GREAT LAKES FISHERY COMMISSION

2021 Summer Meeting

Virtually

August 4, 2021

Minutes

Submitted By:
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Great Lakes Fishery Commission

GREAT LAKES FISHERY COMMISSION
2200 Commonwealth Blvd, Suite 100
Ann Arbor, Michigan 48105
Great Lakes Fish Health Committee

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LIST OF ATTENDEES

Danielle Godard	Wisconsin Department of Natural Resources
Nicole Nietlisbach	Wisconsin Department of Natural Resources
John Dettmers	Great Lakes Fishery Commission
Andy Noyes	New York State Department of Environmental Conservation
Brian Niewinkski	Pennsylvania Fish & Boat Commission
Dave Meuninck	Indiana Department of Natural Resources
Gary Whelan	Michigan Department of Natural Resources
Isaiah Tolo	Minnesota Department of Natural Resources
Kenneth Philips	U.S. Fish and Wildlife Service
John Coll	U.S. Fish and Wildlife Service
Paula Phelps	Minnesota Department of Natural Resources
Sunita Khatkar	Fisheries and Oceans Canada
Coja Yamashita	Pennsylvania Fish & Boat Commission
Tom Loch	Michigan State University
Andy Jarrett	Ohio Department of Natural Resources
Kevin Irons	Illinois Department of Natural Resources
Jeremiah Blaauw	Michigan Department of Natural Resources
Kevin Kayle	Ohio Department of Natural Resources

Guests:

Jan Lovy - New Jersey Division of Fish & Wildlife

Hui-Min Hsu - Wisconsin Veterinary Diagnostic Laboratory

Tony Goldberg & Whitney Thiel - University of Wisconsin

Ed Eisch – Michigan Department of Natural Resources

Jesse Landwehr – Wisconsin Department of Natural Resources

Myron Kebus – State of Wisconsin Chief Fish Veterinarian

GREAT LAKES FISH HEALTH COMMITTEE VIRTUAL MEETING

August 4th, 2021 (EST)

Agenda

SESSION 1

9:00 am	Welcome and Introductions – Chair
9:10 am	GLFC Update – Dettmers
9:20 am	USGS Thiamine Concentrations in Lake Trout Eggs continued coordination of egg collections/samples and transfer of research lead – Dettmers
9:30 am	Lactic acid bacteria (*Vagococcus salmoninarum* and *Carnobacterium maltoaromaticum*) in the Great Lakes, USFWS La Crosse, WI Update – Phillips
9:50 am	Bacteriology – Dr. Hui-Min Hsu
10:15 am	AFS Bluebook Revision Process Update – Loch and Lovy
10:30 am	Baitfish Testing by state summary and storing fish health data – Whelan
11:00 am	**BREAK**
11:15 am	VHSv Research in Sport fishes in Wisconsin -- Dr. Tony Goldberg and graduate student Whitney Thiel
12:00 pm	**BREAK**

SESSION 2

2:00 pm	Fish Disease Risk Assessment – Dr. Myron Kebus
2:15 pm	MSU Research Updates – Loch
2:45 pm	Wild Rose State Hatchery Presentation Tour
3:15 pm	**BREAK**
3:30 pm	Agency Updates
4:00 pm	Interesting Cases
4:20 pm	Winter 2022 Meeting Dates/Location
4:30 pm	**ADJOURN**

Summary of Action Items

If your representative agency or state has not responded to the bait fish testing questionnaire, please send them to Gary as soon as possible.

The GLFHC is asked to share with Gary Whelan how their state or agency is storing fish health data, either via a developed database or internally via spreadsheets.

Dr. Myron Kebus requests input from the GLFHC on the Fish Disease Risk Assessment that he has been developing as it relates to their fish health research, regulations, and surveillance programs.

Gary Whelan will send out the final publication on Great Lakes coregonine parasite research to the committee when it is complete

The committee prefers to meet in person for the winter 2022 meeting, but plans to revisit the in-person option and location for the winter 2022 meeting in November, 2021 given any unknown COVID and/or travel restrictions

SESSION 1

GLFC Update – Dettmers

All past GLFHC meeting minutes are formalized and posted to the GLFHC webpage hosted on the Commissions website at www.glfc.org. To access GLFHC minutes, navigate to "For Our Partners, Joint Strategic Plan Partners, GLFHC", then scroll down to, or click this link: "More publications available through the Publication Search"

GLFC is continuing to work with all the lake committees to develop the ability to conduct in-person meetings again.

USGS Thiamine Concentrations in Lake Trout Eggs continued coordination of egg collections/samples and transfer of research lead – Dettmers

Don Tillitt with the USGS has been working on collecting lake trout eggs to understand the concentration of thiamine and its impact to lake trout populations in the lower Great Lakes. Don is retiring in January 2022 and will be handing over the USGS lead to Brian Lantry. The samples from2020 have not been analyzed yet, so there are no new updates on analyses. USGS is looking for a contractor who can run the samples for them. Brian has reached out to the field crews across all five Great Lakes asking them to collect eggs for thiamine analysis this fall. USGS wants to continue to do these analyses on behalf of the committee. The committee should continue to communicate to their agencies to continue to make effort in collecting eggs for thiamine analysis

Discussion

Invasive goldfish also can contain large amounts of thiaminase.

In Lake Michigan, alewife is a much larger part of steelhead diets than previously thought and could lead to low thiamine for steelhead.

Thiamine treatments have been used to control thiamine deficiency, but MSU is investigating a way to effectively disinfect eggs while also treating them with thiamine. Researchers on the west coast who work on thiamine deficiency add thiamine as they add sperm to fertilize the eggs. This technique seems promising to produce enough thiamine for the eggs during the disinfection process.

Lactic acid bacteria (*Vagococcus salmoninarum* and *Carnobacterium maltoaromaticum*) in the Great Lakes, USFWS La Crosse, WI Update – Phillips

The initial outbreak of *V. salmoninarum* (V. sal) occurred during fall 2017 at the Iron River National Fish Hatchery where coaster brook trout brood eggs were green in color after spawning in the spring. By December, staff saw a spike in fish mortality. There were no apparent effects on lake trout brood at the facility. Necropsy observations showed fish with egg retention, cloudy fluid and fibrous material surrounding the heart, and ascites fluid. Samples for bacterial analysis detected *Vagococcus salmoninarum.* Aquaflor was used to treat but it was unsuccessful in

decreasing mortality. During summer and fall 2018, mortality persisted after evaluating vaccinations. The decision was made to cull all brook trout brood lots at the facility. This decision provided an opportunity to study this pathogen looking at prevalence in males and females, tropism, vertical transmission, and egg disinfection. Ovaries and liver tissue were superior for detecting the pathogen as compared to kidney tissue. A few publications on this work came out in 2020 (Standish et al). During our February 2019 inspection, V. sal was again detected and all brood and future brood were culled. In February 2021, it was detected again in brook trout broodstock. Additional detections occurred in the Keweenaw Bay region. The *Carnobacterium maltaromaticum* previously called *Lactobacilus pisciciola* was detected at Iron River NFH in brook trout brood and was detected at other facilities in the Great Lakes region with no disease or mortality. Fish were treated with Erythromycin and will continue monitoring and validating a duplex qPCR assay and evaluation of V. sal genome. We will be moving the brook trout into new rearing space at Iron River NFH.

Bacteriology – Dr. Hui-Min Hsu

Dr. Hui-Min Hsu works for the Wisconsin Veterinary Diagnostic Laboratory uses Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) to better classify bacteria. Bacterial identification may be done using a phenotypic-based, serological-based and molecular-based approaches. There is minimal preparation work, short process time, low consumables cost, and comparable to 16s rRNA sequencing as well as the ability to create a custom library of bacteria of interest.

Sample mass spectrometry results are compared to the database to determine the relatedness. The ten most closely related bacteria are identified. The pattern recognition is score-based for identifying bacteria to species and/or genus or as “no reliable identification”. Biologists conduct the interpretation of these results. The benefits of this work are that it can be customized for scientists’ needs in terms of identification.

Flavobacterium psychrophilum and psychrophilum-like yellow pigmented bacteria have been isolated from rainbow trout presenting clinical symptoms as well as from other fish that are diseased and fish that appear healthy in Michigan (Loch et al, 2013).

Another study has evaluated the use of MALDI-TOF to identify *Flavobacterium psychrophilum* from *Flavobacterium psychrophilum*-like species. MALDI_TOF technology significantly improved correct identification of psychrophilum species.

MALDI-TOF may also be used to identify or differentiate sub species of bacterium. MALDI-TOF successfully identified important fish pathogens to the subspecies level. However, for *Aeromonas salmonicola*, it was inconclusive.

Benefits of MALDI-TOF include a one protocol fits all (except for Mycobacteria), an extensive database of over 9000 MSPs, and about 3000 bacterial species, it can identify unfamiliar bacteria, and is easier for discovery of new pathogens. Generally genus or species at low confidence for *Aeromonas* spp, *Flavobacterium* spp, and *Pseudomonas* spp. Future improvement will require adding more fish pathogens in the commercial database, differentiation of

taxonomically related bacteria and geographic or species variability among bacterial strains, and working with manufacturer to improve the library. Hui-min can be contacted at Hui-min.hsu@wvdl.wisc.edu

Discussion

PFBC has used this technology for identifying bacteria and believes it is an efficient and effective tool in its fish health analyses.

How do we know if these custom databases are producing quality results or how do we know the results are reliable and what do you ask from the lab in terms of quality control on how accurate the results are? There are a lot of evaluations that go into it to provide accurate results.

AFS Bluebook Revision Process Update – Loch and Lovy

Jan Lovy is the Blue Book Revision Project Manager leading the revision process and updates to the Fish Health Section of the Blue Book, supported by Ken Cain. There are three sections of the fish health section of the Blue Book. Section one covers Diagnostic Procedures for Finish and Shellfish Pathogens, section two focuses on USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections, and section three is a QA/QC Model for Fish Health Labs. Section two is most frequently used for fish health inspections; it was identified as needing more information to provide guidance for a larger variety of facilities such as those that work with finfish, mollusks, and other species. This section has been difficult to add pathogens and new diagnostic tests when needed; during review, agencies requested this section become more adaptable so as to more easily add newer testing protocols and diagnostics. The Blue Book is used by many states, which frequently have Blue Book methods in statute for fish health policy. It is used by states, federal governments, and aquaculture industry; all of these impacted entities need to be considered when revising the Blue Book. The revision process is overseen by a 13-person Steering Committee made up of state, federal, and tribal agencies, as well as industry and two working groups (Natural Resource Agency Working Group and Industry Working Group) to provide feedback during the process.

Specific revisions for the diagnostic testing chapter are expected to include specific criteria for defining pathogens. In addition, a citation guidelines piece will be inserted before both sections 1 and 2. There will be a new section alongside the Inspection Manual that provides focused guidance about certain host groups. If there is any feedback or input on the process please contact the leaders of the Natural Resource Agency Working Group which include co-chairs Gary Whelan (Michigan DNR) or Wade Cavender (Utah DWR), or the project managers, Jan (Jlovy.bluebook@gmail.com) and Ken Cain (Kcain@uidaho.edu).

Discussion

Comprehensive Aquaculture Health Program Standards (CAHPS) may not work for all facilities such as smaller ones that are not state run, and may need to be more robust in the future. It may be more expensive to use a CAHPS system versus annual lot-based system and it wont fit everyone, but the revisions will try to include the needed flexibility.

It's going to be the top 2% of the industry that can afford to use CAHPS.

Baitfish Testing by state summary and storing fish health data – Whelan

Gary Whelan provided summarized responses from eight member states to the questionnaire regarding each state's baitfish industry and how each samples across its state.

About 50% of the responses indicated that the state has some idea about how it would sample its baitfish industry. The other 50% need more time to consider because there is little information about the baitfish industry available within those states.

For the question, "How many baitfish are moving through the state?" About 50% of the responses lacked data about the amount (gallons) of baitfish moving through the state. For others, such as Minnesota, it was estimated to be about 30 million gallons. In Michigan about 40 million gallons move through the state, mostly coming from wild sources; the remaining states don't have the data to provide an estimate.

Several labs have the capacity or could share the responsibility to do the sampling and analysis. A multi-state conservation grant may be pursued to fund this work. There is interest and support in testing baitfish supplies across the country.

Gary will continue working on what a multi-state grant application would look like to provide funding for widespread baitfish testing across the Great Lakes. These types of grant proposals will start with a letter of intent to be reviewed at the fish chief level and then pre and final proposals would be reviewed and decided on.

This was also presented to the CLC and they provided full support in pursuing the testing of baitfish industry across the Great Lakes.

ACTION ITEM: If your representative agency or state has not responded to the baitfish testing questionnaire, please send responses to Gary as soon as possible.

In addition to the questionnaire on baitfish testing, Gary asks, how each agency is storing fish health data; either via a dedicated fish health database designed for your state, an internal Access Database that was designed for your agency, or just storing it across several excel spread sheets?

ACTION ITEM: The GLFHC is asked to share with Gary Whelan how their state or agency is storing fish health data, either via a developed database or internally via spreadsheets

Discussion

Illinois DNR's bait is nearly all imported so in addition to their questionnaire answers, the results should be similar to those found to be imported elsewhere.

Minnesota DNR uses a combination of software for storing data and spreadsheets

USFWS has a new LIMS system - a little cumbersome in the entry side of things, but hopefully better for partner queries.

Ohio DNR is still using spreadsheets as well.

Maurine Purcell is working on a novel approach to create a national database for fish health data

VHSv Research in Sport fishes in Wisconsin -- Dr. Tony Goldberg and graduate student Whitney Thiel

Viral Hemorrhagic Septicemia Virus (VHSv) experimental infection antibody detection and surveillance in Wisconsin fishes.

VHS is a Rhabdovirus that has a high virulence in fish but is not zoonotic. It causes fish die offs, affects more than 40 marine and freshwater fishes, and is a reportable pathogen. Modes of transmission include anglers and ballast water discharges.

The first VHSv detection in the Great Lakes was in 2005 in Lake Ontario. However, it was later found from archived samples as early as 2003 in Lake St. Clair, MI. In 2005 several outbreaks occurred in the Great Lakes region. Federal regulations were established in 2007 for interstate movements between the affected states and provinces of Canada to slow the spread. It was detected from all five Great Lakes by 2010. Since then, regulations have been maintained to reduce the spread of VHS.

Methods of detection include viral detection, which requires lethal sampling of the fish. Antibody detection is a non-lethal sampling method of the fish; however, it is not yet approved for surveillance testing. A competitive blocking ELISA was developed based on the method used by Wilson-Rothering.

The first part of the study sought to understand the kinetics of an immune response and develop a VHS ELISA threshold for northern pike. At 30 days post infection, about 50% mortality in the infected group was observed compared to the control group and was statistically significantly different. For clinical signs of disease, 50% of fish showed signs of disease at 21 days post infection and continued to increase during the remainder of the experiment. ELISA results were measured by percent inhibition = 1%. Mean % inhibition of the control and infected group at each week post infection, the control and infected groups were very similar at 0 to 1 weeks and then differentiated from 2+ weeks post infection. Inputting the ELISA results into a ROC curve allows them to drive sensitivity and specificity of the ELISA test to determine a cutoff value where all fish above a particular % inhibition would be positive and all fish below a certain % inhibition would be negative. To maximize sensitivity, the threshold of the % inhibition can be lowered; if a high specificity were desired, the threshold could be raised, which could be more effective for surveillance testing.

The second part of the project is to find out where VHSv is in Wisconsin by conducting wild fish surveillance. From spring 2016 to spring 2017, four economically important sport fish species were targeted from inland water bodies using a variety of sampling methods. All non-lethal blood samples from fish were taken back to the lab for analysis. Approximately 1600 fish were sampled across 47 water bodies. Bluegill, brown trout, northern pike, and walleye samples were analyzed. The percent inhibition was compared across these four species.

Seropositivity in 2016 and 2017 for all four species across the state shows the highest % inhibition for walleye. Highest seropositive locations were concentrated in the northwest in

2016; in 2017 it shifted to the southeast region. Looking at the changes in seroreactivity between years, seroreactivity decreased from 2016 to 2017 in bluegill, walleye, and northern pike in various locations. They then explored whether there is a geographical pattern in seroprevalence using spatial autocorrelation to determine any connection of seropositivity, and found no pattern in straight line distances. Next, this was looked at by Wisconsin Water Management Units (WMU). Comparison of WMUs found significant differences when grouping the sampling locations into these units. Watershed level may be indicative of how the virus is spreading. For northern pike, the % mean inhibition in 2016 was highest. Decreases in seropositivity from 2016 to 2017 where detected when grouping by watershed or WMU. VHSv is likely spreading through interconnected water bodies. Improvements to management practices includes surveillance testing for selection of broodstock from seronegative water bodies and treatment of hatchery source water with seropositive history. VHSv is not everywhere, as there are areas of high VHSv or VHSv hot spots next to negative water bodies so educating the public about the virus to limit the spread between positive and negative water bodies should be a priority. Future research may include tracking individual fish in the wild and test if positive water bodies are staying positive or track any changes, determine how is VHSv is moving, and looking to see if there are other patterns of spread of the virus in other states/water bodies.

Discussion

Was there any indication of cross-reactivity with other viruses? Previous studies have showed a lack of cross-reaction between the antibodies and several other viruses.

Did VHSv cause any major fish kills in the Mississippi River drainage like it did in Wisconsin water bodies? No fish kills documented in the Mississippi River and no detection of VHSv during regular spring surveillance over the years.

There are also no recent significant fish kills or die offs in Lake St. Clair, MI.

Are there any plans to go back into water bodies that are hot spots and do more monitoring or survey work with next gen sequencing or something other than ELISA? Not in the plans right now but could be another study using other testing methods compared to ELISA.

Given the results, would (agencies/states) change how surveillance for VHSv is conducted?

From an MDNR perspective, several regulations are in place that are reasonably effective in decreasing the movement of potentially infected fish, but the agency has not been able to increase the amount of surveillance for VHSv and does not have the capacity to do it at this point.

Was PCR done alongside competitive ELISA? We found fish that had neutralizing antibodies and VHSv positive by tissue culture but those results only happened during a 6-week post-infection window. Any paired sampling done along the way? No, it was not done for the purpose of this study, but during the development of the ELISA, PCR was used alongside ELSIA in tissue sampling work done by Anna Wilson at University of Wisconsin, Madison.

Godard has shared Thiel's and Anna Wilson's publications on VHSv and ELISA research via email to committee members (Appendix 4).

SESSION 2

Fish Disease Risk Assessment – Dr. Myron Kebus

Dr. Kebus is Wisconsin's chief fish veterinarian, involved in setting the fish health regulations for commercial fish farms as well as for the Wisconsin DNR. Dr. Kebus has been responsible for determining which pathogens warrant attention from a regulatory standpoint, how to list them, and set regulations for them. His office provides more than 300 health certificates annually.

The Fish Disease Risk Assessment adheres to the guidance and requirements of the AFS - Fish Health Section and the Blue Book.

The secure finfish supply risk assessment approach as shown on page 3 (APENDIX item xx.), now involves a National USDA Fish Health Initiative: Commercial Aquaculture Health Program Standards (CAHPS).

ACTION ITEM: Kebus requests input from the GLFHC on the Risk Assessment and approaches as it relates to their fish health research, regulations, and surveillance programs.

Discussion

In terms of endemic pathogens that cause disease in hatcheries but do not cause as much disease in the wild, how is antibiotic resistance considered? There are factors within each pathogen that can make it riskier in the environment than others. Does that become more conservative or is that weighted in your risk assessments? The Risk Assessment has to have flexibility to incorporate changes that can occur as pathogens are better understood and as variants arise over time. Yes, these things are worked in and the group has to come up with how we will address antibiotic resistance in that case.

The involvement with many different stakeholders and perspectives in the development of this risk assessment is important and provides a variety of needs and input across Great Lakes.

MSU Research Updates – Loch

Investigating infectious diseases as a "bottleneck" to lake whitefish (*Coregonus clupeaformis*) recruitment is a project by student Courtney Harrison funded by the Great Lakes Fishery Trust.

It is not well understood whether pathogens are present in reproductive tissues and if so, are they transmitted to offspring, and do infections equate to disease in the offspring? Pathogens do not always equal disease. Currently, *Renibacterium*, *Aeromonas*, and *Carnobacterium* are infectious diseases in Great Lakes lake whitefish.

Harrison's research examines *A. salmonicida-salmonicida* effects on juvenile lake whitefish health. Juveniles (n =5 per group) were reared to use in experimental immersion pilot challenges at a low dose and high dose. Since it was a pilot study, the experiment was not replicated. Two days after fish were immersed in the bacterial suspension, they showed ataxia or swimming aimlessly in the fish tank. Another day later, there were early signs of hemorrhaging, lesions, and then mortality as intercranial hemorrhaging and widespread hemorrhaging advanced. By seven days post challenge in the high dose group, all of the fish had died. By day eight in the low dose group, half the fish died and there were zero mortalities in the control group. The bacterium was not recovered in the negative control fish, whereas it was isolated from fish in both the low dose and high dose groups.

Another study, "Evaluating novel methods for preventing *Aeromonas*-associated mortality in yellow perch" is led by Dr. Megan Shavalier (post-doc) at MSU. She is maintaining 400 yellow perch provided by University of Wisconsin-Milwaukee. Immersion treatments of the vaccine bacterin and sham immersion vaccine have been completed. Two months after vaccination, they will be challenged with *A. salmonicida.* There are plans to test this in the field and expand this work on a larger scale as funds become available.

Flavobacterium psychrophilum (Fpsy) is a large problem causing bacterial cold-water disease (BCWD) and Loch's lab has been working extensively on this bacterium. A PhD candidate, Chris Knupp, is currently working towards methods for improving BCWD diagnosis. Initially, the lab gained an understanding of genetic variation within *F. psychrophilum* and mapped out these genetic variants. Some variants seem to prefer specific host species (i.e., some genetic variation seems to have implications for host species preference). Observations also suggest that some genetic variants may not grow as well on routinely used culture media. With this in mind, 165 Fpsy isolates were examined from the variant genetic map and then looked at three types of basal culture media that are widely used for Fpsy. For some variants, they grew equally well on all three media types, whereas others would grow equally well on two out of three of the media. Out of the three different media types, the TYES media was the best but occasionally the other two media types (OW and EAOCa) were better. Chris looked at the ingredients in the TYES medium as the "best basal medium" for Fpsy growth and then incorporated a range of new components into new experimental media at low and high concentrations to develop a more effective medium. After plating a range of the Fpsy isolates he found that 5 out of 11 media components had significant effects in growth between the high and low dose of the media ingredients. Of those 11 types, decreasing concentrations of some of the ingredients had a better impact on bacterial recovery or growth. Based on these analyses, they devised two new media concentrations with the most effective ingredients for detecting and diagnosing *F. psychrophilum*.

Discussion

Does data so far show TYES standard better than filtered? Anecdotally, we thought filtered TYES was better than autoclaved, but as we have been going through isolates, it is actually performing a bit "worse" than standard TYES, but not significantly worse according to statistics.

Was there a difference in how long it took to culture/isolate colonies between media types?

Chris says "from what I've noted, a/b media (new) gets colony development slightly ahead of TYES/TYES-filtered, maybe by a day (on average)." But of course, we still have more analyses to do for final insights``

Wild Rose State Fish Hatchery Presentation Tour

Jesse Landwehr (hatchery supervisor) and Joe Gaber (hatchery biologist) provided a presentation about the WDNR's Wild Rose State Hatchery.

During 2019, the hatchery provided walleye, brown trout, Chinook salmon, rainbow trout, coho, brook trout, largemouth bass, northern pike, musky, lake trout, splake, and lake sturgeon totaling about 6.8 million fish for stocking throughout the state.

The hatchery provides fingerling fish to local angling groups as a cooperative program that gives a boost to local angling populations and is cost effective for the DNR. About 150K fish have been stocked through this program.

Wild Rose hatchery is centrally located in Wisconsin is at the edge of glacial Lake Wisconsin, with a large aquifer underneath the hatchery. The hatchery started more than 100 years ago and was founded to stock local lakes and streams. From the 1920s to the 1960s, the hatchery vastly expanded as demand for stocking fish increased. Originally most tanks and ponds were supported through artesian flow, but as additional water was needed, pipes were installed to add more flow to the facility. An issue with this, is they were all non-compliant wells, and thus required infrastructure updates and ecological restoration of wetlands and streams in the area for the cold water and cool/warmwater sides of the hatchery.

Addressing the issues prevents the public from entering the production areas for biosecurity reasons, a video was produced to share the production side of the hatchery with the public.

The new facility after renovations is capable of producing 470,000 brown trout for 12 counties, 1.2 M Chinook into Lake Michigan, 500 K Coho Salmon into eight counties, 32 K lake sturgeon in nine counties, 55 K muskellunge in 17 counties, 194 K northern pike in 19 counties, 108 steelhead into three counties, and 138 K walleye in 13 counties for a grand total of almost 2.7 million fish into 42 of the 72 counties of Wisconsin coming out of Wild Rose.

Agency Updates

<u>Ohio Division of Wildlife</u>

Law enforcement division needs training of new personnel for fish health issues and aquaculture and a few other staffing issues. Trying to get more information out to the public on fish health and fish kill issues using the division website.

<u>Illinois</u>

From Illinois Jake Wolf Hatchery, fish health has been great and nothing to report out. Pollution based fish kills but nothing related to fish disease to report on.

DFO

Work has not been affected by COVID this year so far. We have not received any fish kill reports in the past two years.

Michigan DNR

Some mortality with Chinook and Atlantic salmon this year, but not unusual. The Atlantic salmon issues at the Harrietta State Fish Hatchery were probably from a gas/nitrogen issue, that historically has been a problem. There have been some steelhead issues with systemic cold-water infections at both Wolf Lake and Thompson hatcheries. Waiting for medicated feed to arrive to administer to treat.

Work on Great Lakes Coregonines parasites (Muzzall and Whelan) has now been through the editorial process and ready for publication soon. Gary Whelan will send the final publication out to the committee. We are continuing the AFWA discussion of a National Fish and Wildlife Health Initiative.

Professor Bart Gorgoglione at MSU has put in a full proposal to look at Proliferative Kidney Disease (PKD) in salmonids. The Eyes in the Field Program reports are very high this year. There were some very dry and hot periods followed by a very wet period and fluctuations in temperature and precipitation that may have been the cause of an outbreak of koi herpes virus (KHv) in Lake Orion and possibly another Michigan lake. Increasing numbers of melanistic smallmouth bass are being reported. Investigations into lesions in smallmouth bass in Great Lakes waters is being done by a Sea Grant funded student.

Indiana DNR

No bacterial gill disease detected in steelhead and Coho during the early rearing period. A BGD outbreak is normal each year. Survival from swim up to now has been exceptional. Steelhead broodstock operations were finished with no excessive handling mortalities as were seen the last two to three years. There has been some predation by racoons and otters in production facilities. Carp kills were seen last week at the Hardin Reservoir likely from KHv. Samples were taken and are being processed in the lab now.

Wisconsin DNR

EEDv was detected in lake trout and splake at Les Voigt SFH and were stocked in Lake Superior, which is a known EEDV positive waterbody. This was done after consulting with GLFHC. If the same scenario occurs, WDNR will determine if a risk assessment may be needed again. Walleye sex ratio is skewed to females and examining why. Wild fish surveillance program has had some slight changes because previous methods were redundant and some hatchery source waters are being reconsidered. VHSv from the wild was detected in musky broodstock ovarian fluid from the Fox River but was negative in the progeny. VHSv was detected in gizzard shad from mortalities in the Menomonee River in March. KHv was detected from common carp mortalities in Beaver Dam Lake and an AciVH1-like virus was detected in

sturgeon in the Wisconsin River via PCR. We welcomed a new fish health technician, Willow Smith.

Minnesota DNR

Isaiah Tolo just started with the MN DNR two months ago. Continuing VHSv surveillance for the state to maintain VHSv disease free status. New regulatory changes have allowed MN to reduce VHSv testing. No detections of VHSv for the 14th year since MN DNR started screening for it. Several fish kills this season but not many adequate samples for pathological examination, most may be attributed to hot and dry weather. We are looking to improve how we respond to fish kills in the wild. There was one case of koi herpes virus (KHv) and carp edema virus, but they often occur as co-infections. There were disease issues in cool water hatcheries so we did not meet the quota for musky production this year. Samples were taken from small fish, so there was an inadequate tissue sample collection for a full analysis, but saw some gram-positive cocci bacterium with 2 to 4% mortality every day over the course of a week or two.

USFWS, LaCrosse

Teresa took a position at headquarters as division chief and her last day as regional chief was this week so we are looking to fill her position. Majority of the updates are provided in Ken's presentation on *V. salmoninarum.*

USFWS, Lamar

The region has been on pace with normal production/surveillance work. There was one mortality of an eight-inch bloater and necropsy was conducted. A wet summer so far. General wild fish health analyses on brook trout, and surveillance for gill lice was done for the region.

Winter 2022 Meeting Dates/Location

February 1-2, 2022 potentially at the GLFC office in Ann Arbor, MI if an in-person meeting is achievable. The in-person option will be revisited in November.

GREAT LAKES FISH HEALTH COMMITTEE
TECHNICAL ADVISORS

August 2021

Bacteriology
Diane Elliot (U.S. Geological Survey)
Hui-Min Hsu (Wisconsin Veterinary Diagnostic Laboratory)
Thomas Loch (Michigan State University)

Virology
James Winton (U.S. Geological Survey)
Tom Waltzek (University of Florida)

Molecular
Nick Phelps (University of Minnesota)
Sharon Clouthier (Fisheries and Oceans Canada)

Nutrition
Wendy Sealey (U.S. Fish and Wildlife Service)
Ann Gannam (U.S. Fish and Wildlife Service)

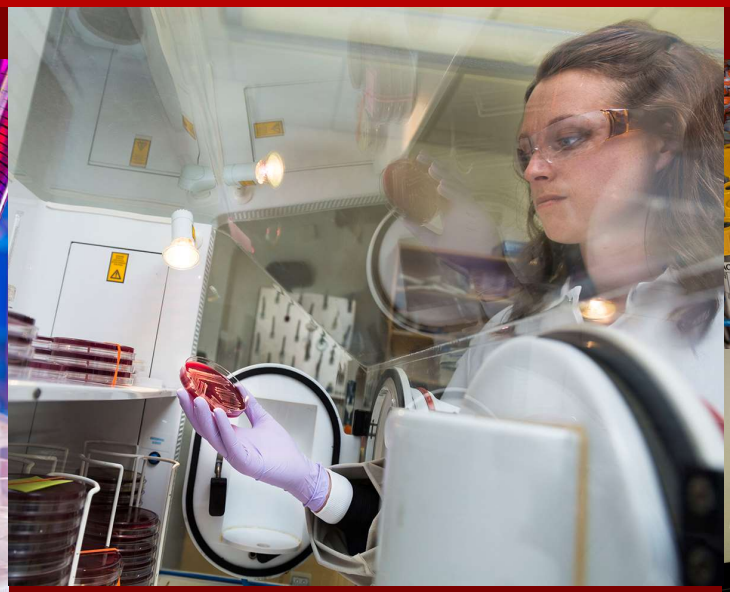
Quantitative Fish Health Data Analysis
Dominic Travis (University of Minnesota)
Travis Brenden (Michigan State University)

Epidemiology
Lori Gustafson (U.S. Department of Agriculture)

Parasitology
David J. Marcogliese (Environment Canada)

Thiamine Deficiency
Jacques Rinchard (SUNY Brockport)
Brian Lantry (USGS)





Wisconsin Veterinary Diagnostic Laboratory

UNIVERSITY OF WISCONSIN–MADISON

Use of MALDI-TOF Mass Spectrometry for Identification of Bacterial Fish Pathogens

Hui-Min Hsu DVM PhD

MALDI-TOF MS

Matrix Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry

Bacterial Culture and Isolation

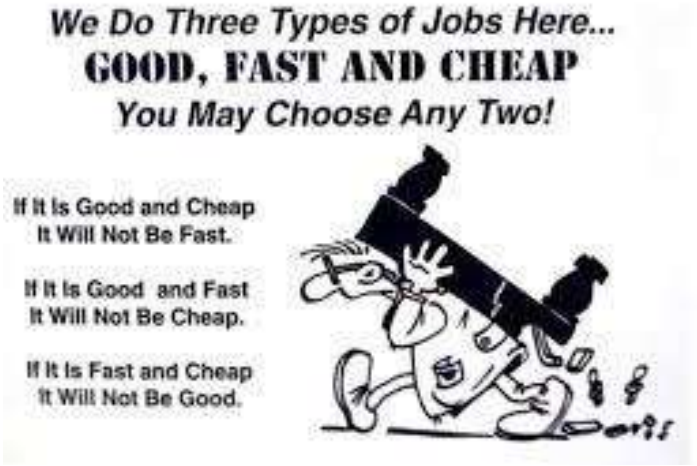
We need isolates!

- Identify and characterize bacteria of interest
- Perform antimicrobial susceptibility tests
- Make autogenous vaccines
- Test development
- Research work

Bacterial Identification

- Phenotypic-based approach
  - Physical characteristics

    colony morphology, hemolytic patterns, presence of pigments, Gram staining
  - Conventional tube biochemical tests
  - Commercial kits (API 20E, API 20Strep): miniature biochemical tests
  - Automated ID system (Biolog)
- Serological-based approach
  - Agglutination test
  - Fluorescent antibody test
- Molecular-based approach
  - PCR
  - 16S rRNA sequencing “gold standard”



MALDI-TOF MS

Proteomics-based approach

VITEK MS (bioMerieux Inc.)

MALDI Biotyper (Bruker Daltonics Inc.)

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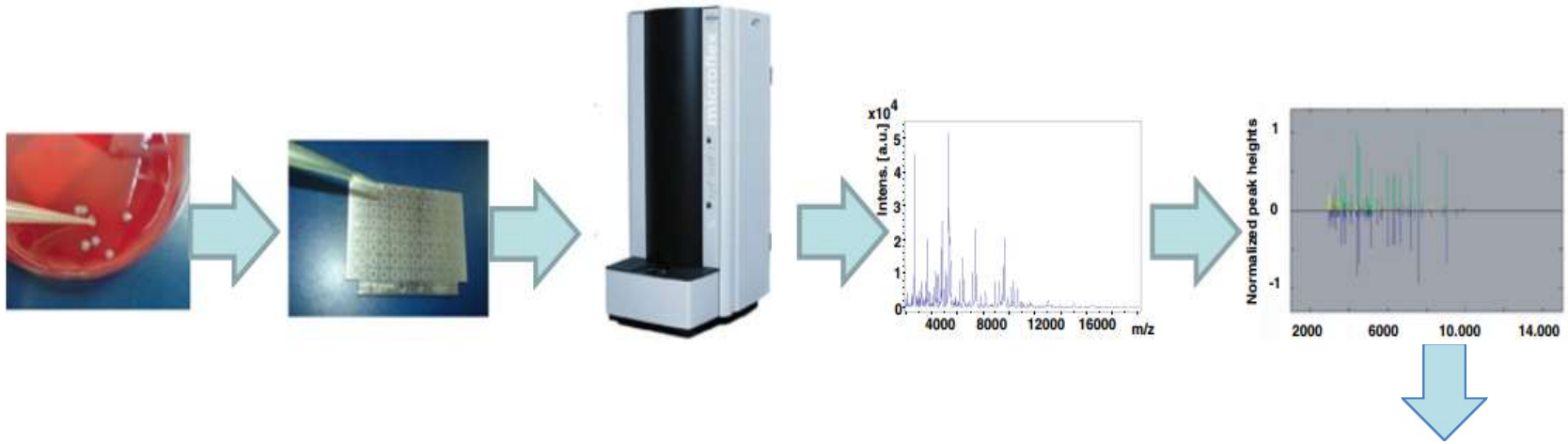
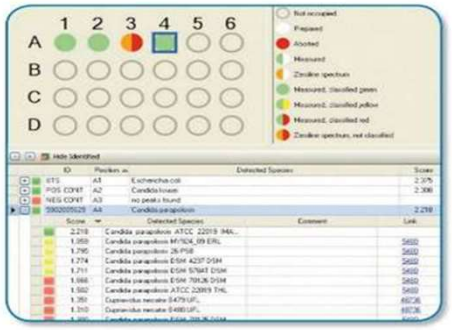
Workflow



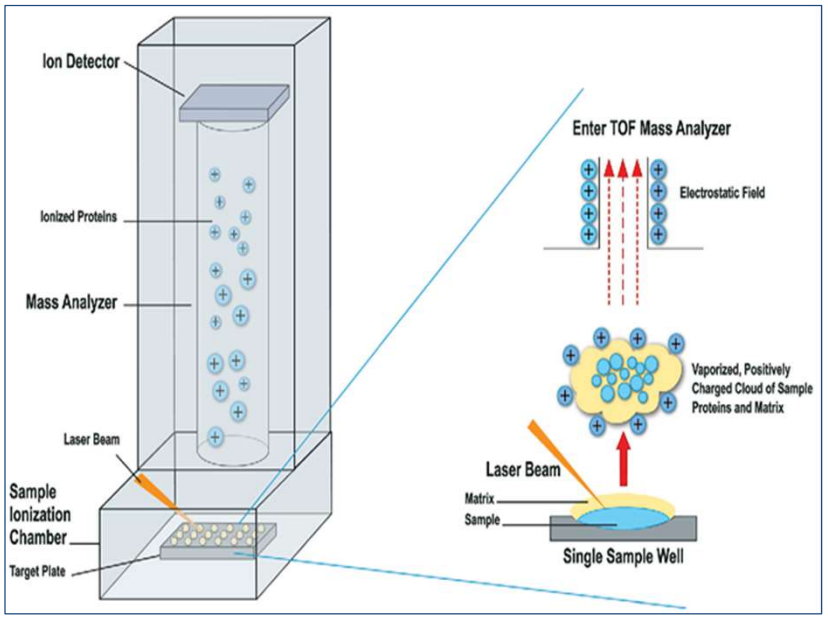
- Minimum preparation work
- Short process time
- Low consumables cost
- Comparable to 16S rRNA sequencing



(Source of images: Clinical Chemistry 61:1 2015)

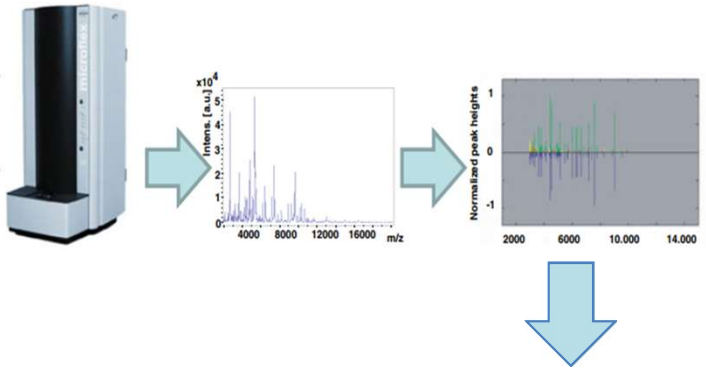
The Principle of **MALDI-TOF** (Matrix Assisted Laser Desorption/Ionization-Time of Flight) Mass Spectrometry

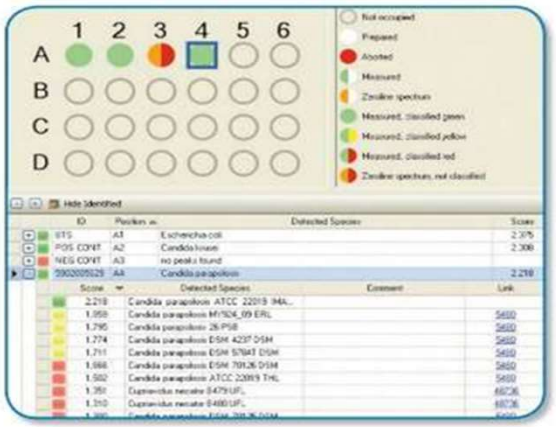
- Sample is treated with an energy-absorbent compound (matrix)
- Sample and matrix co-crystalize on drying
- Sample is loaded and vacuum is established
- Sample proteins are ionized with laser beam
- Ions are separated and accelerated into a vacuum tube based on the mass-to-charge ratio (m/z)
- "Time of flight" is measured and analyzed
- Characteristic mass spectrum is generated



(Source of image: Clinical Chemistry 61:1 2015)

Analysis





- Compare sample MS to the database to determine the relatedness
- 10 most closely related bacteria are identified
- Pattern recognition and score based
  - 2.0-3.0 Species identification (green)
  - 1.7-1.99 Genus identification (yellow)
  - < 1.70 No reliable identification (red)

(Source of image: Clinical Chemistry 61:1 2015)

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Result Interpretation

Species level ID

	Score	Detected Species
🟢	2.19	Plesiomonas shigelloides LMG 12657
🟢	2.18	Plesiomonas shigelloides LMG 4240
🟢	2.13	Plesiomonas shigelloides LMG 4245
🟢	2.10	Plesiomonas shigelloides LMG 4243
🟡	1.92	Plesiomonas shigelloides DSM 8224T
🔴	1.63	Plesiomonas shigelloides CCM 5860
🔴	1.55	Plesiomonas shigelloides DSM 8224T
🔴	1.41	Plesiomonas shigelloides CCM 1991
🔴	1.40	Plesiomonas shigelloides CCM 1996
🔴	1.37	Escherichia coli ATCC 25922 THL

Genus level ID or Species ID at low confidence

	Score	Detected Species
🟢	2.24	Aeromonas veronii DSM 17676 HAM
🟢	2.19	Aeromonas ichthiosmia DSM 6393T
🟢	2.16	Aeromonas veronii CECT 4257T DSM
🟢	2.14	Aeromonas veronii CECT 5761T DSM
🟢	2.11	Aeromonas veronii DSM 7386T HAM
🟢	2.07	Aeromonas hydrophila CECT 839T
🟢	2.02	Aeromonas jandaei CECT 4228T DSM
🟢	2.01	Aeromonas hydrophila ssp hydrophila
🟢	2.01	Aeromonas veronii DSM 11576T HAM
🟢	2.01	Aeromonas veronii 0807M090438 IBS

More Examples…

Genus level ID

	Score	Detected Species
🟡	1.99	Flavobacterium hydatis DSM 2063T
🟡	1.86	Flavobacterium saccharophi um DSM
🟡	1.83	Flavobacterium hydatis DSM 2063T
🟡	1.78	Flavobacterium hibernum DSM 12611T
🔴	1.67	Flavobacterium pectinovorum DSM
🔴	1.54	Bacteroides fragilis MB_9009_05 THL
🔴	1.44	Flavobacterium saccharophi um DSM
🔴	1.43	Flavobacterium aquidurense DSM
🔴	1.43	Flavobacterium johnsoniae DSM 2064T
🔴	1.42	Flavobacterium chungangerse CIP

No reliable ID

	Score	Detected Species
🔴	1.50	Vibrio albensis LMG 4406T HAM
🔴	1.40	Klebsiella oxytoca ATCC 700324 THL
🔴	1.37	Vibrio harveyi VN_02940 AWIH
🔴	1.35	Vibrio diazotrophicus VN_02828 AWIH
🔴	1.33	Vibrio vulnificus RV_1 LBK
🔴	1.32	Vibrio diazotrophicus VN_03198 AWIH
🔴	1.30	Vibrio harveyi VN_02926 AWIH
🔴	1.27	Vibrio pectenicida LMG 19642T HAM
🔴	1.27	Klebsiella aerogenes ATCC 13048T THL
🔴	1.27	Vibrio vulnificus DSM 10143T DSM

Benefits of MALDI-TOF MS

AND can be Customized

Users can create custom library of bacteria of interest

Flavobacterium psychrophilum and *Flavobacterium psychrophilum-like* yellow pigmented bacteria

- *Flavobacterium psychrophilum*, etiological agent of bacterial coldwater disease and rainbow trout fry syndrome

- Other *Flavobacterium* species isolated from diseased RBT presenting similar clinical symptoms (Zamora et al, 2012, 2013, 2014)
  - *F. plurextorm*, *F. tructae*, *F. collinsii*, *F. oncorhynchi*, and *F. piscis*
- Diverse Flavobacteria isolated from diseased and apparently healthy fish in Michigan (Loch et al, 2013)
  - 32 clusters of *Flavobacterium* spp
  - 10 clusters of *Chryseobacterium* spp
  - *Flavobacterium oncorhynchi, F. araucananum, Chryseobacterium viscerum, C. piscicola, C. chaponense*

Flavobacterium psychrophilum

- Presumptive identification:
  - Grow on selective medium, but not BHI or TSA
  - Grow at 15-20°C, not at 30°C
  - Presence of flexirubin pigment
- Definitive identification: FAT, PCR

Source: AFS-FHS Bluebook

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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Differentiation of *Flavobacterium psychrophilum* from *Flavobacterium psychrophilum*-like species by MALDI-TOF mass spectrometry

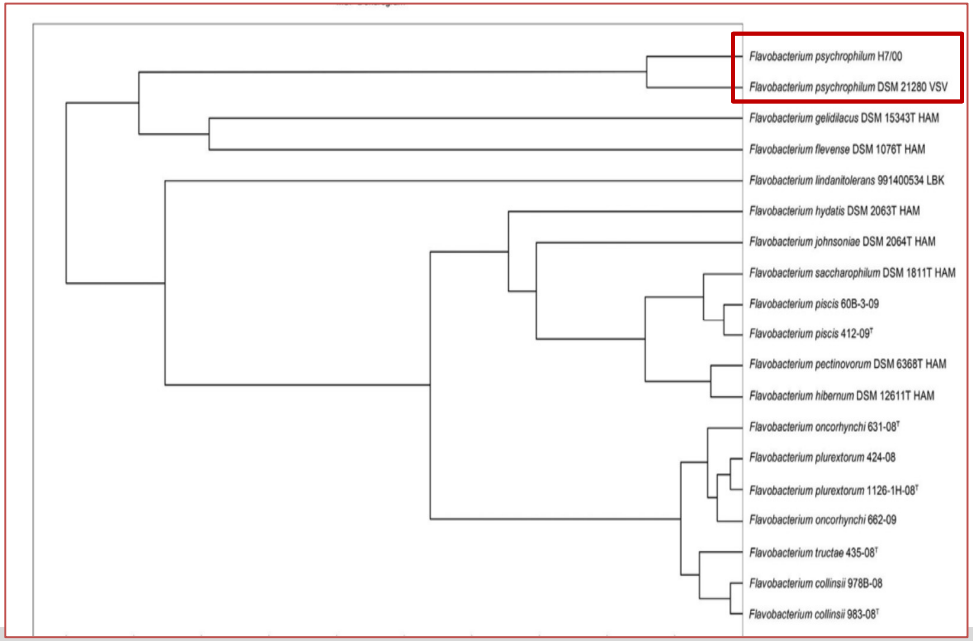
Marta Pérez-Sancho[a], Ana Isabel Vela[a,b], Tom Wiklund[c], Markus Kostrzewa[d], Lucas Domínguez[a,b], José Francisco Fernández-Garayzábal[a,b,*]

Initial evaluation (n=64)
19% Correct ID at low confidence level
76% Unreliable ID (scores<1.699)
5% Misidentification

Construct custom library of reference strains and incorporate into manufacturer's database

F. psychrophilum, F. plurextorum, F. piscis, F. oncorhynchi, F. tructae, F. collinsii

Verification (n=53)
88% Correct ID (incl. all *F. psychrophilum*)
4% Correct ID at Low confidence
6% Unreliable ID
2% Misidentification



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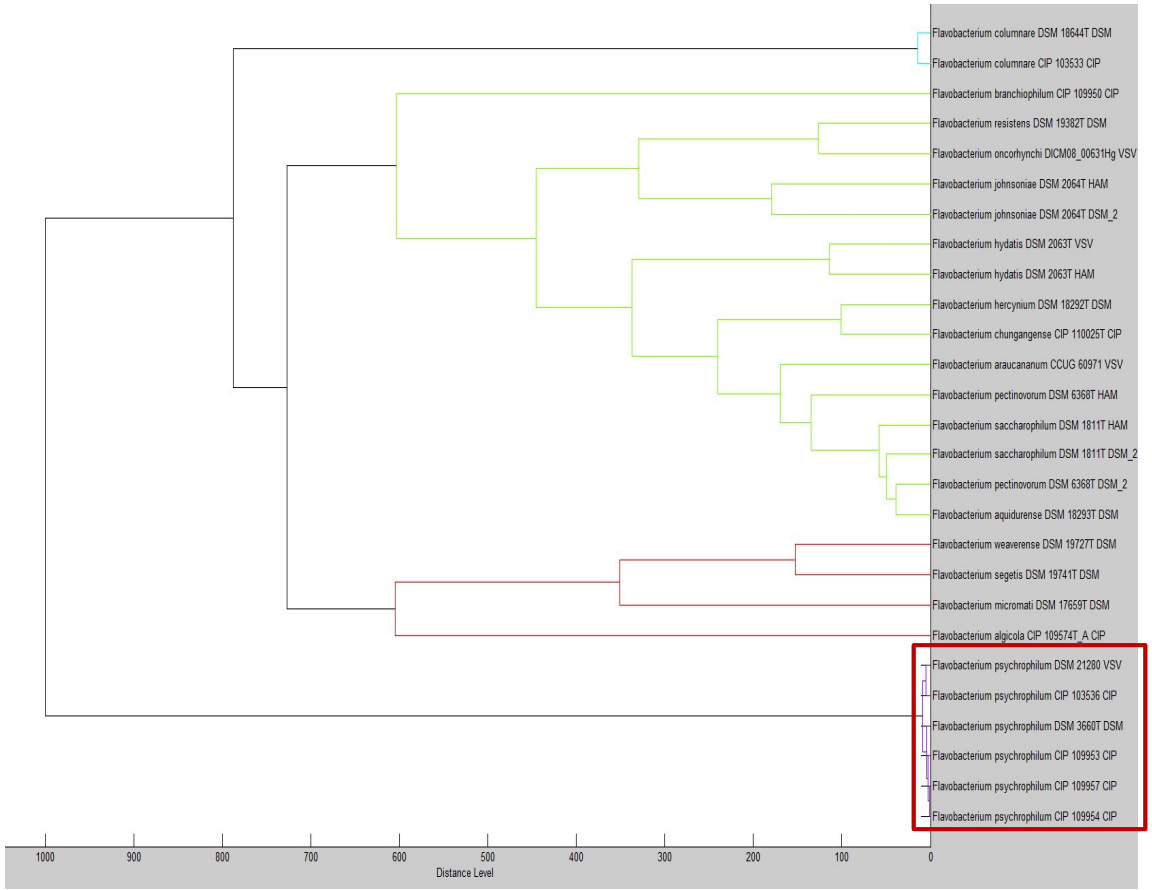
Flavobacterium species

In current Bruker's database (updated July 2021)

- 83 MSPs
- 68 *Flavobacterium* spp



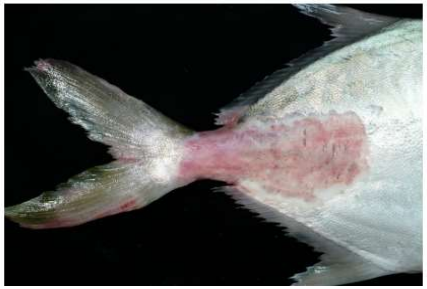
Photobacterium damselae

Subsp. *piscicida*

- Obligate pathogen
- Acute bacterial septicemia with very little gross pathology

Subsp. *damselae*

- Normal inhabitant of environment
- Skin lesions
- Zoonotic potential



Presumptive Diagnosis: Biochemical phenotype (API)

Confirmatory Diagnosis: Multiplex PCR

Source: AFS-FHS Bluebook

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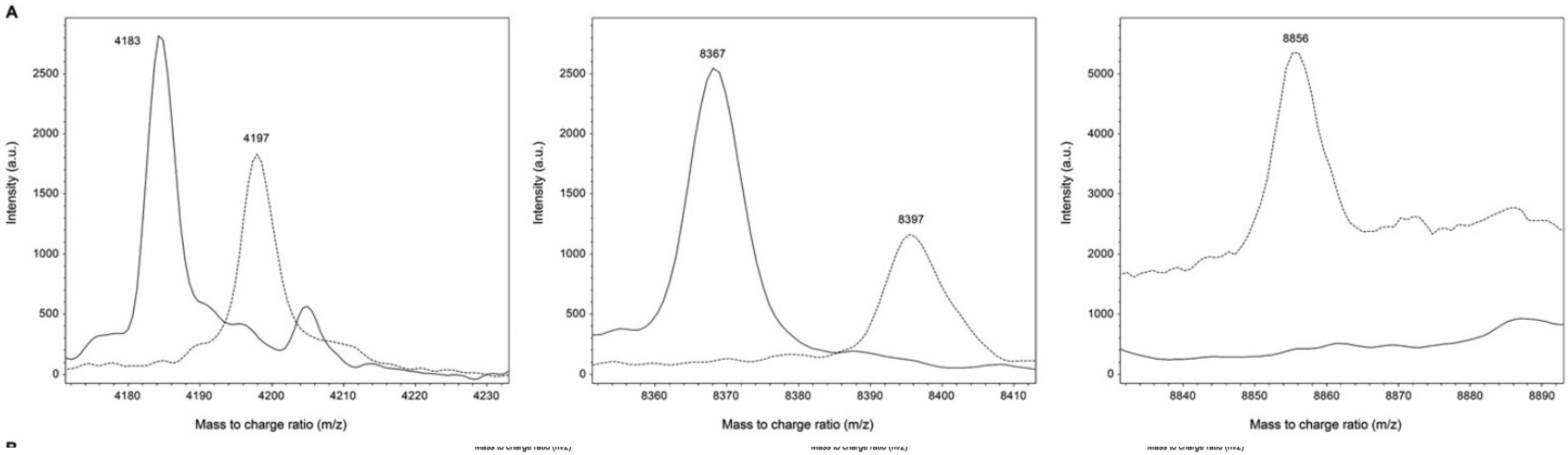
Subspecies level identification

Differentiation of *Photobacterium damselae* subspecies using Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in fish isolates

Marta Pérez-Sancho [a,b], Isabel Vela Ana [a,c], Marian Awad [d], Markus Kostrzewa [d], Lucas Domínguez [a,b,c], José Francisco Fernández-Garayzábal [a,c,*]

Aquaculture



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240, Bull. Eur. Ass. Fish Pathol., 40(6) 2020

MALDI-TOF MS: a diagnostic tool for identification of bacterial fish pathogens

Identification with each lab's standard technique for identification	No of isolates tested	Identification with MALDI-TOF Best match (no. identified)	Score value in MALDI-TOF
Aeromonas salmonicida subsp. *salmonicida**	6	*Aeromonas salmonicida* (4) *Aeromonas bestiarum* (1) *Aeromonas eucrenophila* (1)	2.145 – 2.302 2.214 2.174
Aeromonas salmonicida atypical*	7	*Aeromonas salmonicida* (4) *Aeromonas bestiarum* (3)	2.13-2.171 1.917-2.214
*Flavobacterium columnare**	17	*Flavobacterium columnare*	2,135 – 2,417
*Flavobacterium psychrophilum** NVI 2016-60-459	59 4	*Flavobacterium psychrophilum* *Flavobacterium psychrophilum*	2.204 – 2.544 2.275-2.509
*Renibacterium salmoninarum**	15	*Renibacterium salmoninarum*	2.036 -2.4
Vibrio anguillarum, serotype I and II	7	*Vibrio anguillarum*	2.123 – 2.318
Vibrio vulnificus	3	*Vibrio vulnificus*	2.218 – 2.418
Vibrio splendidus	18	*Vibrio tasmaniensis* *Vibrio gigantis* *Vibrio chagasii*	1.784- 2.035
Yersinia ruckeri	11	*Yersinia ruckeri*	1.997 – 2.239
Pleisiomonas shigelloides	5	*Pleisiomonas shigelloides*	2.311 – 2.447
Pseudomonas anguilliseptica	1	*Pseudomonas anguilliseptica*	2.356
Hafnia alvei	1	*Hafnia alvei*	2.448

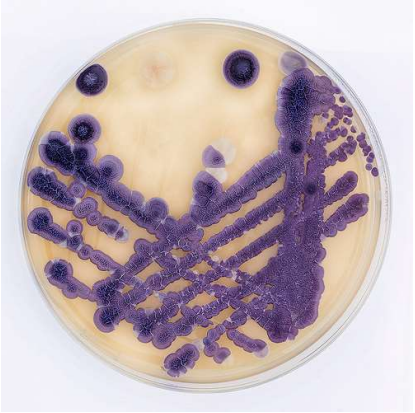
- Successfully identify important fish pathogens to species level

  Flavobacterium columnare, Flavobacterium psychrophilum, Renibacterium salmoninarum, Psedomonas anguillisepticum, Yersinia ruckeri, Hafnia alvei, Plesiomonas shigelloides, Vibrio anguillarum, Vibrio vulnificus.

- Inconsistent results for ***Aeromonas salmonicida***
- No reliable ID for ***Vibrio splendidus***

My favorite things about MALDI

- Easy preparation, fast turn-around time, cost effectiveness
- One protocol fits all (except Mycobacteria)
- Extensive database: >9000 MSPs, ~3000 bacterial species
- Identify unfamiliar bacteria
- Easier for discovery of new pathogens



Common Fish Bacteria through MALDI-TOF MS

- Species level ID

  Chryseobacterium scophthalmum, C. pisicola, C. indologens,

  C. piscium, C. chaponense,

  Edwardsiella tarda, Flavobacterium psychrophilum,

  Hafnia alvei, Plesiomonas shigelloides*, *Shewaunella putrefaciens,

  Yersinia ruckeri

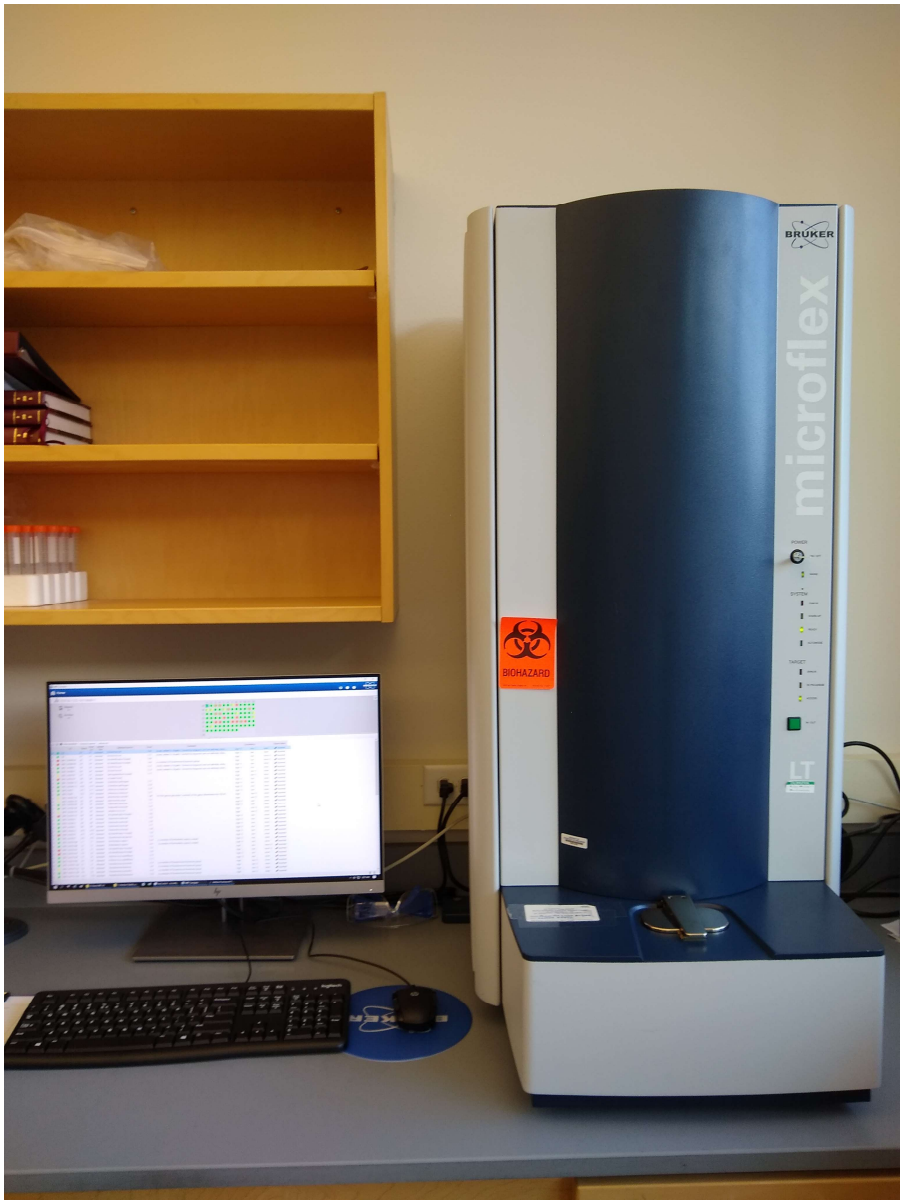
- Genus or species at low confidence

  ***Aeromonas* spp, *Flavobacterium* spp , *Pseudomonas* spp**

Challenges and Future Improvement

- Not all fish pathogens are covered in the commercial database
- Differentiation of taxonomically related bacteria
- Geographic or species variability among bacterial strains

- Need more studies and to work with manufacturer to improve the library.



Questions?

hui-min.hsu@wvdl.wisc.edu

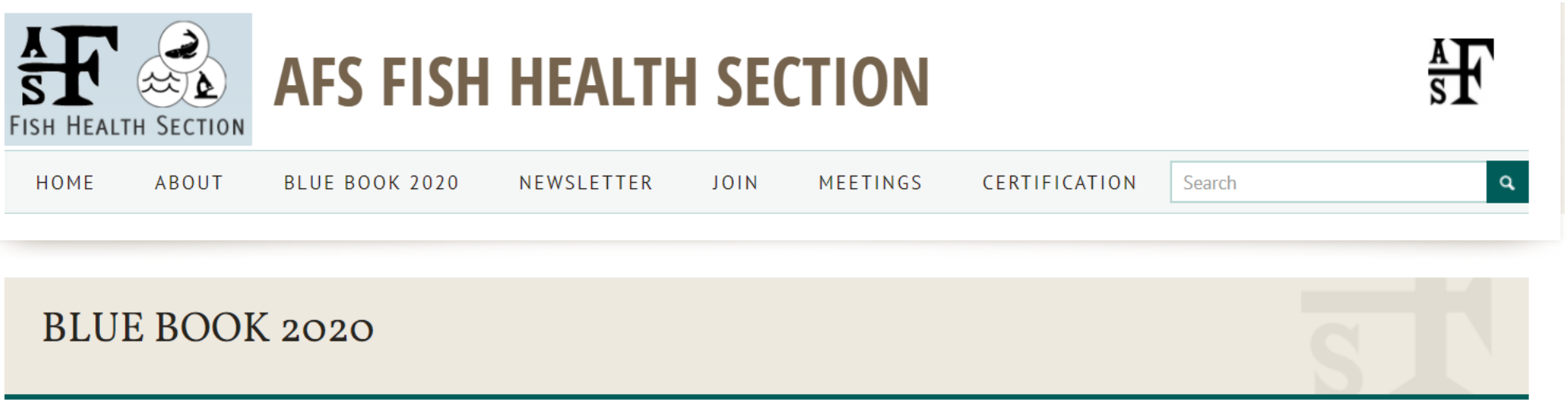
Updates on the AFS Blue Book Revision project

Jan Lovy, Ph.D.
Research Scientist in Aquatic Animal Health
Blue Book Revision Project Manager

AFS FISH HEALTH SECTION

Blue Book in need of revisions

Appendix 3



- Revision was requested, *Ad hoc* committee reviewed need for revisions
- Section 2 is most frequently utilized for fish health inspections
  - Focused on finfish / salmonids
  - Risk-based approaches for inspections
  - Difficult to make substantial updates

SECTION 1

Diagnostic Procedures for Finfish and Shellfish Pathogens

SECTION 2

USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

SECTION 3

QA/QC Model for Fish Health Labs

Who uses the Blue Book and how?

- Blue Book issues technical guidance (best advice) for aquatic animal health screening and diagnostic testing

- Though unintended to be viewed as a regulatory document, many states have Blue Book methods in statute or fish health policy
  - Should not be used as stand-alone regulation
  - Regulators must identify pathogens, species, movement restrictions

- Used by states, federal gov, and aquaculture industry held to this guidance
  - Important to consider all entities impacted
  - Though revisions will continue to be guidance, must consider the impacts to entities that utilize Blue Book methods

Blue Book revision project- communication

- Revision process is overseen by a 13- person Steering Committee
  - USDA-APHIS, NOAA, USFWS, USGS, States (3), Tribes (1), Industry (2), AFS FHS President (past), Academic, Project Managers

- Two working groups to provide feedback during the process
  - Natural Resource Agency Working Group
  - Industry Working Group

ASSOCIATION of FISH & WILDLIFE AGENCIES

U.S. FISH & WILDLIFE SERVICE
DEPARTMENT OF THE INTERIOR

NATIONAL Aquaculture ASSOCIATION

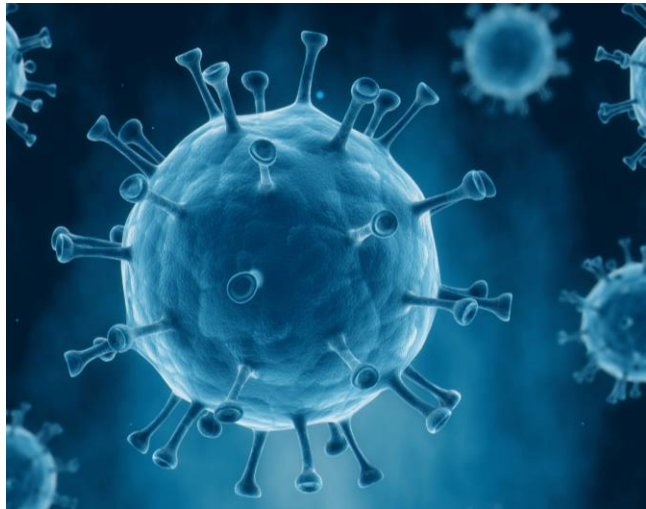
Blue Book intended revisions

Diagnostic testing chapter

- No clear guidance on when a diagnostic test may be incorporated into Section 2 (Inspection Section) of the Blue Book
  - Assays in aquatic animal testing rarely meet validation according to OIE
  - What is required of an assay before incorporation into the Blue Book?

- A tiering system for assays published in the Blue Book
  - Appropriate use of assays (diagnostic, screening, surveillance of wild)
  - How does sensitivity and specificity influence population testing?

- A diagnostic work group has been developed to assemble this chapter
  - Provide best technical guidance document
  - Will be reviewed by the SC and different working groups



Diagnostic testing chapter

Appendix 3

- Defining testing criteria prior to both sections 1 and 2

*Defining pathogen testing criteria

PREFACE

INTRODUCTION

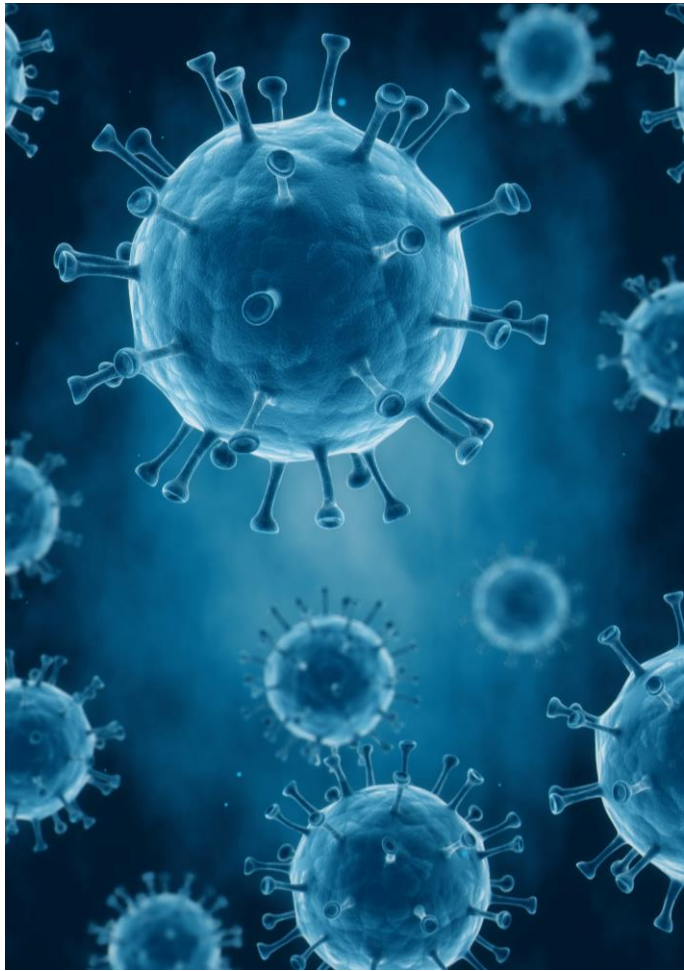
CITATION GUIDELINES

SECTION 1

Diagnostic Procedures for Finfish and Shellfish Pathogens

SECTION 2

USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections



New sections alongside Inspection Manual

Appendix 3

SECTION 2

USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

- New sections providing focused guidance on certain host groups
  - Mollusks
  - Crustaceans
  - Finfish (more robust coolwater and warmwater fish)
- Chapter structure to give perspective on these species in North America
  - End use (food, live movements, wild populations)
  - Table on host susceptibilities to pathogens
- Biosecurity guidance and recommendations
- Risk-based approaches for inspections (CAHPS)
- Standardized testing protocols (new and cross-referencing current Section 2)

Provide feedback!

Natural Resource Agency Working Group

- Chairs of this working group are Gary Whelan and Wade Cavender

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AFS FISH HEALTH SECTION

- As members of the FHS you can provide additional input

Project Managers

- Reach out to us:
- Jan Lovy: Jlovy.bluebook@gmail.com
- Ken Cain: Kcain@uidaho.edu

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ARTICLE

Assessment of a Serologic Diagnostic Test and Kinetics of Antibody Development in Northern Pike Experimentally Infected with Viral Hemorrhagic Septicemia Virus

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Abstract

Viral hemorrhagic septicemia virus (VHSV) is an ongoing cause of disease and mortality in freshwater fishes across the Great Lakes region of the Midwestern United States. Antibody detection assays such as enzyme-linked immunosorbent assay (ELISA) are nonlethal serological methods that can have significantly shorter turnaround times than the current validated viral detection diagnostic methodology for VHSV: cell culture with confirmation by polymerase chain reaction (PCR). This study evaluated an ELISA that detects nonneutralizing antinucleocapsid antibodies to VHSV in Northern Pike *Esox lucius*. Juvenile Northern Pike were experimentally infected with VHSV by intraperitoneal injection. The infected fish were monitored for 12 weeks for signs of disease, and weekly serum samples were obtained. An analysis of the survival data showed that mortality occurred significantly more quickly in inoculated fish than in control fish. Fish that were infected by injection showed a significant increase in antibody response by 2 weeks postinfection. However, variation in the rate and pattern of antibody response among the infected fish was high at any given point. The optimum window for detecting antibodies in Northern Pike is 2–12 weeks postinfection, which generally follows the median time to appearance of clinical signs (21 d postinfection). The receiver-operating characteristic curve analysis showed the ELISA to have a sensitivity of 80.5% and a specificity of 63.2% in Northern Pike, but these values can be adjusted by choosing different percent inhibition cutoffs, which may facilitate the use of the test for specific management goals. The results of this study offer insights into the disease progression and immune kinetics of VHSV, including interindividual variation, which will aid in the management of this economically important virus.

*Corresponding author: tony.goldberg@wisc.edu
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Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus Rhabdoviridae: *Novirhabdovirus* that is affecting a diversity of fish species worldwide (Wolf 1988; Kim and Faisal 2011; Millard and Faisal 2012; Millard et al. 2014; Wilson-Rothering et al. 2014, 2015). A freshwater strain, Great Lakes VHSV-IVb was first detected in Muskellunge *Esox masquinongy* in Lake Saint Clair, Michigan, in 2003, and has since been associated with large-scale mortality in 31 freshwater fish species (Kim and Faisal 2010a, 2010b; Faisal et al. 2012; Olson et al. 2013). Because VHSV is a reportable pathogen according to many state and federal agencies, fish are tested for VHSV as a part of routine fish health inspections and disease surveillance efforts. The current and most commonly used approved presumptive diagnostic testing method for VHSV is virus isolation in cell culture with a confirmatory polymerase chain reaction (PCR); however, other options for confirmatory diagnosis are available (Batts and Winton 2014). Viral cell culture requires tissue samples (kidney, spleen, heart, or brain) or ovarian fluids to be cultured on susceptible cell lines (e.g., *Epithelioma papulosum cyprini* or endothelial progenitor cells) for 14 d (although a positive result may appear sooner), followed by a 14-d blind passage prior to confirmatory PCR testing (Batts and Winton 2014; OIE 2018). This virus detection method involves lethal sampling, has up to a 4-week turnaround time for results, and detects current infection but not prior exposure. Rapid antibody detection methods, such as serologic methods, are nonlethal and could reduce turnaround time significantly. In addition, while both virus detection and serologic methods are useful for disease detection, serologic methods can improve surveillance and provide a better indication of the true prevalence of infection within a population (OIE 2018).

In the past decade, enzyme-linked immunosorbent assay (ELISA) methods have been developed to confirm prior exposure to VHSV in the United States by detecting antibodies in fish serum (Millard and Faisal 2012; Millard et al. 2014; Wilson-Rothering et al. 2014, 2015). Enzyme-linked immunosorbent assay compares favorably with other antibody-detecting diagnostic tools. For example, the competitive ELISA that was developed by Millard et al. (2014), found 78.4% agreement with plaque neutralization testing. In 2014, Wilson-Rothering et al. published an antinucleocapsid-blocking ELISA that is able to detect nonneutralizing VHSV antibodies with greater sensitivity and specificity than a virus neutralization assay does. Although ELISA is gaining momentum as a useful diagnostic tool for VHSV, there are still knowledge gaps. Notably, the diagnostic performance characteristics of VHSV ELISA remain poorly understood for many fish species that are susceptible to VHSV, as do the kinetics of the antibody response that ELISA measures.

Although in principle the blocking ELISA can be applied to any species of fish, the performance characteristics of the assay in Northern Pike have not been assessed nor have cutoffs for diagnostic testing been determined. Therefore we examined antibody development over the course of disease in experimentally infected Northern Pike to examine the rate and timing of antibody development compared with the appearance of clinical signs, establish an inhibition cutoff threshold in Northern Pike, and thereby assess the applicability of the test to this economically important sport fish.

METHODS

Fish.— The fish were obtained at 6 months posthatch from the Wild Rose State Fish Hatchery in Wisconsin, USA. All of the fish were confirmed free of significant pathogens, including VHSV, by following the certified protocols in the American Fisheries Society Bluebook testing guidelines (Batts and Winton 2014).

The Northern Pike were allowed to acclimate for 6 months[1] in 200-L circular plastic tanks at a maximum density of 15 fish per tank in a recirculating system consisting of cycled deionized water with supplemental filtration and aeration. The deionized water was treated with a water conditioner (SeaChem Laboratories, Madison, Georgia) to remove residual hardness and to seed the biofilter with beneficial bacteria. The ammonia source for the fishless cycle was ammonium chloride (MilliporeSigma, Saint Louis, Missouri). Frequent water testing was conducted to ensure a completed cycle prior to adding the fish to the tanks. After the fish were added to tanks, an automated light timer maintained a 12 h light : 12 h dark cycle. Daily water changes of at least 5% of the total tank volume were performed according to the recommendations by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin–Madison (IACUC approval V005768-A01). The fish were fed 2.0 mm pellets (Bio-Oregon, Westbrook, Maine) by an automatic feeder three times per day throughout the study. Water temperature was recorded daily and lowered from 17°C to 11 ± 1°C at a rate of 1°C per day before the start of the infection trials to mimic the temperature at which VHSV is most infective (Hershberger et al. 2013). Ammonia, nitrite, nitrate, and pH in each tank were tested and recorded weekly (the water quality parameters were maintained at approximately 0.25, 1.0, 20 mg/L, and 7.2, respectively).

Prior to VHSV exposure, the fish were anesthetized with a dose of 100 mg/L tricaine methanesulfonate

[1]The fish were held for this amount of time prior to experimental infection to ensure acclimatization and to obtain the necessary administrative clearances.

(MS-222; Syndel USA, Ferndale, Washington) that was buffered 1:1 (volume basis) with sodium bicarbonate (Millipore-Sigma, Saint Louis, Missouri) and marked with two visible implant elastomer tags (Northwest Marine Technology, Shaw Island, Washington) such that all of the individuals were uniquely identifiable. A baseline blood draw of 0.5 mL from the caudal tail vein of each fish was then performed. The blood samples were collected by using a 22G needle and syringe with the fish on a recirculating wet table, and the samples were then transferred to glass no-additive blood tubes (VWR International, Radnor, Pennsylvania) and inverted several times to induce clotting. The blood samples were allowed to clot overnight at 5°C then centrifuged for 15 min at $1{,}947 \times g$. The serum was separated and stored in cryovials at −80°C until testing by ELISA.

Culture and verification of the Great Lakes strain MI03 of VHSV.— Viral culture and quantification were performed at the La Crosse Fish Health Center in Onalaska, Wisconsin. Briefly, a Great Lakes strain VHSV-IVb isolate (confirmed by a reverse transcription PCR of a 946-base-pair diagnostic portion of the viral nucleoprotein gene and Sanger sequencing prior to the initiation of the study) was propagated by using a multiplicity of infection of less than 0.1 on endothelial progenitor cells that were grown in T75 tissue culture flasks with Minimum Essential Media-10 (Thermo Fisher Scientific, Waltham, Massachusetts) growth media. After a 100% cytopathic effect was observed, the virus stock was harvested from the flasks by scraping to dislodge the cells and media. These suspensions were pooled, centrifuged at $1{,}800 \times g$ for 10–15 min at 4°C to remove cellular debris, aliquoted, and frozen at −80°C. The virus was then quantified by serial dilution, and aliquots of 3.75 mL each suspended in Minimum Essential Media-10 growth medium were stored at −80°C for use in inoculation experiments. Using the tissue culture infectious dose that produced a 50% endpoint (TCID50; the dilution of virus-containing sample that infects 50% of tissue culture samples) the final concentration of the virus was 4.74×10^8 TCID50/mL (Binder 2017).

Experimental infection of Northern Pike.— Acclimatized Northern Pike (12 months old; average length 27.94 cm) were infected with 5×10^5 PFU/mL of VHSV by intraperitoneal injection (IP).[2] The fish were separated into three tanks of six to eight fish each, and six additional fish were kept in a separate tank as controls. The IP fish (23 fish in three tanks) were anesthetized with 100 mg/L MS-222 buffered 1:1 with sodium bicarbonate and then injected with a volume of 0.5 mL per fish. The control fish were mock-infected under the same conditions with cell culture media, Minimum Essential Media-10. After exposure, the fish were maintained at a water temperature of 11 ± 1°C. Daily monitoring during the experimental period included tank water temperature, observing fish for signs of disease, and recording mortalities. Euthanasia was warranted for fish that showed markedly abnormal swimming behavior, severe lethargy, severely decreased gill activity, severe anemia, and excessive bloating and/or hemorrhaging. The fish were euthanized with an overdose of MS-222 (200 mg/L) buffered 1:1 with sodium bicarbonate for 10 min.

Nonlethal blood samples were collected from the caudal vein of surviving fish, including controls, weekly for up to 12 weeks (84 d) postinfection. The blood collection and sample processing were performed as described above for the baseline blood draw. The fish that were euthanized prior to week 12 were only bled prior to euthanasia if the timing aligned with the weekly sampling schedule. On day 84, after the final weekly blood draw, all of the remaining fish were euthanized with an overdose of MS-222.

Antibody detection by competitive ELISA.— The blocking ELISA method that was developed by Wilson-Rothering et al. (2014) was used. Coating antigen was made from purified virus grown on endothelial progenitor cells, the same cell line that was used to culture the virus for experimental infection. Immulon II HB (Fisher Scientific, Hampton, New Hampshire) flat-bottomed 96-well plates were coated with 100 µL of antigen that was diluted at 1:200 in coating buffer in alternating positive and negative antigen rows. The coated plates were stored at −20°C until they were ready to use.

On the day of testing, the serum samples were first thawed at room temperature, then heated for 30 min at 45°C in a water bath to inactivate the complement, then centrifuged at $1{,}947 \times g$ for 15 m, and finally diluted at 1:8 in wash buffer to reduce nonspecific binding. Antigen and blocking buffer from a thawed ELISA plate were then removed, and 50 µL of diluted controls and Northern Pike serum was added to the positive and negative antigen wells. The plate was incubated for 30 min at 37°C, after which 50 µL of monoclonal antibody (Aquatic Diagnostics, Sterling, Scotland; conjugated by American Qualex, San Clemente, California) diluted 1:6,000 in blocking buffer (phosphate-buffered saline) was added to all of the wells containing sera. The plate was incubated for 90 min at 37°C and then washed three times. One hundred microliters of Sure-Blue tetramethylbenzidine substrate (KPL, Gaithersburg, Maryland) was then added to each well, and the plate developed for approximately 15 min at 37°C. One hundred microliters of tetramethylbenzidine Stop Solution (KPL, Gaithersburg, Maryland) was added to terminate the reaction. The ELISA plate was then read

[2]Initially, an additional group of eight Northern Pike was infected by static immersion (SI; 90 min in 30 L of aerated aquarium with 45.86 mL of virus stock, 5 × 105 PFU/mL of VHSV). However, the preliminary data indicated that this group was not successfully infected. Therefore, the SI group was not included in final analyses.

by using an absorbance microplate reader at an optical density (OD) of 450 nm. The OD readings were adjusted to eliminate background by subtracting the readings from the negative antigen wells from those from the positive antigen wells. The ELISA results were reported as percentage of inhibition (%I) and normalized to correct for any overdevelopment of negative samples by multiplying by the value of the negative control OD divided by the highest sample OD on each plate (Wright et al. 1993). The negative control consisted of pooled serum from confirmed-negative hatchery-reared Brown Trout from the Wild Rose State Fish Hatchery in Wisconsin.

Statistical analyses.— The data analyses were performed using R version 3.3.3 (R Core Team 2017). To determine the optimal positive and negative percent inhibition threshold, a receiver-operating characteristic (ROC) curve was constructed. All of the ELISA results (%I) from every fish at each point (including those from the baseline samples) combined with binary viral exposure status of the fish (negative = not exposed to VHSV, positive = exposed to VHSV) were used to form the ROC curve. A threshold %I value was chosen as the cutoff that maximized both sensitivity (true positive results) and specificity (true negative results). Kaplan–Meier survival curves were used to display the survival probability of IP and control fish at postinfection. The groups were compared by using a log-rank test. A *P*-value of less than 0.05 was considered statistically significant. To account for potential confounders (exposure status, survival time, and time to development of clinical signs of disease), a Cox proportional hazard model was also constructed. The Kruskal–Wallis rank sum test was used to analyze the difference in %I between infected and noninfected fish.

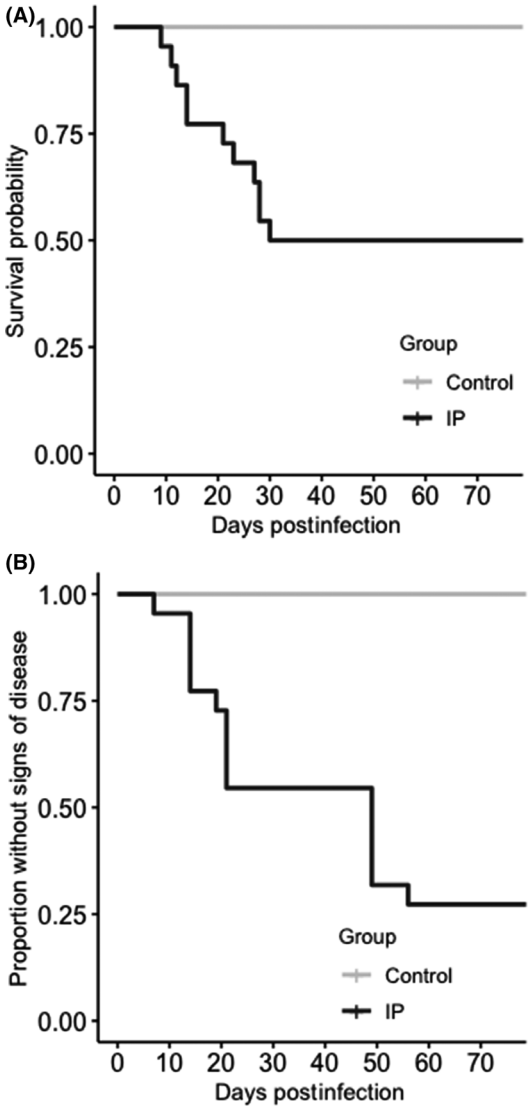

FIGURE 1. **(A)** The survival probabilities for both experimental groups of Northern Pike (control and IP) and **(B)** the Kaplan–Meier curve showing the proportion of Northern Pike without clinical signs of disease over time. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 16.1$, df = 1, $P = 0.00005$). About 50% of IP-infected fish showed clinical signs of disease by 21 d postinfection.

RESULTS

Survival and Development of Clinical Signs of Disease

The survival probabilities for both experimental groups (control and IP) are shown in Figure 1A. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 15.2$, df = 1, $P = 0.00009$). The control group had the highest percentage of survival to the end of the experiment (100%), followed by the IP group with 50% survival. The median survival time for the IP group was 69 d postinfection. The Cox proportional hazard analysis showed a significant difference in risk of death between the control and IP groups ($P = 4.03 \times 10^{-7}$).

Figure 1B shows the probability that a Northern Pike remained nonclinically affected (i.e., did not display clinical signs of disease) for each group over time. No signs of disease were observed in the control group at any point during the postinfection observation period. However, the exposed group showed clinical signs including erythema, exophthalmia, anemia, bloated abdomen, and abnormal swimming behavior. The log-rank tests showed a significant difference between the two groups ($\chi^2 = 16.1$, df = 1, $P = 0.00005$). The infected group showed an increase in the probability of development of clinical signs of disease over time, with about 50% of the Northern Pike displaying signs of disease by 21 d postinfection. The Cox proportional hazard analysis showed a slight decrease in risk of death when signs of disease were observed (Hazard ratio 0.98, 95% confidence interval, 0.97–1.00, $P = 0.02$), i.e., the fish that showed clinical signs of disease survived

longer than those that did not show clinical signs of disease did. As shown in Figure 1, almost 25% of the infected fish had already died during the acute stage of infection (prior to the peak development of clinical signs at 21 d postinfection), likely explaining this observation. The other potential confounders that were examined were not significant.

Kinetics of the Antibody Response

Figure 2 shows the average percentage of inhibition by ELISA for both the IP and control groups over the 12 weeks postinfection, including the baseline samples that were taken prior to infection. The %I for the control fish ranged from 0% to 59.9% (average, 36.0%). At baseline, the fish that were infected by IP injection had %I values that ranged from 5.3% to 66.1% (average, 42.7%), and %I ranged from 0% to 89.0% (average, 51.0%) during the postinfection period. The standard errors for the control and IP groups were 1.53% and 1.44%, respectively. Furthermore, the average rate of change per week for each group was −0.15% and 0.42%, respectively, over the course of 12 weeks.

ELISA Diagnostic Performance Characteristics

Table 1 shows a summary of the VHSV ELISA results, with Northern Pike divided into either infected (IP) or uninfected (control) groups. To avoid false negatives, virus isolation was not performed as a confirmatory test, as viral titers varied at time of death and most likely would be low or even absent (based on the preliminary trials that preceded this study). Given the 41.3% threshold, 112 of 139 serum samples (80.5%) from the IP injection group were positive, as determined by ELISA. The Kruskal–Wallis rank sum test showed a significant difference in the %Is between the infected and the uninfected groups ($\chi^2 = 43.6$, df = 1, $P = 3.9 \times 10^{-11}$). Figure 3A shows the results of the ROC analysis of ELISA sensitivity and specificity. The area under the ROC curve was 0.7613. A %I value of ≥41.3% to demarcate positive samples and <41.3% to demarcate negative samples maximized the accuracy of the assay. With these cutoff values, the ELISA performed at a sensitivity of 80.5% (95% confidence interval, 73–87%) and a specificity of 63.2% (95% confidence interval, 52–73%). The positive predictive value of the ELISA for infected fish was 78% (95% confidence interval, 70–84%) and the negative predictive value was 67% (95% confidence interval, 56–77%). The positive likelihood ratio was 2.19 (95% confidence interval, 1.64–2.92) and the negative likelihood ratio was 0.31 (95% confidence interval, 0.21–0.45).

Figure 3B shows the results of an additional ROC analysis of ELISA sensitivity and specificity that was completed by using a subset of data from only weeks 2–12 during the postinfection period. Using the same threshold as is described above, the area under the ROC curve was 0.7860, with a sensitivity of 83% (95% confidence interval, 74–90%) and specificity of 62% (95% confidence interval, 47–76%).

ELISA Kinetics

Positive ELISA results were detectable during all 12 weeks postinfection for the experimentally infected group. The highest percentage of positive results occurred during weeks 5 and 8 of the postinfection period (Figure 4). A comparison of standard error of the mean over time shows that the average %I of the infected group differed significantly from that of the control group during weeks 2–3, 5–8, and 10–12 postinfection (Figure 2).

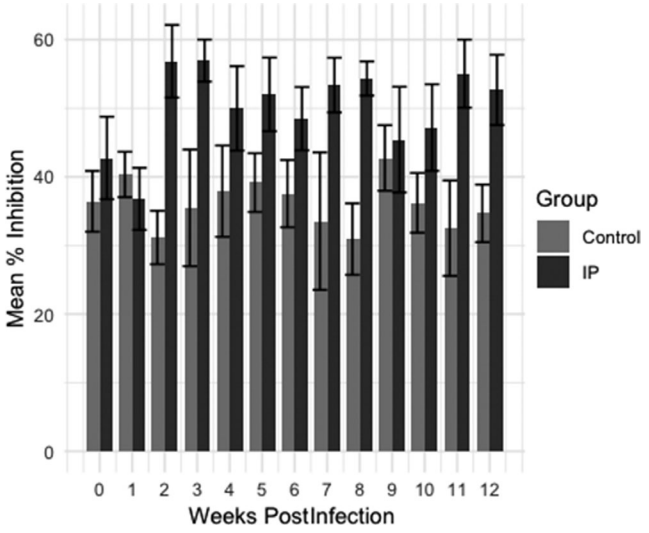

FIGURE 2. Comparison of average percent inhibitions for each experimental group over the course of infection. The error bars represent standard error of the mean.

DISCUSSION

Our results show that Northern Pike that were infected with VHSV by intraperitoneal injection showed a 50% survival rate and the development of a sustained antibody response over the course of a 12-week postinfection period. The survival rates for the infected fish were significantly lower than those of the mock-infected controls

TABLE 1. The 2 × 2 table of VHSV ELISA results for fish of known infection status.

	True positive	True negative	Total
ELISA positive	105	26	131
ELISA negative	115	65	180
Total	220	91	311

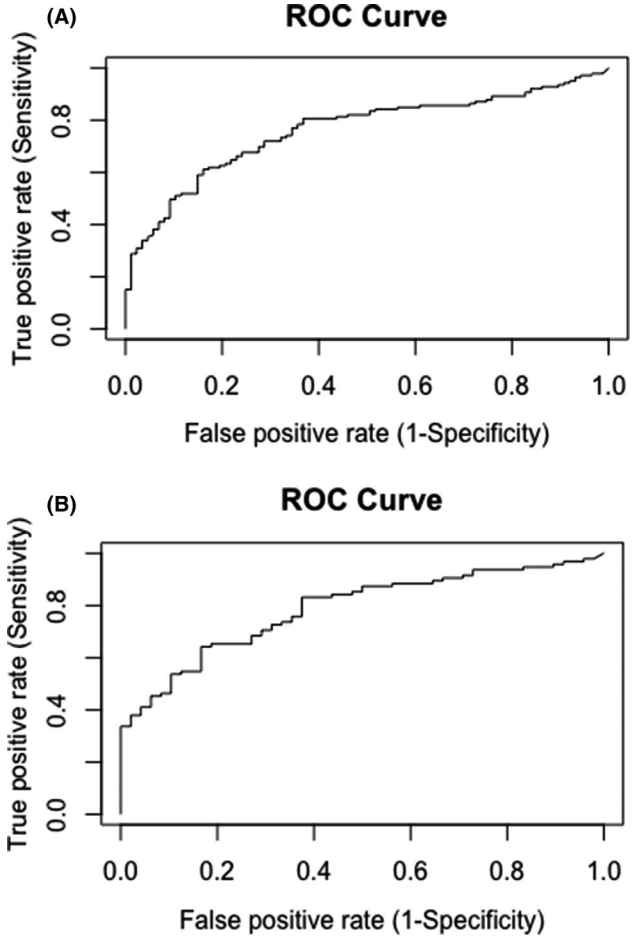

FIGURE 3. **(A)** Receiver operating characteristic (ROC) curve showing the true positive rate for Northern Pike serum that was tested at a 1:8 dilution by VHSV ELISA. The data that were used to form this curve consisted of 139 infected fish and 87 uninfected fish. The area under the curve is 0.7613. At the optimum threshold of 41.3% inhibition, the sensitivity of the ELISA is 80.5% and specificity is 63.2%. If the threshold is altered to maximize specificity, the new threshold is 58.2% inhibition, with a sensitivity of 34.5% and specificity of 95.4%. Also shown is **(B)** an alternative ROC curve showing the true positive rate for the Northern Pike serum that was tested at a 1:8 dilution by VHSV ELISA. A subset of the data from weeks 2–12 postinfection for both controls and IP-infected fish were used to form this curve. The data consisted of 95 infected fish and 48 uninfected fish. The area under the curve is 0.7862.

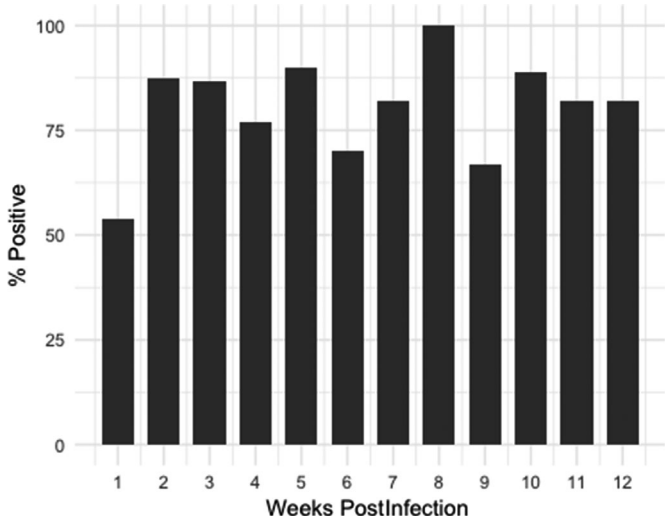

FIGURE 4. Percentage of infected Northern Pike that tested positive by ELISA during each week postinfection. The highest percentage of positive cases occurred during week 5 and week 8 (90% and 100%, respectively) .

(100%). The onset of clinical signs in the majority of infected Northern Pike occurred at 3 weeks postinfection. The globally optimal sensitivity (80.5%) and specificity (63.2%) of the VHSV ELISA was achieved by setting the inhibition cutoff at 41.3%. However, alternative cutoffs can achieve substantially higher sensitivity or specificity, which may be advantageous for certain applications.

It is noteworthy that we successfully replicated the blocking ELISA method by Wilson-Rothering et al. (2014) with a few minor alterations and used it to detect the presence of nonneutralizing anti-VHSV antibodies in experimentally infected Northern Pike, which were not a species that was used to develop or assess the method. Notable alterations in our protocol included increasing the serum sample test dilution and the centrifugation of the serum prior to testing. Both of these changes helped to reduce background (nonspecific binding) in the negative antigen wells. Nonspecific binding seemed to occur more frequently in the serum from Northern Pike than has been observed in other species that have been tested previously. Only 6 of 78 control samples (7.6%) had background ODs > 0.1 in the negative antigen well, whereas 33 of 148 fish (22.2%) that were infected by IP injection had background ODs > 0.1 at the 1:8 dilution. The high level of nonspecific binding that was observed in this study could have been caused by the inoculation procedure. In a study conducted by Güven et al. (2014), for example, nonspecific binding in a human autoantibody ELISA correlated with inflammatory markers in serum.

An inhibition threshold of 41.3%, which maximized overall test accuracy, yielded a sensitivity of 80.5% and a specificity of 63.2%. These values are lower than the values of 96.4% and 88.2%, respectively, that were reported by Wilson-Rothering et al. (2014). These differences could indicate factors that are unique to different esocid species, in that Northern Pike were not included in the study by Wilson-Rothering et al. (2014). Similarly, Millard et al. (2014) found that competitive ELISA and a plaque neutralization test had strong agreement but a lack of a gold standard in that study precluded a formal assessment of sensitivity and specificity. Therefore, it is noteworthy that

the area under the ROC curve improved marginally when the fish from weeks 2–12 postinfection were analyzed separately (Figure 3; The area under the curve was 0.7613 and 0.7862, respectively), indicating that ELISA sensitivity and specificity improves when fish are tested during the optimum window of antibody detection. Using the 41.3% cutoff for Northern Pike reported herein would favor sensitivity (i.e., detecting true positives) over specificity (i.e., detecting true negatives), although neither value is ideal. However, depending on the purpose for which the test is used, alternative cutoffs could be chosen. For example, the cutoff could be lowered to increase sensitivity in a situation where it is important to maximize detection of positive fish (e.g., prior to translocating fish into a VHSV-free water body), albeit at the expense of elevating the false positive rate. Otherwise, the cutoff could be raised to increase specificity in a situation where false positive results would be costly (e.g., prior to destroying fish or eggs). The choice of a cutoff should, in other words, be dictated by the purpose of testing.

Fish that were infected by the IP route developed detectable antibodies by 1 week postinfection with the most consistency in detectable positive results from 2 to 12 weeks postinfection. Notably, we documented high interindividual variation in ELISA positivity both among fish and within fish over time. Therefore, the ELISA assay in this study is more suited to assessing the population status of VHSV exposure rather than assessing the infection history of an individual fish. Indeed, its best use for management may be to compare the sero-status of species and populations over space and time.

In this light, our results also show that clinical signs and ELISA positivity rates peak at approximately 2–8 weeks postviral exposure. This timing corresponds to the dynamics of the disease in natural populations, which is surely more complex, and provides some calibration for the interpretation of positive results. For example, the ELISA assay described herein would probably be less useful for assessing exposure of Northern Pike to VHSV immediately after the introduction of the disease but more useful several weeks or months afterwards. Given the time limits of our study, the duration for which Northern Pike remain ELISA-positive following VHSV exposure remains unclear, as do the physiological and environmental factors that might affect that duration. Nevertheless, even an approximate knowledge of the timing of VHSV exposure in natural populations of Northern Pike (or other species) could improve management decisions, especially given the potentially rapid turnaround time of the assay.

Conclusion

A competitive ELISA method that was developed by Wilson-Rothering et al. (2014) for detecting antibodies to VHSV is repeatable and performs with moderate sensitivity and specificity in Northern Pike (80.5% and 63.2%, respectively) when a 41.3% inhibition threshold is chosen, but either value can be improved by lowering or raising this threshold, respectively, as warranted by the purpose for which the test is used. In experimentally infected Northern Pike, nonneutralizing anti-VHSV antibodies developed by 1 week postinfection and were detectable through all 12 weeks postinfection, but the highest likelihood of detection occurred from weeks 2 to 8 postinfection, which aligned with the development of clinical signs. The potential uses of this assay in Northern Pike include, but are not limited to, testing wild Northern Pike for general VHSV surveillance, testing wild Northern Pike in hatchery source waters, testing Northern Pike that are used as broodstock to supply hatcheries, or testing of Northern Pike prior to translocation. The study also outlines methods that can be used to identify optimal thresholds and sample dilutions for other situations and species.

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ARTICLE

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.

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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 × *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 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).

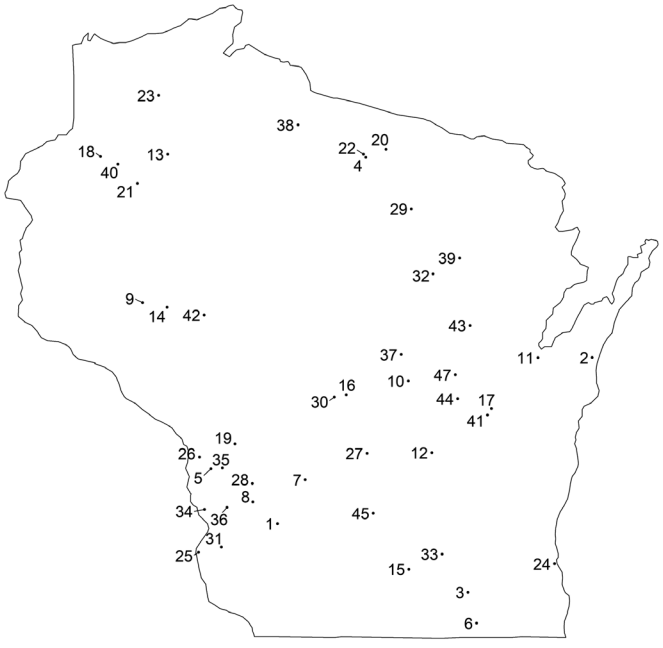

FIGURE 1. Numbered map of Wisconsin water bodies sampled in 2016 and 2017. For water body names and full details, including surveillance results, see Table 1. Location 46 (Lake St. Clair, Michigan) is not pictured here.

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	B (21)	0 (21)	
					2017	B (3)	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	N (9), W (23)	11.1 (9), 8.6 (23)	11.1 (9)
					2017	W (17)	4.1 (17)	
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	B (16), N (8)	6.25 (16), 75 (8)	75 (8)
					2017	B (21), N (30), W (24)	0 (21), 3.3 (30), 4.1 (24)	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	N (5), W (2)	0 (5), 0 (2)	0 (5)
					2017	B (10), N (24)	0 (10), 4.16 (24)	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	B (22), N (5), W (29)	0 (22), 0 (5), 31.0 (29)	0 (5)
					2017	B (20), W (30)	0 (20), 20 (30)	
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	N (7), W (18)	14.2 (7), 5.5 (18)	0 (7)
					2017	N (15), W (19)	0 (15), 5.2 (19)	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	B (11), N (27)	0 (11), 14.8 (27)	14.8 (27)
					2017	B (15), N (6)	0 (15), 0 (6)	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)	

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).

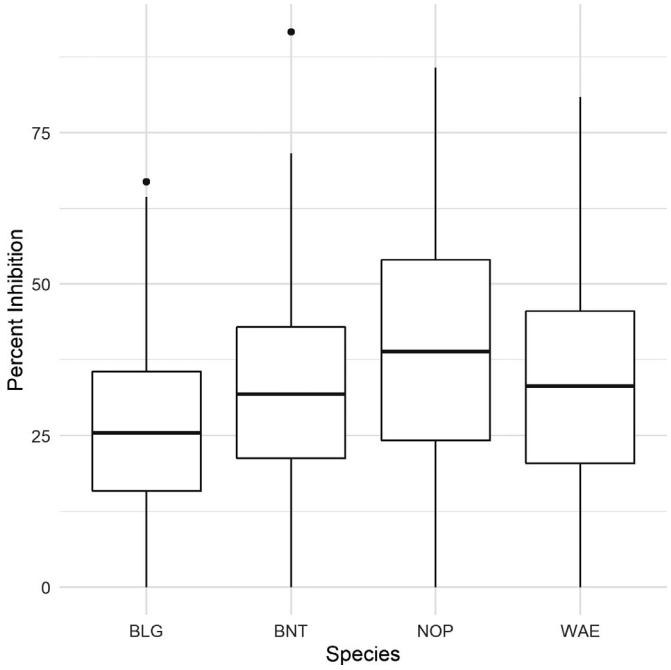

FIGURE 2. Box plot of percent inhibition of the enzyme-linked immunosorbent assay for viral hemorrhagic septicemia virus by species (BLG = Bluegill; BNT = Brown Trout; NOP = Northern Pike; WAE = Walleye). Mean percent inhibition differed among species (Kruskal–Wallis test: $\chi^2 = 107.99$, $df = 3$, $P < 0.0001$), 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

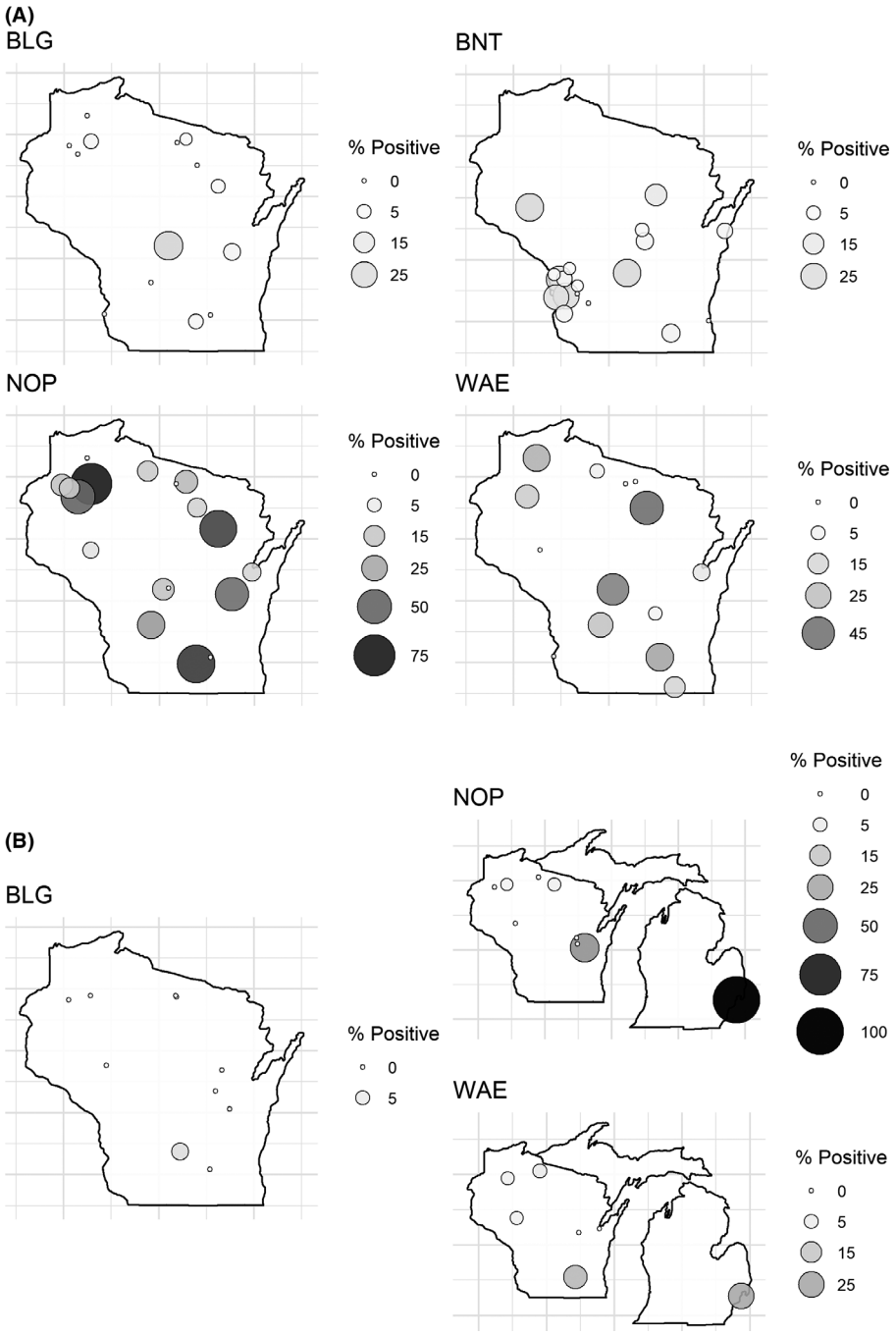

FIGURE 3. Results of surveillance efforts in **(A)** 2016 and **(B)** 2017. Percentages of Bluegill (BLG), Brown Trout (BNT), Northern Pike (NOP), and Walleye (WAE) that tested positive for antibodies to viral hemorrhagic septicemia virus by enzyme-linked immunosorbent assay at each sampling location 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.

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Development and Evaluation of a Blocking Enzyme-Linked Immunosorbent Assay and Virus Neutralization Assay To Detect Antibodies to Viral Hemorrhagic Septicemia Virus

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Viral hemorrhagic septicemia virus (VHSV) is a target of surveillance by many state and federal agencies in the United States. Currently, the detection of VHSV relies on virus isolation, which is lethal to fish and indicates only the current infection status. A serological method is required to ascertain prior exposure. Here, we report two serologic tests for VHSV that are nonlethal, rapid, and species independent, a virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). The results show that the VN assay had a specificity of 100% and sensitivity of 42.9%; the anti-nucleocapsid-blocking ELISA detected nonneutralizing VHSV antibodies at a specificity of 88.2% and a sensitivity of 96.4%. The VN assay and ELISA are valuable tools for assessing exposure to VHSV.

Viral hemorrhagic septicemia (VHS) is one of the most pathogenic viral diseases of fish worldwide and affects a wide range of host species (1–7). Of the four genotypes, the North American strains of VHS virus are designated types IVa and IVb. Type IVa was originally isolated from asymptomatic marine salmonids in the Pacific Northwest in 1988 (8); it is now known to be endemic throughout the northeast Pacific, where it is highly virulent to populations of Pacific herring (*Clupea pallasii*) and other marine fishes (9). A new freshwater strain, type IVb, was isolated from a muskellunge (*Esox masquinongy*) collected from Lake St. Clair, MI, in 2003 (10). This distinctive sublineage has been isolated from 31 species of fish in the Great Lakes (11) and has been associated with significant die-off events of freshwater drum (*Aplodinotus grunniens*), muskellunge (*Esox masquinongy*), gizzard shad (*Dorosoma cepedianum*), round gobies (*Apollonia melanostomus*), and yellow perch (*Perca flavescens*) in the Great Lakes between 2005 and 2008 (2, 10, 12–16). By 2009, the virus had spread to all of the Great Lakes and several inland lakes. The introduction and spread of this pathogen and the threat it poses to a broad range of hosts resulted in increased surveillance of the virus in Wisconsin and other states within the Great Lakes Basin.

Currently, the surveillance methods for VHS virus (VHSV) detection include virus isolation in cell culture, followed most commonly by confirmation by reverse transcription-PCR (RT-PCR). Fish are tested for VHSV according to the guidelines outlined in the American Fisheries Society Fish Health Section Blue Book (17) and the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals (18). Both approved methods detect the virus but do not detect antibodies indicative of previous virus exposure.

Clinical signs of disease are not consistent among VHSV-susceptible species, and VHSV IVb is not always isolated from clinically affected fish, especially salmonids (19, 20). Differences in susceptibility and mortality rates among different populations of yellow perch have been reported recently (21). The clinical signs and severity of infection also depend on water temperature at the time of infection, stress level, host age, and other environmental factors (15, 22). These variables can affect the narrow window of opportunity to detect VHSV by virus isolation; therefore, diseased or recovered individuals may easily be missed during surveillance efforts.

Methods to detect neutralizing antibodies to VHSV have been developed for surveillance using a complement-dependent neutralization test (50% plaque neutralization test [PNT]) and have been highly sensitive and specific for trout (23–25). However, PNT requires overlay and plaque enumeration steps; further, this method is best suited for small sample sizes. A microneutralization format without the use of overlay might lead to a 50% reduction in the resources and labor required to perform the assay. Another advantage of a virus neutralization assay is that the indicator system is a susceptible cell line for the target virus, which makes the assay inherently species independent.

Competitive and blocking enzyme-linked immunosorbent assays (ELISAs) for the detection of antibodies against mammalian viruses have been in use for decades. Indirect ELISAs have been available for VHSV since 1988 (26). A highly sensitive (92%) indirect ELISA for detecting nonneutralizing antibodies for the surveillance of VHS in farmed rainbow trout (*Oncorhynchus mykiss*) has also been described (25), but it requires a species-specific secondary fish antibody. Thus, these tests are not practical for multispecies VHSV surveillance in the wild, because there are at least 31 species known to be susceptible to VHSV IVb.

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Appendix 6

Here, we describe the development and evaluation of a modified virus neutralization (VN) assay and a blocking enzyme-linked immunosorbent assay (ELISA). Both tests were adapted from previously described methods in the World Organisation for Animal Health (OIE) manual (27) and use blood serum samples from uninfected fish and VHS survivors. Although the development of the anti-VHSV nucleocapsid monoclonal antibody used in our ELISA was published in 1988 (26), no competitive ELISA methods have been reported until now. These serological methods broaden the window of detection by demonstrating whether previous exposure to the virus had occurred, which might alleviate the time constraints of surveillance efforts using virus isolation.

MATERIALS AND METHODS

Cell lines. The epithelioma papulosum cyprini (EPC) cell line (American Type Culture Collection, Manassas, VA), originating from fathead minnow (*Pimephales promelas*) epithelial cells (28), was cultured at 25°C according to detailed protocols (18, 29), and the medium was supplemented with tryptose phosphate broth (Teknova; Hollister, CA, USA), 5% fetal bovine serum (FBS) (PAA Laboratories, Inc., Etobicoke, Ontario, Canada), 200 mM L-glutamine (Life Technologies), and buffered with 7.5% sodium bicarbonate solution (Life Technologies). The Chinook salmon embryo (CHSE-214) cell line (American Type Culture Collection, Manassas, VA) was cultured at 20°C, and the medium was supplemented with 10% FBS (PAA Laboratories, Inc.).

Virus isolate. The Great Lakes strain of VHSV (type IVb) was isolated on the EPC cell line. The isolate was obtained from pooled kidney and spleen tissue samples from a freshwater drum in Lake Winnebago during a VHS outbreak in 2007 and confirmed by real-time RT-PCR (30, 31).

Virus propagation and purification. Virus was adsorbed to the EPC cells at a multiplicity of infection (MOI) of 0.01 for 30 min at room temperature and then supplemented with cell culture medium. To propagate virus, flasks were incubated at 15°C for 5 to 7 days or until the first signs of cytopathic effect (CPE) were observed. A plaque-purified stock of VHSV-infected EPC cell supernatant was clarified by the removal of EPC cells, aliquoted for one-time use, and stored at −80°C. Postfreezing, an aliquot was thawed, and the titer of the batch was determined.

CHSE cells were used to propagate virus for ELISA antigen coating. The flasks for propagation were inoculated using the same methods as for the EPC cells but virus was adsorbed for 1 h at room temperature. After two freeze-thaw cycles, cell debris was removed by centrifugation at 4,000 × *g* for 15 min at 4°C in a Sorvall ST40R centrifuge (Thermo) and clarified. The supernatant was purified and concentrated according to the manufacturer's protocols using a Fast-Trap virus purification and concentration kit (Millipore, Billerica, MA). Eluted virus was aliquoted and stored at −80°C. A mock infection was performed in a similar manner to provide cell lysates for determining an optical density baseline in uninfected CHSE cells. Antigen was treated with 10% MEGA-10 detergent (Sigma-Aldrich) for an hour at room temperature prior to diluting in coating buffer for use in the ELISA.

Sera from fish of known infection status. Blood serum samples were obtained from 33 uninfected fish (Table 1), including brown trout (*Salmo trutta*) and yellow perch. A blood serum sample with antibodies to spring viremia of carp virus (SVCV) was obtained from a common carp (*Cyprinus carpio*) (Table 1). The serum samples were collected 4 to 5 months after an SVCV epizootic occurred in May 2002 in Cedar Lake, WI, and tested positive for neutralizing antibodies to SVCV at the Center for Environment, Fisheries, and Aquaculture Science (CEFAS) in Weymouth, United Kingdom, using a competitive ELISA (32). Serum samples were obtained from 28 experimentally infected or wild-caught fish that had survived exposure to VHSV (Table 2), including grass carp (*Ctenopharyngodon idella*), yellow perch, Pacific herring (*C. pallasii*), muskellunge, and freshwater drum. All serum samples were stored frozen at −80°C and then heated to 45°C for 30 min to inactivate complement before use in assays.

TABLE 1 Virus neutralization and blocking ELISA results for VHS-negative group

Serum source	VN titer result	ELISA data	
		% inhibition[a]	Result
Salmo trutta (brown trout) 1[b]	Negative	8.13	Negative
Salmo trutta (brown trout) 2	Negative	31.54	False positive
Salmo trutta (brown trout) 3	Negative	18.88	Negative
Salmo trutta (brown trout) 4	Negative	13.38	Negative
Salmo trutta (brown trout) 5	Negative	19.84	Negative
Salmo trutta (brown trout) 6	Negative	4.65	Negative
Salmo trutta (brown trout) 7	Negative	14.58	Negative
Salmo trutta (brown trout) 8	Negative	20.2	Negative
Salmo trutta (brown trout) 9	Negative	13.50	Negative
Salmo trutta (brown trout) 10	Negative	8.73	Negative
Salmo trutta (brown trout) 11	Negative	13.50	Negative
Salmo trutta (brown trout) 12	Negative	24.73	Negative
Salmo trutta (brown trout) 13	Negative	5.50	Negative
Salmo trutta (brown trout) 14	Negative	8.13	Negative
Salmo trutta (brown trout) 15	Negative	14.34	Negative
Salmo trutta (brown trout) 16	Negative	15.18	Negative
Salmo trutta (brown trout) 17	Negative	9.92	Negative
Salmo trutta (brown trout) 18	Negative	9.90	Negative
Salmo trutta (brown trout) 19	Negative	41.10	False positive
Salmo trutta (brown trout) 20	Negative	11.35	Negative
Salmo trutta (brown trout) 21	Negative	13.03	Negative
Salmo trutta (brown trout) 22	Negative	14.81	Negative
Salmo trutta (brown trout) 23	Negative	16.49	Negative
Salmo trutta (brown trout) 24	Negative	5.14	Negative
Salmo trutta (brown trout) 25	Negative	20.13	Negative
Salmo trutta (brown trout) 26	Negative	14.34	Negative
Salmo trutta (brown trout) 27	Negative	34.29	False positive
Salmo trutta (brown trout) 28	Negative	24.73	Negative
Salmo trutta (brown trout) 29	Negative	2.39	Negative
Salmo trutta (brown trout) 30	Negative	0.24	Negative
Perca flavescens (yellow perch) 1[c]	Negative	12.66	Negative
Perca flavescens (yellow perch) 2	Negative	27.48	False positive
Perca flavescens (yellow perch) 3	Negative	23.90	Negative
Cyprinus carpio (common carp)[d]	Negative	31.45[e]	Negative

[a] Results determined positive at ≥25% inhibition for test sera diluted 1:2 and ≥35% inhibition for undiluted test sera.
[b] Brown trout 1 to 30 were captive broodstock from Westfield, Wisconsin State Fish Hatchery that were never exposed to VHSV.
[c] Yellow perch 1 to 3 were lab-reared at the Great Lakes Water Institute in Milwaukee, WI, and never exposed to VHSV.
[d] Wild-caught from Cedar Lake, WI, following spring viremia of carp virus (SVCV) epizootic in May 2002. The serum sample was positive for neutralizing antibodies to SVCV at the Weymouth Laboratory, Weymouth, United Kingdom, using standard methods (40).
[e] Result from ELISA with undiluted serum, in which the positive threshold is ≥35% inhibition.

All yellow perch used in the study were hatched and reared at the University of Wisconsin, Milwaukee (UWM) School of Freshwater Sciences (SFS) Aquaculture Research Facility, according to previously described methods (33). They were exposed to VHS virus strain IVb (MI03) by intraperitoneal (i.p.) injection at a titer of 1×10^4 PFU/fish (J-Z fish) or 1×10^2 PFU/fish (H-Y fish). At 28 days post-VHSV injection, all yellow perch exhibited mild clinical signs of the disease, such as exophthalmia and hemorrhaging. The fish euthanized on day 64 appeared healthy, exhibiting no clinical signs of VHS, and all plaque assay results were negative.

Hyperimmunized Pacific herring (*C. pallasii*) were produced from laboratory-reared specific-pathogen-free (SPF) colonies (34). Briefly, SPF herring ≥5 years of age were immersed in waterborne VHSV (1.5×10^3

TABLE 2 Real-time RT-PCR, virus neutralization, and ELISA results for VHS-positive group

Serum source	VHS PCR result	VN titer result	ELISA data	
			% inhibition[a]	Result
Ctenopharyngodon idella (grass carp) 1[b]	Negative	Negative	75.12	Positive
Ctenopharyngodon idella (grass carp) 2[b]	C_T, 39.5	Negative	58.38	Positive
Perca flavescens (yellow perch) HI-14[c]	C_T, 36.5	1:16	72.97	Positive
Perca flavescens (yellow perch) J2-13[d]	Negative	p1:16[j]	78.26	Positive
Perca flavescens (yellow perch) J1-13/J3-11[d]	C_T, 37.8	p1:16[j]	46.96	Positive
Perca flavescens (yellow perch) H4 A[c]	C_T, 35.7	p1:16[j]	36.92	Positive
Perca flavescens (yellow perch) H4 B[c]	C_T, 35.7	p1:16[j]	54.04	Positive
Perca flavescens (yellow perch) H4 C[c]	C_T, 38.6	Negative	55.93	Positive
Perca flavescens (yellow perch) J4 A[d]	C_T, 32.6	Negative	42.30	Positive
Perca flavescens (yellow perch) J4 B[d]	Negative	Negative	27.62	Positive
Perca flavescens (yellow perch) Z1-2[e]	Negative	Negative	81.42	Positive
Perca flavescens (yellow perch) Z2-1[e]	Negative	Negative	95.99	Positive
Perca flavescens (yellow perch) Z2-2[e]	Negative	Negative	57.22	Positive
Perca flavescens (yellow perch) Y1-2[f]	Negative	Negative	49.47	Positive
Perca flavescens (yellow perch) Y3-1[f]	Negative	Negative	47.60	Positive
Perca flavescens (yellow perch) Y3-3[f]	Negative	Negative	56.15	Positive
Clupea pallasii (Pacific herring) 140[g]	Negative	p1:16[j]	42.22	Positive
Clupea pallasii (Pacific herring) 141[g]	Negative	Negative	56.22	Positive
Clupea pallasii (Pacific herring) 142[g]	Negative	p1:16[j]	56.91	Positive
Clupea pallasii (Pacific herring) 143[g]	Negative	Negative	41.23	Positive
Clupea pallasii (Pacific herring) 144[g]	Negative	Negative	30.95	Positive
Clupea pallasii (Pacific herring) 145[g]	Negative	1:32	27.23	Positive
Clupea pallasii (Pacific herring) 146[g]	Negative	p1:16[j]	43.88	Positive
Clupea pallasii (Pacific herring) 147[g]	Negative	Negative	26.15	Positive
Clupea pallasii (Pacific herring) 148[g]	Negative	p1:16[j]	34.67	Positive
Clupea pallasii (Pacific herring) 149[g]	C_T, 38.9	Negative	41.82	Positive
Esox masquinongy (muskellunge)[h]	Negative	1:80	7.94	False negative
Aplodinotus grunniens (freshwater drum)[i]	Negative	1:16	32.32	Positive

[a] Results determined positive at ≥25% inhibition for test serum diluted 1:2.
[b] Injected intraperitoneally (i.p.) with 200 μl of 10^6 PFU/fish VHSV IVb, serum collected 21 days post-i.p. injection.
[c] Injected i.p. with 1×10^2 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[d] Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[e] Injected i.p. with 1×10^4 PFU/ml VHSV IVb, held at 12°C, serum collected 64 days post-i.p. injection.
[f] Injected i.p. with 1×10^2 PFU/ml VHSV IVb, held at 12°C, serum collected 28 days post-i.p. injection.
[g] Hyperimmunized Pacific herring were exposed to 1.5×10^3 PFU/ml VHSV IVa by waterborne immersion for 1 h (day 0). The survivors were reexposed by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors after 112 days.
[h] Survived infection with VHSV IVb. The reference serum was received already diluted at 1:20 and used as the starting dilution for the VN assay. A new aliquot was obtained and used at 1:2 in the ELISA.
[i] Wild-caught on Lake Winnebago in Wisconsin on 9 May 2012. Kidney and spleen tissues tested positive for VHSV by real-time PCR according to previously described methods (31).
[j] p1:16, partial neutralization at this dilution.

PFU/ml) for 1 h. The survivors were reexposed to VHSV by i.p. injection after 49 days (2.9×10^2 PFU/fish) and 77 days (2.8×10^1 PFU/fish). Serum samples were collected from the hyperimmunized survivors 112 days after the initial waterborne exposure.

Virus neutralization. The VN assay to detect VHSV-neutralizing antibodies was modified from the mammalian VN assay protocol based on previously described methods (27). The VHSV VN assay was performed as follows: first, epithelioma papulosum cyprini (EPC) cells were preseeded onto sterile microtiter plates, typically 2 days prior to inoculation to achieve 100% confluence. Next, 50 μl of 100 × the 50% tissue culture infective dose ($TCID_{50}$) of virus (35, 36) was mixed with 2-fold serial dilutions of serum starting at 1:16 in 96-well cell culture microtiter plates (BD Biosciences, San Jose, CA) and incubated at 15°C for 24 h. A back titration plate with 10-fold dilutions of the working dilution of virus ($100 \times TCID_{50}$) was included to confirm the correct virus concentrations. Serum controls (serum without virus) were performed for each sample as well as an antibody positive and negative control on each plate. The cells were treated with 7% polyethylene glycol (PEG) for 10 min (37). Lastly, serum-virus mixtures were inoculated onto the PEG-treated cells, covered, and incubated at 15°C for 5 days. The virus neutralization titers were read as the last serum dilution showing protection of the cell monolayer.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. Mouse anti-VHSV nucleocapsid monoclonal IgG antibody (Aquatic Diagnostics, Stirling, Scotland) (26) was purchased for use in the blocking ELISA. The specificity of the anti-VHSV nucleocapsid monoclonal antibody to VHSV nucleocapsid was assessed by performing a Western blot, as previously described (38, 39). Spring viremia of carp virus (SVCV) is a rhabdovirus that is closely related to VHSV and that also causes disease during the spring season. The lysates were obtained from an isolate circulating during an SVCV epizootic in wild common carp (*C. carpio*) in northwestern Wisconsin (40). VHSV and SVCV lysates were separated on 4 to 20% gradient gels by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) using a wet transfer Mini Trans-Blot cassette according to the manufacturer's protocols in the Mini-PROTEAN Precast Gels Instruction Manual and Application Guide (Bio-Rad), with the following modifications. The membranes were soaked in Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBS-T) with 5% StartingBlock (PBS) blocking buffer (Thermo) overnight at 4°C. The

membranes were then incubated with anti-VHSV nucleocapsid monoclonal antibody diluted 1:100 in StartingBlock (PBS) blocking buffer at room temperature for 1 h with constant agitation. Three 5-min wash steps with TBS-T were performed after each antibody incubation step. The membranes were then incubated with peroxidase-rabbit anti-mouse IgG (H+L) (Invitrogen) at 1:1,000 in StartingBlock (PBS) blocking buffer for 1 h. A CN/DAB substrate kit (Thermo) was used for chromogenic detection of horseradish peroxidase-bound antibodies and stopped with deionized water.

Blocking enzyme-linked immunosorbent assay. A blocking ELISA was developed using modifications to a previous ELISA method (41). The anti-VHSV nucleocapsid monoclonal antibody utilized in our assay was previously shown to lack neutralizing activity (26). The antibody was purified and conjugated to horseradish peroxidase (HRP) using a commercial laboratory (American Qualex, San Clemente, CA). Alternating rows of purified MEGA-10 detergent-treated VHSV antigen and mock-infected MEGA-10 detergent-treated antigen diluted 1:100 in carbonate-bicarbonate buffer (pH 9.6) (Sigma) were adsorbed to 96-well Immulon 2 HB microtiter plates (Thermo) for 24 h at 21°C in an EchoTherm IN20 incubator (Torrey Pines Scientific) and then blocked with 200 μl StartingBlock (PBS) blocking buffer for 2 h at 20°C. Antigen and blocking buffer were aspirated from the wells using an ELx405 microplate washer (BioTek). Fifty microliters of fish test serum (either straight or diluted 1:2) was added to the wells containing VHSV antigen and mock-infected antigen and incubated for 30 min at 37°C. Directly after incubation (without washing or removal of test sera), 50 μl of the HRP-conjugated monoclonal antibody, diluted 1:5,000 in StartingBlock (PBS) blocking buffer, was added to the wells and incubated with the test sera for an additional 90 min at 37°C. The plates were then washed 3 times with phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 (Sigma) to remove unbound antibodies. PBS was made by diluting 18.46 g of FTA hemagglutination buffer (BD, Chicago, IL) in 2 liters of deionized water. SureBlue 3,3′,5,5′-tetramethylbenzidine (TMB) 1-component microwell peroxidase substrate (KPL, Gaithersburg, MD) was used as an enzyme substrate and chromogen for development of the assay. One hundred microliters of enzyme substrate was added to each well, and the assay was developed for 15 min at 37°C. The reaction was terminated by adding 100 μl of 1% HCl TMB Stop Solution (KPL, Gaithersburg, MD) per well and the optical density (OD) at 450 nm was measured in an ELx808 absorbance microplate reader (BioTek). Multiple modified checkerboard experiments were performed to determine the optimal working dilution for the HRP-conjugated monoclonal antibody and the antigen concentrations for the coating plates.

The serum samples were tested both undiluted and at a 1:2 dilution in PBS wash solution. All OD readings for the samples and controls were adjusted by subtracting the background OD levels in the mock-infected wells. The percent inhibition (%I) was calculated using the formula $\%I = 100 - (100 \times \text{sample}_{OD}/\text{negative control}_{OD})$.

The presence of blue color after incubation with enzyme substrate indicated an absence of anti-nucleocapsid antibodies in a well. A higher concentration of anti-VHSV nucleocapsid serum antibodies in a well resulted in the absence of blue color and therefore higher percent inhibition of the mouse anti-VHSV nucleocapsid monoclonal binding to the VHSV antigen.

ROC analysis. A receiver operating characteristic (ROC) analysis was performed over a range of possible percent inhibition cutoff points for the ELISA (42). The thresholds were based on the percent inhibition values for the infected and uninfected fish.

Viral RNA analysis of serum by real-time RT-PCR. Two published real-time RT-PCR assays were used to detect viral RNA. At the Great Lakes Water Institute, University of Wisconsin (UW)-Milwaukee, RNA was extracted, and real-time PCR (21, 43) was performed on all yellow perch (Table 1). At UW-Madison, RNA was extracted, and real-time RT-PCR was performed (31) on the remaining fish. Any samples crossing the cycle threshold before cycle 40 were considered positive.

RESULTS

Prevalence of neutralizing antibodies in control serum by VN assay. The VN assay was modified from a previously described protocol (27). The results from the pilot studies (not shown) indicated that 24-h incubation of the virus and serum prior to inoculation onto the cells produced more significant neutralization of the virus versus a 30-min or 1-h incubation described in previously developed plaque neutralization test protocols for VHSV I and IVb (24, 44). The pilot studies also showed considerable toxicity at dilutions of 1:2 to 1:8. Thus, an initial dilution of 1:16 was used. Serum samples showing partial or complete protection were considered positive and were designated p1:16 (partial) or 1:16 (complete). If no protection was observed at 1:16, a serum sample was considered to be negative.

Neutralizing antibody titers were not detected in any of the serum samples from fish in the VHS-negative group ($n = 34$). Low VHSV-neutralizing titers were detected in 43% (12/28) of the fish from the VHS-positive group (Table 2), with titers ranging from p1:16 to 1:80. Thus, the VN assay had a specificity of 100% (95% confidence interval, 89.6% to 100%) and a sensitivity of 42.9% (95% confidence interval, 24.5% to 62.8%).

The addition of naive brown trout serum as complement was evaluated in our VN assay and was found to have no effect on neutralization (data not shown). A methylcellulose overlay was also evaluated for the isolation of plaques but was not necessary in reading the last serum dilution showing protection of the monolayer for determining the neutralizing antibody titer in the VN assay (data not shown). Three antibody-positive controls and one antibody-negative control were used to compare results with and without the addition of overlay, and no difference in antibody titer was observed.

Cross-reactivity of anti-VHSV nucleocapsid monoclonal antibody with SVCV. A Western blot under reduced conditions showed staining only with the nucleocapsid protein of VHSV using the anti-VHSV nucleocapsid monoclonal antibody, showing the specificity of the antibody to this protein (results not shown). No staining occurred with the SVCV lysate in a Western blot using the anti-VHSV nucleocapsid monoclonal antibody, indicating no cross-reactivity between our monoclonal detection antibody and SVCV. Specifically, these results show that there is no cross-recognition between the linear epitopes of the N proteins of VHSV and SVCV.

Analysis of anti-VHSV nucleocapsid monoclonal antibody in ELISA. A blocking ELISA is well suited for testing diagnostic samples from wildlife species because a secondary antibody is not required. At the time of assay development, no effective monoclonal antibody against the VHSV glycoprotein was available commercially. The anti-VHSV nucleocapsid monoclonal antibody used in this study was commercially available and effective. The anti-VHSV nucleocapsid monoclonal antibody has an advantage in that it detects persistent antibodies directed against the nucleocapsid. ELISA plates coated with intact viral particles revealed incomplete blocking. Treating the virus with MEGA-10 detergent prior to coating the plates was a critical step to allow for accurate identification of infected and noninfected fish. Presumably, this treatment reveals the target epitope of the nucleocapsid protein and allows the binding of the anti-nucleocapsid monoclonal antibody (45–47).

The efficacy of the anti-VHSV nucleocapsid monoclonal anti-

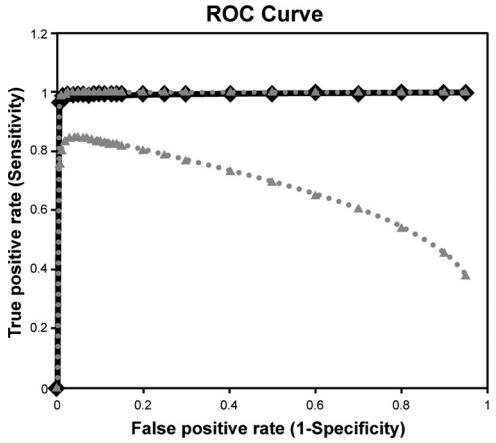

FIG 1 Receiver operating characteristic (ROC) curve of blocking ELISA using 1:2 diluted serum from VHSV-infected fish ($n = 28$) and uninfected fish ($n = 34$). The dashed lines indicate upper and lower 95% confidence intervals for the true-positive fraction, and the solid line indicates the curve for the true-positive rate.

body was evaluated by testing serum samples from the 34 uninfected and 28 previously infected fish. The serum samples were tested both undiluted and at a 1:2 dilution. The serum samples were tested at a 1:2 dilution to eliminate high background issues with hemolyzed serum.

Thirty of 34 serum samples (88.2%) from the VHS-negative group fish diluted 1:2 were negative by ELISA (Table 1). Twenty-seven of the 28 serum samples (96.4%) from the VHS-positive group fish diluted 1:2 were positive by ELISA (Table 2).

ROC analysis for ELISA. A receiver operating characteristic (ROC) curve was performed to derive the optimal percent inhibition threshold for detecting VHSV antibodies in fish serum (Fig. 1). Undiluted samples were considered positive at 35 to 100% inhibition and negative at <35% inhibition. The samples tested at a 1:2 dilution were considered positive at 25 to 100% inhibition and negative <25%, based on the ROC analysis. The area under the ROC curve was 0.994, confirming that the ELISA diagnostic performance characteristics under these thresholds were well correlated with the true status of each serum sample. These cutoff values demonstrated 88.2% specificity (95% confidence interval, 72.5% to 96.6%) and 96.4% sensitivity (95% confidence interval, 81.6% to 99.4%) with serum diluted 1:2. The percent inhibition values from uninfected fish ranged from 0.24% to 41.1% (average, 15.66%) and 7.94% to 95.99% (average, 49.21%) for previously infected fish. The positive predictive value of the ELISA for experimentally infected fish is 87.1% (95% confidence interval, 70.2% to 96.3%) and the negative predictive value is 96.8% (95% confidence interval, 83.2% to 99.5%) (Table 3 and 4).

Viral RNA detection by real-time RT-PCR. Serum samples were tested for VHSV by RT-PCR to determine if there was viral infection at the time blood was collected for fish exposed to VHSV and to determine if the inhibition of antibody binding was occurring in our tests due to antibodies being complexed with virus in the serum.

VHSV RNA was not detected by real-time RT-PCR in sera from uninfected fish. Sera from yellow perch H1-14, J1-13/J3-11, H4 A, H4 B, H4 C, and J4 A were positive, with threshold cycle (C_T) values ranging from 32.6 to 39.5 (Table 2). Serum from grass carp 2 was positive, with a C_T of 39.5, and that from Pacific herring 149 was positive, with a C_T of 38.9. All other sera from fish in the VHS-positive group tested negative for viral RNA.

TABLE 3 Results of blocking ELISA[a]

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	27	4
Negative	1	30

[a] $n = 62$. The sensitivity is 96.4% and the specificity is 88.2%, both calculated from fish in the VHS-negative and VHS-positive groups.

TABLE 4 Results of VN assay[a]

VHS infection status	No. of known positives ($n = 28$)	No. of known negatives ($n = 34$)
Positive	12	0
Negative	16	34

[a] $n = 62$. The sensitivity is 42.9% and the specificity is 100%, both calculated from fish in the VHS-negative and VHS-positive groups.

DISCUSSION

We successfully developed a virus neutralization assay and a blocking ELISA to detect neutralizing and nonneutralizing antibodies against VHSV, respectively. The VN assay has the advantage of recognizing antibodies that likely confer protective immunity to VHSV and can indicate recent exposure to the virus (25). The blocking ELISA is valuable for identifying nonneutralizing anti-nucleocapsid antibodies, which may persist longer and therefore extend the opportunity to detect VHSV antibodies after initial virus exposure (25, 48). These assays complement viral detection methods by providing a means for determining the exposure histories of wild populations.

Previous studies have described the use of complement-dependent 50% plaque neutralization tests and indirect ELISAs to detect VHSV antibodies in trout (24, 25). These methods are reliable but not practical for screening large populations of fish from multiple species. There are currently no commercially available diagnostic tests in the United States for detecting antibodies to VHSV. Surveillance efforts by virus isolation are labor-intensive, must occur within narrow water temperature windows, and are costly. Although real-time PCR assays are available, these methods generally need to be performed in high-throughput laboratories for large sample sizes. Serological assays, such as our ELISA and VN assay, provide efficient and less costly methods for evaluating the VHSV exposure histories of samples from large wild fish populations or waterbodies.

Our VN assay is different from the traditional 50% plaque neutralization test (24, 44, 49) in that it is performed in a microneutralization format and the antibody titers are read as the last serum dilution showing complete protection of the cell monolayer from VHSV. Additionally, we determined that methylcellulose overlay is not necessary in our VN assay because plaques are not counted to determine the titers. Complement has been shown to enhance neutralization in 50% PNTs when applied to trout serum (23, 48, 50). However, neutralization was not enhanced by the addition of complement in our assay, which may indicate the presence of a different immune mechanism specific to trout. It should be noted that the reduced sensitivity observed in our VN

assay may be due to VHSV forming a complex with neutralizing antibodies in the serum, which reduces the availability of antibodies for binding to virus neutralization epitopes in the VN assay (45–48). However, this concern is obviated by using the nucleocapsid protein as our target, because antibodies with nucleocapsid affinity presumably do not complex with the intact viral particle, which underscores another value of the anti-nucleocapsid ELISA. It should be noted that four of the eight sera (50%) from our VHS-infected group tested positive for viral RNA but negative in the VN assay. This emphasizes the importance of utilizing parallel assays when testing the virus exposure history of fish.

Although previous experiments have determined that homologous strains of VHSV must be used for neutralization epitopes to be recognized (51, 52), serum samples from five Pacific herring hyperimmunized with VHSV type IVa were able to neutralize type IVb virus in the VN assay at low titers of p1:16 to 1:32. There are 21 amino acid differences between the type IVa and IVb glycoprotein sequences. None of these differences occur in two of the identified glycoprotein-neutralizing epitopes. This suggests there are shared neutralizing epitopes between types IVa and IVb (53, 54). Type IVa glycoprotein epitopes may be similar enough to those of type IVb to react in our VN assay. Indeed, a similar phenomenon was noted when Pacific herring vaccinated with the glycoprotein gene isolated from VHSV type Ia were protected from VHSV type IVb (55). Further investigation is needed to determine whether our VN assay may detect different antibody titers in herring exposed to virus that is homologous to that used in the VN assay. Additionally, although we demonstrate the ability to detect antibodies in hyperimmunized Pacific herring that likely experienced artificially high antibody titers, further investigations are needed to determine the sensitivities of these assays in wild Pacific herring or in those surviving more realistic VHSV exposure histories.

Our new blocking ELISA is a suitable nonlethal method for detecting exposure to VHSV. Considering the broad host range of VHSV type IVb (11), the advantage of a species-independent ELISA is significant for the surveillance of VHSV. The assay can measure the concentrations of antibodies directed against the nucleocapsid in any freshwater species since it does not require a secondary antibody. Furthermore, the ability of the monoclonal antibody to bind to a single viral epitope results in high specificity. We demonstrated by Western blotting that the nucleocapsid monoclonal antibody binding was specific to VHSV versus SVCV, another rhabdovirus that is present in Wisconsin. Previous studies showed a lack of cross-reaction between the antibody used herein and spring viremia of carp virus, infectious hematopoietic necrosis virus, pike fry rhabdovirus, or rhabdovirus anguilla (26, 56). According to the manufacturer of the antibody (Aquatic Diagnostics, Stirling, Scotland), no cross-reaction of the antibody occurs with nodavirus, infectious salmon anemia virus, koi herpesvirus, salmon alphavirus (1, 2, and 3), or *Piscirickettsia salmonis* infected cells. We were not able to test positive sera from transboundary VHSV strains (type I, II, and III); however, previous efforts indicate the anti-nucleocapsid monoclonal antibody detects anti-nucleocapsid antibodies against all strains of VHSV (26).

A feature crucial to the function of our ELISA is the treatment of the viral antigen with MEGA-10 detergent prior to coating the plates (47). Repeated trials showed that treatment of the virus with detergent allowed for better attachment of the nucleocapsid-specific antibody to the virus. This result is probably due to the ability of the detergent to expose the nucleocapsid epitope and make it available for antibody binding.

The large difference in sensitivities between our blocking ELISA (96.4%) and VN assay (42.9%) may be attributed to the immune response kinetics at the time of serum collection. Studies have shown that neutralizing antibodies do not persist as long as nonneutralizing antibodies in trout (25, 48), and neutralizing antibody titers peak at 6 weeks postinfection in rainbow trout infected with VHSV I (24) and at 11 to 16 weeks in muskellunge infected with VHSV IVb (44). It was observed that the majority of VHSV-exposed fish with serum samples collected prior to 6 weeks postinfection had no or low neutralizing antibody titers. Those with low titers may have still been clearing virus while producing protective antibodies, indicated by the presence of viral RNA in the serum sample of a portion of our VHSV-exposed fish. Investigation into the kinetics of viral replication and the related immune response in multiple species are therefore important for further study.

A limitation of this study is the number of serum samples available from VHSV-uninfected and -infected fish. We used serum samples from 27 experimentally infected and one wild-caught fish that had VHSV-positive kidney and spleen tissues as tested by real-time RT-PCR for ELISA development. It is also important to note that our threshold for detecting VHSV antibodies by ELISA may require adjustment when evaluating various wild-caught species due to differing environments and susceptibility. In this light, we note that 4 of 34 serum samples diluted 1:2 from the VHS-negative group were positive on the blocking ELISA. There may be nonspecific reactions occurring that more extensive testing would help reconcile. For the purpose of this assay as a surveillance tool, it is more practical to keep a threshold at a level that maximizes sensitivity.

In summary, the blocking ELISA shows high sensitivity and acceptable specificity, whereas the VN assay has unacceptably low sensitivity but high specificity. When used in parallel, the VN assay and ELISAs correctly identified the VHSV exposure status of all known uninfected and infected fish. Our results highlight that the anti-VHSV nucleocapsid monoclonal antibody used in the blocking ELISA is a good indicator of past exposure to VHSV and may be a reliable time-independent and species-independent diagnostic test suitable for nonlethal surveillance of VHSV. Our nonlethal serological assays will be valuable for assessing VHSV exposure history and might reduce the extensive laboratory effort needed to screen fish for VHSV using virus isolation. Use of the VN assay, blocking ELISA, and virus isolation under actual surveillance conditions is needed to fully demonstrate the interplay between the assays. The collection of additional reference samples is required for continued assay validation to further assess the sensitivity and specificity and determine repeatability, robustness, and ruggedness. Our serological assays might supplement existing VHS surveillance protocols, which might have regulatory implications for fish movement between VHSV-positive and -negative locations in certain jurisdictions or geographic regions.

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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

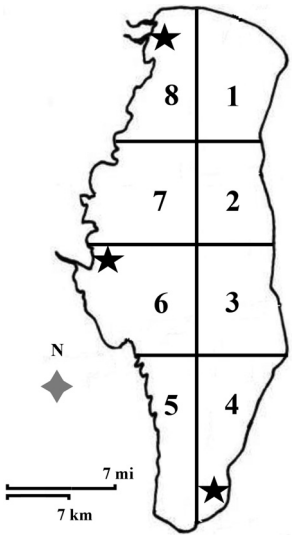

FIG 1 The eight sampling locations in Lake Winnebago, Wisconsin. The stars indicate previous VHSV isolation sites determined by the Wisconsin DNR during prior surveillance efforts.

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-

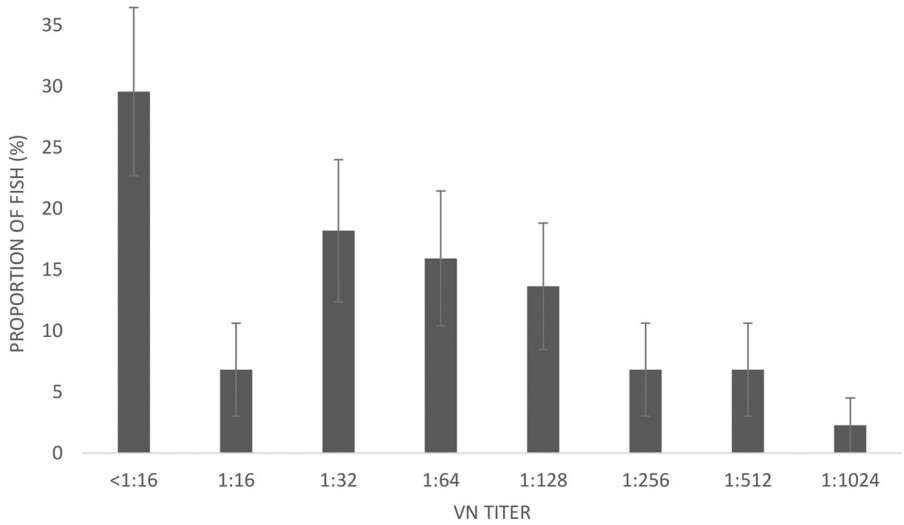

FIG 2 Distribution of neutralizing antibody titers ($n = 44$) against viral hemorrhagic septicemia virus in freshwater drum from Lake Winnebago, Wisconsin. The error bars indicate ±1 standard error of the mean.

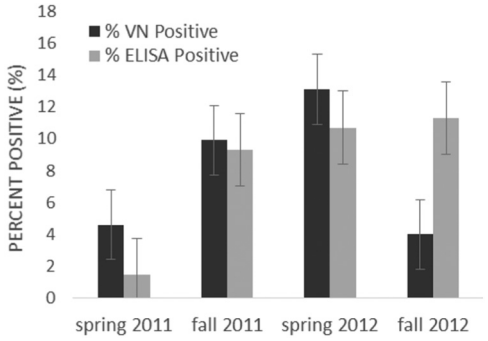

FIG 3 Seroprevalence of freshwater drum from Lake Winnebago, Wisconsin, for viral hemorrhagic septicemia virus in spring 2011 ($n = 130$), fall 2011 ($n = 172$), spring 2012 ($n = 122$), and fall 2012 ($n = 125$). The error bars indicate ±1 standard error of the mean.

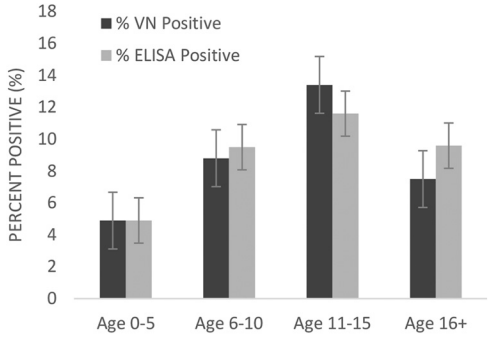

FIG 4 Seroprevalence of freshwater drum from Lake Winnebago, Wisconsin, for viral hemorrhagic septicemia virus by age class (0 to 5 [$n = 205$], 6 to 10 [$n = 137$], 11 to 15 [$n = 112$], and 16 to 28 [$n = 94$] years), based on VN (neutralizing antibodies) and ELISA (nonneutralizing antibodies). The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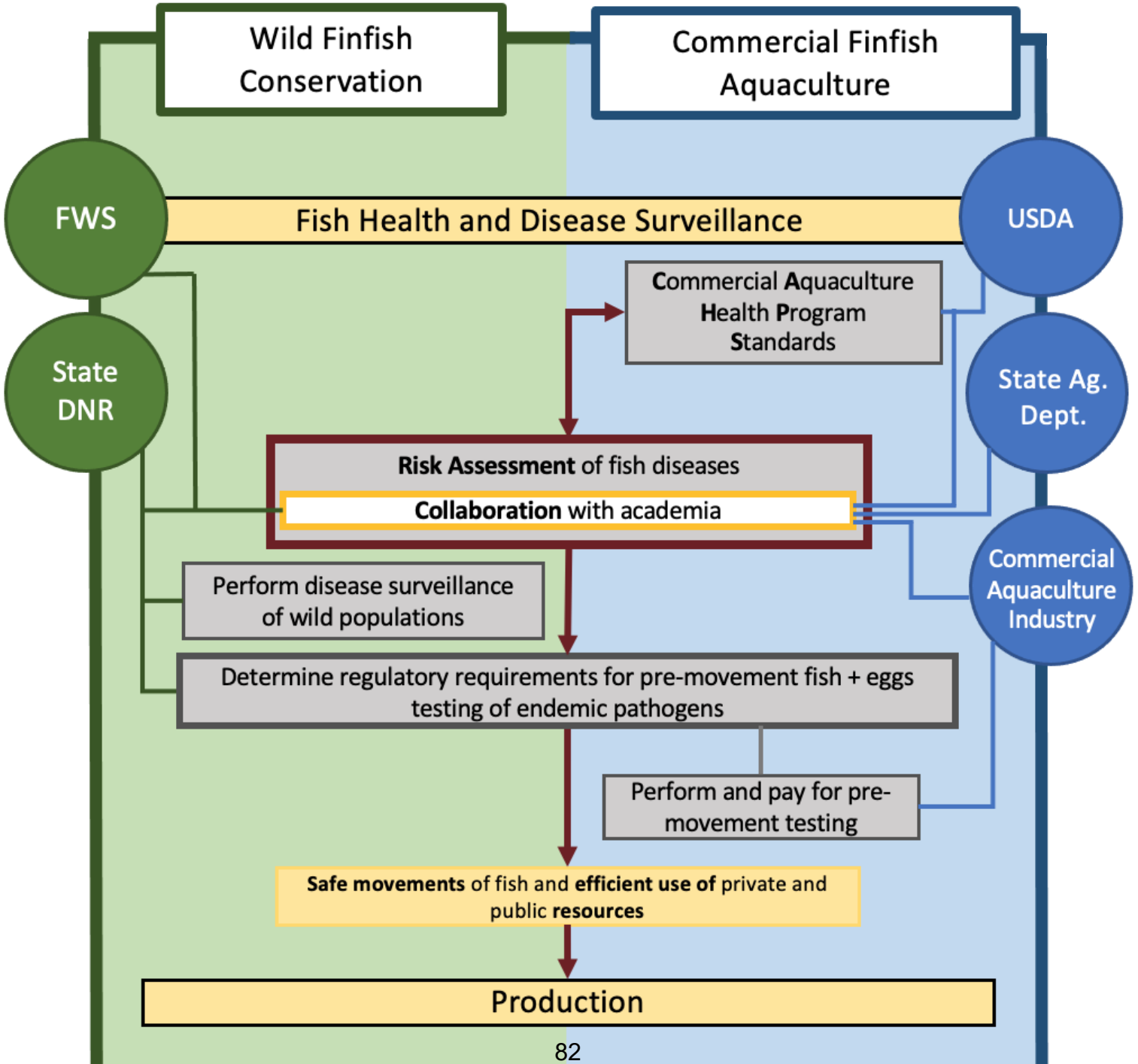
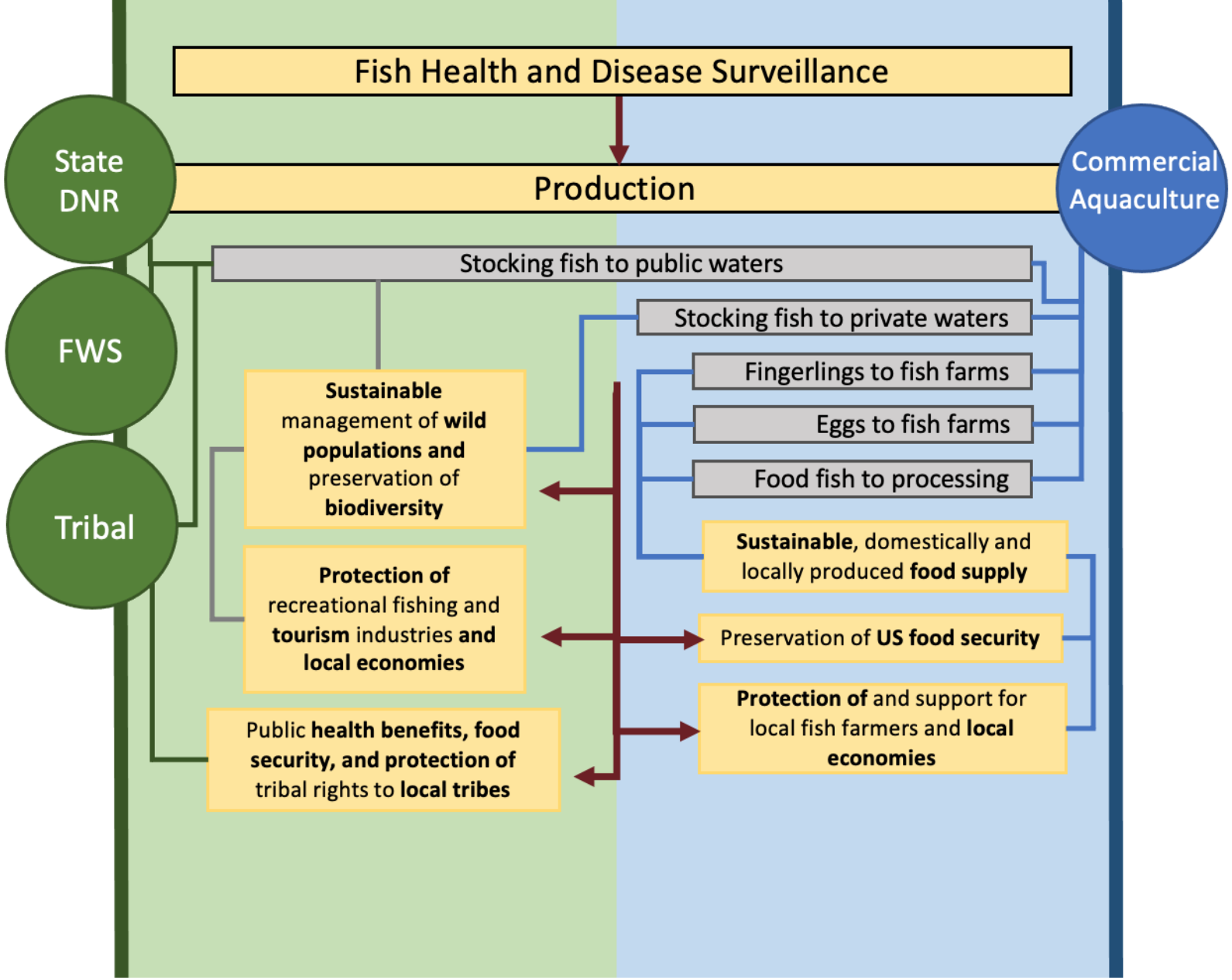
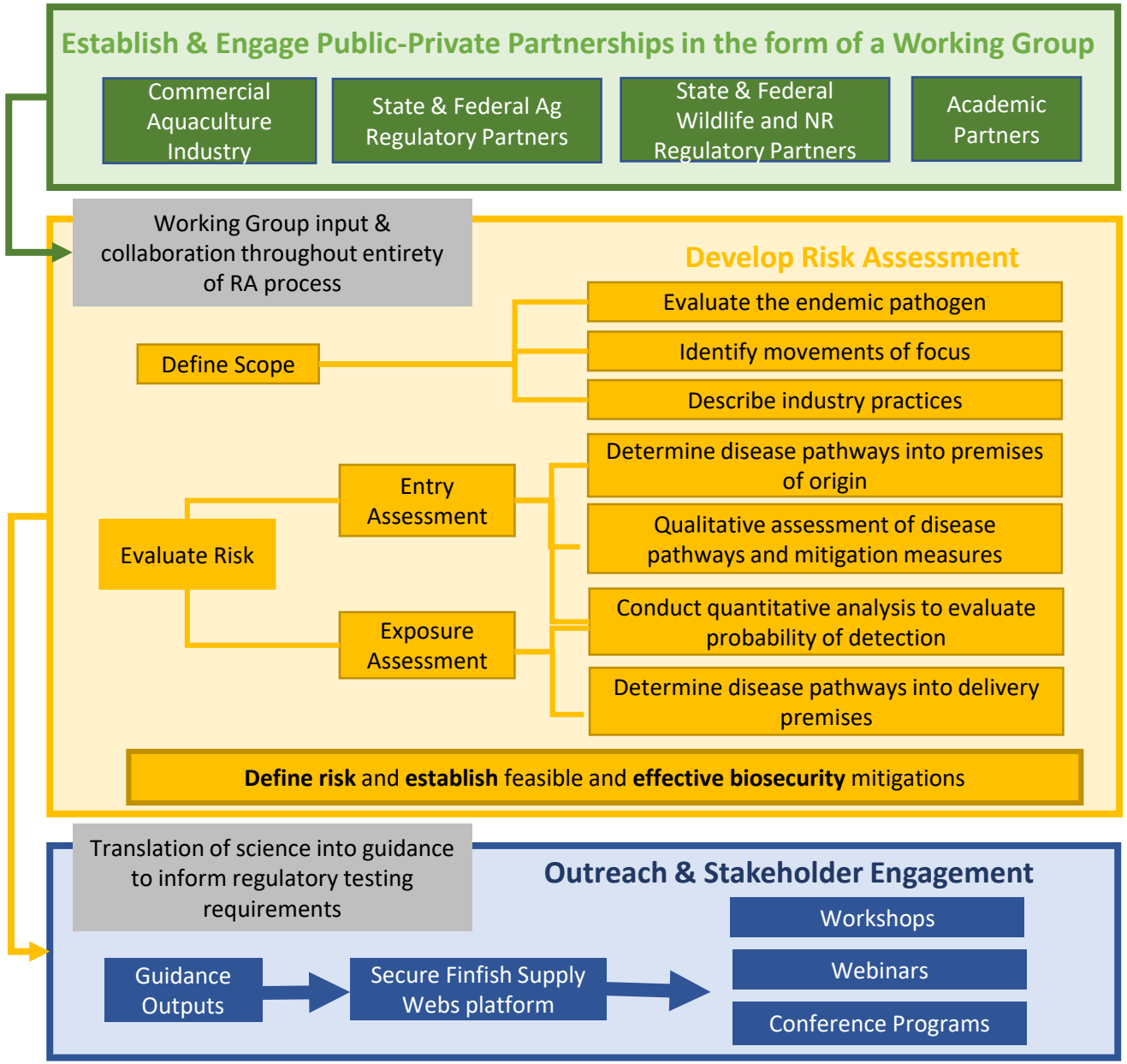

Wild Finfish Conservation
Commercial Finfish Aquaculture
FWS
Fish Health and Disease Surveillance
USDA
Commercial Aquaculture Health Program Standards
State DNR
State Ag. Dept.
Risk Assessment of fish diseases
Collaboration with academia
Commercial Aquaculture Industry
Perform disease surveillance of wild populations
Determine regulatory requirements for pre-movement fish + eggs testing of endemic pathogens
Perform and pay for pre-movement testing
Safe movements of fish and efficient use of private and public resources
Production

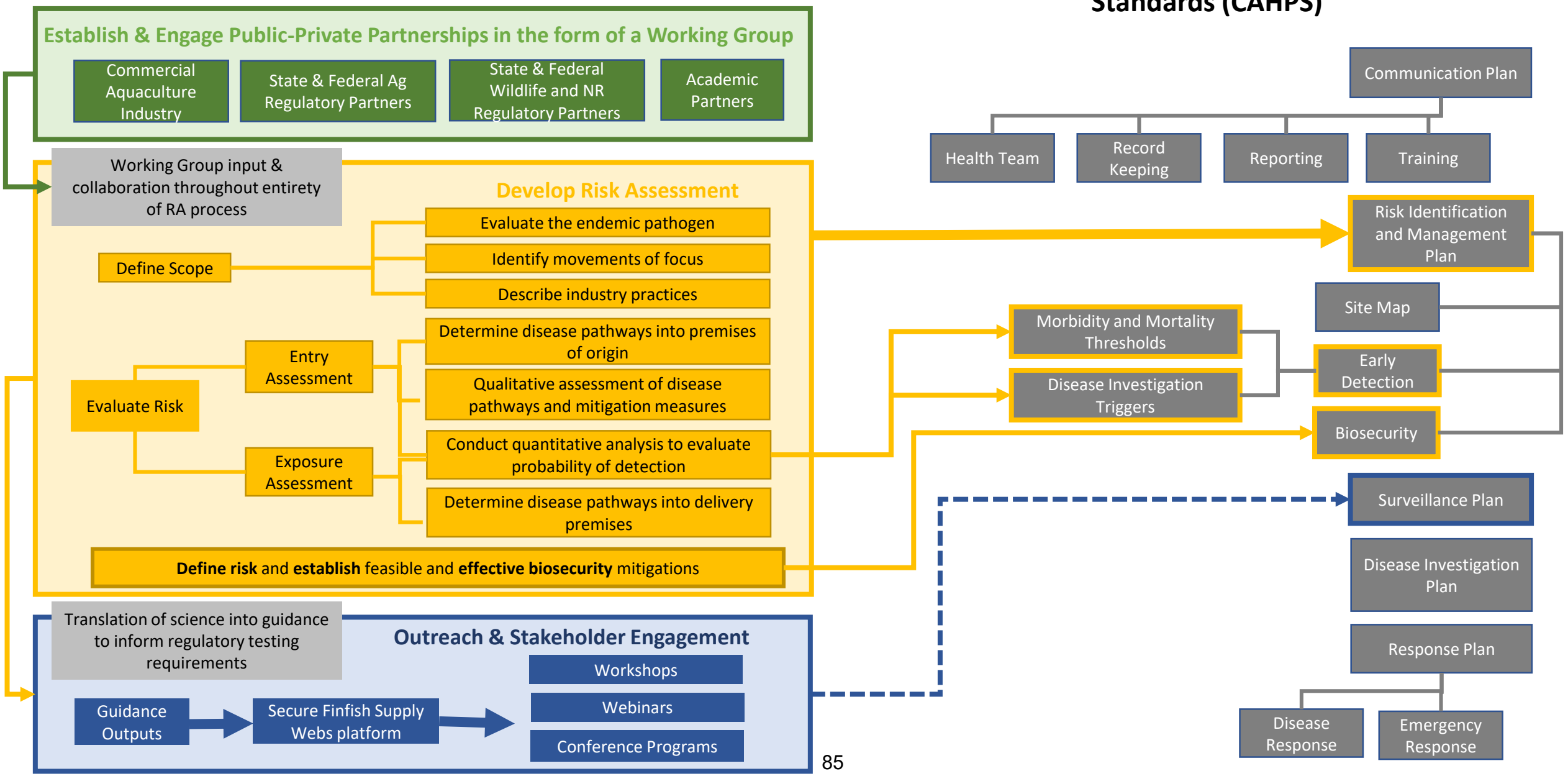

Fish Health and Disease Surveillance
State DNR
FWS
Tribal
Production
Commercial Aquaculture
Stocking fish to public waters
Stocking fish to private waters
Fingerlings to fish farms
Eggs to fish farms
Food fish to processing
Sustainable management of wild populations and preservation of biodiversity
Protection of recreational fishing and tourism industries and local economies
Public health benefits, food security, and protection of tribal rights to local tribes
Sustainable, domestically and locally produced food supply
Preservation of US food security
Protection of and support for local fish farmers and local economies

Secure Finfish Supply – Risk Assessment Approach



Secure Finfish Supply – Risk Assessment Approach

National USDA Fish Health Initiative: Commercial Aquaculture Health Program Standards (CAHPS)

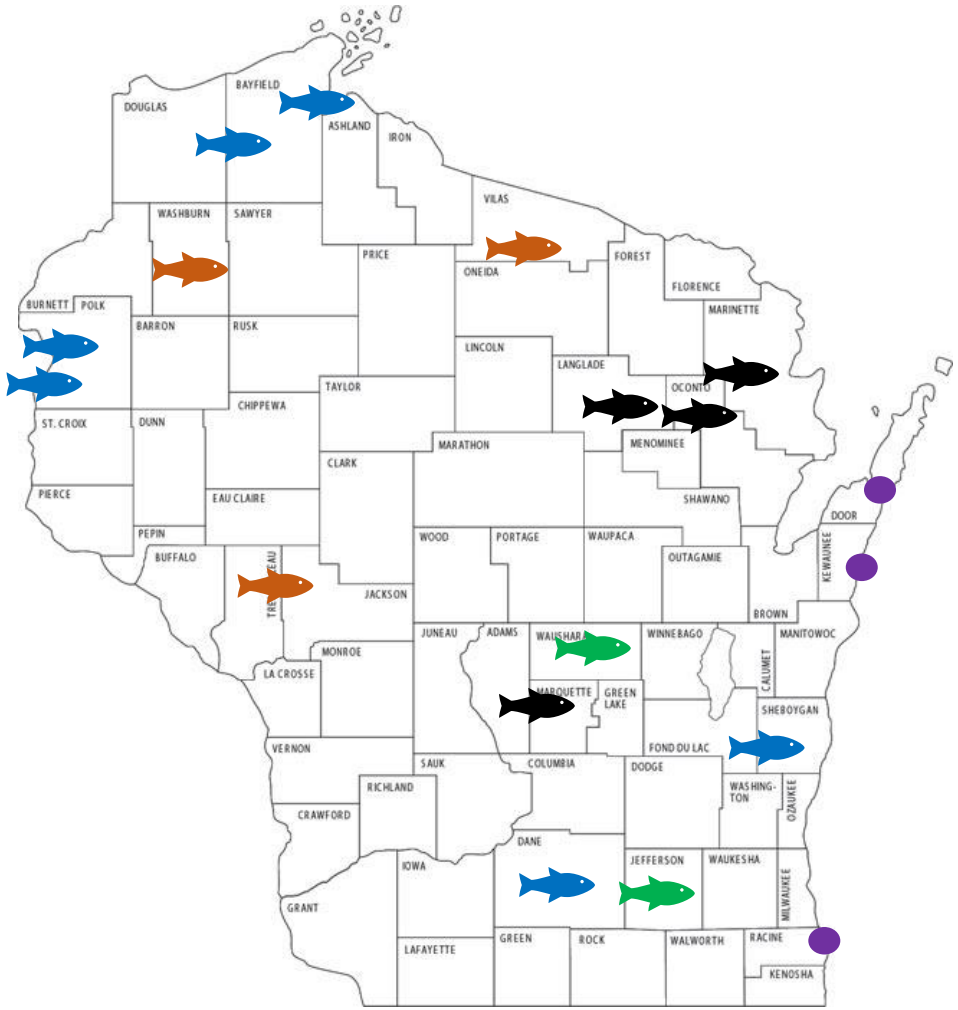


Wisconsin Stocking Program

Jesse Landwehr – Great Lakes Fish Heath Committee

08/04/2021

WDNR Fish Rearing Facilities



2019 Stocking Summary

DNR Production

- 2,767,000 Walleye
- 1,064,000 Brown Trout
- 828,000 Chinook Salmon
- 689,000 Rainbow Trout
- 359,000 Coho Salmon
- 327,000 Brook Trout
- 72,000 Largemouth Bass
- 334,000 Northern Pike
- 190,000 Lake Trout
- 62,000 Musky
- 66,000 Splake
- 59,000 Lake Sturgeon

~6.8 Million

Co-Op Stocking

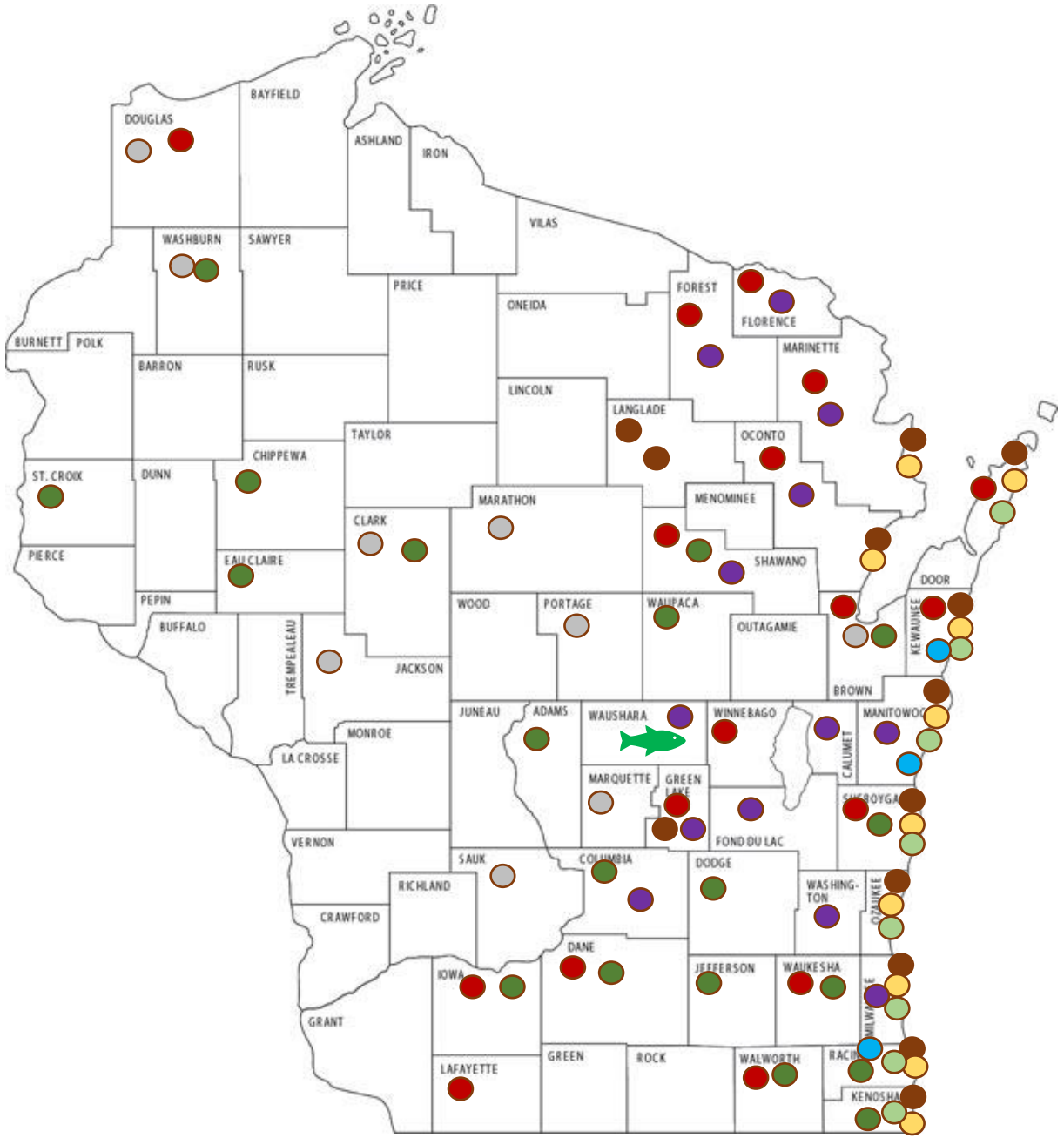
- 74,000 Brook Trout
- 30,000 Lake Trout
- 38,000 Brown Trout
- 8,000 Walleye

~150,000

Total Stocking = ~7 Million Fish

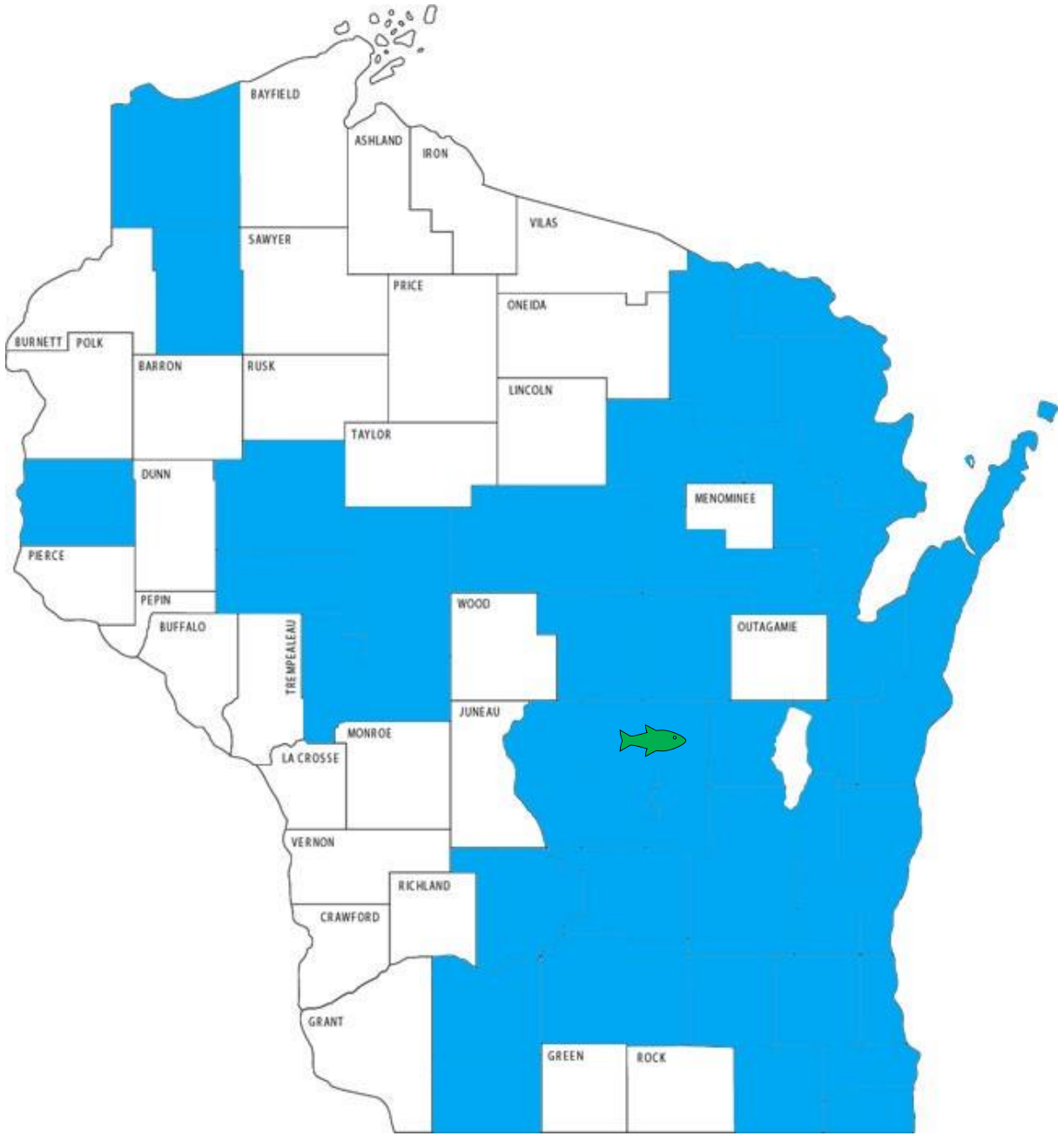
2021-2022 Wild Rose Production Plan

- 470,000 Brown Trout in 12 counties
- 1.2 Million Chinook Salmon in 10 counties
- 500,000 Coho Salmon in 8 Counties
- 32,000 Lake Sturgeon in 9 counties*
- 55,000 Musky in 17 Counties
- 194,000 Northern Pike in 19 Counties
- 108,000 Steelhead in 3 counties.
- 138,000 Walleye in 13 Counties



2021-2022 Wild Rose Production Plan

- 470,000 Brown Trout in 12 counties
- 1.2 Million Chinook Salmon in 10 counties
- 500,000 Coho Salmon in 8 Counties
- 32,000 Lake Sturgeon in 9 counties*
- 55,000 Musky in 17 Counties
- 194,000 Northern Pike in 19 Counties
- 108,000 Steelhead in 3 counties.
- 138,000 Walleye in 13 Counties



Grand total of almost 2.7 Million fish into 42 of the 72 counties if Wisconsin

CONNECT WITH US

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