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Widespread Seropositivity to Viral Hemorrhagic Septicemia Virus (VHSV) in Four Species of Inland Sport Fishes in Wisconsin

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Abstract

Serological assays were conducted for anti-viral hemorrhagic septicemia virus (VHSV) antibodies in four species of fish in Wisconsin (Bluegill *Lepomis macrochirus*, Brown Trout *Salmo trutta*, Northern Pike *Esox lucius*, and Walleye *Sander vitreus*) to examine spatial and temporal distributions of exposure. Sera were tested for non-neutralizing anti-nucleocapsid antibodies to VHSV by blocking enzyme-linked immunosorbent assay (ELISA). Results (percent inhibition [%I]) were analyzed for differences among species, across geographic distance, and among water management units. Positive fish occurred in 37 of 46 inland water bodies tested, including in water bodies far from reported outbreak events. Using highly conservative species-specific thresholds (mean %I of presumptive uninfected fish + 2 SDs), 4.3% of Bluegill, 13.4% of Brown Trout, 19.3% of Northern Pike, and 18.3% of Walleye tested positive for VHSV antibodies by ELISA. Spatial patterns of seropositivity and changes in %I between sampling years were also analyzed. These analyses explore how serology might be used to understand VHSV distribution and dynamics and ultimately to inform fisheries management.

Strain IVb of viral hemorrhagic septicemia virus (VHSV; Rhabdoviridae, *Novirhabdovirus*) emerged in the early 2000s in U.S. waters of the Laurentian Great Lakes (Elsayed et al. 2006; Thompson et al. 2011) and has caused episodes of mortality in more than 30 fish species (Kim and Faisal 2010a, 2010b; Faisal et al. 2012; Olson et al. 2013; Warg et al. 2014; Wilson-Rothering et al. 2015). The Wisconsin Department of Natural Resources (WI DNR) routinely monitors state fish hatcheries, source waters for these hatcheries, broodstock, wild fish, and feeder fish for VHSV, with the goal of preventing viral spread. However, active management of VHSV is critical because the U.S. Department of Agriculture's Animal and Plant Health Inspection Service continues to require states to maintain regulations that reduce the risk of VHSV spread despite lifting the viral hemorrhagic septicemia (VHS) federal order in 2014 (USDA-APHIS 2014).

In Wisconsin, VHSV has been detected only in the Great Lakes, the Lake Winnebago system, and closely connected waters since 2012 (WI DNR 2019). However, those results are based on assays that detect live virus and viral nucleic acids rather than on antibody detection assays, which indicate prior exposure to VHSV. Wilson-Rothering et al. (2015) showed that VHSV antibodies persisted years after a mass mortality event in Freshwater Drum *Aplodinotus grunniens* in Lake Winnebago. Of 548 Freshwater Drum that were tested 5 years after a documented VHSV outbreak, 8.0% were antibody positive by virus neutralization assay and 8.2% were positive by enzyme-linked immunosorbent assay (ELISA), with seven fish testing positive by both assays (Wilson-Rothering et al. 2015). Similarly, Millard and Faisal (2012) detected the presence of neutralizing antibodies in Freshwater Drum, Muskellunge *Esox masquinongy*, Northern Pike *E. lucius*, and Smallmouth Bass *Micropterus dolomieu* sampled over a 6-year period from Lake St. Clair, Michigan, even though virus was detected in only two of the six sampling years. Other studies confirm that VHSV persists in populations even during interepidemic years (Hershberger et al. 2010; Kim and Faisal 2012; Millard and Faisal 2012). For example, Kim and Faisal (2012) documented that a single exposure to VHSV allows surviving fish to shed high titers of virus into the water for 15 weeks postinfection and that shedding can be extended or resumed by exposure to stress. Hershberger et al. (2010) were able to detect VHSV in kidney, spleen, and brain tissues from experimentally infected Pacific Herring *Clupea pallasii* 224 d after exposure.

Currently, the most common method for targeted surveillance testing, as outlined by the American Fisheries Society (AFS) "Blue Book" (Batts and Winton 2020) and the World Organisation for Animal Health (OIE 2019b), is viral isolation in cell culture followed by PCR, which requires lethal sampling of fish tissues and is both cumbersome and time-consuming. However, as recently as 2020, target-specific antibody tests are gaining momentum and are now recommended as surveillance tools by AFS (Batts and Winton 2020). Wilson-Rothering et al. (2014) developed an ELISA that detects nonneutralizing antinucleocapsid antibodies to VHSV across fish species by using nonlethal blood samples. The original publication showed that the test performed well in Brown Trout *Salmo trutta*, Yellow Perch *Perca flavescens*, Grass Carp *Ctenopharyngodon idella*, Pacific Herring, Muskellunge, and Freshwater Drum (sensitivity = 96.4%; specificity = 88.2%), and we (Thiel et al. 2020) recently demonstrated that the test performed adequately in Northern Pike (sensitivity = 80.6%; specificity = 63.2%). However, this test has yet to be used for broad surveillance of wild fish populations.

Here, we present a serosurvey of fish populations across Wisconsin's inland water bodies by using the nonlethal blocking ELISA developed by Wilson-Rothering et al. (2014, 2015). This effort yields the first comprehensive assessment of VHSV exposure and activity in inland Wisconsin water bodies and, to our knowledge, the first such assessment in any state or region. The results of this study should be useful for the management of wild and captive fisheries in Wisconsin and elsewhere.

METHODS

Field sampling.— From March to November 2016 and from March to June 2017, 46 different inland water bodies were sampled across Wisconsin, and sera were collected from 1,662 fish (367 Bluegill *Lepomis macrochirus*, 442 Brown Trout, 450 Northern Pike, and 403 Walleye *Sander vitreus*). Fisheries management districts (FMDs; four management zones based on delineated Wisconsin counties under the direction of fisheries biologists) provided a management-relevant framework for classifying sampling locations, and fish were sampled as equally as possible across and within FMDs by choosing comparable numbers and geographic ranges of locations per district. State fisheries biologists and technicians captured fish by using a variety of methods, including fyke netting, boom shocking, stream shocking, and capture via spawning weir (Zale et al. 2013). Fish were held in aerated tanks and processed on a wet table with water continuously flowing over the gills. Blood samples (between 0.5 and 3.0 mL, depending on the size of the fish; Use of Fishes in Research Committee 2014) were collected from the caudal vein of each fish by using an 18-, 21-, or 22-gauge needle and a 3–5-mL syringe; samples were transferred to a no-additive, red-top glass blood tube (Monoject; VWR International, Radnor, Pennsylvania) and were inverted repeatedly to initiate clotting. All fish were released at the point of capture. Blood samples were stored on ice in the field and at 4°C in the laboratory overnight to allow clotting. Within 24 h after

collection, samples were centrifuged at $3,200 \times g$ for 15 min, and sera were transferred to sterile, 2.0-mL cryovials and stored at $-80°C$.

In March 2017, Lake St. Clair in Michigan experienced an outbreak of VHS in which tens of thousands of fish died, including Gizzard Shad *Dorosoma cepedianum*, Bluegill, Pumpkinseed *L. gibbosus*, Black Crappie *Pomoxis nigromaculatus*, Largemouth Bass *M. salmoides*, Muskellunge, Northern Pike, Freshwater Drum, Common Carp *Cyprinus carpio*, and Yellow Perch, along with common mudpuppies *Necturus maculosus* (Whelan 2017). As of June 2017, the known epidemic region included the St. Clair River, Michigan; Lake Erie; and parts of the Huron River in Ohio (Whelan 2017). To capitalize on this documented VHSV outbreak, the field team collected blood samples from Northern Pike (3 fish) and Walleye (32 fish) with the assistance of the Michigan Department of Natural Resources in May 2017, and samples were processed and stored as described above.

Antibody detection by ELISA. — The ELISA method developed by Wilson-Rothering et al. (2014, 2015), with minor alterations by Thiel et al. (2020), provided the basis for this serological assessment. This blocking ELISA uses a monoclonal antibody (Aquatic Diagnostics, Stirling, Scotland; conjugated by American Qualex, San Clemente, California) against the nucleocapsid protein of the virus (Olesen et al. 1991; Wilson-Rothering et al. 2014). Negative-control samples consisted of pooled sera from confirmed-negative, hatchery-reared Brown Trout from the Wild Rose State Fish Hatchery (Wild Rose, Wisconsin), which regularly tests for VHSV using viral detection methods. Wild-fish serum was tested at a 1:8 dilution (serum : phosphate-buffered saline), and optical density (OD) readings were adjusted by subtracting the OD value contributed by the sera reacting with uninfected cells. Results were reported as percent inhibition (%I), normalized to correct for overdevelopment of negative samples by adjusting results by a factor equal to the negative-control OD divided by the highest sample OD on each plate (Wright et al. 1993).

Because of the management consequences of false-positive results, two complementary and highly specific threshold criteria were used to classify fish as positive. First, Bluegill, Brown Trout, Northern Pike, and Walleye results were considered positive at 2 SDs above the mean %I for presumptive uninfected fishes (OIE 2019a; Bluegill: ≥50.26%I; Brown Trout: ≥50.21%I; Northern Pike: ≥56.48%I; Walleye: ≥48.38%I). Second, alternative positive thresholds were also calculated for Brown Trout and Northern Pike by using a receiver operating characteristic curve based on published results for these species. For Brown Trout, an alternative threshold of ≥25%I was used (Wilson-Rothering et al. 2014). For Northern Pike, an alternative threshold of ≥58.2%I was used (Thiel et al. 2020; note that

the published threshold of ≥41.3%I in experimentally infected Northern Pike was altered to improve results for surveillance purposes, which increased specificity to 95.4% and therefore decreased sensitivity of the assay to 34.5%).

Data analyses. — Statistical analyses were conducted in R version 3.3.3 (R Core Team 2017). Analysis of variance was used to compare mean %I among species, along with a Shapiro–Wilk test to assess for assumptions of normality and a Levene's test to assess homogeneity of variances. Because the assumptions of normality and homogeneity of variances were violated, the nonparametric Kruskal–Wallis rank-sum test with post hoc Dunn's test was used to evaluate differences in %I among species and differences in positivity among seasons. Similarly, for any water body where more than one species was sampled, Spearman's rank-order correlation was used to assess associations between mean %I of water bodies in which the same pairs of species were sampled during the same year. To examine risk factors for seropositivity, multivariate logistic regression was conducted with individual- and location-specific factors as predictors and the serostatus of a fish (positive or negative) assigned based on the most conservative criterion of 2 SDs above the mean %I. Data were analyzed while including effects for clustering by sampling event using the glm function in R. Multiple models were considered using different combinations of variables, and the best model was chosen based on comparison using Akaike's information criterion values. Multiple diagnostic plots were examined to check for linearity of relationships, normality of the distribution of residuals, and variance homogeneity of the residuals as well as to detect influences on regression results. Goodness of fit was assessed with McFadden's pseudo-R^2 (0.440). To examine spatial patterns of VHSV seroreactivity, maps were created using the ggmap package in R base maps for the states of Wisconsin and Michigan (Kahle and Wickham 2013). For analysis of water management units (WMUs) in Wisconsin, the open data shapefile for WMUs was provided by the WI DNR (2018). To test for spatial autocorrelation in %I among sampling sites within each species and year, Moran's index I was used. Additionally, sampling locations were sorted into WMUs and tested for similarity of mean %I within WMUs by using Kruskal–Wallis rank-sum tests.

RESULTS

Descriptive Statistics

Overall, 14.6% of 1,697 fish sampled from 47 water bodies (including those sampled from Lake St. Clair, Michigan) tested positive for VHSV antibodies (using a threshold of 2 SDs above the mean %I for presumptive uninfected fishes). Fish sampled in spring had the highest positivity (15.2%), followed by those sampled in summer (14.7%) and fall (11.2%). There was no significant

difference in positivity among seasons (Kruskal–Wallis test: $\chi^2 = 2.19$, $df = 2$, $P = 0.33$). Percent inhibition ranged from 0.00%I to 91.59%I, with a mean ± SD of 33.06 ± 17.37%I. Two or more species of fish were sampled at 22 of 47 water bodies (Figure 1; Table 1). Water temperature ranged from 2.22°C to 20.94°C. Length and weight of sampled fish ranged from 12.0 to 98.0 cm and from 0.03 to 7.40 kg, respectively.

Comparisons of ELISA Results among Species

Distribution and range of %I did not vary substantially by species (see Figure 2); however, differences in mean %I among species were statistically significant (Kruskal–Wallis rank-sum test: $\chi^2 = 107.99$, $df = 3$, $P < 0.0001$). Post hoc analysis showed that the mean %I for each species varied significantly from those of other species (Dunn's test: all $P < 0.05$), except for Brown Trout and Walleye (Dunn's test: $P = 0.39$). Of all fish tested, Northern Pike had the highest seropositivity (19.9%), followed by Walleye (18.8%), Brown Trout (13.6%), and Bluegill, which had the lowest seropositivity (4.4%). This finding is similar to that reported by Kim and Faisal (2010a) in comparing the susceptibility of representative Great Lakes fishes.

Enzyme-Linked Immunosorbent Assay Results Using Species-Specific Thresholds

The overall number of positive fish of all species tested across Wisconsin was 237 (14.2%) of 1,662 based on the

FIGURE 1. Numbered map of Wisconsin water bodies sampled in 2016 and 2017. For water body names and full details, including surveillance results, see Table 1. Location 46 (Lake St. Clair, Michigan) is not pictured here.

threshold criterion of 2 SDs above the mean %I for presumptive uninfected fishes. Thirty-seven of 46 inland water bodies sampled had at least one seropositive fish. Sixteen Bluegill (4.3%), 60 Brown Trout (13.4%), 87 Northern Pike (19.3%), and 74 Walleye (18.3%) tested positive. At least one seropositive fish was found in 7 of 20 water bodies where Bluegill were sampled, 14 of 18 water bodies where Brown Trout were sampled, 18 of 23 water bodies where Northern Pike were sampled, and 13 of 18 water bodies where Walleye were sampled. The locations with the highest seropositivity for each species in 2016 were Lake Sherwood for Bluegill (33.3%), Elk Creek (Chippewa County) for Brown Trout (30.3%), Lac Courte Oreilles for Northern Pike (75.0%), and Pelican Lake for Walleye (47.0%). The locations in Wisconsin with the highest seropositivity for each species in 2017 were Lake Wisconsin for Bluegill (9.0%), Lake Winnebago (Asylum Bay) for Northern Pike (33.3%), and Rock Lake for Walleye (20%; Brown Trout were not sampled in 2017).

Documented VHS outbreaks have occurred and fish have tested positive for VHSV by virus isolation, PCR, and ELISA serum testing during multiple years between 2005 and 2018 in Lake Winnebago (including Asylum Bay) and between 2003 and 2017 in Lake St. Clair (Faisal et al. 2012; Wilson-Rothering et al. 2015; Whelan 2017; Kamke 2018; WI DNR 2019). In Lake Winnebago, 17 of 65 fish (26.2%) tested positive. In Lake St. Clair, 11 of 35 fish (31.4%) tested positive, making this lake (where the most recent documented VHSV outbreak occurred) the location with the highest seropositivity in our study. See Table 1 for species-specific results at Lake Winnebago and Lake St. Clair.

Use of the alternative %I thresholds based on published values for Brown Trout (≥25.0%I) and Northern Pike (≥58.2%I) expectedly increased the estimated numbers of seropositive Brown Trout and Northern Pike (see Table 1 for results by location). However, the locations in Wisconsin that contained the highest proportions of seropositive fish of each species as determined by the initial threshold criterion (i.e., 2 SDs above the mean) were the same locations that contained the highest proportions of seropositive fish as determined by the alternative threshold criterion (published values). Figure 3 and Supplementary Figure 1 (available in the online version of this article) depict the geographic distribution of seropositive fish based on both threshold values.

Comparison of Locations Tested in Both Field Seasons

Eight locations were sampled in both 2016 and 2017: the Yellow River, Turtle Flambeau Flowage, Rock Lake, Madeline Lake, Lac Courte Oreilles, Fox River, Clear Lake, and Lake Winnebago (Asylum Bay). Supplementary Figure 2 shows the direction and magnitude of the change in average %I at each sampling site for each species. Clear Lake and Lac Courte Oreilles had an increase in mean %I

TABLE 1. Information on Wisconsin water bodies (Figure 1) sampled for Bluegill, Brown Trout, Northern Pike, and Walleye during 2016 and 2017. Lake St. Clair, Michigan, was also sampled and is included here.

Map number[a]	Water body	Latitude	Longitude	WMU[b]	Year(s) sampled	Species sampled[d]	% Positive, established thresholds[e]	% Positive, alternative thresholds[f]
1	Ash Creek	43.2991	−90.4317	Lower Wisconsin	2016	T (5)	0 (5)	20 (5)
2	Besadny Anadromous Fisheries Facility	44.4642	−87.5595	Twin–Door–Kewaunee	2016	T (29)	0 (29)	20 (29)
3	Bluff Creek	42.8175	−88.693	Lower Rock	2016	T (28)	6.8 (28)	44.8 (28)
4	Clear Lake	45.868	−89.6259	Upper Wisconsin	2016 2017	B (21) B (3)	0 (21) 0 (3)	
5	Coon Valley	43.6844	−91.0404	Bad Axe–La Crosse	2016	T (27)	25.9 (27)	55.5 (27)
6	Delevan Lake	42.6014	−88.6152	Lower Rock	2016	W (26)	15.3 (26)	
7	Dutch Hollow Lake	43.6072	−90.1793	Lower Wisconsin	2016	B (1), N (30), W (30)	0 (1), 20 (30), 23.3 (30)	26.6 (30)
8	Elk Creek	43.4522	−90.658	Lower Wisconsin	2016	T (15)	0 (15)	53.5 (15)
9	Elk Creek	44.8502	−91.6639	Lower Wisconsin	2016	T (33)	30.3 (33)	90.9 (33)
10	Emmons Creek	44.3002	−89.2384	Wolf River	2016	T (21)	9.5 (21)	90.4 (21)
11	Fox River	44.4635	−88.0542	Lower Fox	2016 2017	N (9), W (23) W (17)	11.1 (9), 8.6 (23) 4.1 (17)	11.1 (9)
12	Green Lake	43.7971	−89.0236	Upper Fox	2016	W (21)	4.7 (21)	
13	Lac Courte Oreilles	45.8902	−91.4347	Upper Chippewa	2016 2017	B (16), N (8) B (21), N (30), W (24)	6.25 (16), 75 (8) 0 (21), 3.3 (30), 4.1 (24)	75 (8), 3.3 (30)
14	Lake Altoona	44.8169	−91.441	Lower Chippewa	2016	N (26), W (1)	7.69 (26), 0 (1)	7.69 (26)
15	Lake Kegonsa	42.9799	−89.2333	Lower Rock	2016	B (32), N (24)	6.35 (32), 62.5 (24)	62.5 (24)
16	Lake Sherwood	44.2026	−89.8048	Central Wisconsin	2016	B (21), N (4)	33.3 (21), 0 (4)	0 (4)
17	Lake Winnebago	44.1061	−88.4787	Upper Fox	2016	B (23), N (30)	8.69 (23), 46.6 (30)	43.3 (30)
18	Lipsett Lake	45.8738	−92.0485	Saint Croix	2016	N (30)	16.6 (30)	16.6 (30)
19	Little La Crosse River	43.8593	−90.8209	Bad Axe–La Crosse	2016	T (29)	3.4 (29)	41.3 (29)
20	Little Saint Germaine Lake	45.9257	−89.4419	Upper Wisconsin	2016	B (29), N (31), W (13)	3.4 (29), 19.3 (31), 0 (13)	16.1 (31)
21	Long Lake	45.6857	−91.7106	Lower Chippewa	2016	B (1), N (2), W (25)	0 (1), 50 (2), 20 (25)	50 (2)
22	Madeline Lake	45.89	−89.6476	Upper Wisconsin	2016 2017	N (5), W (2) B (10), N (24)	0 (5), 0 (2) 0 (10), 4.16 (24)	0 (5) 4.16 (24)
23	Middle Eau Claire Lake	46.3031	−91.5178	Saint Croix	2016	B (14), N (2), W (28)	0 (14), 0 (2), 28.5 (28)	0 (2)
24	Milwaukee Harbor	43.0175	−87.9026	Milwaukee River	2016	T (2)	0 (2)	0 (2)

TABLE 1. CONTINUED.

Map number[a]	Water body	Latitude	Longitude	WMU[b]	Year(s) sampled	Species sampled[d]	% Positive, established thresholds[e]	% Positive, alternative thresholds[f]
25	Mississippi River Pool 10	43.0978	−91.1536	Bad Axe–La Crosse	2016	B (26), W (3)	0 (26), 0 (3)	
26	Mormon Coulee	43.7655	−91.1437	Bad Axe–La Crosse	2016	T (30)	3.3 (30)	56.6 (30)
27	Neenah Creek	43.7922	−89.6149	Upper Fox	2016	T (31)	29.0 (31)	64.5 (31)
28	Otter Creek	43.581	−90.6624	Lower Wisconsin	2016	T (31)	3.2 (31)	67.7 (31)
29	Pelican Lake	45.5072	−89.2104	Upper Wisconsin	2016	B (17), N (24), W (34)	0 (17), 12.5 (24), 47.0 (34)	8.3 (24)
30	Petenwell Lake	44.1871	−89.9135	Central Wisconsin	2016	N (6), W (29)	16.6 (6), 41.3 (29)	16.6 (6)
31	Plum Creek	43.135	−90.9445	Lower Wisconsin	2016	T (33)	9.0 (33)	84.8 (33)
32	Red River	45.0509	−89.0119	Wolf River	2016	T (24)	16.6 (24)	66.6 (24)
33	Rock Lake	43.0857	−88.9295	Upper Rock	2016 2017	B (22), N (5), W (29) B (20), W (30)	0 (22), 0 (5), 31.0 (29) 0 (20), 20 (30)	0 (5)
34	Rush Creek	43.399	−91.0983	Bad Axe–La Crosse	2016	T (29)	24.13 (29)	79.3 (29)
35	Spring Coulee	43.6901	−90.9358	Bad Axe–La Crosse	2016	T (27)	7.4 (27)	77.7 (27)
36	Tainter Creek	43.4132	−90.8926	Lower Wisconsin	2016	T (27)	25.9 (27)	85.1 (27)
37	Tomorrow River	44.4851	−89.3029	Wolf River	2016	T (21)	4.7 (21)	19.0 (21)
38	Turtle Flambeau Flowage	46.0962	−90.2452	Upper Chippewa	2016 2017	N (7), W (18) N (15), W (19)	14.2 (7), 5.5 (18) 0 (15), 5.2 (19)	0 (7) 0 (15)
39	White Lake	45.163	−88.7696	Wolf River	2016	B (20), N (27)	5 (20), 59.2 (27)	59.2 (27)
40	Yellow River	45.8213	−91.8898	Saint Croix	2016 2017	B (11), N (27) B (15), N (6)	0 (11), 14.8 (27) 0 (15), 0 (6)	14.8 (27) 0 (6)
41	Asylum Bay (Lake Winnebago)	44.0617	−88.5152	Upper Fox	2017	B (9), N (3)	0 (9), 33.3 (3)	0 (3)
42	Eau Claire Lake	44.762	−91.1012	Lower Chippewa	2017	B (2), N (21), W (24)	0 (2), 0 (21), 4.1 (24)	0 (21)
43	Grass Lake	44.6891	−88.6742	Wolf River	2017	B (1)	0 (1)	0 (1)
44	Lake Poygan	44.1754	−88.7869	Upper Fox	2017	N (25)	0 (25)	0 (25)
45	Lake Wisconsin	43.3724	−89.5585	Lower Wisconsin	2017	B (22)	9.09 (22)	0 (25)

TABLE 1. CONTINUED.

Map number[a]	Water body	Latitude	Longitude	WMU[b]	Year(s) sampled	Species sampled[d]	% Positive, established thresholds[e]	% Positive, alternative thresholds[f]
46	Lake St. Clair	42.5473	−82.8272	N/A[c]	2017	N (3), W (32)	100 (3), 25 (32)	100 (3)
47	Nepco Lake	44.3437	−88.8091	Central Wisconsin	2017	B (10), N (29), W (7)	0 (10), 0 (29), 0 (7)	0 (29)

[a]For a numbered map of water bodies, see Figure 1.
[b]Water management unit (WMU) delineated by the Wisconsin Department of Natural Resources (groups of watersheds, basins, and common drainage systems for management purposes).
[c]No WMU is listed for Lake St. Clair because it is in Michigan and WMUs are specific to Wisconsin.
[d]Species (number of fish sampled is shown in parentheses). Species abbreviations are as follows: B = Bluegill, T = Brown Trout, N = Northern Pike, and W = Walleye.
[e]Percent positive (number of fish tested is shown in parentheses) using positive thresholds for each species established from the mean percent inhibition (%I) of uninfected fish (fish with an optical density [OD] ≥negative-control OD) plus 2 SDs. Thresholds were ≥50.26%I for Bluegill, ≥50.21%I for Brown Trout, ≥56.48%I for Northern Pike, and ≥48.38%I for Walleye.
[f]Percent positive using published threshold criteria of ≥25%I for Brown Trout (Wilson-Rothering et al. 2014) and ≥58.2%I for Northern Pike (Thiel et al. 2020).
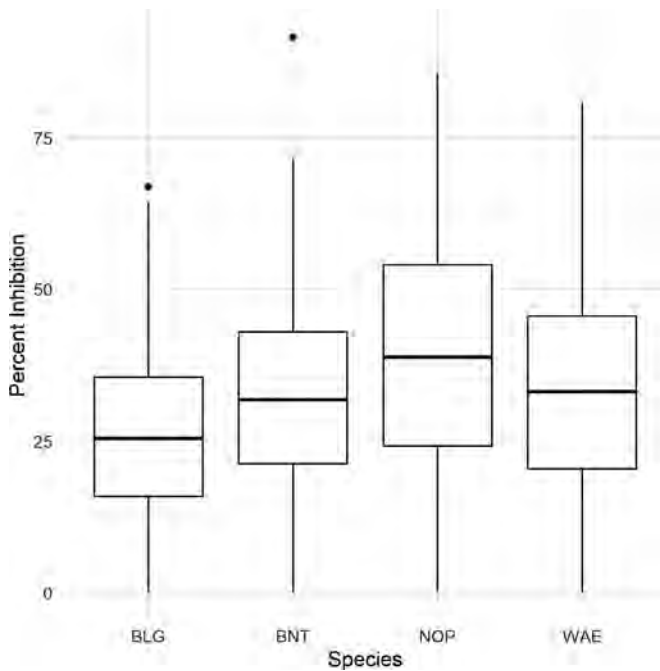
FIGURE 2. Box plot of percent inhibition of the enzyme-linked immunosorbent assay for viral hemorrhagic septicemia virus by species (BLG = Bluegill; BNT = Brown Trout; NOP = Northern Pike; WAE = Walleye). Mean percent inhibition differed among species (Kruskal–Wallis test: $\chi^2 = 107.99$, $df = 3$, $P < 0.0001$), and each species varied significantly from the other species (Dunn's test: all $P < 0.05$) except BNT and WAE (Dunn's test: $P = 0.39$).

for Bluegill only. All other locations and species showed an overall decrease in mean %I from 2016 to 2017.

Risk Factor Analyses

Table 2 depicts the results of multivariate logistic regression for the serostatus of fish based on species, WMU, water temperature, fish TL, and month and year of sampling. With the exception of sampling month, all variables examined were significant predictors of the serostatus of fish. Fish weight was not analyzed because it was strongly correlated with length. Species was the strongest binary predictor of serostatus (adjusted odds ratios between 8.86 and 35.07), followed by WMU, fish TL, and year (adjusted odds ratio = 0.39, reflecting a 2.56-fold decrease from 2016 to 2017). Walleye were at the highest risk of seropositive status, followed by Northern Pike, Brown Trout, and Bluegill. Total length and water temperature were also significant, with fish TL being protective (odds of seropositivity decreased by 0.96-fold for every 1-cm increase in length) and water temperature being a risk factor (odds of seropositivity increased by 1.16-fold for every 1°C increase in water temperature at the time of sampling). Month of sampling was not a significant predictor of serostatus; however, it is notable that July and October had the highest adjusted odds ratios (1.03 and 1.09, respectively).

Mean %I showed no significant association with straight-line distances between water bodies for any species in either sampling year (all $P > 0.190$). However, mean %I differed significantly among WMUs for Bluegill, Brown Trout, Northern Pike, and Walleye in 2016 as well as for Bluegill and Walleye in 2017 (Supplementary Table 1 available in the online version of this article). Maps of mean %I by species and WMU for each sampling year are presented in Supplementary Figure 3.

In 2016 and 2017, we sampled Bluegill, Northern Pike, and Walleye at several of the same water bodies (Figure 3). We found no significant correlation in %I among pairs of species from the same water bodies during the same year (Bluegill and Northern Pike, 2016: Spearman's rank correlation coefficient = −0.006, $P = 0.991$; Bluegill and Walleye, 2016: Spearman's = −0.107, $P = 0.839$; Northern Pike and Walleye, 2016: Spearman's = −0.090, $P = 0.811$; Bluegill and Northern Pike, 2017: Spearman's = −0.6, $P = 0.41$; Bluegill and Walleye, 2017: Spearman's = 0.2, $P = 0.916$; Northern Pike and Walleye, 2017: Spearman's = 0.2, $P = 0.916$).

DISCUSSION

Distribution of VHSV Seropositivity in Wisconsin

Results of ELISA testing suggest that VHSV in Wisconsin has not been localized to the Great Lakes, Green Bay, and Lake Winnebago systems, as was concluded from previous surveillance efforts using viral detection methods (virus isolation followed by PCR confirmation; WI DNR 2019). Fish with high VHSV seroreactivity occurred throughout Wisconsin, with the central, southwestern, and northwestern regions having the highest seroreactivity; even with the most stringent criteria, positive Bluegill, Brown Trout, Northern Pike, and Walleye were documented throughout the state. These findings are consistent with other serologic assessments of VHSV, demonstrating that viral transmission may be active in certain species and locations even when die-offs are not evident (Hershberger et al. 2010; Kim and Faisal 2012; Millard and Faisal 2012; Wilson-Rothering et al. 2015). To the extent that these observations might prove similar in other states and regions, they demonstrate (1) the importance of the addition of serologic testing for VHSV and (2) the likely underestimation of the virus's geographic distribution.

Comparison of Locations Tested in Both Field Seasons

An overall interannual increase in mean %I was found for Bluegill from 2016 to 2017, but an overall decrease in mean %I was observed for the other species during the same period. Although there were sampling differences between years (a limitation of this study), future studies tracking antibody kinetics of individual fish or populations of fish over time (e.g., tracking of sentinel fish or
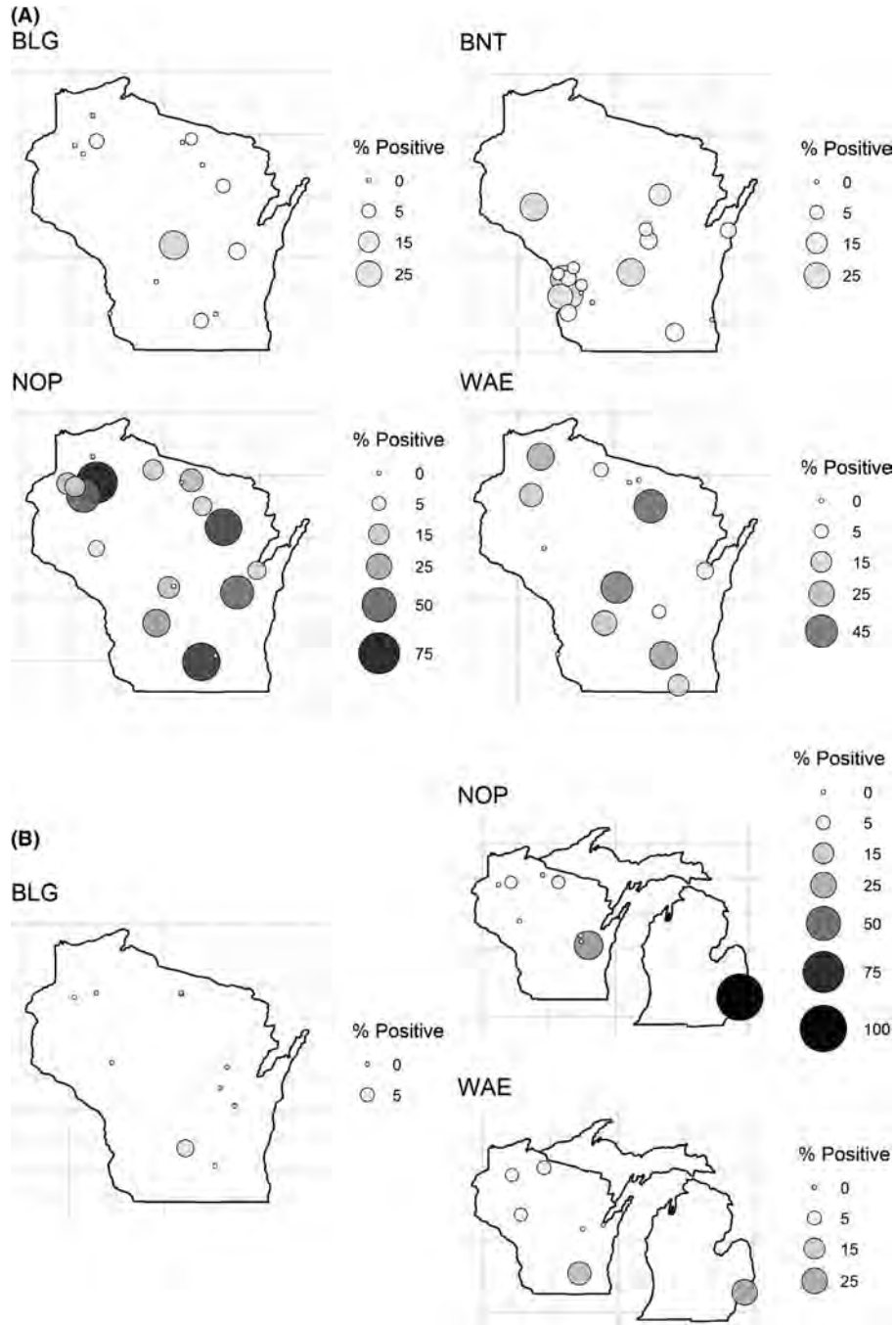
FIGURE 3. Results of surveillance efforts in **(A)** 2016 and **(B)** 2017. Percentages of Bluegill (BLG), Brown Trout (BNT), Northern Pike (NOP), and Walleye (WAE) that tested positive for antibodies to viral hemorrhagic septicemia virus by enzyme-linked immunosorbent assay at each sampling location in Wisconsin are shown. Positive thresholds (percent inhibition [%I]) were ≥50.26%I for BLG, 50.21%I for BNT, 56.54%I for NOP, and 48.38%I for WAE. Size and shading of points reflect the magnitude of percent positive by location on a continuous scale. The same positive thresholds were used for both years. Brown Trout were not sampled in 2017.

populations), in parallel with testing for the virus itself, would help to assess whether temporal changes in VHSV seroreactivity indicate undetected viral transmission (i.e., viral transmission in the absence of fish die-offs), as was shown for Freshwater Drum in Lake Winnebago (Wilson-Rothering et al. 2015).

TABLE 2. Results of multivariate logistic regression (LR) for serostatus of fish (positive or negative) based on species, water management unit (WMU), water temperature, fish TL, and sampling month and year (aOR = adjusted odds ratio).

Source	β	SE (β)	Wald χ^2	P (LR test)	aOR	95% CI of aOR
Species; reference = Bluegill			29.98	<0.001		
Brown Trout	2.18	1.00			8.86	1.2, 65.6
Northern Pike	3.10	1.01			22.22	5.38, 91.68
Walleye	3.56	1.29			35.07	9.51, 129.36
WMU; reference = Bad Axe–La Crosse			28.06	0.003		
Central Wisconsin	−0.58	0.82			0.09	0.02, 0.58
Lower Chippewa	−1.95	0.59			0.56	0.09, 3.56
Lower Fox	−1.61	0.72			0.20	0.04, 1.13
Lower Rock	−1.86	0.89			0.16	0.02, 1.03
Lower Wisconsin	−2.29	0.65			0.10	0.02, 0.52
Milwaukee River	−18.00	1.28			0.00	0, ∞
St. Croix	−1.45	0.76			0.24	0.04, 1.38
Upper Chippewa	−2.82	0.55			0.06	0.01, 0.31
Upper Fox	−1.94	1.16			0.13	0.01, 1.30
Upper Rock	−1.94	0.54			0.14	0.03, 0.69
Upper Wisconsin	−1.00	0.97			0.37	0.08, 1.72
Wolf River	−2.69	0.76			0.07	0.01, 0.54
Fish TL (cm)	−0.04	0.03	8.28	0.003	0.96	0.94, 0.99
Water temperature (°C)[a]	0.15	0.08	4.85	0.025	1.16	1.02, 1.32
Month; reference = Apr			8.38	0.128		
Mar	−1.45	1.47			0.23	0.02, 2.25
May	−1.29	0.43			0.27	0.09, 0.86
Jun	−0.46	1.19			0.63	0.05, 8.36
Jul	0.03	1.37			1.03	0.07, 15.89
Sep	−0.38	0.95			0.68	0.04, 10.93
Oct	0.09	1.37			1.09	0.11, 10.53
Nov	−14.22	1.23			0.00	0, ∞
Year (2016 vs. 2017)	−1.10	0.43	7.98	0.003	0.39	0.18, 0.82

[a]Water temperature on the date of sampling.

Risk Factor Analyses

Species, WMU, fish length, water temperature, and sampling year were statistically significant predictors of VHSV seropositivity. Both individual factors (species and length) and environmental factors (location, year, and temperature) affected the odds of seropositivity. For example, increasing fish length was protective against positive serostatus, perhaps reflecting an increased susceptibility of younger fish or waning immunity over time. Within the range of values examined, water temperature was a risk factor, supporting the observation that VHSV outbreaks (and optimal viral growth and/or higher metabolism) occur in late spring, when water temperatures begin to warm (Kim and Faisal 2010a; Hershberger et al. 2013). Mechanistic explanations for the strong species, geographic, and temporal differences revealed by this analysis remain elusive, but the differences likely reflect combinations of biological and stochastic ecological host–virus dynamics.

There was no significant association between mean %I and straight-line geographic distance between water bodies for any fish species tested. However, mean %I values were not significantly different for water bodies located within the same WMU. Water management units are groups of watersheds delineated by the WI DNR for management purposes based on physiographic and political criteria (WI DNR 2018). Localized movements of fish, water, and possible vectors (Faisal and Winters 2011) within watersheds may better explain the observed patterns of VHSV distribution than long-distance movement of the virus between watersheds (e.g., by boaters or anglers; VHS Expert Panel and Working Group 2010). For example, the watersheds in the WMUs with the highest mean %I for each species in 2016 all had a common major drainage system, the Mississippi River, which is currently considered VHSV free. It is notable that some seronegative water bodies were located very close to seropositive water bodies (Figure 3; Supplementary

Figure 1), suggesting that exposure to VHSV is not uniform within WMUs. Studying the movement of fish and water within such watershed units may provide valuable insights into the spread of VHSV.

Limitations

The ELISA on which these inferences are based has certain limitations. Although blocking ELISA assays are theoretically species independent, significant differences in assay results for different fish species indicate the need for species-specific modifications. For example, nonspecific binding of antibodies was more evident in Northern Pike (47.6% of serum tested had an OD $\geq$0.1 on the negative antigen well) than in Bluegill, Brown Trout, or Walleye (2.7, 2.4, and 3.4%, respectively). Although %I calculations reduce the effects of non-specific binding on our results, there is still a risk of false positives. For this reason, highly conservative thresholds were adopted to maximize specificity (2 SDs above the mean), and alternative positive thresholds were also considered for Brown Trout and Northern Pike based on published data for these species (Wilson-Rothering et al. 2014; Thiel et al. 2020; the published threshold for Northern Pike was altered to increase specificity for surveillance purposes—see Methods for details). The thresholds chosen (Table 1) may change as new data are collected, but the use of such baselines for management decisions is feasible. Unfortunately, published threshold values were unavailable for Bluegill and Walleye. Future studies are needed to establish such thresholds in these and other species (Thiel et al. 2020).

The ELISA would also benefit from additional validation using sera of known-negative wild fish—for example, from water bodies far from VHSV endemic areas—to further increase specificity of the assay and confirm a lack of cross-reactivity between wild-fish sera and the VHSV antigen. Wilson-Rothering et al. (2014) confirmed that the nucleocapsid monoclonal antibody used in this ELISA does not cross-react with spring viremia of carp virus, another rhabdovirus that is native to Wisconsin. Other studies have confirmed a lack of immunologic cross-reactivity between VHSV and spring viremia of carp virus as well as several other common fish rhabdoviruses, including infectious hematopoietic necrosis virus, pike fry rhabdovirus, rhabdovirus anguilla, nodavirus, infectious salmon anemia virus, koi herpesvirus, salmon alphavirus, and *Piscirickettsia salmonis*-infected cells (Aquatic Diagnostics; Lorenzen et al. 1988; Ristow et al. 1991; Wilson-Rothering et al. 2014). However, it cannot be ruled out that yet-undiscovered viruses could be present that cross-react with this assay.

Management Implications

These findings suggest that current testing strategies used for management of VHS may be improved by the further development of serological methods. To our knowledge, there have been no documented declines in the four fisheries addressed in this study for any of the seropositive water bodies, but not all water bodies are monitored closely enough to be certain. The addition of management practices that emphasize active surveillance, longitudinal monitoring of target populations, and using sentinel fish of several species to estimate infection risk might yield actionable data to control the spread of VHSV. As stated in a recent review of the use of serology in finfish (Jaramillo et al. 2017), serological tests detect historical infection and are therefore better at assessing the disease status of a population. Serological tests also have desirable characteristics for use in fish health management applications, such as surveillance studies, which require low sample sizes and are cost-effective, and biosecurity practices to outline disease-free zones.

The results of this study may also help to improve VHSV management in Wisconsin and other locations where future research identifies similar patterns. If, as the data suggest, positive and negative water bodies exist in close proximity, then strategies to contain the local spread of the virus could be enacted and evaluated by using serologic testing. Such strategies could include selecting hatchery broodstock from seronegative inland water bodies (verified through continued serologic monitoring) and treating inflowing hatchery source water from natural water bodies with a history of VHSV seropositivity (Gaumnitz 2003).

Conclusion

Serologic assessments of VHSV exposure in four species of economically important sport fish in Wisconsin (Bluegill, Brown Trout, Northern Pike, and Walleye) demonstrated the value of the addition of serological testing to current testing protocols. Analysis of seroreactivity to VHSV at the level of the water body and fish species indicated that major watershed units differed significantly in seroreactivity, straight-line geographic distance did not predict similarity in VHSV seroreactivity, certain seronegative water bodies were located near seropositive water bodies, and patterns of seroreactivity among fish species from the same water bodies were uncorrelated, suggesting that viral transmission dynamics may be localized. These results demonstrated how increased serologic testing would aid in the understanding of VHSV epidemiology and fisheries management from hatchery systems to wild fish populations.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.