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HETEROGENEITY IN LEVELS OF SERUM NEUTRALIZING ANTIBODIES AGAINST VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB AMONG FISH SPECIES IN LAKE ST. CLAIR, MICHIGAN, USA

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ABSTRACT: The presence of neutralizing antibodies against viral hemorrhagic septicemia virus (VHSV-IVb) was investigated in sera of 13 fish species collected from Lake St. Clair, Michigan, USA, a VHSV-endemic water body. We tested 297 sera collected May 2004–June of 2010, using a complement-dependent 50% plaque neutralization test (50% PNT). Neutralizing antibodies were detected in 23% (67/297) of the samples. The highest overall antibody prevalence (85%, 34/40) and mean positive antibody titer (12,113±11,699 SD) were detected in muskellunge (*Esox masquinongy*). Antibodies were also detected in 50% (15/30) of sampled northern pike (*E. lucius*), 25% (15/61) of freshwater drum (*Aplodinotus grunniens*), and 7% (3/41) of smallmouth bass (*Micropterus dolomieu*). All sera from channel catfish (*Ictalurus punctatus*), lake sturgeon (*Acipenser fulvescens*), quillback (*Carpiodes cyprinus*), rock bass (*Ambloplites rupestris*), shorthead redhorse (*Moxostoma macrolepidotum*), silver redhorse (*M. anisurum*), walleye (*Sander vitreus*), white perch (*Morone americana*), and yellow perch (*Perca flavescens*) were negative. Antibodies in one or more fish species were detected in all sampling years (2004, 2006, 2007, 2009, and 2010), whereas in parallel sampling periods, VHS virus was detected only in 2006 and 2009. Our results suggest the continued presence of VHSV-IVb in the Lake St. Clair ecosystem, and underscore the importance of assessing immune responses of fish populations to determine prior virus exposure.

Key words: Antibodies, Lake St. Clair, muskellunge, serologic surveillance, viral hemorrhagic septicemia virus.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is a pathogenic fish virus belonging to the family *Rhabdoviridae*, genus *Novirhabdovirus*. In spring-summer of 2005 and 2006, a novel North American VHSV sublineage (designated IVb) was implicated in fish die-off events in Lake St. Clair, Lake Erie, and Lake Ontario. Mortality episodes involved numerous fish species including freshwater drum (*Aplodinotus grunniens*; Lumsden et al., 2007), round gobies (*Neogobius melanostomus*; Groocock et al., 2007), muskellunge (*Esox masquinongy*), gizzard shad (*Dorosoma cepedianum*), yellow perch (*Perca flavescens*), and others (Winton et al., 2008; Kim and Faisal, 2011). Archived samples date the presence of VHSV-IVb in the Lake St. Clair ecosystem to at least 2003 (Elsayed et al., 2006). The discovery of this reportable virus in the Great Lakes initiated fish movement restrictions and surveillance testing for purposes of zoning and monitoring the spread of the virus. Within the next 5 yr, VHSV-IVb was isolated from all five Great Lakes, the St. Lawrence River, and several inland lakes in Michigan, New York, Ohio, and Wisconsin, USA (Kim and Faisal, 2011).

Fish are tested for VHSV following guidelines of the American Fisheries Society (Batts and Winton, 2010) and the World Organisation for Animal Health (OIE, 2009). Laboratory detection of VHSV requires isolation in cell culture followed by confirmation of the isolated

virus, most commonly by reverse transcriptase polymerase chain reaction (RT-PCR). Although this approach is currently considered the gold standard for VHS diagnosis, populations that exhibit low infection prevalences or those that have recovered and cleared the virus, can be missed.

Fish surviving infection with VHSV (Olesen and Jørgensen, 1986) and other pathogenic rhabdoviruses, including infectious hematopoietic necrosis virus (IHNV; Amend and Smith, 1974; Jørgensen et al., 1991) and spring viremia of carp virus (SVCV; Ahne, 1986), mount an adaptive immune response that includes the production of neutralizing antibodies. Survivors demonstrate enhanced resistance to disease upon subsequent exposures (LaPatra et al., 1993; Kocan et al., 2001; Ahne et al., 2002). Antibodies to VHSV remain in sera for an extended period after the virus is no longer detectable, and have been used in epizootiologic studies to identify fish populations that previously have been infected (LaPatra, 1996).

In Lake St. Clair, following the initial large-scale mortality events in spring-summer 2006, and despite surveillance testing, the virus was not isolated again until a smallmouth bass (*Micropterus dolomieu*) die-off occurred in June 2009. One logical explanation for the absence of VHSV outbreaks in Lake St. Clair in 2007, 2008, and 2010, is that fish residing in this infected zone might have developed some immunity to the virus. We sought to determine if fish in Lake St. Clair have neutralizing antibodies against VHSV-IVb and compare the levels of these antibodies among resident fish species.

MATERIALS AND METHODS

Field serum samples

Most fish were captured using trap nets set in Anchor Bay, Lake St. Clair (Fig. 1) by personnel of the Michigan Department of Natural Resources (MDNR) and the Aquatic Animal Health Laboratory (AAHL, Michigan State University, East Lansing, Michigan, USA). In May 2010, blood samples were collected by caudal venipuncture from channel catfish (*Ictalurus punctatus*, $n=25$), freshwater drum ($n=10$), muskellunge ($n=21$), northern pike (*Esox lucius*, $n=16$), quillback (*Carpiodes cyprinus*, $n=7$), rock bass (*Ambloplites rupestris*, $n=24$), shorthead redhorse (*Moxostoma macrolepidotum*, $n=6$), silver redhorse (*M. anisurum*, $n=13$), smallmouth bass ($n=25$), walleye (*Sander vitreus*, $n=10$), white perch (*Morone americana*, $n=12$), and yellow perch ($n=3$). Lake sturgeon (*Acipenser fulvescens*, $n=15$) were sampled by the MDNR the first week of June 2010. Blood was sampled nonlethally from all 13 species and fish were released, with the exception that 11 of the 21 muskellunge were euthanized following blood collection to obtain tissues (kidney, spleen, and heart) for VHSV testing. Gametes were also collected from all mature muskellunge for VHSV testing. Water temperature in May 2010 was 9–15 C.

Archived sera from muskellunge ($n=6$) and freshwater drum ($n=40$) in May 2007 and muskellunge ($n=10$) in 2009, originated from apparently healthy fish collected for VHSV surveillance. Serum samples from northern pike in May 2004 ($n=10$), all species in May 2006 ($n=28$), and smallmouth bass in June 2009 ($n=16$), were collected as part of targeted sampling efforts during, or immediately following, mortality events in Lake St. Clair. Most fish were captured alive and immediately transported on ice to the AAHL. Upon arrival, blood was collected and tissues (kidney and spleen) and reproductive fluids (from ripe fish) were sampled for VHSV testing.

For all captured muskellunge, weight and total length were recorded and a dorsal fin ray clip was taken for age estimation (Clark et al., 2004) by MDNR researchers. Blood samples from all fish were kept at 4 C overnight and then centrifuged (2,500 × G, 10 min, 4 C). Sera were aliquoted and stored at −80 C until testing.

Cell line and virus isolate

The *epithelioma papulosum cyprini* (EPC) cell line (Fijan et al., 1983) was cultured at 25 C in Earle's minimum essential medium (EMEM; Life Technologies, Carlsbad, California, USA) supplemented with 10% tryptose phosphate broth (TPB; Becton, Dickinson and Company, Sparks, Maryland, USA), 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA), 2 mM L-glutamine (Life Technologies), and buffered with 7.5% sodium bicarbonate. The Great Lakes strain of VHSV-IVb (Elsayed et al., 2006) was propagated on the EPC cell

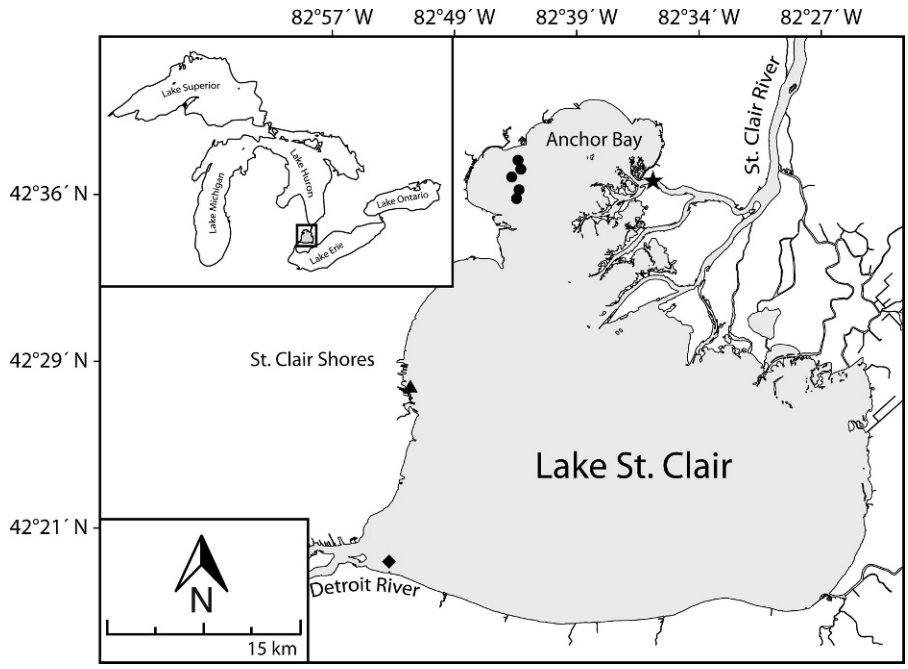

FIGURE 1. Lake St. Clair (Lake Erie watershed, USA) showing sampling sites. Most fish were captured using trap nets (●) positioned between 42°39′N, 82°46′W and 42°37′N, 82°46′W in Anchor Bay. Lake sturgeon (*Acipenser fulvescens*) were captured using survey setlines (★: 42°37′N, 82°37′W). Smallmouth bass (*Micropterus dolomieu*) from a mortality event in June of 2009 (▲: 42°29′N, 82°53′W), and a single muskellunge (*Esox masquinongy*) collected May 2006 (◆: 42°21′N, 82°54′W), were caught using scoop nets.

line and aliquots of supernatant were stored at −80 C for use in the 50% plaque neutralization test (PNT). The virus titer was determined by plaque assay as described in Batts and Winton (1989).

Virus isolation

All serum samples, tissues, and reproductive fluids collected were tested for VHSV in accordance with suggested procedures (Batts and Winton, 2010). Serum samples were diluted 1:20 (v/v) in EMEM with 10% TPB, 14 mM tris buffer, penicillin (100 U/ml), streptomycin (100 μg/ml; Life Technologies), gentamicin sulfate (100 μg/ml; Sigma-Aldrich, St. Louis, Missouri, USA), and amphotericin B (2.5 μg/ml; Lonza, Walkersville, Maryland, USA). Tissues and reproductive fluids were diluted 1:4 (w/v), homogenized, and centrifuged (2,500 × G, 30 min, 4 C). Homogenate supernatants and serum samples were inoculated onto EPC cells grown to confluency in cell culture medium (EMEM with 10% TPB, 5% FBS, 2 mM L-glutamine, 14 mM tris, and 100 U penicillin, 100 μg streptomycin, 100 μg gentamicin sulfate, and 2.5 μg amphotericin B/ml). Plates were incubated at 15 C for two successive passages and any samples exhibiting cytopathic effects (CPE) were subject to confirmation using RT-PCR.

Neutralizing antibody detection

A complement-dependent 50% PNT (LaPatra et al., 1993) was used with some modifications to detect VHSV-IVb neutralizing antibodies in fish sera. Samples were heat inactivated for 30 min at 45 C and serial twofold dilutions, at a starting dilution of 1:20, were mixed with an equal volume of VHSV-IVb (2.0×10^4 plaque-forming units/ml). Serum-virus mixtures were incubated for 30 min at 18 C with constant gentle motion. An equal volume of unheated naïve sera from lake trout (*Salvelinus namaycush*), diluted 1:30, was added as a source of complement and the incubation period was repeated. Mixtures were adsorbed for 1 hr at 15 C to 7% polyethylene glycol pretreated EPC cells in duplicate wells of 24-well tissue culture plates. Fluid was then removed and cells overlaid with 1% methylcellulose in cell culture medium (with 2× concentrated EMEM).

After incubation for 5 days at 15 C, the cells were fixed and stained with 0.5% crystal violet in formalin. Titers are reported as the reciprocal of the highest serum dilution that reduced the number of virus plaques by 50% compared to the negative control. Each time the assay was performed, positive and negative control sera were included.

Complement source

Naïve sera used in the 50% PNT were collected from adult lake trout that had been raised at the Michigan State University Research Containment Facility (MSU UCRF). Prior to blood collection, fish were anesthetized using 0.1mg/ml tricaine methanesulphonate (MS-222, Argent Chemical Laboratories, Redmond, Washington, USA) buffered with sodium bicarbonate. Blood was processed as previously described, and aliquots of pooled sera were stored in liquid nitrogen until immediately before use.

Naïve sera examination

Serum samples from three groups of naïve muskellunge, originating from hatcheries with no history of VHSV exposure, were tested with the 50% PNT to determine if innate neutralization activities against VHSV-IVb exist in sera of unexposed fish. Fish were maintained at 11±2 C in a flow-through system at the MSU UCRF and fed fathead minnows (*Pimephales promelas*) raised in a farm with no prior history of VHSV. Blood was collected weekly from Groups I (n=30; ~4 mo old) and II (n=46; ~6 mo old) for 9 wk. Fish from these groups were euthanized at the time of blood collection with an overdose of MS-222 (0.25 mg/ml) and organs tested in cell culture to confirm the absence of VHSV. Blood samples were collected nonlethally from Group III (n=7, ~22 mo old) muskellunge every 6 wk for six sampling periods (total of 42 sera).

Specificity of VHSV-IVb neutralizing antibodies

To test the specificity of the serum neutralizing activities against VHSV-IVb, six Lake St. Clair muskellunge sera (titers of 20–20,480) and a single freshwater drum serum (titer of 40) were tested with IHNV (strain 220-90; LaPatra et al., 1994). Serum samples were tested in parallel with VHSV-IVb and IHNV using the 50% PNT conditions as described previously.

Statistical analysis

Differences in the occurrence of antibodies between species, and between mature versus immature muskellunge, were compared using a two-tailed Fisher's exact test. Pearson's correlation was used to determine if antibody titers in sera of muskellunge were associated with weight or length. Significant differences were accepted at $P<0.05$.

RESULTS

Virus isolation

All serum samples from Lake St. Clair fish were negative for VHSV. Tissues collected in 2004, 2007, and 2010, and all reproductive fluids from muskellunge also were negative. VHSV was isolated in cell culture from tissues of 19 of 28 fish sampled in 2006. All muskellunge, northern pike, rock bass, and shorthead redhorse were VHSV-positive, as were 55% of freshwater drum and 20% of silver redhorse. Most of these fish exhibited external and internal petechial hemorrhages, exophthalmia, and congestion of internal organs. VHSV was also isolated from tissues of smallmouth bass (25%; 4/16) collected during a mortality event in June 2009. The identity of VHSV was confirmed in all cell culture-positive samples by RT-PCR.

Innate neutralization of VHSV by sera of naïve muskellunge

Most (89%) naïve muskellunge sera did not neutralize VHSV-IVb at the starting serum dilution of 1:20 (titer <20; Table 1). Titers of 20–80 were observed in sera of 13 of 118 muskellunge (11%). Therefore, only neutralization titers >80 are considered antibody-positive for wild muskellunge and its congener, northern pike. A specific cutoff value for other fish species could not be ascertained because of the absence of samples collected from naïve fish. Results for these species are reported at titers of ≥20.

Neutralization specificity of fish sera

Neutralization titers ranging from 20 to 20,480 in muskellunge sera and 40 in a freshwater drum serum did not cross-react with IHNV at titers of ≥40 (Table 2).

TABLE 1. Distribution of neutralizing titers against viral hemorrhagic septicemia virus in 118 serum samples collected from naïve juvenile muskellunge (*Esox masquinongy*). Group I (~4 mo., 17 cm±1 SD, 19 g±4 SD), Group II (~6 mo., 16 cm±2 SD, 17 g±5 SD), Group III (~22 mo., 32 cm±1 SD, 148 g±21 SD).

Titer	Group I (n=30)	Group II (n=46)	Group III (n=7)[a]	Overall (%)
<20	30	40	35	105 (89)
20	0	1	5	6 (5)
40	0	4	2	6 (5)
80	0	1	0	1 (1)

[a] Blood was collected nonlethally at 6-wk intervals (6 time points; 42 total sera).

Neutralizing antibodies against VHSV-IVb

Blood samples were collected from a total of 297 Lake St. Clair fish representing 13 species (Table 3). Neutralizing antibodies against VHSV-IVb were detected in 23% (67/297) of sera, and in all study years (2004, 2006, 2007, 2009, 2010). Four species, muskellunge, northern pike, freshwater drum, and smallmouth bass, were antibody-positive. The overall antibody prevalence in muskellunge (85%; 34/40) was significantly higher than northern pike (50%; 15/30; P=0.0032), freshwater drum (25%; 15/61; P<0.0001), and smallmouth bass (7%; 3/41; P<0.0001). Excluding archived sera, fish sampled in 2010 followed a similar trend; muskellunge exhibited the highest antibody prevalence (86%; 18/21), followed by northern pike (63%; 10/16), freshwater drum (20%; 2/10), and smallmouth bass (12%; 3/25). All sera from channel catfish, lake sturgeon, quillback, rock bass, shorthead and silver redhorses, walleye, white perch, and yellow perch were negative (titers <20).

TABLE 2. Serum neutralization specificity of Lake St. Clair muskellunge (1–6; *Esox masquinongy*) and freshwater drum (7; *Aplodinotus grunniens*). Sera were tested in parallel with viral hemorrhagic septicemia virus (VHSV-IVb) and infectious hematopoietic necrosis virus (IHNV) using a 50% plaque neutralization test (50% PNT).

	50% PNT titers to:	
Serum no.	VHSV-IVb	IHNV
1	20	<20
2	320	<20
3	320	<20
4	1,280	20
5	10,240	<20
6	20,480	<20
7	40	<20

Neutralization titers reached the highest levels in muskellunge; 91% (31/34) of antibody-positive individuals had titers of 2,560–40,960 (Fig. 2). In comparison, maximum titers for other antibody-positive species did not exceed 1,280 (northern pike and freshwater drum) and 320 (smallmouth bass). Mean positive (±SD) titers were higher for muskellunge (12,113±11,699) compared to northern pike (608±449), freshwater drum (201±339), and smallmouth bass (213±92). Among muskellunge, the prevalence of neutralizing antibodies was significantly higher in reproductively mature fish than immature fish (Table 4; P<0.0001). Antibody titer in this species was not correlated with length (n=40, r=0.24, P=0.14), or weight (n=39, r=0.18, P=0.27).

For some species, lower antibody prevalences were detected during VHSV-positive sampling events. The lowest yearly antibody prevalence for muskellunge (33%, 1/3) was detected in 2006 when this species was VHSV-positive. In 2006, anti-VHSV antibody prevalence in freshwater drum (0%; 0/11) was also lower compared to 2007 (33%; 13/40) and 2010 (20%; 2/10). Similarly, none of the smallmouth bass from a VHSV-positive mortality event in 2009 were antibody-positive, whereas the following year, 12% (3/25) had antibodies.

TABLE 3. Prevalence (%) of viral hemorrhagic septicemia virus (VHSV-IVb) neutralizing antibodies among Lake St. Clair fish species.

Species[a]	No. antibody-positive/No. tested (prevalence [%])[b]						Mean positive titer[c]	SD[c]
	2004	2006	2007	2009	2010	Overall		
Muskellunge	—	1/3 (33)	6/6 (100)	9/10 (90)	18/21 (86)	34/40 (85)[d]	12,113	11,699
Northern pike	2/10 (20)	3/4 (75)	—	—	10/16 (63)	15/30 (50)[e]	608	449
Freshwater drum	—	0/11	13/40 (33)	—	2/10 (20)	15/61 (25)[f]	201	339
Smallmouth bass	—	—	—	0/16	3/25 (12)	3/41 (7)	213	92
Channel catfish	—	—	—	—	0/25	0/25	NA	NA
Lake sturgeon	—	—	—	—	0/15	0/15	NA	NA
Quillback	—	—	—	—	0/7	0/7	NA	NA
Rock bass	—	0/3	—	—	0/24	0/27	NA	NA
Shorthead redhorse	—	0/2	—	—	0/6	0/8	NA	NA
Silver redhorse	—	0/5	—	—	0/13	0/18	NA	NA
Walleye	—	—	—	—	0/10	0/10	NA	NA
White perch	—	—	—	—	0/12	0/12	NA	NA
Yellow perch	—	—	—	—	0/3	0/3	NA	NA
Overall	2/10 (20)	4/28 (14)	19/46 (41)	9/26 (35)	33/187 (18)	67/297 (23)	NA	NA

[a] Scientific names are provided in text.

[b] Dashes indicate no sera were collected.

[c] NA = Not applicable.

[d] Significantly higher antibody prevalence compared to northern pike ($P=0.0032$), smallmouth bass, and freshwater drum ($P<0.0001$).

[e] Significantly higher antibody prevalence compared to smallmouth bass ($P<0.0001$) and freshwater drum ($P=0.0192$).

[f] Significantly higher antibody prevalence compared to smallmouth bass ($P=0.0332$).

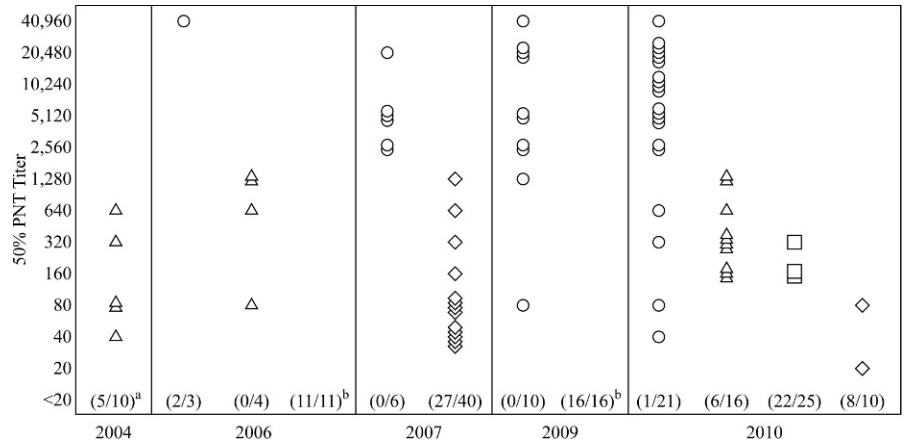

FIGURE 2. Antibody titers measured by a 50% plaque neutralization test (50% PNT) against viral hemorrhagic septicemia virus (IVb) from Lake St. Clair fish species that were antibody-positive: muskellunge (○ *Esox masquinongy*), northern pike (△ *E. lucius*), smallmouth bass (□ *Micropterus dolomieu*), freshwater drum (◇ *Aplodinotus grunniens*). Neutralization titers of >80 are considered positive for muskellunge and northern pike. [a] Number of sera with titers of <20/number sampled. [b] All freshwater drum in 2006 and smallmouth bass in 2009 had titers of <20.

DISCUSSION

Neutralizing antibodies are targeted against viral glycoproteins and are an essential component of the protective immune response of fish against rhabdoviruses (Engelking and Leong, 1989; Lorenzen et al., 1990). Muskellunge that survive infection with VHSV-IVb develop neutralizing antibodies in their sera (Millard and Faisal, 2012), and are partially resistant to disease, despite high levels of shed virus in surrounding water (Kim and Faisal, 2012). In this context, fish residing in a VHSV-endemic water body, such as Lake St. Clair, could be intermittently re-exposed to VHSV, without experiencing disease outbreaks. In this study, we detected evidence of an acquired immune response in sera of four of the 13 fish species sampled, albeit at different prevalences and titers.

The majority of Lake St. Clair muskellunge (85%) and northern pike (50%) were positive for VHSV-IVb antibodies. Neutralizing antibodies were detected in fewer sera from freshwater drum (25%) and smallmouth bass (7%), and were completely absent in sera from channel catfish, lake sturgeon, quillback, rock bass, shorthead redhorse, silver redhorse, walleye, white perch, and yellow perch. Interspecific

TABLE 4. Maturity, sex, age, and size of Lake St. Clair muskellunge (*Esox masquinongy*) included in this study.[a]

	Sex			Range (mean±SD)			No. antibody-positive (>80)/ No. sampled
	M	F	ND	Age (yr)	Length (cm)	Weight (g)	
Immature[b]	3	0	1	2–3 (3±1)	45–73 (61±12)	452–2,200 (1,370±717)	0/4[c]
Mature	23	11	0	6–20 (11±3)	88–127 (107±11)	4,000–13,800 (8,285±3,079)	34/34

[a] Table excludes two muskellunge (titers of <20) captured in 2006 due to incomplete measurements.

[b] Classification of immature fish was based upon the absence of ripe gametes and was confirmed by examination of reproductive organs. In one immature muskellunge, sex could not be determined (ND).

[c] Antibody prevalence of immature muskellunge was significantly less than that of mature muskellunge ($P<0.0001$).

variation in antibody prevalence could be related to differences in species' ecologic niches, which would influence the frequency of exposure to the virus. Esocids (muskellunge and northern pike) are top predators in the Lake St. Clair ecosystem. It is possible that their high trophic position provides more opportunities for virus exposure through ingestion of VHSV-infected prey fish. Neutralizing antibody prevalence also might be influenced by species' susceptibility to the virus, or differences in the immune mechanisms employed against VHSV-IVb. Of the 13 species sampled, seven (muskellunge, northern pike, freshwater drum, smallmouth bass, rock bass, and shorthead and silver redhorses) were positive for VHSV in this investigation, and the remaining species, with the exception of lake sturgeon and quillback, are also known to be naturally susceptible (USDA–APHIS, 2008). The dose of VHSV-IVb necessary for infection and disease in these species, however, is variable. It is possible that a relatively low virus concentration in the water could infect and induce a neutralizing antibody response by species of high susceptibility (e.g., muskellunge; Kim and Faisal, 2010b); whereas species that are less susceptible or resistant (e.g., lake sturgeon; M. Faisal, unpubl. data) might not become infected. However, because the experimental susceptibility among species included in this study is known only for muskellunge, yellow perch, and smallmouth bass (Kim and Faisal, 2010a, b), a correlation between occurrence of antibodies and susceptibility cannot be made at the present time. The absence of antibodies detectable by the 50% PNT in the nine aforementioned antibody-negative species does not necessarily imply the lack of an immune response. VHSV induces both cell-mediated and humoral defenses, including non-neutralizing antibodies that would not be detectable by the 50% PNT (Lorenzen et al., 1999).

Antibody prevalence was lower in immature muskellunge (ages 2–3) than reproductively mature fish (ages 6–20). Juvenile muskellunge (4 mo old) produce neutralizing antibodies to VHSV-IVb under laboratory conditions (Millard and Faisal, 2012). Thus, the absence of antibodies in immature Lake St. Clair muskellunge is not due to an inability to mount a neutralization response. Rather, it could be due to having fewer opportunities for virus exposure. Spawning congregations, for example, have been identified as a likely route for VHSV transmission, due to increased fish densities and increased virus concentration in the water from infected individuals (Hershberger et al., 2010; VHSV Expert Panel and Working Group, 2010). The absence of serum antibodies in immature muskellunge, as well as the small sample size, could also suggest an increased mortality rate upon VHSV exposure of young muskellunge. Intraspecific variation in neutralization titers could also be attributed to exposure dose, as well as time postexposure.

Antibody-positive status for muskellunge and northern pike was defined as having a neutralization titer of >80. This is because when we tested several groups of naïve juvenile muskellunge, with no history of VHSV-IVb exposure, a few individuals exhibited low levels of innate virus neutralization. The nature of the low level neutralization remains to be elucidated; however, some virus inhibitors have been reported in sera of unexposed rainbow trout (*Oncorhynchus mykiss*; Dorson and de Kinkelin, 1974; Park and Reno, 2005). The absence of neutralization activities in the majority of nonesocid fish sera tested in this study led us to believe that such VHSV inhibitors might not be present in sera of these species. Thus, titers of ≥ 20 were considered positive. By setting the definition of antibody-positive slightly higher for esocid fish, the number of antibody-positive fish might be underestimated by seven individuals.

Evidence for the presence of immunoglobulins in several antibody-positive Lake St. Clair muskellunge was confirmed using

an indirect ELISA that utilizes a monoclonal anti-muskellunge IgM antibody (Millard, Kaattari, and Faisal, data not shown). In our analysis, we also confirmed that virus neutralization activity in muskellunge sera was specific to VHSV, because no cross-reactivity with IHNV was observed at titers >20. Rainbow trout sera with antibodies against VHSV-I also do not cross-react with IHNV or SVCV (Olesen and Jørgensen, 1986).

Virology results from this study, as well as additional testing of tissue samples of Lake St. Clair fish by the AAHL, confirm the inability to isolate VHSV during the summers of 2007 and 2010. Antibodies, however, were detected in all sampling years. The duration of virus in tissues, induction and duration of the immune response, and outcome of rhabdovirus infection are influenced by several factors, including virus dose, water temperature, and various properties of the host (LaPatra, 1998; Lorenzen and LaPatra, 1999). When Kim (2010) infected muskellunge held at 11 C with VHSV-IVb by immersion, virus titers peaked within the first 2 wk postinfection (pi). Most fish sampled later in the disease course had no, or substantially lower, virus titers in tissues, however, virus was detectable in some fish up to 9 wk pi. At higher temperatures, VHSV is cleared from tissues more rapidly (Jørgensen, 1982; Goodwin and Merry, 2011). Antibody titers peak later in the disease course, by approximately 6 wk in rainbow trout infected with VHSV-I at 13 C (Olesen and Jørgensen, 1986), and by 11–16 wk in muskellunge infected with VHSV-IVb at 11 C (Millard and Faisal, 2012). The duration of VHSV-neutralizing antibodies is not fully understood, but antibodies remain detectable in some fish 1 yr pi (Olesen and Jørgensen, 1986).

In 2006, when fish were collected immediately following a VHSV outbreak, the virus was readily isolated in cell culture. Antibodies were only detected in 14% of the serum samples collected from these fish, and all antibody-positive individuals were also virus-positive, indicating an early convalescent stage of infection. Neutralizing antibodies were detected in >85% of muskellunge sampled in May of 2007, 2009 and 2010, indicating that the majority of adult muskellunge have survived encounters with the virus. Given the high proportion of antibody-positive individuals, it seems likely that adult muskellunge have established immunity to the virus.

In the absence of acute disease outbreaks, serologic testing would aid in the identification of fish populations that previously have been infected with VHSV. Blood samples can be taken nonlethally, which avoids the unnecessary sacrifice of valuable and endangered fish stocks. Our results suggest that muskellunge is a good species to sample nonlethally as an indicator of past VHSV-IVb exposure. Assessing immunity among Great Lakes fish stocks would provide a more accurate understanding of VHSV distribution, and could potentially be used to predict the vulnerability of a given population to VHSV outbreaks, both of which would provide valuable information for fisheries managers.

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