Vol. 119: 37–44, 2016
doi: 10.3354/dao02970

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 12

Fathead minnow nidovirus infects spotfin shiner *Cyprinella spiloptera* and golden shiner *Notemigonus crysoleucas*

Ashley Baird[1], Mohamed Faisal[1,2,*]

[1]Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI 48824, USA

[2]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, 1129 Farm Lane, Room 173, Michigan State University, East Lansing, MI 48824, USA

ABSTRACT: Since the initial isolation of the fathead minnow nidovirus (FHMNV), concerns have been raised regarding the risks it may pose to other fish species. In this study, 7 fish species resident to the Laurentian Great Lakes were challenged intraperitoneally with 2 doses of FHMNV: $10^{2.8}$ and $10^{4.8}$ median tissue culture infective dose ($TCID_{50}$) ml^{-1}. Infected spotfin shiner *Cyprinella spiloptera* and golden shiner *Notemigonus crysoleucas* suffered morbidity and mortality during the 40 d observation period, while other species, including creek chub *Semotilus atromaculatus*, rainbow trout *Oncorhynchus mykiss*, largemouth bass *Micropterus salmoides* and walleye *Sander vitreus*, showed no clinical signs or mortality. FHMNV was re-isolated on the epithelioma papulosum cyprini cell line from the tissues of infected spotfin shiner and golden shiner, which harbored high numbers of viral RNA copies as measured by quantitative loop-mediated isothermal amplification. Infected spotfin shiner and golden shiner exhibited external petechiae, exophthalmia, oedematous kidneys, and liver pallor. Histopathological analysis revealed multifocal areas of necrosis in the kidney, spleen and liver of infected fish. Spotfin shiner and golden shiner were then infected with 2 doses of FHMNV ($10^{3.5}$ and $10^{3.9}$ $TCID_{50}$ ml^{-1}) by immersion to mimic more natural modes of infection. Spotfin shiner experienced 60% mortality at both doses, while golden shiner did not experience mortality nor develop any clinical signs following a 40 d observation period. Overall, piscivorous fish tested in this study do not seem to be at risk for infection, while cyprinids appear to vary in their susceptibility to the strain of FHMNV used in this study.

KEY WORDS: Fathead minnow nidovirus · FHMNV · Spotfin shiner · Golden shiner · Baitfish · Great Lakes

INTRODUCTION

In 1997, a novel coronavirus of the genus *Bafinivirus* (order *Nidovirales*), the fathead minnow nidovirus (FHMNV), was isolated from a mortality event of fathead minnow *Pimephales promelas* of both wild-type and rosy-red phenotypes on a baitfish farm in Arkansas, USA (Iwanowicz & Goodwin 2002, Batts et al. 2012). The disease caused by FHMNV was systemic in nature with moribund fish exhibiting external petechiae. Rivers' postulates (Rivers 1937) were fulfilled for this virus since fathead minnow experimentally infected via the intraperitoneal (IP) route suffered from high levels of mortality and haemorrhagic diathesis similar to that observed in naturally infected minnows (Iwanowicz & Goodwin 2002).

Since the initial isolation of FHMNV in Arkansas, the virus has been isolated from fathead minnow reared in Wisconsin, Minnesota and Illinois (Batts et al. 2012). In addition, it has been isolated on a single

*Corresponding author: faisal@cvm.msu.edu

occasion from a group of creek chub *Semotilus atromaculatus*, another cyprinid that is resident to the Great Lakes basin (McCann 2012), as well as from hatchery-raised muskellunge in Michigan and Wisconsin in 2011 (Baird 2015). Despite its pronounced morbidity and mortality, little work has been done to assess the pathogenicity and host range of this virus. This is particularly alarming as FHMNV may pose significant risks to native cyprinids, which play a central role in the stability of the foodweb.

In the USA, the baitfish industry was estimated to generate annual revenues of US $170 million shipping about 10 billion fish per year, with fathead minnow and golden shiner *Notemigonus crysoleucas* being the most popular species used (Goodwin et al. 2011). There has been increasing concern over pathogens in baitfish, as we have begun to detect them more frequently. Interestingly, recent studies have determined fathead minnow are statistically more likely ($p = 0.021$) to be infected with a virus compared to other popular baitfish species used (golden shiner and white sucker *Catostomus commersonii*; McCann 2012). Despite this increased detection of pathogens, the baitfish industry is mostly unregulated when it comes to disease screening requirements (Goodwin et al. 2011). FHMNV in particular is currently not listed as an OIE-notifiable disease (OIE 2012), and therefore baitfish are not required to be screened for this virus prior to being transferred to various states and water bodies. To this end, this study was designed to elucidate the potential pathogenicity of FHMNV to representative fish species resident in the Great Lakes basin that may be at risk for FHMNV infection either through the baitfish industry or rehabilitation programs.

MATERIALS AND METHODS

Fish and maintenance

A total of 7 fish species were used in this study. Certified specific pathogen-free fish were obtained from the following organizations: fathead minnow from the Animal and Plant Health Inspection Services, US Department of Agriculture (Ames, IA); spotfin shiner *Cyprinella spiloptera*, golden shiner and largemouth bass *Micropterus salmoides* from the La Crosse Fish Health Center, US Fish and Wildlife Service (Onalaska, WI); and rainbow trout *Oncorhynchus mykiss* from Wolf Lake State Fish Hatchery (Mattawan, MI). Certified specific pathogen-free walleye *Sander vitreus* (gametes collected from St. Mary's River broodstock) were obtained from the Sault Tribe in Chippewa County, MI. In addition, creek chub *Semotilus atromaculatus* that were wild caught using hook and line from a stream in Kalamazoo, MI, were included in this study. Prior to use in experimental infection, 5 fish from each species were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories) (25 mg ml^{-1}) and tested for the presence of pathogens including FHMNV, as described below.

Experimental fish were allowed to acclimate to laboratory conditions for 3 wk, during which time the temperature was progressively lowered to 11 ± 1°C before the experiment was initiated. All fish were initially housed in separate 420 l fiberglass tanks in a continuous flow-through system supplied with oxygenated and facility-chilled well water with outflow uniting into a common tract entering an ultraviolet sterilization unit (Aquafine Corporation). Experimental fish were held at the Michigan State University-Research Containment Facility, East Lansing, MI, in accordance with the International Animal Care and Use Committee (IACUC) guidelines (Approval number 03/14-046-00).

Cell culture and virus propagation

Virus stocks of the FHMNV strain MUS-WL originally isolated from muskellunge (Baird 2015) were produced using the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983), aliquoted, and kept at −80°C until used. To determine the virus concentration, a median tissue culture infective dose ($TCID_{50}$) assay was performed on EPC cell lines and calculated as described by Reed & Muench (1938). Titrated virus stocks were aliquoted in cryogenic vials (Corning) and kept at −80°C until used.

Cell lines were grown and maintained in 150 cm^2 tissue culture flasks (Corning) at 25°C using growth formulation of Earle's salt-based minimal essential medium (MEM) (Invitrogen Life Technologies) supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich).

Experimental infection of fathead minnow

This experiment was performed to serve as a positive control for virus susceptibility and to guide the

choice of 2 virus concentrations to be used for susceptibility screening. Certified disease-free fathead minnow were infected with FHMNV using a standard IP injection protocol to ensure all fish were simultaneously infected with 5 different doses of the virus ranging from $10^{0.8}$ to $10^{4.8}$ $TCID_{50}$ ml^{-1}. Six tanks total were used, the first tank being a negative control tank which received only sterile media and then 5 'infected' tanks (one for each dose; $10^{0.8}$, $10^{1.8}$, $10^{2.8}$, $10^{3.8}$, or $10^{4.8}$ $TCID_{50}$ ml^{-1}). Immediately after being anesthetized with MS-222 (100–150 ppm), 100 µl of each dose level was administered to each of 10 fish. Fish were then recovered from anesthesia in a 72 l polyethylene tank (Aquatic Eco-Systems) in a continuous flow-through system. All fish were monitored every 8–12 h daily for an observation period of 60 d postinfection (PI). Representative visceral organs were collected from freshly dead and moribund fish and processed for viral testing as described in 'Tissue processing for viral isolation'.

Experimental infection by IP-injection for susceptibility screening

Each species was challenged with FHMNV using a standard IP injection protocol to ensure all fish were given a known dose of the virus. Three groups of fish per species (n = 10) were used in this experiment; the first group received 100 µl of $10^{2.8}$ $TCID_{50}$ ml^{-1} (low dose group), while the second group received the same volume of $10^{4.8}$ $TCID_{50}$ ml^{-1} (high dose group). The third group received 100 µl of sterile media (negative control group). All fish were monitored daily for an observation period of 40 d PI. Tanks were monitored every 8 to 12 h daily for moribund or freshly dead fish. Kidney and spleen were collected and processed for viral testing. If infected fish did not experience any mortality by the end of the study period, fish tissues were collected in pools (5 fish per pool) and processed for viral testing.

Experimental infection of spotfin shiner and golden shiner by immersion

Based on the initial screening, spotfin shiner and golden shiner were selected for testing the pathogenicity of FHMNV by immersion. Certified disease-free spotfin shiner and golden shiner were experimentally infected with 2 virus doses ($10^{3.5}$ and $10^{3.9}$ $TCID_{50}$ ml^{-1}) by an immersion protocol previously described by Kim & Faisal (2012). During the infection process, 10 individual fish from each species per treatment were immersed in 6 l of diluted virus in aerated glass aquaria (~19 l) for 1 h. A third fish group from each species was immersed in 6 l of diluted sterile tissue culture medium and were considered the negative control groups. Following the treatment period, fish were removed from their respective aquaria and rinsed with chilled freshwater before being transferred to their round 72 l experimental tanks. Experimental fish were observed for morbidity and mortality for up to 40 d PI. Samples were taken for virus testing and histopathology as described for the IP-infection experiments.

Tissue processing for viral isolation

Tissue samples were diluted with Earle's salt-based MEM, supplemented with 12 mM Tris buffer (Sigma Chemical), penicillin (100 IU ml^{-1}), and amphotericin B (250 µg ml^{-1}, Invitrogen) to produce a 1:4 dilution of original tissues w/v. Samples were then homogenized using a Biomaster Stomacher (Wolf Laboratories) at the high speed setting for 2 min. Following homogenization, samples were centrifuged at 5000 rpm (2711 × *g*) for 30 min, and the supernatant from each individual sample was inoculated into triplicate wells of a 96-well plate containing EPC cells grown with MEM supplemented with 5% fetal bovine serum (Hyclone). Infected plates were incubated at 15°C for up to 21 d and observed for the formation of cytopathic effects (CPE).

RNA extractions and quantitative loop-mediated isothermal amplification (qLAMP)

Total RNA was extracted from 100 µl of the supernatant from processed fish tissue using the Qiagen RNeasy mini kit (Qiagen). The number of viral copies was quantified using a qLAMP assay specific to FHMNV that was developed by Zhang et al. (2014). The reaction was carried out in a 25 µl reaction mixture containing 2.5 µl 10× isothermal amplification buffer, 1.5 µl of $MgCl_2$ (100 mM), 5 µl of betaine (5 M), 1.75 µl of deoxynucleoside triphosphate (dNTP) (20 mM), 2 µl each of FIP and BIP primers (20 mM), 1 µl of LF and LB primers (20 mM), 0.5 µl of F3 and B3 primers (10 mM), 2.25 µl of RNA free water, 1 µl of *Bst* DNA polymerase (New England Biolabs), 0.5 µl of MLV Reverse Transcriptase (Invitrogen), 1 µl Calcein, and 0.5 µl $MnCl_2$ (10 mM).

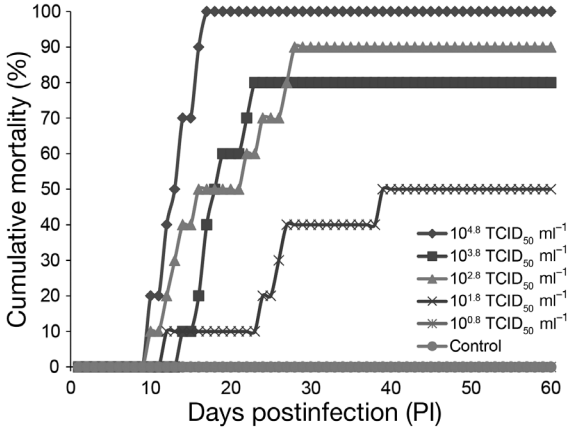

Fig. 1. Mortality of fathead minnow *Pimephales promelas* (n = 10 per treatment) infected by intraperitoneal injection with 5 doses of fathead minnow nidovirus. TCID$_{50}$: median tissue culture infective dose

Histopathology

Lesions that were grossly observed from freshly dead and severely moribund (euthanized with an overdose of MS-222; 25 mg ml^{-1}) fish as well as kidney, spleen, and liver tissues were sampled for histopathology. Organs were fixed in 10% buffered formalin and embedded in paraffin prior to sectioning. Tissue sections (5 µm) were stained with haematoxylin and eosin (H&E) as detailed in Prophet et al. (1992). Tissue alterations were determined by microscopical examinations under a light microscope (Olympus BX41) equipped with camera (Olympus DP25) and image software (Olympus DP25-BSW version 2.2).

RESULTS

Experimental infection of fathead minnow

Representative samples of fish species used in experimental infection studies proved to be free of viruses. FHMNV-infected minnow began experiencing mortality around 10 d postinfection (Fig. 1). By the end of the 60 d study period, the fish with the highest dose of $10^{4.8}$ TCID$_{50}$ ml^{-1} had reached 100% mortality, those with a dose of $10^{3.8}$ TCID$_{50}$ ml^{-1} had reached 80% mortality, those with a dose of $10^{2.8}$ TCID$_{50}$ ml^{-1} had reached 90% mortality, and those with a dose of $10^{1.8}$ TCID$_{50}$ ml^{-1} had reached 50% mortality. The group with the lowest dose of $10^{0.8}$ TCID$_{50}$ ml^{-1} and the control group both experienced no mortality (Fig. 1). Based on the mortality curves obtained for each virus concentration, the median lethal dose (LD$_{50}$) was calculated to be $10^{1.8}$ TCID$_{50}$ ml^{-1}. Based on this experiment, doses of $10^{2.8}$ and $10^{4.8}$ TCID$_{50}$ ml^{-1} were chosen for consequent FHMNV susceptibility scanning of additional species.

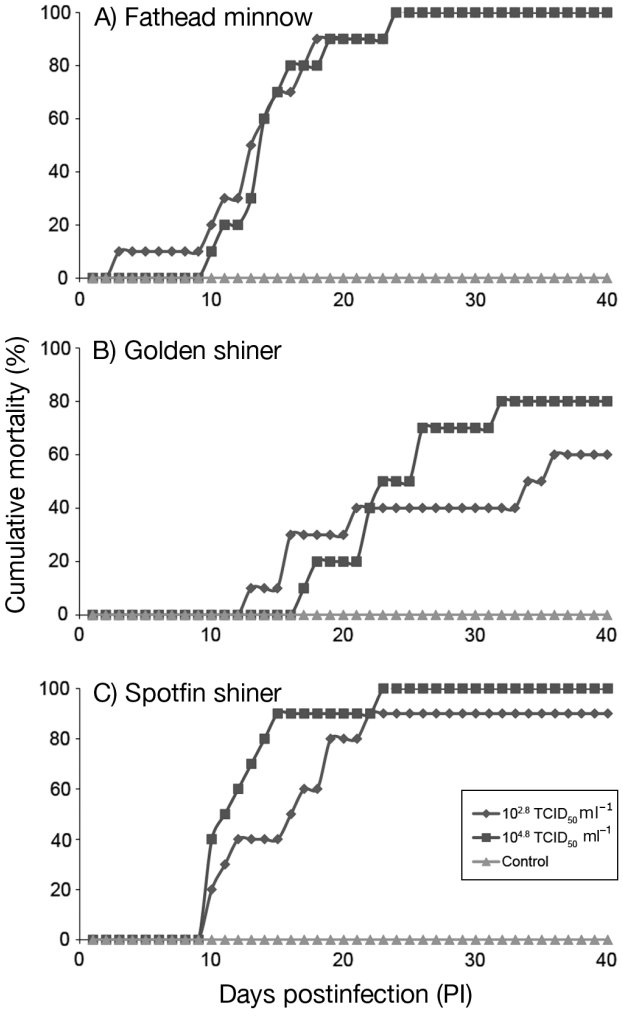

Fig. 2. Mortality curves for experimentally challenged fish species (n = 10 for each treatment group) that were susceptible to fathead minnow nidovirus infection by intraperitoneal injection. Each species was infected with 100 µl of a low ($10^{2.8}$ median tissue culture infective dose [TCID$_{50}$] ml^{-1}), high ($10^{4.8}$ TCID$_{50}$ ml^{-1}) and control (sterile media) dose (400×)

Experimental infection by IP-injection

Fathead minnow. Most mortality occurred between 10 and 20 d PI (Fig. 2A). Moribund fish exhibited behaviors of erratic swimming, lethargy, and increased opercular movements. Externally, dead/moribund fish showed gill pallor, exophthalmia, and

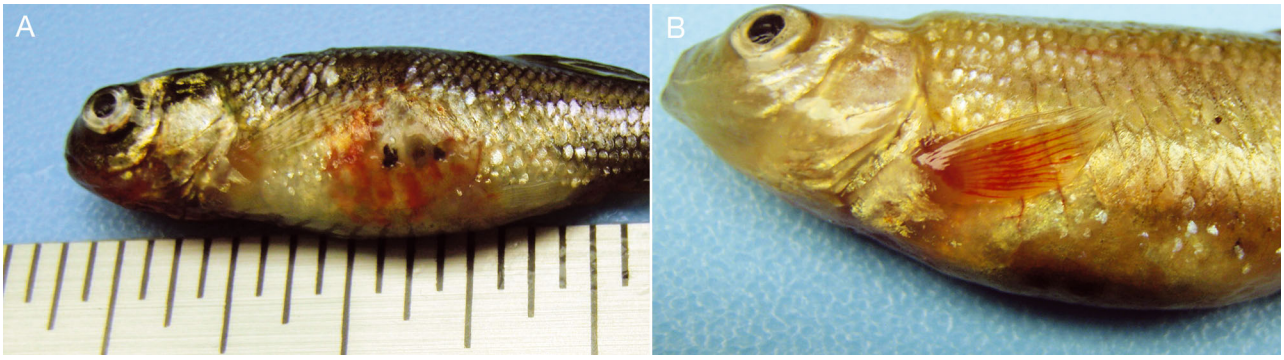

Fig. 3. Fathead minnow *Pimephales promelas* infected by fathead minnow nidovirus, exhibiting (A) a lesion 1–5 mm in diameter on the flank containing moderate diffuse haemorrhage and (B) enlarged blood vessels in the pectoral fin

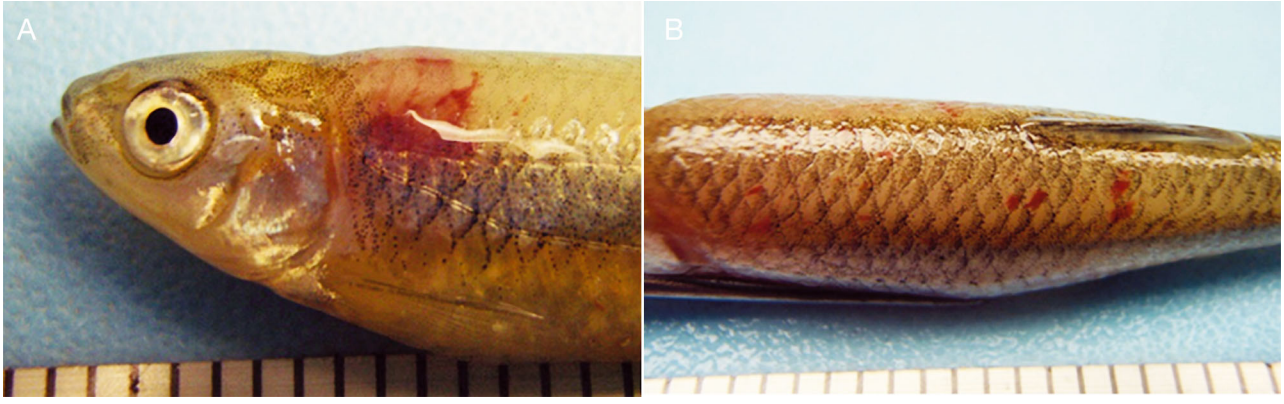

Fig. 4. Golden shiner *Notemigonus crysoleucas* infected by fathead minnow nidovirus, exhibiting (A) severe diffuse haemorrhage just posterior to the head and (B) moderate multifocal petechiae along the spine just below the dorsal fin on the left side

petechiae and ecchymoses throughout the eyes, fins and skin (Fig. 3). Internal examination of infected fish revealed enlarged spleens and oedematous kidneys. Several individuals also exhibited pale livers with multifocal petechiae throughout. All tissue samples from infected fish were positive for FHMNV by cell culture and qLAMP. No virus was detected from any control fish either by cell culture or qLAMP.

Golden shiner. FHMNV was pathogenic to golden shiner when infected by IP injection with mortality reaching up to 80% at the end of the observation period. Infected golden shiner from the high dose group began experiencing mortality at 13 d PI, and by 36 d PI, the low dose group had reached 60% mortality while the high dose group had reached 80% mortality (Fig. 2B). Clinical signs were consistent with what was observed in fathead minnow and included petechial and ecchymoses throughout the eyes, skin and fins (Fig. 4) as well as liver pallor. All individuals were positive for FHMNV by qLAMP, with virus copies reaching up to 5×10^5 copies mg^{-1} of tissue in the low dose group and 3×10^6 copies mg^{-1} of tissue in the high dose group. FHMNV was also reisolated from infected fish using EPC cells. No virus was detected from control fish.

Spotfin shiner. Mortalities began at 9 d PI, sharply increasing and reaching 90% in the low dose group by 22 d PI and 100% mortality in the high dose group by 23 d PI (Fig. 2C). FHMNV-infected spotfin shiner showed similar clinical signs to that of infected fathead minnow and golden shiner, including petechiae throughout the eyes, fins and skin (Fig. 5) as well as splenic enlargement. Liver pallor was also observed in several individuals. All infected fish were positive for FHMNV by qLAMP, with virus copies ranging between $10^{5.9}$ and $10^{6.7}$ viral copies mg^{-1} of tissue. The virus was reisolated on EPC cells from infected fish.

Other species tested. Creek chub, walleye, rainbow trout and largemouth bass did not exhibit any mortality following experimental infection. All tissues including those from control fish tested negative for FHMNV both by cell culture and qLAMP methods.

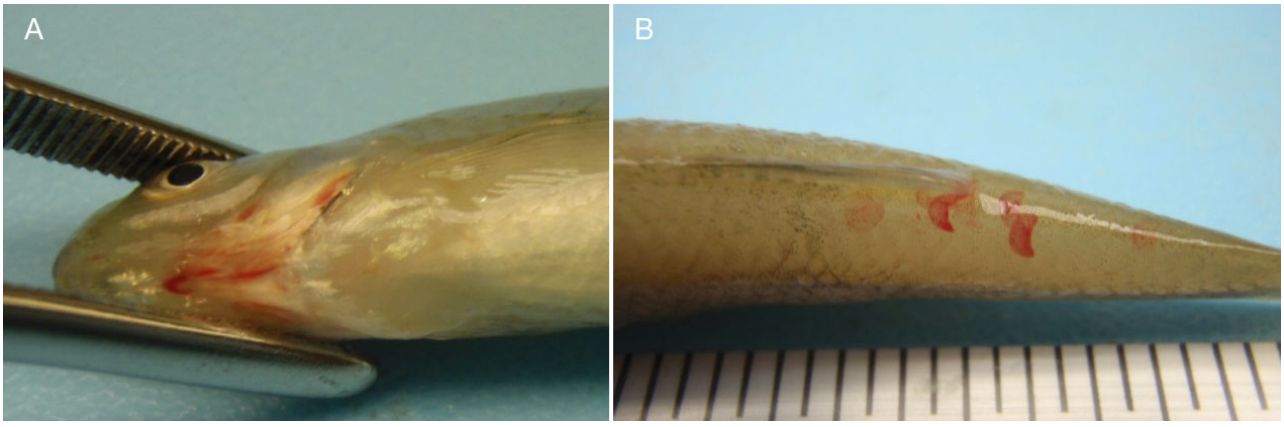

Fig. 5. Spotfin shiner *Cyprinella spiloptera* infected by fathead minnow nidovirus, exhibiting (A) moderate ecchymoses on the isthmus and (B) severe multifocal ecchymoses along the spine just caudal to the dorsal fin

Experimental infection of spotfin shiner and golden shiner by immersion

Infected spotfin shiner experienced mortality beginning from Day 11 PI. By the end of the 40 d study period, both low and high immersion dose groups had reached 60% mortality (Fig. 6). Except for one fish, FHMNV was detected from all infected individuals from the low dose group by qLAMP, with copy numbers detected at levels up to 10^4 copies mg^{-1} of tissue for both doses. FHMNV was reisolated from the visceral organs of 60% of infected spotfin shiners using EPC cells.

Golden shiner infected with FHMNV by immersion did not show any clinical signs of infection and did not exhibit any mortality. One pool of golden shiner tissues from the high dose group tested positive for FHMNV by qLAMP; however, all other pools tested negative for the presence of the virus. All golden shiner tissue pools were also negative for FHMNV by cell culture.

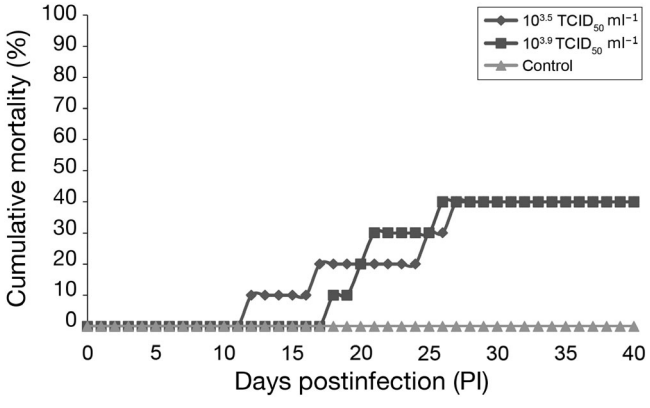

Fig. 6. Cumulative percent mortality for spotfin shiner *Cyprinella spiloptera* infected with either $10^{3.5}$ median tissue culture infective dose ($TCID_{50}$) ml^{-1} or $10^{3.9}$ $TCID_{50}$ ml^{-1} of fathead minnow nidovirus by immersion and a negative control (sterile media) that received diluted tissue culture medium

Histopathology of FHMNV-infected fish

Interestingly, spotfin shiner, golden shiner, and fathead minnow experimentally infected with FHMNV via IP injection and immersion exhibited similar histopathological alterations in the form of multifocal necrosis in the liver, haematopoietic interstitium of the anterior kidney, and splenic stroma (Fig. 7). Similar histopathological lesions were observed in spotfin shiner infected by immersion.

DISCUSSION

Results obtained from this study demonstrate that representative Great Lakes native fish species vary in their susceptibility to FHMNV. The spotfin shiner seems to be susceptible to this virus since morbidity and mortality occurred following experimental infection, not only by intraperitoneal injection but also by immersion. While IP injection studies yield some evidence for susceptibility, immersion challenges are necessary to deem a species 'susceptible' per World Organization for Animal Health (OIE) requirements (OIE 2012). Golden shiner showed some evidence for susceptibility since they exhibited both morbidity and mortality following experimental infection by IP injection; however, this was not evidenced during immersion trials. All other species screened, including creek chub, rainbow trout, largemouth bass, and

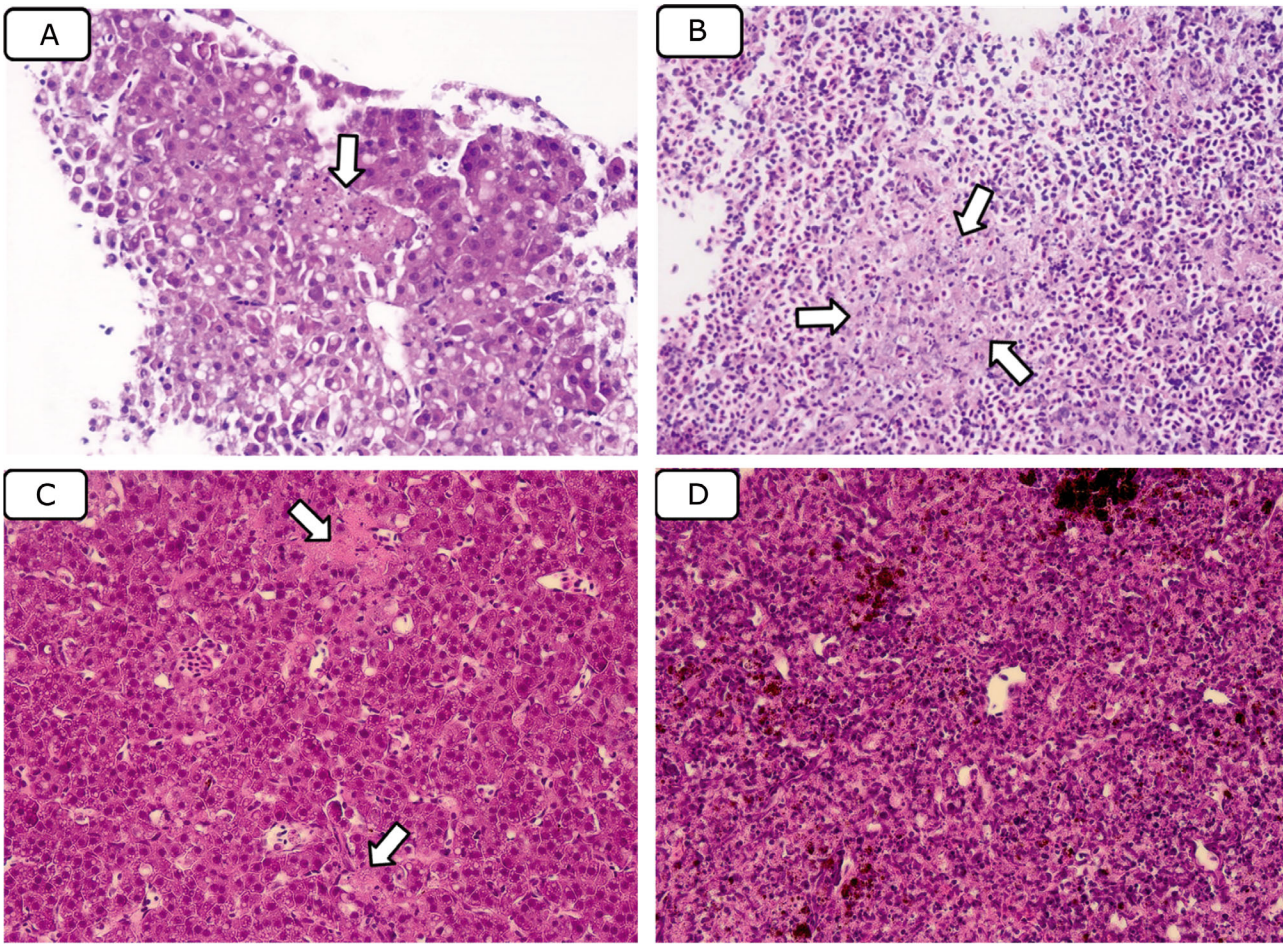

Fig. 7. Haematoxylin and eosin-stained tissue sections from spotfin shiner *Cyprinella spiloptera* infected with fathead minnow nidovirus by (A,B) intraperitoneal injection and (C,D) immersion. (A) Liver exhibiting multifocal hepatocellular necrosis (arrow, 400×). (B) Spleen exhibiting multifocal areas of necrosis (arrows, 400×). (C) Liver exhibiting multifocal hepatocellular necrosis (arrows, 400×). (D) Diffuse necrosis of the interstitial tissue in the anterior kidney (400×)

walleye, were considered not susceptible, at least not at the 2 virus concentrations used in this study, as they did not experience any morbidity or mortality by IP injection. It is noteworthy that in a parallel experiment, fathead minnow bathed in comparable FHMNV concentrations and kept under the same conditions suffered 100% mortality within 3 wk PI (data not shown).

Both fathead minnow and spotfin shiner infected with FHMNV exhibited similar clinical signs and histopathological alteration in hematopoietic tissues, suggesting that the pathogenic mechanism employed by FHMNV is similar in both species. These findings were also consistent with what has been observed in both experimentally and naturally FHMNV-infected muskellunge (Baird 2015). While golden shiner that were experimentally infected with the MUS-WL FHMNV by IP injection exhibited similar clinical signs and histopathology, infection by immersion failed, indicating that FHMNV is unable to overcome the golden shiner's intact body natural barriers.

Creek chub used in this study did not succumb to FHMNV when infected by the IP route, despite the fact that FHMNV was previously isolated from a single group of creek chub during a routine disease screening of baitfish dealers in Wisconsin (McCann 2012). This discrepancy can be explained in 2 ways. We were only able to obtain wild caught creek chub; therefore, there is a possibility that these fish had been exposed to FHMNV in their natural habitat prior to their capture and may have already built up an immune response to the virus. Another possibility is that creek chub may be not susceptible to the muskellunge-adapted FHMNV strain used in this study.

FHMNV does not appear to cause risks to other piscivorous species tested (largemouth bass, walleye, and rainbow trout). All of these species tested negative for any traces of FHMNV both by qLAMP and cell culture. One potential reason for this is that these piscivorous species have an innate resistance to FHMNV. There are a variety of different molecules necessary for nidoviruses to attach to host cells (Siddell & Snijder 2008), so it is entirely possible that host cells of these species do not have the necessary receptors for FHMNV attachment. While walleye, rainbow trout and largemouth bass seem to be safe from FHMNV infection, this pathogen is still a significant problem for muskellunge, which support a large sport fishing industry in the Great Lakes basin (Baird 2015).

FHMNV's ability to infect multiple indigenous cyprinids is particularly alarming for several reasons. These species are very important ecologically as a food source for larger piscivorous species. Furthermore, fathead minnow, golden shiner and spotfin shiner are critical components of the baitfish industry, which is of high economic importance. Most of the fish used in this industry are cultured on baitfish farms; however, approximately 20% are still caught from the wild (Goodwin et al. 2011). Frequently, baitfish are shipped across state lines and then distributed either by retail or wholesale networks (Goodwin et al. 2011). This mixing and spreading of various baitfish species in combination with lack of disease screening requirements creates ideal conditions for FHMNV to spread. In addition, many sport fishermen are unaware that dumping their bait buckets at their various fishing destinations (Litvak & Mandrak 1993) may contribute to the spread of pathogenic fish viruses.

The Great Lakes region in particular is a major importer of baitfish species to be used both for recreational fishing and as a forage source in rehabilitation programs for native piscivorous species. Currently, baitfish are not required to be certified free of FHMNV prior to their introduction to the Great Lakes watershed (Michigan Department of Natural Resources, www.michigan.gov/dnr). The results of this study underscore the importance of disease screening requirements for baitfish, as well as increased education and regulation regarding baitfish use. An outbreak of FHMNV in the Great Lakes could have devastating impacts on native cyprinid populations, as well as on wild and hatchery raised muskellunge. FHMNV poses serious risks to Great Lakes native cyprinids, the baitfish industry, as well as muskellunge rehabilitation programs. Research needs to be completed to determine if and to what extent FHMNV has spread throughout the Great Lakes basin in order to develop effective managerial strategies.

Acknowledgements. The United States Fish & Wildlife Service (USFWS) generously provided funding for this project (Grant No. USFWS F12AP00632). The authors thank Professor Scott Fitzgerald for his valuable consultation in histopathology.

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Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: June 15, 2015; Accepted: January 25, 2016
Proofs received from author(s): March 21, 2016