleen. Only 9 of the 35 lots (25.7%) that exhibited behavioral changes and/or external or internal clinical signs were found to be infected by one of the viruses that were the focus of this study.

Virus Isolation and Identification

Virus isolation using the EPC cell line, followed by molecular confirmation, revealed that a total of 18 of 90 lots (20.0%) were positive for one of three viruses: FHMMNV (9 of 90 lots), GSRV (8 of 90 lots), and VHSV (1 of 90 lots). The low species-specific sample sizes by source (i.e., in-state and out-of-state) limited our ability to detect differences in viral prevalence between sources using ANOVA with the appropriate statistical power. Fathead minnow nidovirus was isolated from six out-of-state source lots of Fathead Minnow (6 of 30) and three lots of Emerald Shiners (3 of 42), two of which originated from in-state sources. Golden shiner reovirus was isolated from only out-of-state-sourced baitfish. Specifically, GSRV was isolated from 5 of 11 Golden Shiner lots and 3 of 30 Fathead Minnow lots. The OIE-reportable VHSV was also isolated from one in-state source lot of Emerald Shiners (1.1%; Table 3). Within that lot, VHSV was confirmed in 2 of 12 pooled samples of Emerald Shiners by using RT-PCR. The intensity of the VHSV-positive pooled samples was $1.4 \times 10^8$ to $3.4 \times 10^8$ per mg of tissue. Further phylogenetic analyses demonstrated that the two VHSV isolates belonged to VHSV genotype IVb (96.9–97.8% similarity in the N-gene), as they fell into a well-supported cluster containing VHSV genotype IVb isolates only (Figure 2). However, the two Emerald Shiner isolates were also distinct from the other genotype IVb sequences, as evidenced by the formation of a well-supported subclade (Figure 2). None of the Sand Shiner, Spottail Shiner, Northern Redbelly Dace, Blacknose Dace, or Weed Shiner tissue samples developed any CPE on EPC cells (Table 3).

Gross Pathology and Histopathology in Emerald Shiners with Viral Hemorrhagic Septicemia Virus Only

Viral hemorrhagic septicemia virus was detected in one lot of Emerald Shiners. As mentioned above, we were unable to isolate any virus other than VHSV from this lot. It was apparent that this group of Emerald Shiners was diseased, as many fish (~25%) died en route to the laboratory, and many more died during overnight holding. In several Emerald Shiners that were found to be VHSV positive, we observed moderate to severe gill pallor; unilateral exophthalmia; shallow to deep dermal erosion and/or ulceration on the trunk (Figure 3A), ventrum, and

| TABLE 2. Primers used to confirm viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), fathead minnow picornavirus (FHMPV), and white sucker bunyavirus (WSBV) via reverse-transcription PCR. |
|---|
| Virus | Gene targeted | Expected amplicon size (base pairs) | Primer | Sequence (5’ to 3’) |
| VHSV | N-gene | 811 | Ncon-F | GGGGACCCCAGACTGT |
| | | | Ncon-R | TCTCCTGTCACCTTGATCC |
| SVCV | G-gene | 606 | F1 | TCTTGGAGCCAAAATAGCTCARRTC |
| | | | R2 | AGATGGTATGGGACCCCAATACATHACNCAY |
| | | | R4 | CTGGGGTTTCCNCCTAAAGTYG |
| FHMPV | 3D-gene | 821 | FHMPVmul-F | ACAATGAGRAGTTTTCATGCC |
| | | | FHMPVmul-R | AATCACGCTGTCATGAAGTCTC |
| WSBV | M-gene | 222 | WSBV-F | CATGCATCTACGGAATGTGG |
| | | | WSBV-R | CCTGTGCCCAGTAGAGAACG |
TABLE 3. Prevalence of six viruses (golden shiner reovirus [GSRV], fathead minnow nidovirus [FHMNV], viral hemorrhagic septicemia virus [VHSV], spring viremia of carp virus [SVCV], white sucker bunyavirus [WSBV], and fathead minnow picornavirus [FHMPV]) examined in baitfish lots (60 fish/lot) in Michigan.

| Fish species            | Number of positive lots/total lots examineda | Number of positive virus lots |
|-------------------------|--------------------------------------------|-------------------------------|
|                         |                                            | GSRV | FHMNV | VHSV | SVCV | WSBV | FHMPV |
| Golden Shiner           | 5/11                                       | 5    | 0     | 0    | 0    | 0    | 0     |
| Fathead Minnow          | 9/30                                       | 3    | 6     | 0    | 0    | 0    | 0     |
| Emerald Shiner          | 4/42                                       | 0    | 3     | 1    | 0    | 0    | 0     |
| Sand Shiner             | 0/3                                        | 0    | 0     | 0    | 0    | 0    | 0     |
| Spottail Shiner         | 0/1                                        | 0    | 0     | 0    | 0    | 0    | 0     |
| Northern Redbelly Dace  | 0/1                                        | 0    | 0     | 0    | 0    | 0    | 0     |
| Blacknose Dace          | 0/1                                        | 0    | 0     | 0    | 0    | 0    | 0     |
| Mixed (Weed Shiner and  |                                            | 0    | 0     | 0    | 0    | 0    | 0     |
| Sand Shiner)            |                                            | 0    | 0     | 0    | 0    | 0    | 0     |
| Total                   | 18/90                                      | 8/90 | 9/90  | 1/90 | 0/90 | 0/90 | 0/90  |

*aCytopathic effects in the case of VHSV, SVCV, and WSBV are dominated by virally damaged cells and cell detachment, while GSRV, FHMPV, and FHMNV form giant cells (syncytia), rounding, and cell aggregation. All positive samples were molecularly confirmed.

caudal peduncle; and/or petechial to ecchymotic hemorrhages of the mandible, maxilla, vent, ventrum, caudal peduncle, opercula, and isthmus (Figure 3B). Ascites was also observed in several fish that were VHSV-positive, as was mild to severe hemorrhagic enteritis and intestinal swelling; renal edema and hemorrhage (Figure 3C); and friable, swollen livers with diffuse petechial hemorrhage. Some of the fish that exhibited these signs tested negative for VHSV. Approximately 30% of the Emerald Shiners in this lot were VHSV-free and exhibited no gross signs or histopathological changes externally or internally.

Microscopical examination of stained tissue sections from Emerald Shiners that were VHSV-positive revealed diffuse intramuscular hemorrhage and concurrent multifocal necrosis (Figure 4A), significant hemorrhage and necrosis in the pancreas (Figure 4B) and adipose tissue, and intestinal hemorrhage. Notable histopathological changes in the kidneys included degeneration and necrosis of the renal tubules (Figure 4C), glomerular necrosis that was also accompanied by distension of Bowman’s space, and renal interstitial edema and necrosis. In the liver, VHSV-infected Emerald Shiners showed hepatic vacuolation, multifocal hepatocellular necrosis, and degeneration, whereas in the ventricle, trabecular necrosis was observed.

**Pathological Signs and Histopathology of Fathead Minnow Nidovirus-Infected Baitfish**

The isolates of FHMNV were initially identified by formation of syncytia, rounded cells, and cell aggregation of infected EPC cell line and were confirmed using the FHMNV-specific LAMP assay. Three Fathead Minnow lots and one Emerald Shiner lot that were identified as positive for FHMNV (4 of 9 positive lots) exhibited clinical signs of disease. Behavioral changes of FHMNV-infected fish included lethargy, erratic swimming, and loss of equilibrium. External disease signs in FHMNV-infected fish included petechial to ecchymotic hemorrhages at the base of fins, isthmus, opercula, ventrum, vent, dorsum, caudal peduncle, mandible, and maxilla (Figure 5A); unilateral or bilateral exophthalmia; abdominal distension; and shallow to deep erosion and/or ulceration of the skin, especially on the ventral and caudal peduncle surface. Internally, FHMNV-positive fish exhibited ascites; clear,
distended gall bladder; darkened, granular, friable, and enlarged spleen; hemorrhage of the liver; hemorrhagic enteritis along with distension; and/or petechial to diffuse hemorrhage of kidneys. Apparently healthy fish were observed in three Fathead Minnow lots and two Emerald Shiners that were confirmed as FHMNV-positive by RT-LAMP.

Stained and sectioned tissues from FHMNV-positive Fathead Minnow and Emerald Shiners were examined for histopathological changes, which revealed widespread intestinal hemorrhage and necrosis (Figure 6), degeneration and sloughing of the intestinal mucosal epithelium (Figure 6), renal tubular degeneration, renal interstitial edema, and renal interstitial necrosis.

**Pathological and Histopathological Lesions of Golden Shiner Reovirus-Infected Baitfish**

The GSRV isolates were confirmed using LAMP assay after the presence of CPE in infected EPC cells (e.g., giant cell formation and cell aggregation). External gross observations were noted in four of eight GSRV-positive lots (one Fathead Minnow lot and three Golden Shiner lots) and included unilateral exophthalmia; focal to diffuse hemorrhage on fins, opercula, ventrum, caudal peduncle, isthmus, mandible, and maxilla; and abdominal distension. Internally, we observed the presence of ascites; petechial to ecchymotic hemorrhage within the liver (Figure 5B); darkened, friable, and enlarged spleen; hemorrhagic enteritis and concurrent swelling; and renal hemorrhage, swelling, and edema. No behavioral or pathological changes were observed in the four additional lots that were GSRV-positive (two Fathead Minnow lots and two Golden Shiner lots).

Histopathological changes in GSRV-infected Golden Shiners and Fathead Minnow included congestion and edema within the spleen, along with multifocal necrosis of the splenic stroma. In the liver, diffuse hemorrhage, multifocal to diffuse hepatocellular necrosis, and edema were observed.

**DISCUSSION**

Our findings clearly demonstrated that baitfish obtained from retail bait vendors harbor OIE-notifiable and baitfish-associated viruses that are not only infectious to cypri- nids but also to a wide range of other fish species (Faisal et al. 2012, 2016). Surprisingly, some of the infected fish exhibited pronounced external signs of disease and yet remained for sale, with the possibility of ultimately finding their way into numerous waterbodies. This study clearly documents that the end of the trade custody chain (i.e., baitfish retail shops) represents a source for the potential introduction of viruses to public waters via angler-pur- chased and potentially released baitfish. This situation highlights the need for regulatory intervention to implement health inspection of the baitfish supply chain. Considering the ease with which viruses can be disseminated due to their wide host range and the ecological niche of these prey fish species, one would expect that VHSV, FHMNV, and GSRV will continue their expansion—not only in the Great Lakes but also in the inland lakes and waterways of Michigan. The potential increased risk from VHSV and FHMNV spreading via the baitfish trade is

FIGURE 3. External and internal clinical signs in an Emerald Shiner from which viral hemorrhagic septicemia virus was isolated: (A) deep dermal hemorrhagic ulceration on the dorsum; (B) diffuse hemorrhage on the isthmus and opercula; and (C) severely edematous, hemorrhagic kidney. No other viruses were isolated from this fish. [Color figure can be viewed at afsjournals.org.]
indeed alarming, particularly because of the pathological consequences of these viruses. Additionally, since 61 of the 159 fish species residing in the Great Lakes basin are threatened or endangered (GLFC 2016), an expansion of viruses with wide host range via the baitfish trade could exacerbate the tenuous status of the fish stocks that are at risk. In the same context, many important fish species that are the focus of stock enhancement programs at state and federal hatcheries in the Great Lakes region are fed with baitfish purchased from private sources prior to their stocking in public waters. The Muskellunge is one such species that is fed baitfish in hatcheries and has proven susceptible to both VHSV and FHMNV (Faisal et al. 2012, 2016). As a consequence, this practice may expose a highly susceptible fish species to potentially lethal, debilitating viral infections, and widespread stocking of these fish may lead to further expansion into new geographic areas.

A major finding of this study was the detection of the OIE-reportable VHSV in Emerald Shiners (Faisal et al. 2012). Although the two VHSV Emerald Shiner isolates were proven to belong to genotype IVb, which has been commonly detected in the Laurentian Great Lakes since 2003, they formed a well-supported subclade that demarcated them from the other VHSV-IVb sequences included in the phylogenetic analysis. Whether the two Emerald Shiner isolates form a distinct genomovar remains to be elucidated. In a previous study, Thompson et al. (2011) compared the partial G-gene sequence of 108 VHSV-IVb isolates from the Great Lakes and found the genetic diversity among the isolates to be relatively low; however, those authors were able to identify 11 unique sequences. Although the two Emerald Shiner isolates from the present study closely resembled one another, some sequence variation was observed, indicating the occurrence of continuous genetic alterations in the G-gene among isolates retrieved from the same fish host. The possibility that in the future, new isolates of VHSV-IVb could be spread to VHSV-free waterbodies through baitfish is indeed alarming.

The coronavirus FHMNV was isolated from Fathead Minnow and Emerald Shiners, both of which are important baitfish sold in the United States. This is also alarming because the prevalence by lot in these species reached 12.5% (9 positive lots of 72 lots examined), and the number of positive pools within these lots reached as high as 11 of 12 pools. As the name of the virus implies, the Fathead Minnow is believed to be the primary host. However, several other baitfish species were reported to contract the infection naturally (e.g., Creek Chub Semotilus atromaculatus; McCann 2012) or experimentally (e.g., Spotfin Shiner Cyprinella spiloptera and Golden Shiner; Baird and Faisal 2016), indicating the relatively wide host range of the virus among cyprinids. Thus, the relatively high prevalence of FHMNV in baitfish retail stores constitutes a potential hazard for cyprinid populations in the receiving waterbodies. In this study, FHMNV was identified both in apparently healthy fish (5 of 9 positive lots)

**FIGURE 4.** Hematoxylin-and-eosin-stained tissue sections of Emerald Shiners from which viral hemorrhagic septicemia virus was isolated (scale bar = 25 μm): (A) multifocal necrosis (arrowhead) and diffuse intramuscular hemorrhage (arrow); (B) pancreatic hemorrhage and necrosis (arrowheads); and (C) multifocal renal tubular necrosis (arrows). [Color figure can be viewed at afsjournals.org.]
and in fish exhibiting clinical signs. This indicates that a definitive diagnosis should be based on virus isolation and confirmation by molecular or serological testing. The risk of FHMMNV extends also to Muskellunge raised in hatcheries, where baitfish constitute a primary food source. Indeed, Muskellunge are vulnerable to FHMMNV infection and can contract the virus by cohabitation and predation on infected fish (Baird and Faisal 2016; Faisal et al. 2016). Although FHMMNV has been reported in Michigan previously (Faisal et al. 2016), infected baitfish can disseminate it further to otherwise virus-free waterbodies. This study reports, for the first time, that Emerald Shiners are also susceptible to FHMMNV.

The Aquareovirus C, GSRV, was present in 5 of 11 (~45%) Golden Shiner lots and 3 of 30 (10%) Fathead Minnow lots examined. Half of the positive virus lots (4 of 8) exhibited external and internal hemorrhages and tissue alterations. The host range of this virus is not currently known and requires further study. Goodwin et al. (2006) pointed out that crowded conditions and a poor environment can lead to acute epizootics involving this virus. McCann (2012) demonstrated that a GSRV strain that was isolated from a baitfish sample could be lethal to Fathead Minnow after experimental intraperitoneal injection. The Chinese grass carp reovirus, another member of the Aquareovirus C group that closely resembles GSRV, has been detected in moribund and healthy Golden Shiners, Fathead Minnow, Grass Carp Ctenopharyngodon idella, and Creek Chub (McEntire et al. 2003; Goodwin et al. 2006). Similar to VHSV and FHMMNV, the potential for widespread GSRV dissemination by baitfish to indigenous cyprinids in the Laurentian Great Lakes basin is concerning.

Despite its presence in Lake Hamilton, Ontario (Garver et al. 2007), and in other Midwestern states like Minnesota (Phelps et al. 2012), Wisconsin (Dikkeboom et al. 2004), and Illinois (Warg et al. 2007), SVCV was not found in this study. In a recent study, SVCV was identified as the cause of morbidity and mortality with varying degrees of pathogenicity in baitfish species (e.g., Fathead Minnow and Golden Shiner) after waterborne immersion (Boonthai et al. 2017b) and in Emerald Shiners and White Suckers via intraperitoneal infection (Misk et al. 2016). Fathead minnow picornavirus and WSBV were not detected in this study; however, their pathogenicity and the risk they pose to piscivorous fish need to be assessed. In the same context, molecular evidence has demonstrated the presence of other viruses in a common baitfish species, the Golden Shiner—namely a novel piscine-myocarditis-like virus (Mor and Phelps 2016a) and a novel totivirus (Mor and Phelps 2016b). However, the potential pathogenicity of these novel viruses to Golden Shiners or other fish species is currently unknown.

In conclusion, we documented a relatively high prevalence of viruses in baitfish sold in retail stores, which clearly indicates that the baitfish trade could be contributing to the expansion of viruses to wild fish populations in the Great Lakes basin (McCann 2012). It is likely that the high virus prevalence reflects the stressful conditions under
which these fish are maintained, including high-density storage, limited or no feeding, and frequent handling. It likely also reflects the lack of basic biosecurity practices at bait shops, including the removal of moribund and dead fish, based on mortalities observed in tanks during collections. The prevalence of FHMMNV and the existence of VHSV in the retail bait shops combined with the high frequency of baitfish use among anglers result in a high risk for new introductions (Drake and Mandrak 2014). This risk is particularly elevated when considering that anglers have been documented to release unused baitfish at rates as high as over 40% (Litvak and Mandrak 1993; Kilian et al. 2012). As such, there is a need for managerial intervention to abrogate or slow down the rate of viral expansions from this vector. In Michigan, with regard to all management practices, additional enforcement, and an increased outreach campaign to ensure that pathogenic viruses do not spread further throughout the Great Lakes region via the baitfish trade.

ACKNOWLEDGMENTS

We are grateful to the Great Lakes Restoration Initiative for generous funding (Grant Number 751B5500047). We appreciate Emily Giuliano, Cheryl Benson, Ed Baker, and Jan VanAmberg (MDNR Fisheries Division) and Roger Greil (Lake Superior State University) for assistance with baitfish collections. There is no conflict of interest declared in this article.

REFERENCES

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410.

Baird, A., and M. Faisal. 2016. Fathead minnow nidovirus infects Spotfin Shiner Cyprinella shiner spiloptera and Golden Shiner Notemigonus crysoleucas. Diseases of Aquatic Organisms 119:37–44.

Baird, A. M. 2015. Risks of the emerging coronavirus, fathead minnow virus (Order: Nidovirales), on representative Great Lakes fish species. Master’s thesis. Michigan State University, East Lansing.

Boonthai, T., S. J. Herbst, G. E. Whelan, M. G. Van Deuren, T. P. Loch, and M. Faisal. 2017a. The Asian fish tapeworm (Sichyzocotyle acheilognathi) is widespread in baitfish retail stores. Parasites and Vectors 10:618.

Boonthai, T., T. P. Loch, I. Standish, and M. Faisal. 2017b. Susceptibility of representative Great Lakes fish species to the North Carolina strain of spring viremia of carp virus (SVCV). Journal of Aquatic Animal Health 29:214–224.

Brabec, J., R. Kuchta, T. Scholz, and D. T. J. Littlewood. 2016. Paralogues of nuclear ribosomal genes convey phylogenetic signals within the invasive Asian fish tapeworm lineage: evidence from next-generation sequencing data. International Journal of Parasitology 46:555–562.

Bush, A. O., K. D. Lafferty, J. M. Lotz, and A. W. Shostak. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology 83:575–583.

Dikkeboom, A. L., C. Radi, K. Toohey-Kurth, S. Marcquenski, M. Engel, A. E. Goodwin, K. Way, D. M. Stone, and C. Longshaw. 2004. First report of spring viremia of carp virus (SVCV) in wild Common Carp in North America. Journal of Aquatic Animal Health 16:169–178.

Drake, D. A. R., and N. E. Mandrak. 2014. Bycatch, bait, anglers, and roads: quantifying vector activity and propagule introduction risk across lake ecosystems. Ecological Applications 24:877–894.

Faisal, M. 2007. Health challenges to aquatic animals in the globalization era. Pages 120–135 in W. W. Taylor, M. G. Schechter, and L. G. Wolfson, editors. Globalization: effects on fisheries resources. Cambridge University Press, Cambridge, UK.

Faisal, M., A. Baird, A. D. Winters, E. V. Millard, S. Marcquenski, H. M. Hsu, A. Hemings, P. Bochsler, I. Standish, T. P. Loch, M. R. Gunn, and J. Warg. 2016. Isolation of the fathead minnow nidovirus from Muskelunge experiencing lingering mortality. Journal of Aquatic Animal Health 28:131–141.

Faisal, M., M. Shavalier, R. Kim, E. V. Millard, M. R. Gunn, A. D. Winters, C. A. Schulz, A. Eissa, M. V. Thomas, M. Wolgamoood, G. E. Whelan, and J. Winton. 2012. Spread of the emerging viral hemorrhagic septicemia virus strain, genotype IVb, in Michigan, USA. Viruses 4:734–760.

Fijan, N., D. Sulimanovic, M. Bearzotti, D. Muzinic, L. O. Zwillenberg, S. Chilmonczyk, J. F. Vautherot, and P. de Kinkelin. 1983. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp Cyprinus carpio. Annales de l’Institut Pasteur Virologie 134:207–210.

Garver, K. A., A. G. Dowlow, J. Richard, T. F. Booth, D. R. Bennic, and B. W. Souter. 2007. First detection and confirmation of spring viremia of carp virus in Common Carp, Cyprinus carpio L., from Hamilton Harbour, Lake Ontario, Canada. Journal of Fish Diseases 30:665–671.

GLFC (Great Lakes Fishery Commission). 2016. Fish management: the Great Lakes fishery. GLFC, Ann Arbor, Michigan.

Goodwin, A. E., D. K. Nayak, and R. S. Bakal. 2006. Natural infections of wild Creek Chubs and cultured Fathead Minnow by Chinese grass carp reovirus (golden shiner virus). Journal of Aquatic Animal Health 18:35–38.

Boonthai, T., I. Standish, and M. Faisal. 2017b. Susceptibility of representative Great Lakes fish species to the North Carolina strain of spring viremia of carp virus (SVCV). Journal of Aquatic Animal Health 29:214–224.
Hutchings, H. C. 2015. Characterization and risk assessment of a novel virus isolated from White Sucker fish (Catostomus commersonii) in Wisconsin. Master’s thesis. University of Wisconsin, La Crosse.

Iwanowicz, L. R., and A. E. Goodwin. 2002. A new bacilliform fathead minnow rhabdovirus that produces syncytia in tissue culture. Archives of Virology 147:899–915.

Kilian, J. V., R. J. Klauda, S. Widman, M. Kashiwagi, R. Bourquin, S. Weglein, and J. Schuster. 2012. An assessment of a bait industry and angler behavior as a vector of invasive species. Biological Invasions 14:1469–1481.

Litvak, M. K., and N. E. Mandrak. 1993. Ecology of freshwater baitfish use in Canada and the United States. Fisheries 18(12):6–13.

Misk, E., K. Garver, E. Nagy, S. Isaac, L. Tubbs, P. Huber, L. Al-Hussine, and J. S. Lumsden. 2016. Pathogenesis of spring viremia of carp virus in Emerald Shiner Notropis atherinoides Rafinesque, Fathead Minnow Pimephales promelas Rafinesque and White Sucker Catostomus commersonii (Lacépède). Journal of Fish Diseases 39:729–739.

Mor, S. K., and N. B. D. Phelps. 2016a. Detection and molecular characterization of a novel piscine myocarditis-like virus from baitfish in the USA. Archives of Virology 161:1925–1931.

Mor, S. K., and N. B. D. Phelps. 2016b. Molecular detection of a novel totilivirus from Golden Shiner (Notemigonus crysoleucas) baitfish in the USA. Archives of Virology 161:2227–2234.

Mor, S. K., N. B. D. Phelps, M. Barbknecht, M. A. Hoffman, and S. M. Goyal. 2015. A multiplex RT-PCR assay for the detection of fish picornaviruses. Journal of Virological Methods 221:131–134.

OIE (World Organization for Animal Health). 2006. OIE manual of diagnostic tests for aquatic animals, 5th edition. OIE, Paris.

Plumb, J. A., D. E. Bowser, J. M. Grizzle, and A. J. Mitchell. 1979. Fish viruses: a double-stranded RNAicosahedral virus from a North American cyprinid. Journal of the Fisheries Research Board of Canada 36:1390–1394.

Prophet, E., B. Mills, J. Arrington, and L. H. Sobin. 1992. Laboratory methods in histotechnology. Armed Forces Institute of Pathology, American Registry of Pathology, Washington, D.C.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

USDA (U.S. Department of Agriculture) National Agriculture Statistics Service. 2006. 2002 census of agriculture: census of aquaculture (2005), volume 3, part 2. USDA, AC-02-SP-2, Beltsville, Maryland.

USFWS (U.S. Fish and Wildlife Service) and AFS–FHS (American Fisheries Society–Fish Health Section). 2016. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens. AFS–FHS, Bethesda, Maryland.

Zar, J. H. 1999. Biostatistical analysis. Prentice-Hall, New York.