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Temporal Variation in Viral Hemorrhagic Septicemia Virus Antibodies in Freshwater Drum (*Aplodinotus grunniens*) Indicates Cyclic Transmission in Lake Winnebago, Wisconsin

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Viral hemorrhagic septicemia virus (VHSV) is an emerging pathogen that causes mass mortality in multiple fish species. In 2007, the Great Lakes freshwater strain, type IVb, caused a large die-off of freshwater drum (*Aplodinotus grunniens*) in Lake Winnebago, Wisconsin, USA. To evaluate the persistence and transmission of VHSV, freshwater drum from Lake Winnebago were tested for antibodies to the virus using recently developed virus neutralization (VN) and enzyme-linked immunosorbent (ELISA) assays. Samples were also tested by real-time reverse transcription-PCR (rRT-PCR) to detect viral RNA. Of 548 serum samples tested, 44 (8.03%) were positive by VN (titers ranging from 1:16 to 1:1,024) and 45 (8.21%) were positive by ELISA, including 7 fish positive by both assays. Antibody prevalence increased with age and was higher in one northwestern area of Lake Winnebago than in other areas. Of 3,864 tissues sampled from 551 fish, 1 spleen and 1 kidney sample from a single adult female fish collected in the spring of 2012 tested positive for VHSV by rRT-PCR, and serum from the same fish tested positive by VN and ELISA. These results suggest that VHSV persists and viral transmission may be active in Lake Winnebago even in years following outbreaks and that wild fish may survive VHSV infection and maintain detectable antibody titers while harboring viral RNA. Influxes of immunologically naive juvenile fish through recruitment may reduce herd immunity, allow VHSV to persist, and drive superannual cycles of transmission that may sporadically manifest as fish kills.

Viral hemorrhagic septicemia virus (VHSV) is an emerging pathogenic virus that threatens populations of marine and freshwater fish throughout the world and was recently introduced into the Great Lakes Basin, affecting at least 31 species (1–7). Clinical signs of VHS include hemorrhage, exophthalmia, anemia, and abdominal distension. The virus is transmitted through urine or reproductive fluids and can remain viable in the water for up to 14 days (8). One laboratory-controlled experiment demonstrated the virus to be viable in untreated freshwater for up to 40 days at 4°C (9).

Viral hemorrhagic septicemia virus was first detected in farmed rainbow trout in Europe in 1938 (10). The virus was isolated from Coho and Chinook in U.S. Pacific coastal waters during the late 1980s (8), representing the first documentation of the virus in the United States. VHSV was later detected in mummichog (*Fundulus heteroclitus*), brown trout (*Salmo trutta*), striped bass (*Morone saxatilis*), Pacific herring (*Clupea pallasi*), shiner perch (*Cymatogaster aggregate*), and threespine sticklebacks (*Gasterosteus aculeatus*) in marine environments along the Atlantic and Pacific coasts of North America (11, 12). In 2003, a new freshwater strain of VHSV, type IVb, was isolated from spawning muskellunge from Lake St. Clair (1). In 2005 to 2007, large-scale epizootics of wild fish populations occurred in Lakes Ontario, Erie, and St. Clair; Little Lake Butte des Morts; and Lake Winnebago in Wisconsin, and virus was isolated from fish in Lakes Huron and Michigan. Little Lake Butte des Morts and Lake Winnebago are inland lakes approximately 60 km from Lake Michigan and connected to Lake Michigan by the Fox River, which flows into the bay of Green Bay (1–4, 6, 13, 14). The large-scale epizootics that occurred throughout the Great Lakes region affected wild populations of muskellunge (*Esox masquinongy*), freshwater drum (*Aplodinotus grunniens*), round gobies (*Neogobius melanostomus*), smallmouth bass (*Micropterus dolomieu*), and yellow perch (*Perca flavescens*) (1–4, 6, 13).

The source of VHSV introduction into the Great Lakes Basin remains unclear, but ship ballast water discharge, contaminated live well water from recreational boating, and shedding of virus by migratory fish have been implicated (15, 16). Surveys using tissue culture and real-time reverse transcription (rRT)-PCR indicate that VHSV is widely dispersed throughout the Laurentian Great Lakes (17) and could have spread through multiple routes (5, 14,

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15, 18). The introduction of VHSV and potential economic impacts associated with the spread of the virus led to increased surveillance in Wisconsin and other states within the Great Lakes Basin. Surveillance efforts targeted susceptible fish species in select water bodies. In Wisconsin, the majority of VHSV isolations have occurred from fish kills and diagnostic cases, rather than in samples collected for surveillance (Wisconsin Department of Natural Resources [DNR], unpublished data).

Many fish species important to recreational angling are susceptible to the virus, including muskellunge (*E. masquinongy*), northern pike (*Esox lucius*), and largemouth bass (*Micropterus salmoides*). A recent study used VHSV antibody detection by complement-dependent 50% plaque neutralization test (50% PNT) to show the presence of neutralizing antibodies. VHSV antibody prevalence ranged from 7% to 85% in 13 fish species collected from a water body in Lake St. Clair, Michigan, where VHSV is endemic (19). To date, however, such studies have been infrequent due to lack of availability of reliable serological diagnostic tests. Consequently, it has been difficult to ascertain the proportion of wild fish that have been infected by VHSV and have survived. It has also been difficult to infer whether the virus persists in wild fish populations in postepizootic years.

Our study focuses on a naturally abundant, VHS-susceptible species in Lake Winnebago, Wisconsin, that is not commercially important: the freshwater drum. This population experienced a fish kill in 2007 in which hundreds of freshwater drum were observed dead from late April to late May (13). Formerly, methods for VHSV detection were restricted to virus isolation and rRT-PCR; both methods detect virus but do not indicate past exposure or immunity to the virus. By measuring the antibody response to VHSV, our study sheds new light on patterns of past exposure to VHSV in Lake Winnebago drum.

MATERIALS AND METHODS

Sampling of freshwater drum. A total of 548 freshwater drum were obtained by Wisconsin DNR personnel from Lake Winnebago, Wisconsin, via bottom trawl assessments in fixed locations conducted in the spring and fall of 2011 and 2012 (water temperatures were between 12.5 and 15.5°C). Lake Winnebago is the largest inland lake in Wisconsin at 55,728 ha with an average depth of 4.7 m (20). Collection sites were divided into eight locations on the lake (Fig. 1), with four central locations, two northern locations, and two southern locations. Fall samples were collected with a balloon trawl, as previously described (20), towed at 5 min per haul at a speed of 6.6 kilometers per hour, resulting in sampling of 0.405 ha. Spring samples were collected with a smaller (12-ft head rope) trawl towed at 4 kilometers per hour for various time periods, depending on catch rates. Trawls were performed in at least three different locations on the lake during each sampling period, including locations where VHSV had been isolated from fish during outbreaks in 2007.

At least 60 adult drum and 60 juvenile or young-of-the-year drum were obtained during each sampling period. Sample sizes were based on calculations from binomial probability distributions indicating that a sample size of 60 fish would yield a 95% probability of sampling at least one VHS-infected individual, given a minimum infection prevalence of 5% (21). Randomly selected drum from each trawl and age group were anesthetized by immersion in 50 mg/liter Tricaine-S methanesulfonate (MS-222; Western Chemical, Inc.) for 5 min. At least 1 ml of blood was collected from the caudal vein of each fish using 18- or 22-gauge needles and 5- to 10-ml syringes. The needle was then removed from the syringe, and the blood was slowly dispensed into a no-additive red-top glass blood tube (Monoject), which was gently rolled to stimulate clotting. The blood tubes were stored at ambient temperature for 2 to 6 h and then centrifuged

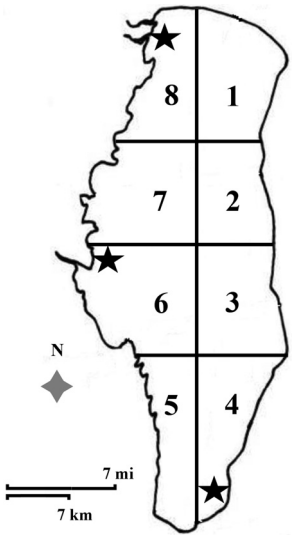

FIG 1 The eight sampling locations in Lake Winnebago, Wisconsin. The stars indicate previous VHSV isolation sites determined by the Wisconsin DNR during prior surveillance efforts.

at 1,000 × *g* for 15 min. The serum was removed with sterile, disposable pipettes into 2-ml cryovials (Corning) and stored at −80°C. After blood collection, the fish were euthanized by immersion in 200 mg/liter MS-222 for 10 min and immediately placed on ice in separate labeled plastic bags. The serum was heated at 45°C for 30 min to inactivate complement (22).

The fish were necropsied at the Wisconsin Veterinary Diagnostic Laboratory (WVDL) within 24 h of collection. The necropsy procedures included the use of separate sterile instruments for each fish and each tissue to prevent cross-contamination of samples. Samples of gill, gonad, liver, spleen, kidney, heart, and brain were collected from each fish and stored in separate sterile cryovials at −80°C. Length, weight, and any external clinical signs of disease were recorded for each fish. To estimate age, the sagittal otoliths were removed from each fish at the time of collection, wiped dry, and placed in coin envelopes. The otoliths were cut in half along a transverse plane through the nucleus (23) using a Pfingst 189/220 circular saw blade mounted on a Dremel rotary tool. The newly exposed surface of each otolith half was polished with wetted 1,000-grit sandpaper and placed in a dish of plumber's putty with the polished surface facing up. The otoliths were viewed under a dissecting microscope at ×1 to ×2 magnification, and the annuli were illuminated using a 0.08-mm-diameter fiber optic light (Dolan-Jenner Industries; model BMY2724) with immersion oil used to improve image clarity. The annuli were enumerated by a single experienced reader.

Viral-RNA extraction and VHSV detection by real-time RT-PCR. A highly sensitive rRT-PCR assay that targets the viral nucleocapsid gene was used to detect viral RNA (24). We added individual tissue samples (approximately 100 mg) to 1 ml of ice-cold phosphate-buffered saline (PBS) in MagNA Lyser Green Beads tubes (Roche) and homogenized them at 6,500 rpm for 30 s in the MagNA Lyser instrument (Roche). We extracted viral RNA from tissue homogenates using the MagMax-96 Viral RNA Isolation Kit (Ambion; 1836) according to the manufacturer's instructions. We then sealed and stored the extraction plates at −20°C until PCR testing.

For PCR, we used the Quantitect Probe RT-PCR kit (Qiagen) containing 2× QuantiTect Probe RT-PCR master mix, RNase-free water, and QuantiTect RT Mix. We included a negative-extraction control, a no-template control, and a positive-amplification control in each PCR run. The thermal cycle profile was 30 min at 50°C and 15 min 95°C, followed by 40 cycles of 15 s at 94°C, 40 s at 60°C (with endpoint data collection), and 20 s at 72°C on an ABI Prism 7500 machine (24). We adjusted the cycle

TABLE 1 Summary statistics for Lake Winnebago freshwater drum[a]

Fish status	Sample size	Sample size by sex (M/F/U)	Age (yr)	Length (mm)	Wt (kg)
VN negative	504	243/184/77	8.8 (6.80)	292.1 (51.3)	0.32 (0.26)
VN positive	44	18/19/7	12.9 (8.39)	318.1 (71.2)	0.46 (0.54)
ELISA negative	503	233/189/81	8.9 (6.9)	291.2 (50.1)	0.31 (0.23)
ELISA positive	45	25/19/1	11.4 (6.96)	316.2 (65.9)	0.45 (0.54)
Total	548	261/203/84	9.1 (6.98)	293 (52.0)	0.32 (0.27)

[a] Age, length, and weight values are means (standard deviations). M, male; F, female. U indicates undetermined sex of immature fish.

threshold (C_T) to 10% of the plateau of the standard amplification curve, so that the results were considered positive at a threshold (C_T value) of 40 cycles.

Detection of neutralizing antibodies by virus neutralization assay. We used a recently developed virus neutralization (VN) assay to detect neutralizing antibodies in serum (22). Twofold serial dilutions of serum were mixed with 100 times the 50% tissue culture infective dose ($TCID_{50}$) of the virus (25, 26) and incubated at 15°C for 24 h. The serum-virus mixtures were then inoculated onto 7% polyethylene glycol (PEG)-treated epithelioma papulosum cyprinid (EPC) cells and incubated at 15°C for 5 days (27). We tested all the sera at a starting dilution of 1:16 to minimize the effects of hemolysis and to maximize sensitivity (22).

Detection of nucleocapsid antibodies by blocking ELISA. We used a newly developed blocking enzyme-linked immunosorbent assay (ELISA) to detect antibodies against the nucleocapsid of VHSV in sera (22). The blocking ELISA uses a monoclonal antibody (Aquatic Diagnostics, Sterling, Scotland) directed against the nucleocapsid (N) protein of the virus and is conjugated to horseradish peroxidase (HRP). We first performed ELISA with undiluted test serum. In cases where hemolysis or debris in the serum resulted in high background, we ran the ELISA again with serum diluted at 1:2 in PBS. We accepted the results from the 1:2-diluted serum if the background was indeed reduced. We considered ELISA results from undiluted serum to be positive at ≥35% inhibition and results from diluted sera to be positive at ≥25% inhibition. Positive thresholds were determined from percent inhibitions of known positive and negative sera and the results of a receiver operating characteristic (ROC) curve analysis (22).

Multivariate predictors of seroprevalence. A mixed-effects logistic-regression model with a random effect for season was fitted to examine location, age, size (length and weight), and sex as predictors of seropositivity in Lake Winnebago drum. Analyses were run in R (28).

RESULTS

VHSV detection by real-time RT-PCR. Of 551 freshwater drum collected over the four sampling periods for which tissues were suitable for testing, the rRT-PCR method detected VHSV RNA in spleen and kidney tissues from a single fish (0.18% prevalence). This female fish (422-mm total length; 0.92 kg; 24 years old) was collected on 9 May 2012. The spleen tissue had a C_T of 38.4, and the kidney tissue had a C_T of 38.7. Virus was not detected in any other tissues from the fish.

To confirm these results, we used rRT-PCR to retest 140 drum tissues extracted as described above, and also at 1:10 dilutions to determine if PCR inhibition might have occurred when extracted tissues were tested undiluted. All reextracted and retested tissues were confirmed negative. Retesting of the single positive female drum confirmed the individual's positive status.

Detection of neutralizing antibodies by virus neutralization assay. We detected neutralizing antibodies in 44 of 548 (8.03%) fish collected during the study (Table 1). The seroprevalences of neutralizing antibodies varied significantly among the collection periods ($\chi^2 = 9.81$; df = 3; $P = 0.01$) and were highest in the fall of 2011 (9.9%) and the spring of 2012 (13.1%) and lowest in the spring of 2011 (4.6%) and the fall of 2012 (4%) (see Fig. 3). Neutralizing antibody titers ranged from 1:16 to 1:1,024 in 1- to 28-year-old fish, with a mean titer of approximately 1:128 (Fig. 2). Sera showing partial neutralization of 50% or more at 1:16 were considered positive and designated <1:16 (partial); sera showing complete protection from viral infection of the cells (no plaques or cytopathic effect) were designated 1:16. We observed partial neu-

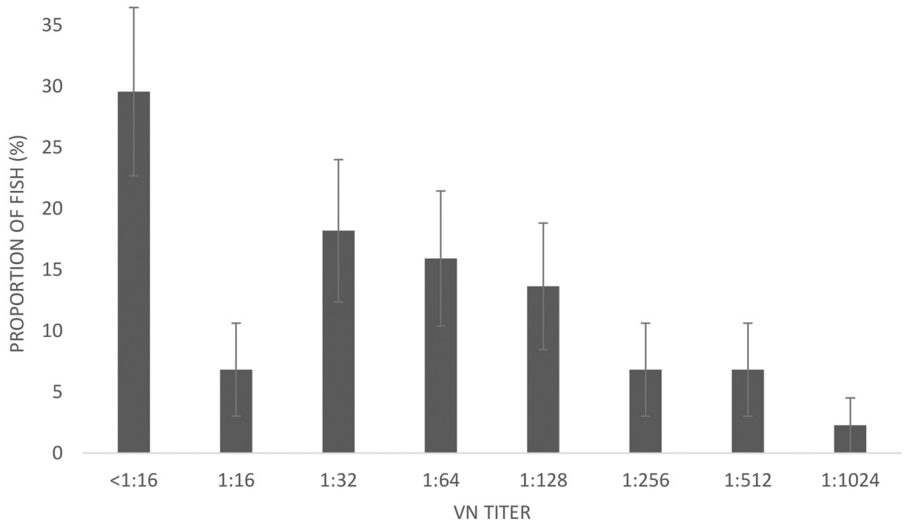

FIG 2 Distribution of neutralizing antibody titers ($n = 44$) against viral hemorrhagic septicemia virus in freshwater drum from Lake Winnebago, Wisconsin. The error bars indicate ±1 standard error of the mean.

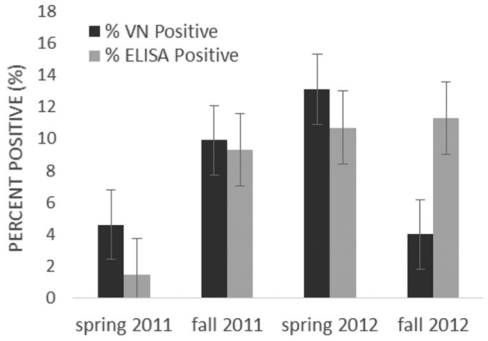

FIG 3 Seroprevalence of freshwater drum from Lake Winnebago, Wisconsin, for viral hemorrhagic septicemia virus in spring 2011 ($n = 130$), fall 2011 ($n = 172$), spring 2012 ($n = 122$), and fall 2012 ($n = 125$). The error bars indicate ±1 standard error of the mean.

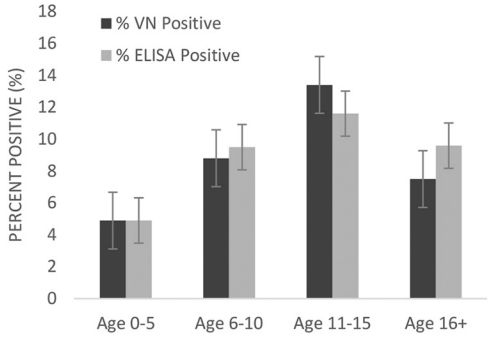

FIG 4 Seroprevalence of freshwater drum from Lake Winnebago, Wisconsin, for viral hemorrhagic septicemia virus by age class (0 to 5 [$n = 205$], 6 to 10 [$n = 137$], 11 to 15 [$n = 112$], and 16 to 28 [$n = 94$] years), based on VN (neutralizing antibodies) and ELISA (nonneutralizing antibodies). The error bars indicate ±1 standard error of the mean.

tralization in dilutions at 1:16 for 12/44 (29.5%) neutralizing antibody-positive drum.

The highest prevalence of neutralizing antibody was observed in the three oldest age classes in the fall 2011 sample. The second-highest neutralizing antibody prevalence was observed in the same age classes sampled in spring 2012. Neutralizing antibody titers were found in fish 6 to 23 years old collected in spring 2011, 1 to 28 years old in fall 2011 and spring 2012, and 2 to 28 years old in fall 2012. Furthermore, the single fish with VHSV RNA detected in the spleen and kidney tissues also had a low neutralizing antibody titer of 1:16.

Detection of nucleocapsid antibodies by blocking ELISA. ELISA results were positive in 8.21% (45/548) of the fish collected during the 2-year study. Seroprevalences varied significantly among the sampling periods ($\chi^2 = 10.48$; df = 3; $P = 0.02$) and were higher in the fall of 2011 (9.3%), the spring of 2012 (10.7%), and the fall of 2012 (11.3%) than in the spring of 2011 (1.5%) (Fig. 3). Inhibition ranged from 25.48% to 72.12% for samples considered positive when sera were tested at a 1:2 dilution. Increasing antibody prevalence with increasing age class was also observed for anti-nucleocapsid antibody prevalence, similar to neutralizing antibody prevalence (Fig. 4). In this case, however, the antibody prevalence declined slightly from the 11- to 15-year-old age class to the 16- to 28-year-old age class. Additionally, the fish with VHSV RNA detected in its tissues also tested positive by ELISA, undiluted and at a 1:2 dilution. Although freshwater drum sera were not available from known VHS-negative locations, sera from other species were evaluated for specificity. Spring viremia of carp virus (SVCV) antibody-positive sera were collected from Cedar Lake, Wisconsin, following an epizootic in 2002, prior to detection of VHSV in Wisconsin, and one sample was tested in our ELISA. As previously described (22), this serum tested negative, demonstrating the high specificity of our ELISA.

Multivariate predictors of seroprevalence. Our generalized linear mixed model indicated that neutralizing antibody positivity was significantly associated with higher fish age (z score = 2.71; $P = 0.006$) (Fig. 4) and collection from the northwest part of Lake Winnebago (z score = 4.19; $P = 0.001$). Age and collection from the northwest part of Lake Winnebago together accounted for 9.27% of the variation in neutralizing antibody positivity.

ELISA positivity was significantly associated with higher fish age (z score = 2.26; $P = 0.02$), higher fish weight (z score = 2.20; $P = 0.03$), and sampling in fall 2011 and spring 2012 ($\chi^2 = 10.35$; df = 3; $P = 0.02$). Weight and sex together accounted for 5.43% of the variation in ELISA positivity. ELISA-based seroprevalences were approximately 9% for males, 9% for females, and 1% for immature fish (sex undetermined), indicating a significantly higher prevalence of ELISA antibodies in mature fish than in immature fish. Age was not significant in the multivariate model for ELISA, likely because of confounding effects with sex or weight. Total antibody positivity (VN and ELISA) varied significantly by season ($\chi^2 = 12.8$; df = 3; $P = 0.005$), with higher seroprevalences occurring during the last three sampling seasons. Antibody positivity increased with age (z score = 3.83; $P < 0.001$) and was significantly associated with collection from the northwest location in Lake Winnebago (z score = 2.09; $P = 0.04$). Overall, age and collection from the northwest location together accounted for 5.39% of the variation in total antibody positivity (combined neutralizing and anti-nucleocapsid antibodies).

Although not all locations on the lake were sampled during each season, the population of freshwater drum in Lake Winnebago is well mixed, so the locations sampled are considered representative of the population as a whole (Wisconsin DNR biologists, personal communication). Thus, our results should not be markedly affected by uneven geographic sampling among sampling periods.

DISCUSSION

The freshwater drum is a VHSV-susceptible species that has experienced mass mortality events due to VHS (1–3, 5, 6, 13). Further, VHSV was responsible for a fish kill in the species in Lake Winnebago in 2007. By applying a newly developed VN assay and blocking ELISA for detecting antibodies against VHSV to freshwater drum collected from Lake Winnebago, we offer the first insights into patterns of VHSV type IVb seropositivity related to the demographic characteristics of a wild fish population. Our results demonstrate that 16.2% of freshwater drum sampled during our study had either neutralizing (anti-glycoprotein) antibodies (8.03%), nonneutralizing (anti-nucleocapsid) antibodies (8.21%), or both (1.3%). Furthermore, our results demonstrate differences in seroprevalence across seasons, years, and age classes and collectively suggest that VHSV transmission may still be ongoing in Lake Winnebago and that the virus is present even in postepizootic years. We note that 83.8% of the fish sampled tested negative for

both neutralizing and nonneutralizing antibodies. It is unlikely that other viral protein antibodies were present.

We hypothesize that the pattern of increased VHSV seroprevalence after the spring 2011 collection may reflect natural oscillations in VHSV transmission due to the reproductive patterns of freshwater drum. Seasonal breeding in the species leads to the annual recruitment of immunologically naive, young-of-the-year fish each May through June (29), typically following the window of water temperatures most suitable for VHSV replication. This influx of immunologically naive hosts may create susceptible populations that drive VHSV transmission, leading to superannual cycles of seropositivity, as observed in our data. Although we expected to see a similar pattern of decreased antibody prevalence in spring 2012, we did not see such a pattern. The average young-of-the-year catches per drag of the trawl net were 83.24 for 2010 and 10.92 for 2011. This decrease in year class strength from 2010 to 2011 preceded increased antibody prevalence in spring 2010, implying that recruitment may have contributed to the patterns observed (data provided by the Wisconsin DNR).

We also observed a significant effect of age on seropositivity, with older fish being more likely to have neutralizing antibodies. Increasing weight of the fish was also positively associated with anti-nucleocapsid antibodies. The increase in serum antibodies in older fish indicates increased probability of exposure to the virus with time and, perhaps, increased protection (30, 31). The correlation between higher antibody titers and weight likely reflects a similar relationship, where older fish attain higher weights and are exposed to antibodies for a longer time. Additionally, we suspect that younger fish had lower antibody prevalence because they had not yet been exposed or their immune systems had not yet responded to infection.

We were surprised to observe spatial structuring in seropositivity within Lake Winnebago. One location in the northwestern part of the lake had higher seroprevalence than other locations. This location is approximately 16 km north of Asylum Bay, where the drum fish kill was observed in 2007 (13). These results could indicate spatial structuring in the Lake Winnebago drum population or spatial heterogeneity in the distribution of VHSV. Regardless, our results demonstrate that the distribution of seropositivity to VHSV can vary geographically within a water body, so that sampling more than one location may be necessary to ascertain the serostatus of a population.

Our results suggest that VHSV transmission may be active in Lake Winnebago even when fish kills are not observed. The interannual differences in antibody prevalence that we observed would be expected in the case of viral transmission and subsequent declining immunity. There may also be a seasonal pattern of infection influenced by environmental factors, such as temperature, contact rates, and stress during spawning (32). This conclusion is directly supported by our detection of VHSV RNA in a single female drum in spring 2012. The fish had viral RNA in kidney and spleen, a low neutralizing antibody titer, and a low anti-nucleocapsid antibody response, collectively suggesting clearing of the virus as a protective immune response was mounting. We note that no clinical signs of VHS or any other disease were observed grossly or by necropsy in this fish or any other fish sampled during the study. Our detection of only one such fish out of approximately 500 indicates that VHSV infection must be very rare or very transient. Considering the wide host range of VHSV type IVb, other species besides freshwater drum may serve as reservoirs for the virus. Persistent VHSV type IVa infections have been demonstrated in Pacific herring surviving previous exposures to the virus, likely resulting from chronic infections in those individuals or transmission from fish to fish (33). Nevertheless, individuals may shed the virus and transmit it to immunologically naive individuals, such as young of the year, perhaps seeding outbreaks when herd immunity wanes.

Outbreaks of VHS have not been reported in Lake Winnebago since the first detection of VHSV in freshwater drum in 2007. The suggestion that viral transmission may be ongoing in Lake Winnebago raises management concerns for that water body and others. If the seasonality of drum reproduction does indeed reduce herd immunity in the years following a VHS outbreak, then superannual cycles of VHS should be expected. This scenario argues strongly against the assumption that lakes, once affected by VHSV, should not be monitored or should be considered to have achieved a "new equilibrium." On the contrary, our data suggest ongoing, cyclical VHSV transmission; low-level transmission even in postepizootic years; and VHSV in adult drum even in the presence of a neutralizing antibody response. Our results also suggest that seropositive fish ages 0 to 5 may confer sufficient herd immunity to limit viral shedding to below the threshold needed to cause disease. Regardless, drum and other seasonally breeding species with significant age structuring may be at particular risk for future outbreaks.

Because our assays are species independent, the methods we describe can be applied to any fish species in any location, thus offering a useful new tool for VHSV surveillance. Our methods should be of particular interest to fishery managers because blood can be drawn from anesthetized fish, whereas accepted standard tissue culture techniques require fish to be euthanized. The nonlethal tests described in this study can also be used to assess whether fish have been exposed to VHSV and have developed antibodies, while tissue culture techniques demonstrate only whether sampled fish are infected with the virus. In our study, we were able to detect antibodies in almost 90 fish, while only a single fish tested positive by rRT-PCR.

One limitation of our study is lack of knowledge about the duration of the VHSV antibody response in wild fish. It would be valuable to assess VHSV antibody response in controlled settings, where environmental variables can be manipulated (e.g., temperature and water quality). In the meantime, we encourage the adoption of serologic diagnostics, in addition to methods of direct viral detection, for management and control of VHSV in Wisconsin and elsewhere. Populations of seasonally breeding fish showing age structure in seroprevalence and superannual cycles of seroprevalence should be monitored closely. This is especially important in years following high recruitment when seroprevalence declines, presumably indicating a corresponding decline in herd immunity, increased probability of VHSV transmission, and increased risk of VHS-associated epidemic mortality.

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