Article Title

Widespread seropositivity to viral hemorrhagic septicemia virus in four species of inland sport fishes in Wisconsin

Whitney A Thiel, *University of Wisconsin- Madison, Robert P. Hanson Laboratories, 1656 Linden Drive, Madison, WI 53706, USA*

Kathy L Toohey-Kurth, *Professor of Clinical Diagnostic Microbiology, University of California- Davis, 105 W Central Ave, San Bernardino, California 92408, USA*

David Giehtbrock, *NR Program Manager, Wisconsin Department of Natural Resources, 2801 Progress Road, Madison, WI 53716, USA*

Bridget B Baker, *Clinical Veterinarian & Deputy Director - WATER Lab, Wayne State University, 101 Integrative Biosciences Center, 6135 Woodward Ave., Detroit, MI 48202, USA*

Megan Finley, *Aquatic Veterinarian, Washington Department of Fish and Wildlife, 3860 Highway 97A, Wenatchee, WA 98801, USA*

& Tony L Goldberg* *John D. MacArthur Chair, Professor of Epidemiology, Department of Pathobiological Sciences, School of Veterinary Medicine and Associate Director for Research, UW-Madison Global Health Institute, University of Wisconsin-Madison, Robert P. Hanson Laboratories, 1656 Linden Drive, Madison, WI 53706, USA*

*Author to whom correspondence should be sent.

Suggested Running Head: Widespread seropositivity to VHSV in WI

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/AAH.10120

DR. TONY GOLDBERG (Orcid ID : 0000-0003-3962-4913)

Article type : Article

Corresponding author mail id: tony.goldberg@wisc.edu

Article Title

Widespread seropositivity to viral hemorrhagic septicemia virus in four species of inland sport fishes in Wisconsin

Whitney A Thiel, University of Wisconsin- Madison, Robert P. Hanson Laboratories, 1656 Linden Drive, Madison, WI 53706, USA

Kathy L Toohey-Kurth, Professor of Clinical Diagnostic Microbiology, University of California- Davis, 105 W Central Ave, San Bernardino, California 92408, USA

David Giehtbrock, NR Program Manager, Wisconsin Department of Natural Resources, 2801 Progress Road, Madison, WI 53716, USA

Bridget B Baker, Clinical Veterinarian & Deputy Director - WATER Lab, Wayne State University, 101 Integrative Biosciences Center, 6135 Woodward Ave., Detroit, MI 48202, USA

Megan Finley, Aquatic Veterinarian, Washington Department of Fish and Wildlife, 3860 Highway 97A, Wenatchee, WA 98801, USA

& Tony L Goldberg* John D. MacArthur Chair, Professor of Epidemiology, Department of Pathobiological Sciences, School of Veterinary Medicine and Associate Director for Research, UW-Madison Global Health Institute, University of Wisconsin-Madison, Robert P. Hanson Laboratories, 1656 Linden Drive, Madison, WI 53706, USA

*Author to whom correspondence should be sent.

Suggested Running Head: Widespread seropositivity to VHSV in WI

Abstract

Serological assays were conducted for anti-viral hemorrhagic septicemia virus (VHSV) antibodies in four species of fishes in Wisconsin: Bluegill Lepomis macrochirus (Rafinesque) Brown Trout Salmo trutta (Linnaeus), Northern Pike Esox lucius (Linnaeus), and Walleye Sander vitreus (Mitchill) to examine spatial and temporal distributions of exposure. Sera were tested for non-neutralizing anti-nucleocapsid antibodies to VHSV by blocking ELISA. Results (percent inhibition; %I) were analyzed for differences among species, across geographic distance, and among Water Management Units (WMUs). 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 plus two standard deviations), 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 inform fisheries management.

Introduction

Strain IVb of viral hemorrhagic septicemia virus (VHSV; Rhabdoviridae, Novirhabdovirus) emerged in the early 2000s in the US 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). In Wisconsin, the 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 (USDA-APHIS) continues to require States to maintain regulations to reduce the risk of spread of VHSV despite lifting the 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, these results are

based on assays that detect live virus and viral nucleic acids, and not 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 (Rafinesque) in Lake Winnebago. Of 548 Freshwater Drum 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 (Mitchill), Northern Pike E. lucius (Linnaeus), and Smallmouth Bass Micropterus dolomieu (Lacepède) sampled over a 6-year period from Lake Saint Clair, Michigan, USA, even though virus was detected in only 2 of the 6 sampling years. Other studies confirm that VHSV persists in populations even in inter-epidemic 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 post-infection, and that shedding can be extended or resumed by exposure to stress. Hershberger et al. (2010) were able to detect VHSV 224 days post-exposure in kidney, spleen, and brain tissues from experimentally infected Pacific Herring Clupea pallasii (Valenciennes).

Currently, the most common method for targeted surveillance testing as outlined by the American Fisheries Society (AFS) Blue Book and World Organisation for Animal Health (OIE), is viral isolation in cell culture followed by polymerase chain reaction (PCR), which requires lethal sampling of fish tissues (Batts and Winton 2020; OIE 2019b) 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). In 2014, Wilson-Rothering et al. published an ELISA assay that detects nonneutralizing anti-nucleocapsid antibodies to VHSV across fish species using non-lethal blood samples. The original publication showed the test to perform well in Brown Trout Salmo trutta (Linnaeus), yellow perch Perca flavescens (Mitchill), grass carp Ctenopharyngodon idella (Valenciennes), pacific herring, muskellunge, and freshwater drum (sensitivity of 96.4% and specificity of 88.2%), and we recently showed the test to perform adequately in Northern Pike (Thiel et al., 2020; sensitivity and specificity of 80.6% and 63.2%, respectively). This test has, however, 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 using the non-lethal 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, 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.

[A] Methods

[C] Field sampling.— From March to November of 2016 and March to June of 2017, 46 different inland water bodies were sampled across Wisconsin, and sera were collected from 1662 fish (367 Bluegill Lepomis macrochirus (Rafinesque), 442 Brown Trout, 450 Northern Pike, and 403 Walleye Sander vitreus (Mitchill). 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 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 mL and 3.0 mL, depending on the size of the fish; Uses of Fishes in Research Committee 2014) were collected from the caudal vein of each fish using an 18-, 21-, or 22-gauge needle and 3 to 5 mL syringe, then transferred to a no-additive red-top glass blood tube (Monoject; VWR International, Radnor, Pennsylvania) and inverted repeatedly to initiate clotting. All fish were released at their point of capture. Blood samples were stored on ice in the field and at 4° C in the lab overnight to allow clotting. Within 24 hours following collection, samples were centrifuged at 3200 x g for 15 minutes and sera were transferred to sterile 2.0 mL cryovials and stored at -80°C.

In March 2017, Lake Saint Clair in Michigan experienced an outbreak of VHS in which tens of thousands of fish died, including Gizzard Shad Dorosoma cepedianum (Lesueur), Bluegill, Pumpkinseed Lepomis gibbosus (Linnaeus), Black Crappie Pomoxis nigromaculatus (Lesueur), Largemouth Bass Micropterus salmoides (Lacepède), Muskellunge, Northern Pike, Freshwater Drum, Common Carp Cyprinus carpio (Linnaeus), Yellow Perch, and Mudpuppies

Necturus maculosus (Rafinesque) (Whelan 2017). As of June 2017, the known epidemic region included the St. Clair River in 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 and Walleye (3 and 32 fish, respectively) with the assistance of Michigan DNR in May 2017, and processed and stored samples as described above.

[C] Antibody detection by ELISA.— The ELISA method developed by Wilson-Rothering et al. (2014, 2015) with minor alterations (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 in 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 two standard deviations (SD) above the mean %I for presumptive uninfected fishes (OIE 2019a; Bluegill ≥50.26 %I, Brown Trout ≥50.21 %I, Northern Pike ≥56.48 %I, and Walleye ≥48.38%). Second, alternative positive thresholds were also calculated for Brown Trout and Northern Pike using a ROC curve based on published results from these species. For Brown Trout, an alternative threshold of ≥25%I was used (Wilson-Rothering et al. 2014), and for Northern Pike an alternative positive threshold of ≥58.2%I was used (Thiel et al. 2020)[1].

[C] Data analyses.— Statistical analyses were conducted in R version 3.3.3 (R Core Team 2017). Analysis of variance (ANOVA) 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

[1] Note 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%.

homogeneity of variances. Because assumptions of normality and homogeneity of variances were violated, the non-parametric Kruskal-Wallis rank sum test with post-hoc Dunn-test was used to evaluate differences in %I among species and 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 where the same pairs of species were sampled in the same year. To examine risk factors for seropositivity, multivariate logistic regression was conducted with individual and location-specific factors as predictors and serostatus of a fish (positive or negative) assigned based on the most conservative criterion of +2 SD above mean %I. Data were analyzed 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 AIC values. Multiple diagnostic plots were examined to check for linearity of relationships, normality of the distribution of residuals, homogeneity of variance of residuals, and to examine influences on regression results. Goodness-of-fit was assessed with McFadden's pseudo R squared (0.440). To examine patterns of VHSV seroreactivity spatially, maps were created using the ggmap package in R base maps for the States of Wisconsin and Michigan (Kahle and Wickham 2013), and for analysis of Water Management Units (WMUs) in Wisconsin, the open data shapefile for WMUs was provided by the Wisconsin DNR (WI DNR 2018). To test for spatial autocorrelation in %I among sampling sites within each species and year, Moran's I was used. Additionally, sampling locations were sorted into WMUs and tested for similarity of mean %I within WMUs using Kruskal-Wallis rank sum tests.

[A] Results

[B] Descriptive Statistics

Overall, 14.6% of 1,697 fish sampled from 47 water bodies (including those sampled from Lake Saint Clair, MI) tested positive for VHSV antibodies (using a threshold of two SD above the mean %I for presumptive uninfected fishes). Fish sampled in spring had the highest positivity (15.2%), followed by summer (14.7%), and finally fall (11.2%). There was no significant difference in positivity among seasons (Kruskal-Wallis Test χ^2 =2.19, df=2, P=0.33). Percent inhibition (%I) ranged from 0 to 91.59, with a mean and SD of 33.06 and 17.37, respectively. Two or more species of fish were sampled at 22 of 47 water bodies (Figure 1 and

Table 1). Water temperature ranged from 2.22 to 20.94 ºC. Length and weight of fish sampled ranged from 12.0 cm to 98.0 cm and 0.03 kg to 7.40 kg, respectively.

[B] Comparisons of ELISA results among species

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

[B] ELISA results using species-specific thresholds

The overall number of positive fish of all species tested across Wisconsin was 237 of 1662 (14.2%), based on the threshold criterion of two SD 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).

Lake Winnebago (including Asylum Bay) in Wisconsin and Lake Saint Clair in Michigan are locations where documented VHS outbreaks have occurred and where fish have tested positive for VHSV by virus isolation, PCR, and ELISA serum testing in multiple years between 2005 to 2018 and 2003 to 2017, respectively (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 Saint Clair, 11 of 35 fish (31.4%) tested positive, making Lake Saint Clair

(where the most recent documented VHSV outbreak occurred) the location with the highest seropositivity in our study. For species specific results at these two locations, see Table 1.

Using the alternative thresholds for Brown Trout and Northern Pike based on published values (%I ≥ 25.0 and %I ≥ 58.2%, respectively) expectedly increased estimates of 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., two SD 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 show the geographic distribution of seropositive fish by both threshold values.

[B] 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 by species. Clear Lake and Lac Courte Oreilles had an increase in mean %I in Bluegill only. All other locations and species showed an overall decrease in mean %I from 2016 to 2017.

[B] Risk factor analyses

Table 2 shows results of multivariate logistic regression for serostatus of fish based on species, WMU, water temperature, length of fish, month and year sampled. All variables examined were significant predictors of serostatus of fish, except month sampled. Fish weight was not analyzed because it strongly correlated with length. Species was the strongest binary predictor of serostatus (adjusted odds ratios between 8.86 and 35.07), followed by WMU, length of fish, and year (adjusted odds ratio of 0.39, reflecting a 2.56-fold decrease from 2016 to 2017). Walleye were at highest risk of seropositive status, followed by Northern Pike, Brown Trout, and Bluegill. Total length and water temperature were also significant, with total length protective (odds of seropositivity decreased by 0.96-fold for every increase in 1 cm of length) and water temperature 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 sampled 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 values >0.190). However, mean percent inhibition 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). Supplementary Figure 3 shows maps of mean %I by species and WMU for 2016 and 2017.

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 in the same year: in 2016, Bluegill and Northern Pike (ρ=-0.006, P=0.991), Bluegill and Walleye (ρ=-0.107, P=0.839), and Northern Pike and Walleye (ρ=-0.090, P=0.811); and in 2017, Bluegill and Northern Pike (ρ= -0.6, P=0.41), Bluegill and Walleye (ρ= 0.2, P=0.916), and Northern Pike and Walleye (ρ=0.2, P=0.916).

[A] Discussion

[B] 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, yet 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 the importance of the addition of serologic testing for VHSV and the likely underestimation of the virus's geographic distribution.

[B] Comparison of locations tested in both field seasons

An overall inter-annual 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 populations), in parallel with testing for the virus itself, would help assess whether temporal changes in VHSV seroreactivity indicate undetected viral transmission (i.e. viral transmission in the absence of fish die-offs), as has been shown for freshwater drum in Lake Winnebago, Wisconsin (Wilson-Rothering et al. 2015).

[B] Risk factor analyses

Species, WMU, length of fish, water temperature, and year sampled were statistically significant predictors of VHSV seropositivity. Both individual factors (species, 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 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 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 statistically significantly similar for water bodies located within the same WMU. WMUs are groups of watersheds delineated by the Wisconsin 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 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 have 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.

[B] 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, non-specific binding of antibodies was more evident in Northern Pike (47.6% of serum tested had an OD ≥0.1 on the negative antigen well) than in Bluegill, Brown Trout, or Walleye (2.7%, 2.4%, and 3.4% respectively). Although percent inhibition 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 (two SD 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[2]). The thresholds chosen (Table 1) may change as new data are collected but 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 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 (SVCV), another rhabdovirus native to Wisconsin. Other studies have confirmed lack of immunologic cross reactivity between VHSV and SVCV, 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, Stirling, Scotland; Lorenzen et al. 1988; Ristow et al. 1991; Wilson-Rothering et al. 2014). However, it cannot be ruled out that as-yet undiscovered viruses could be present that cross-react with this assay.

[2] The published threshold was altered to increase specificity for surveillance purposes. See methods section for details.

[B] Management implications

These findings suggest that current testing strategies used for management of VHS may be improved by the further development of serological methods. Although, to the researchers' knowledge, there have been no documented declines in the four fisheries addressed in this study for any of the seropositive water bodies, though 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 paper 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; they also have desirable characteristics for use in fish health management applications, such as surveillance studies which require low sample size and are cost-effective and biosecurity practices to outline disease free zones.

The results of this study may also help 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 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).

[B] 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 differ significantly in seroreactivity, straight-line geographic distance does not predict similarity in VHSV seroreactivity, certain seronegative water bodies are located near seropositive water bodies, and patterns of seroreactivity among fish species from the same water bodies are 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.

[A] Acknowledgements

We thank the Wisconsin Department of Natural Resources - Bureau of Fisheries Management for invaluable assistance throughout this project, and especially the Fisheries Biologists and Fish Health Program staff and volunteers for support during field sampling efforts. We thank Gary Whelan, Todd Wills, and the Michigan DNR Fisheries Division staff for aiding in the collection of fish samples from Lake St. Clair, Michigan. We also thank the Virology Section of the Wisconsin Veterinary Diagnostic Laboratory for use of laboratory space and generous assistance with serological methods, Anna Wilson-Rothering for invaluable technical advice and assistance, Peter McIntyre for helpful discussions, and Evan Jones and Caleb Wyss-Williams for assistance with field sample collection. This work was supported by The University of Wisconsin Sea Grant Institute through the National Oceanic and Atmospheric Administration (grant number NA14OAR4170092).

[A] References

1. Batts, W. N., and J.R. Winton. 2020. Viral Hemorrhagic Septicemia Virus. In AFS–FHS (American Fisheries Society–Fish Health Section) FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2016 edition. AFS-FHS, Bethesda, MD.
2. Elsayed, E., W. Batts, M. Thomas, G. Whelan, W. Batts, and J. Winton. 2006. Isolation of viral haemorrhagic septicaemia virus from muskellunge, Esox, masquinogy (Michill), in Lake St. Clair, Michigan, USA reveals a new sublineage of the North American genotype. Journal of Fish Diseases. 29:611-219.

3. Faisal, M., and A. Winters. 2011. Detection of Viral Hemorrhagic Septicemia Virus (VHSV) from Diporeia spp. (Pontoporiidae, Amphipoda) in the Laurentian Great Lakes, USA. Parasites & Vectors. 4:2.
4. Faisal, M., M. Shavalier, R.K. Kim, E.V. Millard, M. R. Gunn, A. D. Winters, C. A. Schulz, A. Eissa, M. V. Thomas, M. Wolgamood, G. E. Whelan, and J. Winton. 2012. Spread of the Emerging Viral Hemorrhagic Septicemia Virus Strain, Genotype IVb, in Michigan, USA. Viruses. 4:734–760.
5. Hershberger, P., J. Gregg, C. Grady, L. Taylor, and J. Winton. 2010. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. Diseases of Aquatic Organisms. 93:43-49.
6. Hershberger, P., M. Purcell, L. Hart, J. Gregg, R. Thompson, L. Garver, and J. Winton. 2013. Influence of temperature on viral hemorrhagic septicemia (Genotype Iva) in Pacific herring, Clupea pallasii Valenciennes. Journal of Experimental Marine Biology and Ecology. 444:81–86.
7. Jaramillo, D., E. Peeler, E. Laurin, I. Gardner, and R. Whittington. 2017. Serology in Finfish for Diagnosis, Surveillance, and Research: A Systemic Review. Journal of Aquatic Animal Health. 29:1-14.
8. Gaumnitz, L. 2003. Taking stock of state hatcheries: The art and science of raising fish is tricky in a tight economy. Wisconsin Natural Resources Magazine (February).
9. Kahle, D., and H. Wickham. 2013. ggmap: Spatial Visualization with ggplot2. The R Journal. 5:144-161. Available: http://journal.r-project.org/archive/2013-1/kahle-wickham.pdf
10. Kamke, K. 2018. Final test results confirm VHS as cause of this spring's fishkill in Lake Winnebago. Wisconsin Department of Natural Resources Fisheries (June 28). Available: https://dnr.wi.gov/news/releases/article/?id=4556
11. Kim, R., and M. Faisal. 2010a. Comparative susceptibility of representative Great Lakes fish species to the North American viral hemorrhagic septicemia virus Sublineage IVb. Diseases of Aquatic Organisms. 91:23–34.
12. Kim, R., and M. Faisal. 2010b. Experimental studies confirm the wide host range of the Great Lakes viral haemorrhagic septicemia virus genotype IVb. Journal of Fish Diseases. 33:83–88.

13. Kim, R., and M. Faisal. 2012. Shedding of viral hemorrhagic septicemia virus (Genotype IVb) by experimentally infected muskellunge (Esox masquinongy). Journal of Microbiology. 50:278–284.
14. Lorenzen, N., N. Olesen, and P. Jorgensen. 1988. Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. Diseases of Aquatic Organisms. 4:35-42.
15. Millard, E. V., and M. Faisal. 2012. Heterogeneity in levels of serum neutralizing antibodies against viral hemorrhagic septicemia virus genotype IVb among fish species in Lake St. Clair, Michigan, USA. Journal of Wildlife Diseases. 48:405-415.
16. OIE (World Organisation for Animal Health). 2019a. Principles and methods of validation of diagnostic assays for infectious diseases. In OIE Manual of Diagnostic Tests for Aquatic Animals, 2019 edition. OIE, Paris, France.
17. OIE. 2019b. Viral Haemorrhagic Septicaemia. In OIE Manual of Diagnostic Tests for Aquatic Animals, 2019 edition. OIE, Paris, France.
18. Olesen, N. J., N. Lorenzen, and P. E. V. Jørgensen. 1991. Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). Diseases of Aquatic Organisms. 10:31–38.
19. Olson, W., E. Emmenegger, J. Glenn, C. Simchick, J. Winton, and F. Goetz. 2013. Expression kinetics of key genes in the early innate immune response to Great Lakes viral hemorrhagic septicemia virus IVb infection in yellow perch (Perca flavescens). Developmental and Comparative Immunology. 4:11–19.
20. R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Available: https://www.R-project.org/. (December 2019)
21. Ristow, S., N. Lorenzen, and P. Jorgensen. 1991. Monoclonal-antibody-based immunodot assay distinguishes between viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV). Journal of Aquatic Animal Health. 3:176-180.
22. Thiel, W., K. Toohey-Kurth, B. Baker, M. Finley, and T. Goldberg. 2020. Assessment of a Serologic Diagnostic Test and Kinetics of Antibody Development in Northern Pike Experimentally Infected with Viral Hemorrhagic Septicemia Virus. Journal of Aquatic Animal Health. 32:3-10.

458 23. Thompson, T., W. Batts, M. Faisal, P. Bowser, J.W. Casey, K. Phillips, K.A. Garver, J.
459 Winton, and G. Kurath. 2011. Emergence of Viral hemorrhagic septicemia virus in the North
460 American Great Lakes region is associated with low viral genetic diversity. Diseases of
461 Aquatic Organisms. 96:29-43.
462 24. United States Department of Agriculture Animal and Plant Health Inspection Service
463 (USDA-APHIS). 2014. APHIS to Lift Viral Hemorrhagic Septicemia Federal Order.
464 Stakeholder Announcement, Washington.
465 25. Use of Fishes in Research Committee (joint committee of the American Fisheries Society,
466 the American Institute of Fishery Research Biologists, and the American Society of
467 Ichthyologists and Herpetologists). 2014. Guidelines for the use of fishes in research.
468 American Fisheries Society, Bethesda, MD.
469 26. VHS Expert Panel and Working Group. 2010. Viral Hemorrhagic Septicemia Virus (VHSV
470 IVb) risk factors and association measures derived by expert panel. Preventative Veterinary
471 Medicine. 94:128-139.
472 27. Warg, J., E. Cornwell, A. Cruz, R. Getchell, C. Giray, et al. 2014. Detection and surveillance
473 of viral hemorrhagic septicemia virus using real-time RT-PCR. I. Initial comparison of four
474 protocols. Diseases of Aquatic Organisms. 111:1-13.
475 28. Whelan, G. 2017. Spring 2017 St. Clair- Detroit River Corridor Fish Mortality. Fisheries
476 Division, Michigan Department of Natural Resources.
477 29. Wilson-Rothering, A., T. Goldberg, S. Marcquenski, W. Olson, F. Goetz, P. Hershberger, L.
478 Hart, and K. Toohey-Kurth. 2014. Development and evaluation of a blocking enzyme-linked
479 immunosorbent assay and virus neutralization assay to detect antibodies to viral hemorrhagic
480 septicemia virus. Clinical and Vaccine Immunology. 21:435–442.
481 30. Wilson-Rothering, A., S. Marcquenski, R. Koenigs, R. Bruch, K. Kamke, D. Isermann, A.
482 Thurman, K. Toohey-Kurth, and T. Goldberg. 2015. Temporal variation in viral hemorrhagic
483 septicemia virus antibodies in freshwater drum (Aplodinotus grunniens) indicates cyclic
484 transmission in Lake Winnebago, Wisconsin. Journal of Clinical Microbiology. 53:2889–
485 2894.
486 31. Wisconsin Department of Natural Resources. 2018. Water Management Units. WiDNR Open
487 Data. Available: https://data-wi-dnr.opendata.arcgis.com/datasets/water-management-
488 units?geometry=-102.52%2C41.647%2C-76.241%2C47.136&page=3

32. Wisconsin Department of Natural Resources. 2019. Fishing Wisconsin: Viral Hemorrhagic Septicemia fish virus. Available: https://dnr.wi.gov/topic/fishing/vhs/#three

33. Wright, P.F., E. Nilsson, E. Van Rooij, M. Lelenta, and M. Jeggo. 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Scientific and Technical Review of the Office International des Epizooties. 12:435-450.

34. Zale, A., D. Parrish, and T. Sutton. 2013. In Fisheries Techniques, Third Edition. American Fisheries Society, Bethesda, MD.

Table Captions

Table 1: Information on water bodies sampled for Bluegill, Brown Trout, Northern Pike, and Walleye during 2016 and 2017. For numbered map of water bodies see Figure 1.

Table 2: Results of multivariate logistic regression for serostatus of fish (positive or negative) based on species, Water Management Unit (WMU), water temperature, total length of fish, month and year sampled.

Supplementary Table 1: Results of Kruskal-Wallis rank sum tests of differences in %I among Water Management Units.

Figure Captions

Figure 1: Numbered map of water bodies sampled in 2016 and 2017. For water body names and full details including surveillance results see Table 1. Location 46, Lake Saint Clair, MI, is not pictured here.

Figure 2: Box plot of spread of percent inhibition of VHSV ELISA by species. Mean percent inhibition differs among species (Kruskal-Wallis test χ^2 107.99, df=3, P<0.0001) and each species varies significantly from each other (Dunn test, all P values <0.05) except Brown Trout to Walleye (Dunn test P=0.39).

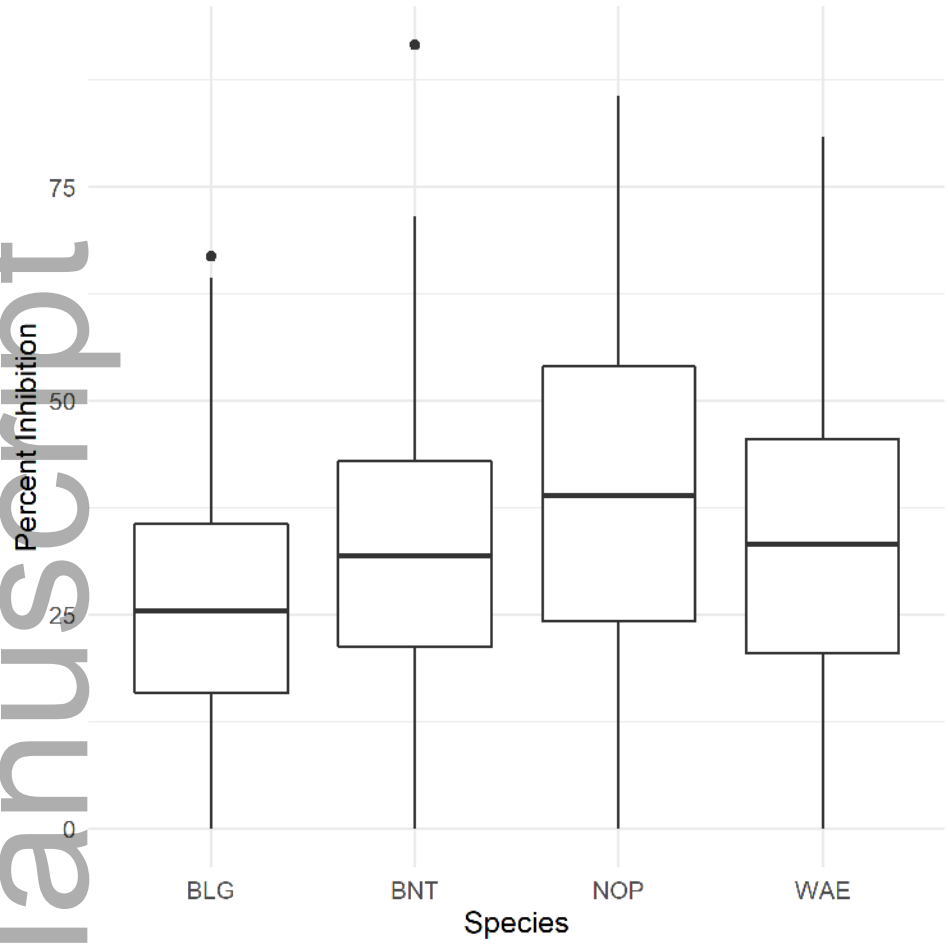
Figure 3: A) Results of 2016 surveillance efforts. Percent of Bluegill, Brown Trout, Northern Pike, and Walleye that tested positive for antibodies to VHSV by ELISA at each sampling location. Positive thresholds for each species are ≥50.26%, 50.21%, 56.54%, and 48.38% inhibition, respectively. Size and shading of points reflects magnitude of percent positive by location on a continuous scale. B) Results of 2017 surveillance efforts using the same positive thresholds described in Figure 3a. Brown Trout were not sampled in 2017.

Supplementary Figure 1: A) Percent positive at each sampling site for Northern Pike during 2016 field season using an alternative threshold. Alternative positive threshold for Northern Pike based on ROC curve developed during experimental infection trials (Thiel et al. 2020; 95.4% specificity and 34.4% sensitivity). A threshold of ≥58.2% inhibition was chosen to maximize specificity for surveillance purposes. B) Percent positive at each sampling site for Brown Trout during 2016 field season using an alternative published threshold (Wilson-Rothering et al. 2014; 88.2% specificity and 96.4% sensitivity). Percent inhibitions of ≥25% are considered positive.

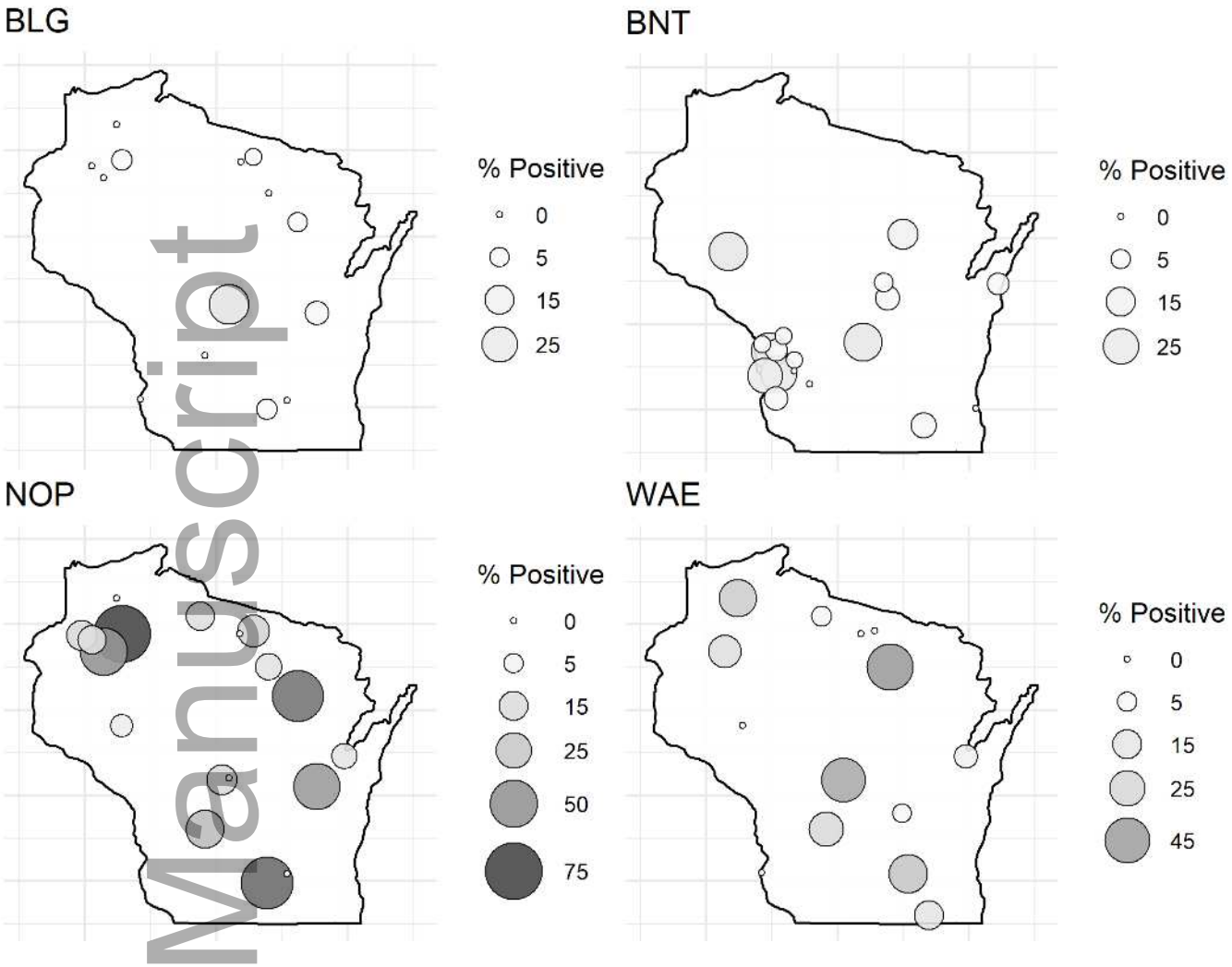
Supplementary Figure 2: Direction and magnitude of change in mean percent inhibition at 8 locations sampled in both 2016 and 2017 separated by species. Sites where mean percent inhibition increased or decreased are represented by upward and downward facing triangles, respectively. Size and shading of triangles reflect absolute value of the change in mean percent inhibition from 2016 to 2017. Brown Trout were sampled only in 2016 and are therefore not included.

Supplementary Figure 3: A) Mean percent inhibition by species and Water Management Unit for 2016. WMUs are numbered as follows, 0=Lower Wisconsin, 1= Upper Rock, 2= Upper Fox, 3=Central Wisconsin, 4=Wolf River, 6=Lower Rock, 8= Bad Axe-La Crosse, 10= Lower Chippewa, 12= St Croix, 13= Upper Chippewa, 14= Upper Wisconsin, 18= Milwaukee, 19= Lower Fox, 22=Twin-Door-Kewaunee. Shading of WMUs reflects mean percent inhibition on a continuous scale. The WMUs with the highest mean %I in 2016 included the Central Wisconsin WMU for Bluegill and Walleye (mean, 42.65%I; SD, 15.82 and mean, 42.50%I; SD, 14.35, respectively), the Lower Chippewa WMU for Brown Trout (mean, 43.58%I; SD, 13.29), and the Lower Rock WMU for Northern Pike (mean, 56.48%I; SD, 27.12). B) Mean percent inhibition by species and Water Management Unit for 2017. Shading of WMUs reflects mean percent inhibition on a continuous scale. The WMUs with the highest mean %I in 2017 were the Lower Wisconsin WMU for Bluegill (mean, 41.14%I; SD, 7.41), the Upper Fox WMU for Northern Pike (mean, 34.15%I; SD 14.68), and the Upper Rock WMU for Walleye (mean, 35.90%I; SD, 14.95).

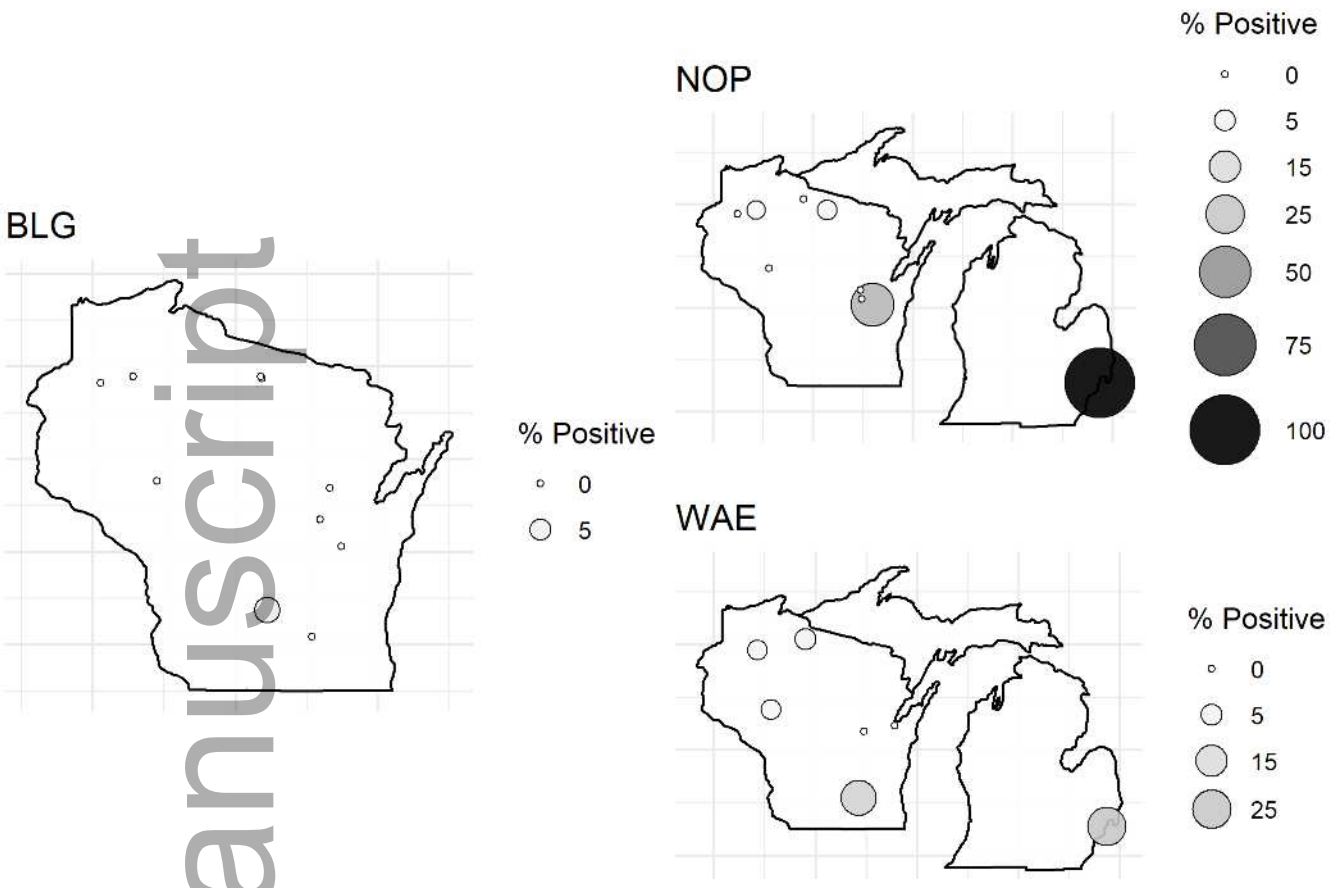
aah_10120_f1.tiff



aah_10120_f2.tiff



aah_10120_f3a.tif



aah_10120_f3b.tif