Elucidating the role of herd immunity in protecting Lake Michigan fish against the Viral Hemorrhagic Septicemia Virus (VHSV)

Project No. 2012.1257
Great Lakes Fishery Trust

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Project No. 2012.1257: Elucidating the role of herd immunity in protecting Lake Michigan fish against the Viral Hemorrhagic Septicemia Virus (VHSV). Mohamed Faisal & Travis O. Brenden. Michigan State University, East Lansing, Michigan.

1) PROJECT ABSTRACT

A novel genotype (IVb) of the highly pathogenic fish virus, viral hemorrhagic septicemia virus (VHSV), emerged in the Great Lakes basin more than a decade ago. Over time, VHSV-IVb has spread throughout the basin, evidenced by mass mortality events involving numerous fish species. With a common goal of learning more about this emerging freshwater genotype, researchers and managers united to investigate pathogenicity, host range, and the comparative susceptibility of Great Lakes fishes. However, there remains a lack of knowledge on the host immune response against VHSV-IVb and no approved preventative measures against this virus exist. This study seeks to address these knowledge gaps and investigate protective measures for eliciting immunity against VHSV-IVb. The study focused primarily on muskellunge (*Esox masquinongy*) due to its high susceptibility and vigorous immune response following VHSV-IVb exposure. Muskellunge were used to elucidate the role of the humoral immune response against VHSV-IVb, First, a monoclonal antibody (mAb), designated 3B10, against muskellunge immunoglobulin (Ig) was developed. The 3B10 mAb allowed for the creation of a muskellunge-specific indirect ELISA to detect anti-VHSV-IVb antibodies. The indirect-ELISA was employed in both a serosurveillance capacity to determine previous viral exposure and to study the humoral immune response following immunization. Following immunization with a DNA plasmid containing the VHSV-IVb glycoprotein (G) gene, muskellunge anti-VHSV binding antibody levels peaked after approximately seven weeks. Knowing that the VHSV-IVb G gene can indeed elicit antibody production, two plasmids, differing only in their promoter sequence, were compared in their ability to elicit protection against VHSV-IVb. One preparation (designated pVHSivb-G) conferred significant protection in muskellunge, resulting in 95% mean relative percent survival (RPS) following a single intramuscular administration, while the other (designated pβ-VHSivb-G) conferred less than 25% mean RPS. Building from these results, we showed that the pVHSivb-G preparation could also elicit significant protection in three salmonid species: rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*). Following these successes with the pVHSivb-G preparation, we continued our work to develop a eukaryotic recombinant vaccine preparation. We were able to express and purify a eukaryotic VHSV-IVb glycoprotein in cabbage looper (*Trichoplusia ni*) larvae using a recombinant baculovirus. The culmination of the studies used the successful vaccination regimen and assays that we developed to investigate whether immunized fish can provide indirect protection to naïve fish. To accomplish this, we designed a flow-through system utilizing viral shedding as the viral exposure source. Co-mingled immunized muskellunge indeed conferred indirect protection to naïve muskellunge, resulting in a decrease in mean mortality from 80.2% to 36.5% when compared to naïve muskellunge housed alone. No protective effect was observed when naïve muskellunge were housed with Chinook salmon (*Oncorhynchus tshawytscha*), a semi-resistant species, indicating that immunity rather than resistance is important in this protective effect. A spatially-explicit individual-based model using Lake Michigan Chinook salmon dynamics and a disease with characteristics similar to VHSV-IVb was used to explore the efficacy of a vaccination program designed around the potential immunization and release of hatchery-propagated individuals in protecting wild fish populations.

Factors that were examined included level of clustering among individuals, infection probability rate, relationship between disease exposure and mortality, number of vaccinated individuals, and whether recovered individuals could resume viral shedding. At a stocking level of 2.4 million fish, vaccination decreased the average infection rate over the last 10 years of the simulations by 28% to 65%, depending on the factors evaluated. Doubling the stocking level resulted in similar protection levels. The largest decrease in infection rate occurred under a condition of low infection, high mortality rate, and high degree of clustering. In conclusion, we have achieved remarkable success in developing and testing an effective vaccine for Great Lakes fish species against the emerging VHSV sublineage. Experimental data and simulation models demonstrated that hatchery fish can be used to confer protection to wild fish populations against this deadly virus.

2) A Narrative Response to GLFT Final Report Questions

Research Final Report
GLFT #2012.1257

PROJECT TITLE:
Elucidating the role of herd immunity in protecting Lake Michigan fish against the Viral Hemorrhagic Septicemia Virus (VHSV)

GRANTEE ORGANIZATION: Michigan State University

PROJECT TEAM:
PI Dr. Mohamed Faisal. Co-PI Dr. Travis Brenden. Drs. Isaac F. Standish and Lori Ivan researchers.

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KEY SEARCH WORDS: VHSV, vaccine, herd immunity, recombinant, Great Lakes fish, individual-based modeling

BACKGROUND/OVERVIEW:

The Great Lakes basin (GLB) hosts a suite of unique resources found nowhere else in the world. GLB fisheries, however, suffer from several recently emerged diseases. One such pathogen is the Viral Hemorrhagic Septicemia Virus (VHSV). This serious fish pathogen has impacted fishery resources as it has spread across the five Great Lakes. The virion of this novirhabdovirus (Family Rhabdoviridae) has a bullet-shaped capsid encased in an envelope. VHSV has a single stranded, negative-sense ribonucleic acid (RNA) genome comprised of 11,184 nucleotides, and contains six open reading frames in the order 3'-N-P-M- G-NV-L-5' (Schütze et al. 1999; Ammayappan & Vakharia 2009). Among these, the G-gene encodes the 72-80 kDa major surface glycoprotein antigen located on the envelope surface and is important for virus attachment (Wagner & Rose 1996). Studies have demonstrated that of all VHSV proteins, the G protein is the major target of cellular and humoral responses in infected fish. The elegant studies of Boudinot et al. (2004) and Utke et al. (2007) provided evidence that fish cytotoxic leukocytes recognize infected cells through the VHSV G-proteins expressed on their surfaces and lyse them. Also, fish humoral antibody responses are directed toward the VHSV G-proteins, thereby preventing the virus from infecting other cells (Lorenzen et al. 1990).

In the early 2000s, VHSV appeared in the Laurentian Great Lakes as a new sublineage of the North American genotype IV (designated IVb), infecting and causing mortality events in a wide range of freshwater fish species. Increased awareness of the virus prompted management and research agencies to increase their efforts to better understand the emerging virus. Specifically,

the Great Lakes Fishery Trust (GLFT) funded a study investigating the biology of the virus, disease course, and host range. Findings of this study paved the way to better understand VHSV-IVb characteristics. It was found that not all Great Lakes fish species are equally susceptible to VHSV-IVb, with muskellunge (*Esox masquinongy*) and largemouth bass (*Micropterus salmoides*) being among the more susceptible species and salmonids being of lesser susceptibility. In the muskellunge, VHSV-IVb disease course can run in one of three forms: acute, subacute, or chronic, depending on the virus dose at exposure (Kim & Faisal 2010a,c 2012). The acute form of the disease progresses quickly and results in high mortalities. Chronically infected fish experience a prolonged disease and less mortality and often result in fish shedding the virus into the surrounding environment for a prolonged period of time. Fish that survived VHSV-IVb produced antibodies that neutralize virus infectivity. Further, we found that VHSV-IVb surviving fish developed a resistance against re-infection, a matter that corresponded well with the levels of circulating, specific VHSV-IVb antibodies. To this end, the aim of this project was to initiate the development of VHSV-antibodies in susceptible fish species in the Great Lakes, including the salmonid fish species which form the backbone of the prosperous sport fishery industry in the basin. In the absence of effective chemotherapy against VHSV, adaptive immunity seems to offer the only alternative to control this deadly disease. **Therefore, our project sought to develop an efficacious VHSV-vaccine to mitigate the potential impacts of this virus on Great Lakes fisheries.**

Human and veterinary medical practices have repeatedly demonstrated that the spread of contagious diseases can be disrupted when a number (not all) of individuals in a population are immune against a particular disease, a phenomenon known as herd immunity. Herd immunity is said to be achieved when a certain proportion of the population is immune to a pathogen, thereby reducing the transmission of the pathogen within the population. The proportion of immune animals in the population is known as the herd immunity threshold (HIT). Studies have shown that the HIT is variable for different diseases, and in part depends on the biology and ecology of infected populations. As a result, determining the HIT can be both difficult and costly. One way to reduce the costs associated in determining the HIT is to construct models that account for factors that influence long-term dynamics of infectious diseases, namely the properties of the virus, the life history traits and dynamics of the infected populations, and the spatial structure of the infected population. Different immunization strategies can then be incorporated in the model to provide insight into how various strategies perform in conferring protection to the population.

In the Laurentian Great Lakes, fishery conservation efforts are based upon propagating healthy fingerlings in state and federal fish hatcheries to a certain age and then stocking them throughout the GLB. For example, Lake Michigan is stocked with more than nine fish species from several agencies. Our study was meant to examine the potentials of using hatchery-propagated fish, following immunization against VHSV-IVb, in protecting susceptible fish species through herd immunity. The costs of eliciting a herd immune response in Great Lakes fish populations under different immunization and release strategies was to be evaluated using a spatially explicit, individual-based, time-discrete modeling framework.

The primary objective goal of this study was to assess the ability of fish immunized against VHSV-IVb to protect susceptible naïve fish species against this emerging VHSV strain. This study was intended to utilize a vaccine preparation (recombinant or DNA G-protein vaccine) for immunizing hatchery-propagated fish to achieve the maximum protection against VHSV.

Immunized fish were then to be tested for their ability to confer herd immunity to other naïve fish of the same or other species. Our project had the following objectives:

Objective 1: To develop a recombinant subunit VHSV-IVb G-proteins. Numerous studies have shown the efficacy of recombinant G protein-based subunit vaccines in protecting rainbow trout (*Oncorhynchus mykiss*) against VHSV genotype Ia in Europe. The PI's laboratory has experience with VHSV-IVb G protein subunit preparation and has successfully used it to produce anti-VHSV hyperimmune serum in rabbits and chickens. In the USA, recombinant subunit vaccines are FDA-approved for use in humans and food animals.

Objective 2: Assessment of VHSV-IVb recombinant subunit protein vaccine on protection against virus challenge. This will involve a comparison of protection and the potency of immune responses elicited by fish inoculated with recombinant proteins produced by either insect or fish cells. This comparison will initially be performed with muskellunge due to its high susceptibility to VHSV (Kim & Faisal 2010c). Moreover, there is much data generated on the susceptibility and disease course of muskellunge in the PI's laboratory. Once a superior vaccine has been selected using muskellunge, three salmonid species [rainbow trout, lake trout (*Salvelinus namaycush*), and brown trout (*Salmo trutta*)] with varying susceptibility to VHSV, will be vaccinated.

Objective 3: To assess elicited immune responses against VHSV-IVb. Humoral and cellular immune responses specific to VHSV will be assessed in serum and mucous samples of immunized fish. Immunological assays will involve virus neutralization test, enzyme-linked immunosorbent assay (ELISA), and cytotoxic leukocyte assays.

Objective 4: To assess herd immunity. Immunized fish will be intermingled with naïve fish susceptible to VHSV such as largemouth bass (*Micropterus salmoides*), yellow perch (*Perca flavescens*) and walleye (*Sander vitreus*). Following VHSV challenge, HIT will be determined using varying proportions of immunized and naïve fish.

Objective 5: To evaluate the feasibility/costs in eliciting a herd immune response in a Great Lakes fish population. An individual-based model will be constructed to evaluate the benefits and costs of eliciting a herd immune response in a Great Lakes fish population through annual stocking of immunized hatchery-propagated specimens. Models will explore different immunization and release strategies and will incorporate uncertainties pertaining to interactions between wild and hatchery-propagated sub-population dynamics (e.g., survival rates).

CHANGES:

i. The production of recombinant proteins in insect, fish and mammalian cells appears limited by both protein insolubility and accumulation of cytotoxic protein. For that reason, we pursued the use of cabbage looper larvae, which allowed us to easily scale up production and obtain a sufficient amount of protein.

ii. Due to the rapid pace of DNA vaccine development against the fish pathogen, we used this approach and developed a DNA vaccine based on the VHSV-IVb G-gene sequence. Vaccination trials included both recombinant proteins and DNA vaccines.

iii. Using the DNA preparation, we were able to develop and optimize a vaccination regimen that allowed us to explore the concept of herd immunity by artificially stimulating immunity in muskellunge in a way in which we could obtain repeatable results. Moreover, this preparation served as a standard which we could then compare to the recombinant preparations that we subsequently examined.

iv. In the design of the herd immunity objective, we made several alterations to the initial concept. First, we determined that co-mingling numerous species in a single flow through tank would be difficult to replicate and would also introduce variability from stress, aggression and a predation risk through the community. By designing the novel flow through tank, we could ensure that tanks were exposed to identical viral concentrations but eliminate some of the variability associated with the previous design. Moreover, by using this design, we could incorporate additional tanks to better determine the cause of the indirect protection that we were observing.

v. In the individual-based model, we had initially proposed modeling Chinook salmon as part of a larger fish community at a relatively coarse scale (e.g., 10-minute grid cells). We instead chose to model Chinook salmon by itself but at a much finer spatial scale (1.8-km^2 grid cell). The finer spatial scale was a better representation of how fish interacted with their environment, but limited our ability to incorporate other species because of computer processing time. When modeling disease transmission in both fish and wildlife populations, it is fairly common for models to be based on a single species.

OUTCOMES:

- This project has produced outstanding data that increased our knowledge on VHSV, immune responses in fish, and, most importantly, it gave the first indication for the presence of herd immunity in fish. Five undergraduates, a doctoral student (now Dr. Isaac Standish) and two post-doctoral fellows (Drs. Thomas P. Loch and Lori Ivan) received rigorous training from this project and were able to produce publications in reputable scientific periodicals.
- Monoclonal antibody against fish immunoglobulin heavy chain was developed and used in developing a sensitive and specific diagnostic assay for detecting post infection antibodies against VHSV. The project finding benefits fishery managers as they enhanced our ability to detect antibody responses in fish.
- Using the cabbage looper instars, we were able to produce copious amounts of recombinant VHSV-G-proteins that conferred considerable immunity to naïve fish upon challenges.
- A DNA vaccine has been developed and tested that was found efficacious in conferring complete protection to a number of Great Lakes fishes.
- Through our modeling efforts, we found that immunization and release of hatchery-propagated individuals can offer a legitimate means of protecting wild fish populations against disease, although the level of protection effect depends on disease dynamics. For example, the greatest level of protection in our study occurred when infection rate was low.
- We also demonstrated through modeling that resumed shedding of viral particles by recovered individuals can be a major factor leading to disease persistence and the overall

need of a vaccination program. From a management standpoint, actions designed around limiting stress factors that can lead to re-shedding may be beneficial.

- The most important outcome of this project is the fact that, through vaccination, a 100% protection can be achieved and that herd immunity exists in fish, and therefore, the hatchery system in the Great Lakes offers a potential method to protect wild fish stocks against disease spread.

RELATED EFFORTS:

This study is built upon a previous GLFT-funded study (08WRGR0006). Parallel to this project, other work funded by a USFWS grant was done to better understand the presence of antibodies against VHSV in Great Lakes fish species. The individual-based modeling conducted as part of this study relied heavily on estimates of Chinook salmon abundances, recruitment levels, and mortality components that resulted from assessment modeling conducted from a previous GLFT funded study (GLFT #2007.950 – Assessing Lake Michigan Salmonine Stocking Policies Using Decision Analysis).

COMMUNICATION/PUBLICATION OF FINDINGS:

Six manuscripts have been published or drafted from this study thus far and are appended:

I. Standish IF, Millard EV, Brenden TO, Faisal M. 2016. A DNA vaccine encoding the viral hemorrhagic septicemia virus genotype IVb glycoprotein confers protection in muskellunge (*Esox masquinongy*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and lake trout (*Salvelinus namaycush*). Virology Journal 13:203 DOI: 10.1186/s12985-016-0662-8.

II. Standish IF, Brenden TO, Faisal M. 2016. Does herd immunity exist in aquatic animals? International Journal of Molecular Sciences 17: 1898. doi:10.3390/ijms17111898.

III. Millard EV, LaPatra SE, Brenden T, Bourke AM, Fitzgerald S, Faisal M. In Press. DNA Vaccination Partially Protects Muskellunge against Viral Hemorrhagic Septicemia Virus (VHSV-IVb). Journal of Aquatic Animal Health.

IV. Faisal M, Standish IF, Vogelbein MA, Millard EV, Kaattari SL. Accepted for publication. Production and characterization of a monoclonal antibody against muskellunge (*Esox masquinongy*) immunoglobulin and development of an indirect ELISA for detection of circulating antibodies against viral hemorrhagic septicemia virus genotype IVb. Fish & Shellfish Immunology.

V. Standish I, Faisal M. In review. A recombinant viral hemorrhagic septicemia virus genotype IVb glycoprotein produced in cabbage looper larvae (*Trichoplusia ni*) elicits antibody response and protection in muskellunge (*Esox masquinongy*). Target Journal: Journal of Aquatic Animal Health.

VI. Ivan, LN, Brenden, TO, Standish, IF, Faisal, M. In preparation. Individual-based modeling of the effectiveness of using vaccinated hatchery fish to prevent disease spread in a wild fish population. Target Journal: Ecological Applications.

Presentations of data developed from tasks of this project:

Isaac F. Standish, Mohamed Faisal: A DNA Vaccine, Based on the Viral Hemorrhagic Septicemia Virus Genotype IVb Glycoprotein Gene Protects Muskellunge (*Esox masquinongy*). 40th Eastern Fish Health Workshop. Spring 2015.

Mohamed Faisal and Isaac Standish: Evidence of herd immunity in fish. The Annual Meeting of the Fish Health Section of the American Fisheries Society. Jackson Hole, Wyoming. July 2016.

Mohamed Faisal and Isaac Standish: Mechanisms of herd immunity in fish against VHSV. The Sixth International Virology Meeting. Hurghada, Egypt. November 29-30, 2016.

Isaac F. Standish. Eliciting Protection and Herd Immunity in Fish Against VHSV-IVb. Defense seminar, Michigan State University. Spring 2016.

Isaac F. Standish. Advances in the Detection & Prevention of VHSV. Great Lakes Fish Health Committee, Michigan State University. Spring 2016.

Isaac F. Standish. Development of a DNA Vaccine Against VHSV-IVb. Phi-Zeta College of Veterinary Medicine Research Day, Michigan State University. Fall 2015.

Isaac F. Standish. Emerging Perspectives of Viral Hemorrhagic Septicemia Virus IVb. Departmental Pathology Seminar, Michigan State University. Fall 2014.

Isaac F. Standish. Immune Response to Viral Hemorrhagic Septicemia in Muskellunge. Phi-Zeta College of Veterinary Medicine Research Day, Michigan State University. Fall 2014.

Lori N. Ivan, Travis O. Brenden, Mohamed Faisal, and Isaac F. Standish. Can vaccinated hatchery fish be used to prevent disease spread? A case study of VHS IVb. 2016 Midwest Fish and Wildlife Conference, Grand Rapids, Michigan. January 2016.

Lori N. Ivan, Travis O. Brenden, Mohamed Faisal, and Isaac F. Standish. Can vaccinated hatchery fish be used to prevent disease spread? A case study of VHS IVb. Great Lakes Fish Health Committee, Michigan State University. Spring 2016.

14. **Compilation reports.** NA

FULL NARRATIVE:

The six appended manuscripts detail the justification, material and methods used, results and discussion. Below is more detailed information on the methods used and results obtained when some of the details were not in the manuscripts to fit in the journal limitation of pages and details.

Objective 1: To develop a recombinant subunit VHSV-IVb G-proteins. The VHSV glycoprotein gene was obtained, amplified, cloned and sequenced. The gene was then sub-cloned into the pAE6 plasmid that we obtained for transfection into the EPC cell line. Initial transfection work has taken place using two commercially available transfection reagents; however, the transfection was not satisfactory. Further to that; we are re-amplifying and cloning a second antibiotic selectivity agent (co-transfection with pAE6 plasmid containing a puromycin resistance gene) which we are optimistic about. This hurdle has not stopped us from immunizing muskellunge by using pAE6 plasmid containing the VHSV glycoprotein gene.

Expression of recombinant VHSV glycoprotein in transfected insect cells was subcontracted to a commercial enterprise (GenScript). The full VHSV G-gene was sent to the company. This gene was cloned into a baculovirus vector as is, transfection and expression of the full gene yielded minimal secreted protein, likely because the glycoprotein was expressed intercellularly. Upon our request, GenScript attempted to re-transfect and express the protein following excision of the transmembrane tail of the glycoprotein. The belief was that removal of this transmembrane anchor would increase the intercellular expression and increase the solubility of the protein into

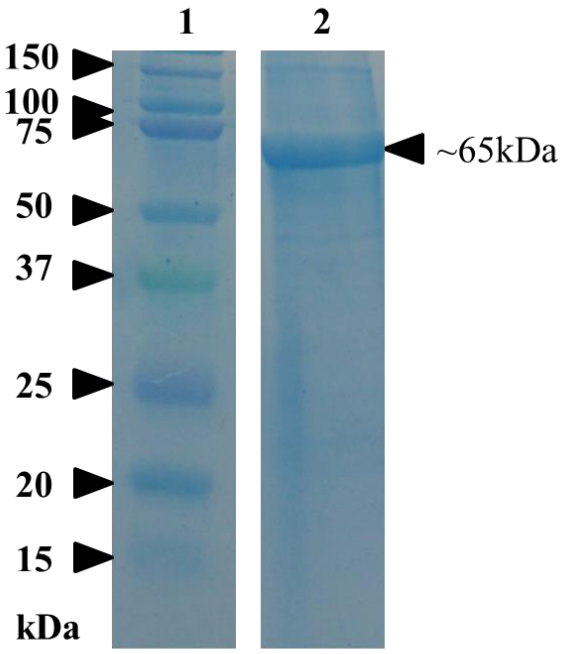
the supernatant and increase yields. We were able to obtain an amount of purified protein sufficient to run some pilot work. Initial experimentation shows minimal anti-VHSV-G gene antibody activity against the recombinant protein, but we are continuing with protection trials. Additionally, in an attempt to obtain recombinant eukaryotic proteins, we outsourced production to a second company (GeneCopoeia) which produces recombinant proteins in mammalian cells. Similarly to our other attempt, the full glycoprotein gene was sent for use in transfecting cells. Initial attempts again showed that little soluble recombinant protein was being produced in mammalian cells and we elected not to proceed on a larger scale.

During our initial work with the baculovirus and insect cell expression system, minimal protein was obtained. However, we devised a different strategy to overcome this obstacle by production of the recombinant glycoprotein in insect larvae instead of a cell line. Whereby, we propagated the recombinant baculovirus vector (which we produced during our initial work with Genscript) containing the VHSV glycoprotein and sent the propagate virus to another company (Frontier Scientific) which houses and infects cabbage looper (*Trichoplusia ni*) larvae. We chose to pursue this more expedited route rather than obtaining the larvae ourselves, as USDA-APHIS permitting is required for transport of live larvae.

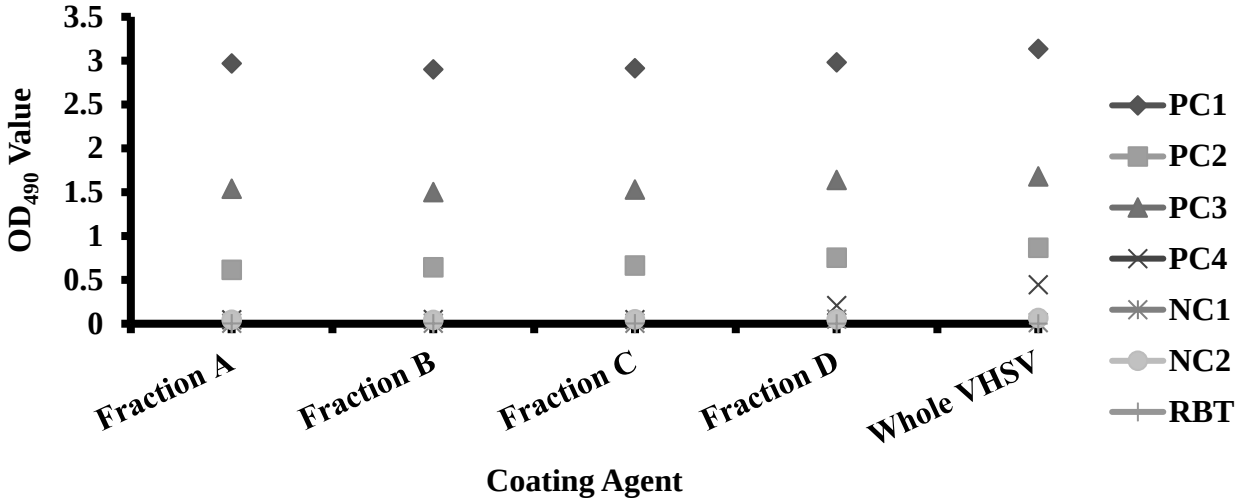
Upon receipt of the frozen larvae, the protein conformation was confirmed using SDS-PAGE and a newly developed indirect ELISA.

Findings: After repeated attempts to transfect our EPC cell line using different vectors and transfection strategies, it appears that immediately following transfection, a cytotoxic element such as the accumulation of protein is inhibiting the stable transfection of our cell lines. This transient transfection has resulted in negligible yields of recombinant glycoprotein.

Use of cabbage looper larvae was much more successful. Following purification, large quantities of highly purified recombinant VHSV glycoprotein were obtained. (Below is an image of SDS page analysis and an arrow indicating the purified glycoprotein.)



To further confirm the three-dimensional shape of the recombinant glycoprotein (rG), either purified recombinant VHSV-IVb glycoprotein (rG) or whole purified VHSV-IVb determined using indirect ELISA. Below are the results of the indirect ELISA, where fractions A and B represent the 4th mL and 5th mL eluted from a single cabbage looper larva following Ni-resin purification. Fractions C and D represent the same elutions from a separate larva. Sera from four positive control (PC), two negative control (NC) muskellunge, and a rainbow trout (RBT) was examined for binding. Note the minimal variation in OD_{490} values between the different coating agents, showing that the recombinant glycoprotein is indeed conformationally correct.



Objective 2: Assessment of VHSV-IVb recombinant subunit protein vaccine on protection against virus challenge. Due to difficulties experienced in the initial production of a eukaryotic glycoprotein, we elected to examine the protective efficacy of a DNA based preparation. We immunized one important hatchery propagated species (muskellunge) with two vectors: pVHSivb-G and pβ-VHSivb-G plasmids, the latter containing a carp β-actin promoter, specifically designed and previously demonstrated to facility efficient gene transcription in fish cells. Muskellunge was selected as the initial experimental fish species due to the wealth of data on this species in the context of VHSV. Moreover, although DNA vaccines are typically reliant on a single intramuscular administration of 10 µg, we investigated the effects of a booster dose of each plasmid. Cox proportional hazards frailty models with tank as a random effect to account for fish deaths within tanks not being independent were also calculated for all trials using PROC PHREG in SAS. Cumulative mortality averaged across tanks within treatments for each trial was used to calculate RPS for each treatment.

$$\text{RPS} = 1 - \left(\frac{\%\ cumulative\ mortality\ of\ vaccinated}{\%\ cumulative\ mortality\ of\ mock\ vaccinated}\right) x\ 100$$

Further, results obtained using this vaccination preparation can still be used in the modeling portion of the project and compared with future vaccination treatments when they are obtained. Vaccinated fish were allowed to develop an immune response for 11-28 weeks, then challenged for both assessment of protection and use in the herd immunity portion of the project.

Based on the results of this initial trial using muskellunge, the pVHSivb-G plasmid preparation that provided superior protection was also used to immunize and assess protection in three hatchery propagated salmonids: rainbow trout, (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*). All fish were allowed a total of 1001° days to react to the antigen prior to either intraperitoneal or immersion VHSV challenge.

The recombinant VHSV-IVb glycoprotein obtained from the cabbage looper larvae was also assessed as a vaccine antigen to elicit a protective response in naïve muskellunge against a VHSV-IVb immersion challenge.

Findings: In four trials using muskellunge, under varying conditions, a single administration of the pVHSivb-G plasmid resulted in 95% RPS after 1880° days incubation, while a booster administration after 940° days resulted in 100% RPS. Conversely, administration of the pβ-VHSivb-G resulted at best in a 25% RPS.

Below is a table of results of the four trials including the number of fish, mean cumulative mortality, mean days to death, hazard ration, RPS and associated *p*-value.

	Fish	**Mean Cumulative % Mortality**	**Mean days to death ± SE**	**HR (95%CI)**	**RPS**	***p*-value**
Trial 1						
pβ-VHSivb-G	29	100%	8.8 ± 0.68	1.55 (0.84-2.88)	-3%	0.423

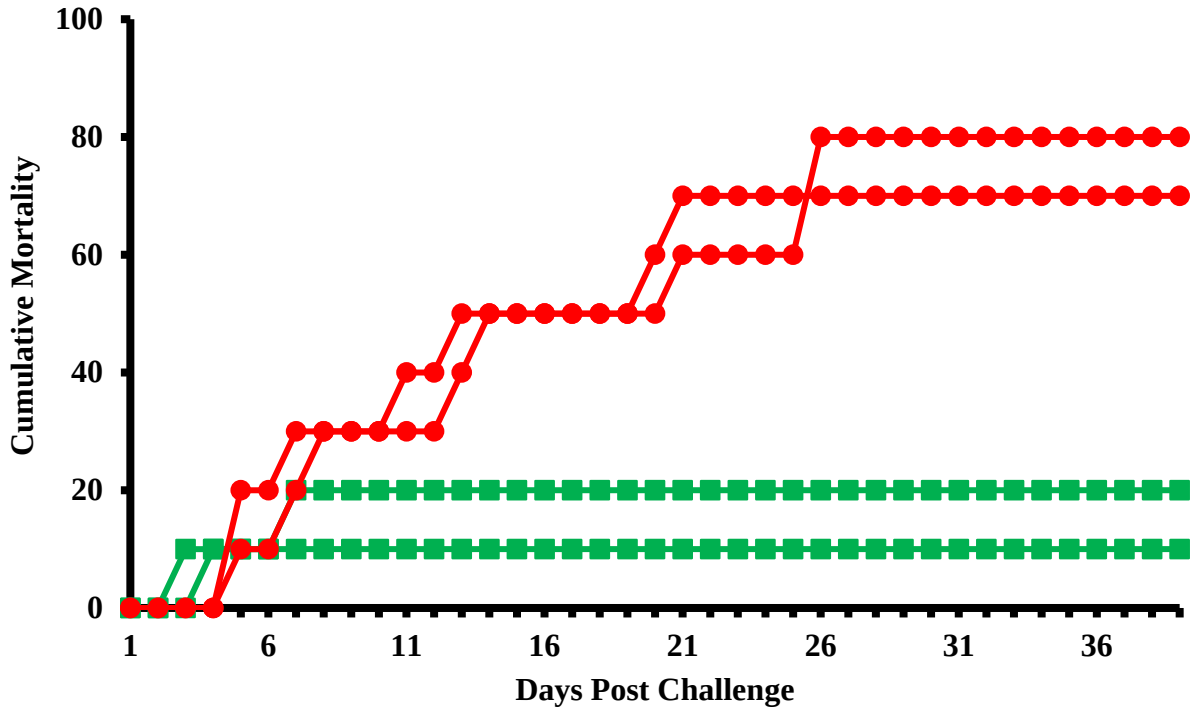
pAE6	30	96.70%	9.0 ± 1.2		NA	NA
Trial 2						
pVHSivb-G	20	5%	NA	0.02 (0.002-0.12)	95%	0.033
pcDNA	20	100%	11.7 ± 1.3		NA	NA
Trial 3						
pβ-VHSivb-G	20	75%	10.2 ± 2.6	0.56 (0.16-1.98)	25%	0.171
pAE6	20	100%	8.8 ± 0.8		NA	NA
pVHSivb-G	20	15%	10.6 ± 0.4	0.07 (0.01-0.35)	85%	0.005
pcDNA	20	100%	9.9 ± 1.0		NA	NA
Trial 4						
pVHSivb-G	20	0%	NA	NA	100%	<0.0001
pcDNA	20	100%	12.8 ± 1.8		NA	NA

Below are the results of trials conducted on the three salmonid species. Fish received the pVHSivb-G plasmid and were allowed to react to the plasmid for 1001° days before challenge. The table includes the number of fish in each treatment, the cumulative mortality, the relative percent survival (RPS), and the associated *p*-value calculated using a two-tailed χ^2-test. Rainbow trout (RBT-1 and RBT-2), brown trout (BNT-1 and BNT-2) and lake trout (LAT-1 and LAT-2).

Treatment	**Fish**	**Cumulative Mortality**	**RPS**	χ^2	***p*-value**
RBT-1					
pcDNA	29	62.1%			
pVHSivb-G	26	26.9%	56.7%	6.83	0.009
pVHSivb-G (2 doses)	13	15.4%	75.2%	7.84	0.005
RBT-2					
pcDNA	35	8.6%			
pVHSivb-G	17	0.0%	100.0%	1.55	0.214
pVHSivb-G (2 doses)	31	0.0%	100.0%	2.78	0.095
BNT-1					
pcDNA	26	23.1%			
pVHSivb-G	38	5.3%	77.2%	4.48	0.034
pVHSivb-G (2 doses)	18	16.7%	27.8%	0.27	0.604
BNT-2					
pcDNA	28	10.7%			
pVHSivb-G	25	4.0%	62.7%	0.85	0.356
pVHSivb-G (2 doses)	25	4.0%	62.7%	0.85	0.356
LAT-1					
pcDNA	26	30.7%			
pVHSivb-G	34	0.0%	100.0%	12.07	0.0005
pVHSivb-G (2 doses)	27	0.0%	100.0%	9.79	0.0018

LAT-2					
pcDNA	26	34.6%			
pVHSivb-G	34	0.0%	100.0%	13.85	0.0002
pVHSivb-G (2 doses)	27	0.0%	100.0%	11.26	0.0008

The protective efficacy of the recombinant glycoprotein was also assessed in muskellunge. In one trial (displayed below), the two mock vaccinated replicate tanks (●) experienced 70% and 80% cumulative mortality. Meanwhile, replicate tanks of muskellunge that received the rG protein (■) experienced 10% and 20% cumulative mortality. This resulted in a mean RPS of 80%, indicating significant protection ($\chi^2 = 14.5$, df = 1, $p < 0.0001$).



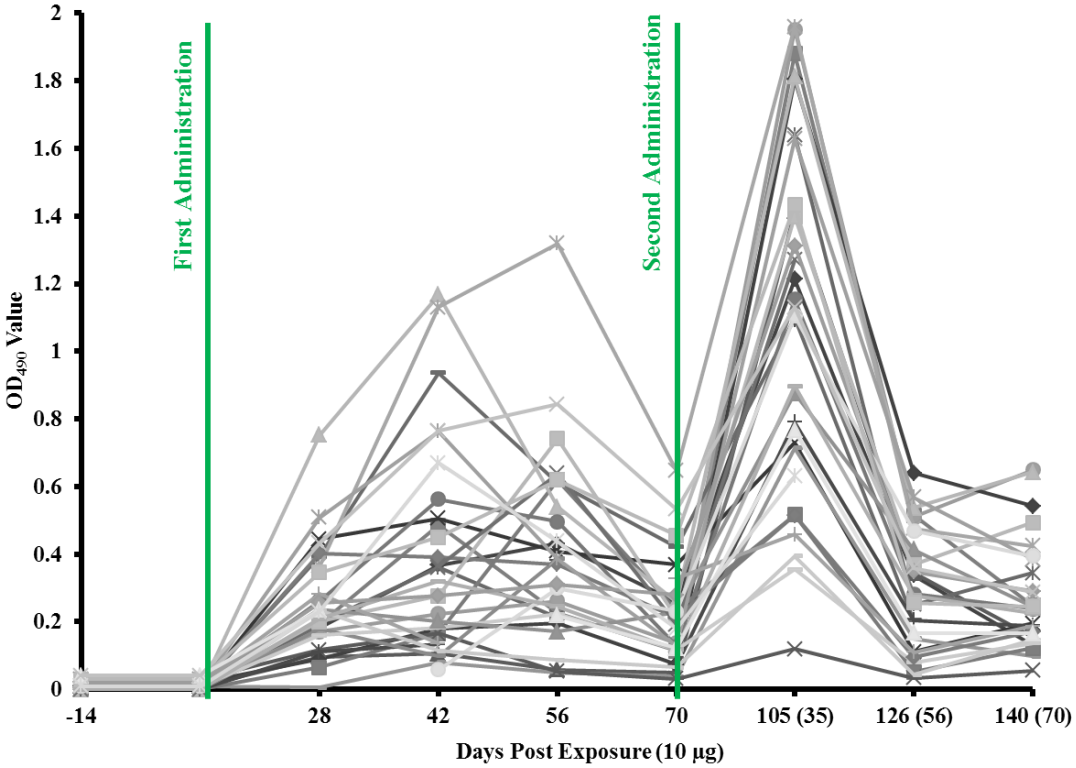
Objective 3: To assess elicited immune responses against VHSV-IVb. To assess the humoral response against VHSV-IVb monoclonal antibody (designated 3B10) against the immunoglobulin (Ig) of muskellunge (*Esox masquinongy*) was developed. The 3B10 mAb was used to develop and optimize an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-VHSV-IVb antibodies. Using the indirect ELISA, anti-VHSV-IVb antibodies were detected in sera from juvenile muskellunge vaccinated against the VHSV-IVb glycoprotein.

In addition to examining the humoral response in immunized muskellunge, we also assessed the cell mediated response following vaccination. We have developed and optimized a novel assay

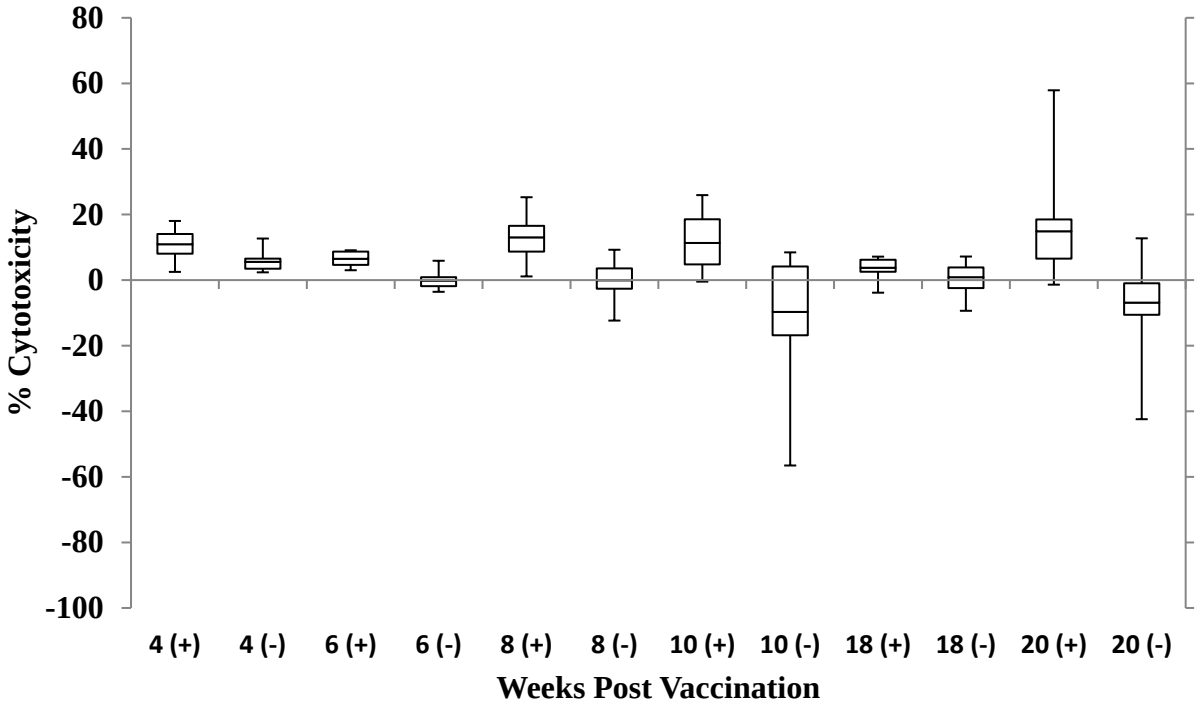
for the examination of the cellular immune response against VHSV following administration of the pVHSivb-G plasmid vaccine. This novel assay involved extensive optimization as it has never been utilized with VHSV genotype IVb or muskellunge. The assay examines the cytotoxicity of peripheral blood mononuclear cells (PBMC) in pit-tagged individual vaccinated muskellunge. Following the isolation of PBMC through gradient centrifugation, cells are added to EPC cells, some of which are infected with VHSV. We then measure the cytotoxicity by quantifying the lactose dehydrogenase (LDH) released into the supernatant. By comparing these values to the LDH release values in non-infected cells we can determine specific cytotoxicity. Further, we can compare this cytotoxicity in G gene vaccinated individuals to mock vaccinated individuals. Additionally, we were able to track these responses over time to examine the dynamics of this response.

Indirect ELISA was also conducted on sera samples collected from wild mature muskellunge from the Detroit River from 2012 to 2015. Samples were collected during the performance of routine health examines in collaboration with ongoing gamete collection by the Michigan Department of Natural Resources. The number of muskellunge sampled varied each year, with 26 in 2012, 27 in 2013, 38 in 2014 and 45 in 2015, for a total of 134. Following collection, blood was transported to MSU at 4°C, then processed and stored as previously described. Trends within the optical density (OD) values were analyzed by year using a general linear model procedure (PROC GLM) in SAS (SAS Institute Inc. 2010).

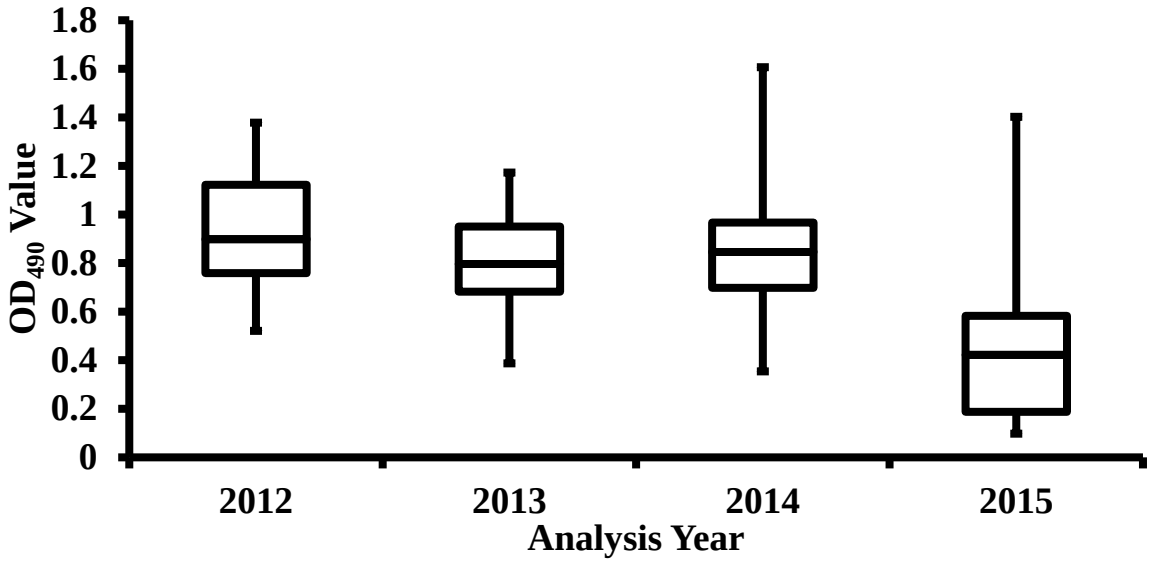
Findings: To assess elicited immune responses against VHSV-IVb.



Displayed above are the indirect ELISA OD values indicating anti-VHSV antibodies generated following immunization with the pVHSivb-G plasmid. In these captive muskellunge (n =32), prior to immunization the mean OD value was 0.010 (SD = 0.008) (above). By 28 days following immunization, the mean OD value had increased significantly in serum samples collected from the same individuals to 0.244 (SD = 0.161) (paired t-test; t = 8.207, df = 28, p < 0.0001). At 42 days post administration, the mean OD value had increased to 0.388 (SD = 0.301). Though, OD values peaked in sera obtained 56 days following exposure (0.395, SD = 0.267). OD values had substantially decreased by 70 days post inoculation (0.215, SD = 0.150), at which point muskellunge were given a second administration of the pVHSivb-G vector. Thirty-five days following a second administration, mean OD values had significantly increased (1.098, SD = 0.506) when compared to the 70 day OD values (p <0.0001, t = 9.084, df = 28). By 56 and 70 days after the second exposure, the mean OD value had again decreased to 0.293 (SD = 0.174) and 0.274 (SD = 0.158), respectively. This steep increase in circulating anti-VHSV antibody levels within 7 weeks of secondary exposure to the pVHSivb-G vector is indicative of an adaptive response and possible B cell proliferation.



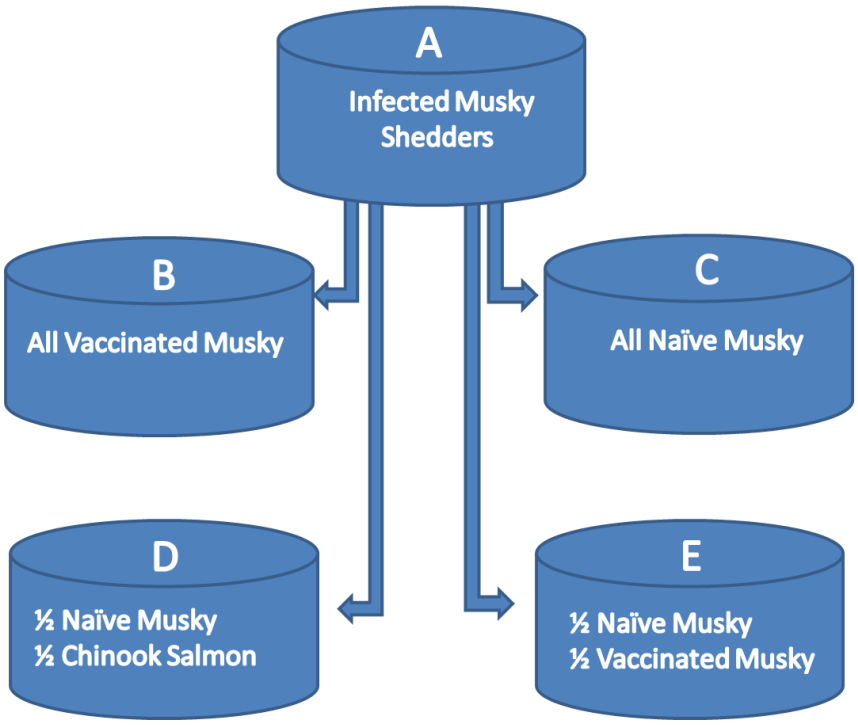
The associated % cytotoxicity of peripheral mononuclear cell following immunization appears to follow a similar trend. Pictured below is the specific cytotoxicity of peripheral cells against either VHSV infected (+) or uninfected (-) EPC cells over time following immunization with the pVHSivb-G plasmid.



Levels of anti-VHSV antibodies were also assessed in 134 wild adult muskellunge collected from the Detroit River, a VHSV-IVb endemic water body which connects Lake St. Clair to Lake Erie. The results demonstrate relatively high levels of anti-VHSV-IVb antibodies, and clear differences between OD values between naïve muskellunge and wild Detroit River muskellunge. Detroit River muskellunge OD values ranged from 0.089 to 1.61, with a mean of 0.703 (SD = 0.341); more than 10-fold higher than those obtained by sera collected from naïve individuals.

The indirect ELISA also provided the opportunity to quantify and examine how the OD values of Detroit River muskellunge population have changed over time. For example, in 2012, the mean OD value for the sampled population was 0.899 (SD = 0.250), 0.796 (SD = 0.229) in 2013, and 0.845 (SD = 0.257) in 2014. However, in 2015, when the greatest number of individual muskellunge were examined (n = 45), the mean OD value had decreased to 0.422 (SD = 0.321). Statistical analyses of sera collected in all four years indicated significant downward trend in antibody levels with an estimated slope of -0.152 (p <0.0001, df =1, F = 42.4).

Objective 4: To assess herd immunity. The experimental layout consisted of multiple flow-through tanks where viral exposure was achieved via shedding from VHSV-IVb infected muskellunge housed in a tank supplying water to other tanks. The other tanks contained different combinations of immunized muskellunge and naïve muskellunge, largemouth bass (*Micropterus salmoides*), or Chinook salmon (*Oncorhynchus tshawytscha*). An example of the tank layout is pictured below. Differences between cumulative mortality, circulating anti-VHSV antibody levels, and viral concentration in the water, as well as the terminal viral concentrations in the posterior kidney of survivors, were compared between fish co-mingled together or housed alone.



Differences in viral shedding rates of infected muskellunge among the trials were tested using one-way analysis of variance following $\log_e + 1$ transformation of the shedding rates. Each of the response measures from the experimental fish described above were analyzed using generalized linear mixed-effect models using PROC GLIMMIX in SAS.

Findings: To assess herd immunity. A total of eight trials were conducted. The highest cumulative mortalities in the experiment involved naïve muskellunge housed alone [average mortality (AM) across trials (AM = 80.2%)] or in combination with largemouth bass (AM = 76.9%) and Chinook salmon (AM = 95.8%). Largemouth bass housed alone (AM = 39.6%) or in combination with naïve muskellunge (AM = 53.8%) experienced the next highest mortality rates. Conversely, vaccinated muskellunge and Chinook salmon experienced the lowest mortality rates during the experiment, with mortality rates in all cases being less than 16.7%. Below are the individual trial results of cumulative mortalities, viral re-isolations and RPS.

Housing Treatment	Cumulative Mortality	Re-isolations	RPS
Trial 1			
Naïve muskellunge	91.7%	11/11	NA
Vaccinated muskellunge	16.7%	2/2	81.9%
Naïve muskellunge w/ vaccinated muskellunge	100.0%	6/6	-9.2%
Vaccinated muskellunge w/ naïve muskellunge	50.0%	3/6	45.5%
Largemouth bass w/ naïve muskellunge	66.7%	4/4	27.3%
Naïve muskellunge w/ largemouth bass	100.0%	6/6	-9.2%
Trial 2			

Naïve muskellunge	100.0%	12/12	NA
Vaccinated muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	83.3%	5/5	16.6%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%
Chinook salmon w/ naïve muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ Chinook salmon	100.0%	6/6	0.0%
Trial 3			
Naïve muskellunge	100.0%	12/12	NA
Vaccinated muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	14.3%	1/1	85.7%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%
Largemouth bass w/ naïve muskellunge	42.9%	3/3	57.1%
Naïve muskellunge w/ largemouth bass	57.1%	4/4	42.9%
Trial 4			
Naïve muskellunge	35.7%	5/5	NA
Vaccinated muskellunge	0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	28.6%	2/2	20.0%
Vaccinated muskellunge w/ naïve muskellunge	0%	NA	100.0%
Trial 5			
Naïve muskellunge	83.3%	10/10	NA
Vaccinated muskellunge	8.3%	1/1	90.0%
Naïve muskellunge w/ vaccinated muskellunge	0.0%	NA	100.0%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%
Largemouth bass w/ vaccinated muskellunge	0.0%	NA	100.0%
Vaccinated muskellunge w/ largemouth bass	16.7%	1/1	80.1%
Chinook salmon w/ naïve muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ Chinook salmon	100.0%	6/6	-20.0%
Largemouth bass	75.0%	9/9	10.0%
Trial 6			
Naïve muskellunge	100.0%	12/12	NA
Vaccinated muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	16.7%	1/1	83.3%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%
Largemouth bass w/ vaccinated muskellunge	0.0%	NA	100.0%
Vaccinated muskellunge w/ largemouth bass	0.0%	NA	100.0%
Chinook salmon w/ naïve muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ Chinook salmon	100.0%	6/6	0.0%
Largemouth bass	66.7%	8/8	33.3%
Trial 7			
Naïve muskellunge	100.0%	12/12	NA
Vaccinated muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	16.7%	1/1	83.3%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%

Largemouth bass w/ vaccinated muskellunge	33.3%	2/2	66.6%
Vaccinated muskellunge w/ largemouth bass	33.3%	1/2	66.6%
Largemouth bass	8.3%	1/1	91.7%
Trial 8			
Naïve muskellunge	30.0%	3/3	NA
Vaccinated muskellunge	0.0%	NA	100.0%
Naïve muskellunge w/ vaccinated muskellunge	33.3%	2/2	-11.1%
Vaccinated muskellunge w/ naïve muskellunge	0.0%	NA	100.0%
Largemouth bass w/ vaccinated muskellunge	0.0%	NA	100.0%
Vaccinated muskellunge w/ largemouth bass	0.0%	NA	100.0%
Chinook salmon w/ naïve muskellunge	33.3%	2/2	-11.1%
Naïve muskellunge w/ Chinook salmon	83.3%	5/5	-177.7%
Largemouth bass	8.3%	1/1	82.3%
Chinook salmon	8.3%	1/1	82.3%

Below are pairwise comparisons of survivorship between tank housing treatments. The degrees of freedom, *t*-statistic and *p*-value are included for each comparison. The odds ratio (OR) represent the odds of mortality occurring in housing treatment 1 compared to treatment 2.

Housing Treatment 1	Housing Treatment 2	df	*t*-statistic	*p*-value	Odds Ratio
largemouth	largemouth w/ naïve muskellunge	22.1	0.31	0.758	1.70
largemouth	largemouth w/ vaccinated muskellunge	30.9	1.51	0.141	9.45
largemouth w/ naïve muskellunge	largemouth w/ vaccinated muskellunge	31.3	0.90	0.375	5.56
naïve muskellunge	naïve muskellunge w/ vaccinated muskellunge	26.3	2.82	0.009	15.96
naïve muskellunge	vaccinated muskellunge	42.3	5.08	<0.001	483.50
naïve muskellunge	naïve muskellunge w/ Chinook	44	-1.15	0.256	0.16
naïve muskellunge	naïve muskellunge w/ largemouth	30.5	0.64	0.525	3.07
naïve muskellunge w/ Chinook	naïve muskellunge w/ largemouth	43.1	1.34	0.186	19.79
naïve muskellunge w/ Chinook	naïve muskellunge w/ vaccinated muskellunge	44	2.86	0.006	102.91
vaccinated muskellunge	vaccinated muskellunge w/ largemouth	42.5	-1.51	0.139	0.10
vaccinated muskellunge	vaccinated muskellunge w/ naïve muskellunge	44	-0.21	0.835	0.73

Below are the pairwise comparisons of circulating binding anti-VHSV antibody OD values in surviving muskellunge. The t-statistic, degrees of freedom, and *p*-value for each of the housing combinations are included for each comparison.

Housing Treatment 1	Housing Treatment 2	t-value	df	*p*-value
Naïve muskellunge	Naïve muskellunge w/ vaccinated muskellunge	0.78	10.7	0.453
Naïve muskellunge	Vaccinated muskellunge	-0.78	8.0	0.456
Naïve muskellunge	Vaccinated muskellunge w/ naïve muskellunge	-4.12	9.3	0.002
Naïve muskellunge w/ vaccinated muskellunge	Vaccinated muskellunge w/ naïve muskellunge	-5.31	11.1	<0.001
Vaccinated muskellunge	Vaccinated muskellunge w/ largemouth	0.06	11.0	0.957
Vaccinated muskellunge	Vaccinated muskellunge w/ naïve muskellunge	-3.98	7.7	0.004
Vaccinated muskellunge w/ largemouth bass	Vaccinated muskellunge w/ naïve muskellunge	-3.51	13.0	0.004

Objective 5: To evaluate the feasibility/costs in eliciting a herd immune response in a Great Lakes fish population. All the methods and results of this objective are fully described in Appendix VI so additional details are not provided here.

CONCLUSIONS:

Objective 1: To develop a recombinant subunit VHSV-IVb G-proteins. Ultimately, success came through the use of a recombinant baculovirus vector to infect cabbage looper larvae (*Trichoplusia ni*), from which purified protein was obtained.

Objective 2: Assessment of VHSV-IVb recombinant subunit protein vaccine on protection against virus challenge. Using DNA based preparations, we showed that the plasmid promoter sequence of a DNA vector can have a profound influence on protection. Under identical conditions, in muskellunge, the VHSV-IVb G gene under the control of the CMV promoter elicited much greater protection (mean RPS = 85%) than expression using a carp β-actin promoter (mean RPS = 25%).We demonstrate even highly susceptible species such as muskellunge can be nearly 100% protected against VHSV.

The pVHSivb-G preparation could also elicit protection to salmonid species with lower VHSV-IVb susceptibility, with significant protection observed in each of the species examined. Additionally, we show that a long incubation period clearly results in the development of a protective immune response, and mimics culture conditions, when fry and fingerling salmonids are raised in hatcheries for an extended period of time prior to stocking. In our study, we also examine one vs. two administrations of the pVHSivb-G plasmid, there was no significant increase in protection following a second administration.

Additionally, in muskellunge, the recombinant glycoprotein (rG) appeared to elicit a protective immune response and provided significant protection in muskellunge. Moreover, this preparation may represent a more innocuous preparation more apt for FDA approval.

Objective 3: To assess elicited immune responses against VHSV-IVb. The indirect ELISA was highly successful in detecting both the primary and secondary antibody responses in vaccinated muskellunge; underscoring the potential application of the developed ELISA in determining the kinetics of teleostean antibody response and efficacy of vaccines. There was a dramatic increase in OD values through the first six weeks and peaked OD values were observed eight weeks post vaccination, indicating a strong B cell response. However, most surprising was the dramatic increase following the secondary antigen exposure. By seven weeks into the secondary response, the OD values had increased five-fold, showing the first administration primed the immune system and suggests an adaptive response and antigenic memory.

The indirect ELISA was also capable of detecting antibodies in 129 of 134 of muskellunge sampled from the Detroit River, part of a known VHSV enzootic zone. This is despite the fact that the last time that this virus was isolated in this area was in 2009 (Faisal et al. 2012). This matter indicates that either the anti-VHSV antibodies are long lived or fish have been exposed to VHSV-IVb more recently. Given the decline we detected between previous years and 2015, it

does seem to suggest fish may have not been exposed as recently as in the past. This data highlights the application of the indirect ELISA in serosurveillance studies; we can detect water bodies where fish have been exposed without lethally sampling fish. Furthermore, we can monitor immune responses of fish in endemic areas, which may be used in the future to predict whether the populations are at risk for an outbreak.

Objective 4: To assess herd immunity. In this study, we used a novel design to examine the concept of aquatic herd immunity against VHSV. We introduced virus into the system using shedding that was elicited following an IP infection of muskellunge, which we believe more closely mimics natural infection.

Throughout trials, in tanks containing co-mingled naïve and vaccinated muskellunge there were fewer instances of VHSV detection in tank water as well as lower mean viral concentration when compared to the tanks containing naïve muskellunge alone, possibly indicating decreased viral transmission or viral shedding. Further, the vaccinated muskellunge co-mingled in this tank exhibited a vigorous humoral response with significantly higher OD values than those obtained from vaccinated muskellunge alone. This heightened immune response likely resulted from increased viral exposure from co-mingling with susceptible muskellunge.

Though we demonstrated that this protective effect appears to be extended to additional susceptible species, largemouth bass, there were no significant differences in survivorship or viral concentrations within the water or tissues. However, we do demonstrate a semi-resistant species such as Chinook salmon was incapable of providing indirect protection. This finding allows us to show the importance of acquired immunity rather than just a decrease in the number of susceptible individuals within a tank is integral to protection. Moreover, the mean cumulative mortality of naïve muskellunge housed with Chinook salmon actually increased.

Based on the data we have generated in this study, it has become evident that herd immunity does exist in aquatic systems. We have demonstrated that naïve muskellunge are protected simply by co-mingling with immunized muskellunge. Further, we demonstrated that this protection can be extended to additional susceptible species. Finally, we show that the same protection does not result from the addition of a semi-resistant species, indicating that protection may be more than just physical in nature and is reliant on immunity.

Objective 5: To evaluate the feasibility/costs in eliciting a herd immune response in a Great Lakes fish population. Our modeling suggests that, under various assumptions of infection probability, mortality probability and clustering, vaccinated hatchery fish can significantly reduce infection rates in at-large fish populations, but eliminating the disease from a population may not be feasible particularly when recovered individuals may still infect susceptible individuals. Vaccinating hatchery fish may be a mechanism to prevent and/or minimize disease spread in at-risk populations when disease is persistent and mortality rates are high. We recommend further model-based evaluations of factors that can influence the efficacy of vaccination programs designed around the immunization and release of hatchery-propagated individuals in protecting wild fish populations against disease outbreaks; we believe it is likely that interest in using this type of vaccination program to protect wild fish populations will increase in the future. In particular, we believe it would be beneficial to explore how efficacy of

vaccination programs can be affected when behavior and dynamics of stocked individuals differ from that of wild conspecifics and how imperfect protection can influence the protective effect from the vaccination program. Given that disease dynamics integrate characteristics of the environment, pathogen, and host, we are of the opinion that spatially-explicit IBMs offer the best modeling platform for evaluating the effectiveness of vaccination programs in fish populations.

DISCUSSION OF RESULTS: A full version of the discussion and references is found in the appended manuscripts.

The emergence of VHSV-IVb in the Great Lakes basin necessitated the development of novel biological reagents for surveillance efforts. In this study, we developed a mAb against muskellunge Ig, allowing us to better characterize the immune response of this species, a highly susceptible species to this virus sublineage. We also gained unique insight into the structure of the muskellunge Ig molecule. Since muskellunge is indigenous to the entire Great lakes basin, the newly developed 3B10 mAb will have multiple uses in pathogen serosurveillance and the study of teleost immunology in general.

Though the initial plan was to use immunopurified immunoglobulin (Ig) from high-titered TNP-KLH immunized muskellunge, screening of resultant clones yielded negative results. In subsequent attempts, 20% SAS precipitate of naïve muskellunge seems to have produced semi-purified Ig and led to the successful development of 3B10 hybridomas that exhibited strong anti-muskellunge Ig antibody production. The western blot using purified muskellunge Ig and 3B10 demonstrated that the generated mAb primarily recognizes the Ig heavy chain. The molecular weight of the muskellunge Ig heavy chain is ~73 kDa. 3B10 specificity does not appear to be limited to muskellunge, as the mAb also reacts with northern pike (*Esox lucius*) and their hybrid: the tiger muskellunge. The fact that 3B10 reacts strongly, not only with muskellunge but also with northern pike and common carp (*Cyprinus carpio*), is advantageous as it extends the potential application of the 3B10 mAb in different serological assays of epidemiological significance.

Based on experiments performed in this study, the newly developed mAb allowed the development of an indirect ELISA which is simple and highly reproducible. We demonstrate the use of the indirect ELISA by detecting anti-VHSV-antibodies in one group of vaccinated fish as well as two groups of wild fish sampled from two VHSV enzootic areas. Additionally, the stringent optimization of our assays yielded optimal OD values very similar to those reported from similar indirect-ELISA results examining antibodies against other fish pathogens. Furthermore, coating with whole purified VHSV-IVb, appears beneficial in our assay when compared to other preparations; increasing efficiency as well as the available epitopes and allowing for the detection of the complete community of anti-VHSV antibodies.

The indirect ELISA was highly successful in detecting both the primary and secondary antibody responses in vaccinated muskellunge; underscoring the potential application of the developed ELISA in determining the kinetics of teleostean antibody response and efficacy of vaccines. This data highlights the application of the indirect ELISA in serosurveillance studies; we can detect water bodies where fish have been exposed without lethally sampling fish. Furthermore, we can monitor immune responses of fish in endemic areas, which may be used in the future to predict whether the populations are at risk for an outbreak. In summary, an anti-muskellunge mAb has

been developed which will be invaluable in the further study of this important North American species. Though we have applied this mAb in an indirect ELISA to detect anti-VHSV-IVb antibodies, the mAb has numerous applications in the study of the immune response of esocids against other pathogens.

This study further demonstrated that a DNA containing the VHSV-IVb G gene downstream of a CMV promoter can confer protection in muskellunge. Indeed, the time following exposure to an antigen and the water temperature have been demonstrated to have a significant effect on host immune responses in fish. The highest RPS (100%) was observed in trial 4 when muskellunge received two administrations of the pVHSivb-G preparation, while a single administration in trial 2 under otherwise similar conditions resulted in a mean of 95% RPS. Our finding that the carp β-actin promoter resulted in a 0-25% RPS suggests that there can be considerable variability among different promoter systems in different species.

We further demonstrate several novel aspects of post vaccine efficacy. For example, the indirect ELISA demonstrates that the pVHSivb-G plasmid elicited the development of a significant circulating VHSV-binding antibody response, which peaked around seven weeks post vaccination. In this study, the incubation period and subsequent challenge we utilized with muskellunge did not appear to correspond with peak anti-VHSV binding antibody levels. Peak binding antibodies levels may not correspond with peak protection; however, the magnitude of the response does appear to indicate the development of an adaptive response and involving long lived plasma cells. The details of the kinetics of the primary and secondary humoral response provides a valuable input for future vaccination and release strategies if this preparation is approved for use in aquaculture.

The finding that some vaccinated muskellunge, while protected, may also actively shed VHSV-IVb into the water column following exposure is troublesome; however, shedding was restricted to a small subset of vaccinated fish that survived the challenge with a relatively high virus dose. In the aquatic environment, it is highly unlikely that fish will be exposed to this viral concentration even in VHSV endemic waters.

In summary, we have described the successful use of a DNA vector containing the VHSV-IVb G gene in eliciting a protective immune response and the development of circulating anti-VHSV antibodies. We demonstrate even highly susceptible species such as muskellunge can be nearly 100% protected against VHSV. We demonstrate the usefulness of such a preparation for the protection of valuable species or broodstock. Certainly, there needs to be a more thorough examination of post vaccine efficacy and additional vector design before this preparation can be widely used. However, aquatic DNA vaccines have been certified and are in use in other countries. Regardless, the results we have obtained and the vaccination model we have developed can be used to examine the protection in other species such as Great Lakes salmonids and serve as a standard by which we can compare other vaccine preparations. Additionally, the development of an efficacious vaccine preparation allows us to more thoroughly study the VHSV-IVb immune response and novel concepts such as aquatic herd immunity.

Despite their relatively low susceptibility, field studies demonstrated that salmonids may contract VHSV-IVb infection. Salmonids experience moderate mortality and morbidity upon exposure to

VHSV-IVb and can act as a vehicle for viral dissemination, as evidenced by its isolation from apparently healthy fish. Faced with these facts and the growing presence of salmonid aquaculture facilities and hatcheries throughout the Great Lakes basin, we opted to determine if the pVHSivb-G preparation, originally designed for muskellunge, a highly susceptible species to VHSV-IVb, can also protect salmonids. Our results indeed demonstrated that we were able to minimize mortalities and provide significant protection to all four species. In heavily stocked aquaculture populations, a reduction in mortality by as little as 5% can lead to a profitable operation and vice versa.

Like other fish vaccines, the DNA vaccine was administered intramuscularly and resulted in significant protection in the three salmonid species representatives following a single administration, a matter that corroborates well with the previous study using muskellunge. Further, the greatest RPS was observed in both of the lake trout trials, where none of the 122 lake trout that received either one or two administrations of pVHSivb-G plasmid died following challenge.

In summary, we can now recommend the use of such a preparation for the protection of propagated salmonids prior to stocking into Great Lake waters where VHSV-IVb now appears to have become endemic. Though salmonids are less susceptible to genotype IVb, obtaining a protective vaccine will provide researchers and managers with a potent tool that can be used to limit transmission of VHSV-IVb.

We were also successful in producing a eukaryotic recombinant VHSV-IVb glycoprotein through the use of a recombinant baculovirus and cabbage looper larvae. The use of a 5' polyhistidine tag facilitated purification of the protein, and the ~65kDa indicates the glycoprotein is glycosylated. This finding led to our initiation of the vaccine trial where we determined that the rG was able to elicit a protective response in muskellunge and resulted in a mean of 80% RPS, which is nearly identical to protection reported (80.2% RPS) using a recombinant baculovirus system in rainbow trout.

Through the use of a eukaryotic larvae expression system, we have overcome the obstacles that previous studies have encountered; namely, obtaining conformationally correct protein. The ultimate success of our eukaryotic larvae system is highlighted when you compare the survivorship we demonstrate to previous studies.

In addition to its use as a protective vaccine antigen, the rG recombinant protein has applications in the development of serological assays. For example, when the purified recombinant glycoproteins were coated onto an ELISA plate alongside whole purified VHSV, there was minimal variation in observed OD values of positive muskellunge sera between coating treatments. This provided further evidence that the rG protein epitopes were conformationally correct and that anti-VHSV-IVb antibodies could not distinguish between the recombinant glycoprotein and glycoproteins on the whole virion. However, the whole VHSV virion overall did result in higher OD values. Though, this would be expected since antibody populations in the sera may be reacting to other viral epitopes on the VHSV virion while only limited epitopes are available for antibody binding in the wells coated with the rG protein. In summary, this study

illustrated the successful use of a recombinant baculovirus in a eukaryotic expression system to obtain purified VHSV-IVb glycoprotein.

We also used a novel study design to examine the concept of aquatic herd immunity against VHSV. The initial results demonstrate that when only half of the population is composed of vaccinated muskellunge, the naïve muskellunge experienced significant protection, demonstrating that, indeed, immune muskellunge provide significant indirect protection to naïve muskellunge. Using the other findings, we can begin to elucidate the cause of protection.

Based on the data we have generated in this study, it has become evident that herd immunity does exist in aquatic systems. We have demonstrated that naïve muskellunge are protected simply by co-mingling with immunized muskellunge. Further, we demonstrated that this protection can be extended to additional susceptible species. Though, the protective mechanism of cohabitation appears to be due in part to an overall decreased transmission, our findings also present the possibility of active viral neutralization by the vaccinated individuals; an effect that may be influenced by the apparent booster effect on the secondary humoral response detected in immunized muskellunge when housed with naïve muskellunge. In this fashion, cohabitation with naïve individuals may contribute to establishment of the critical immune threshold.

In summary, we have demonstrated that aquatic herd immunity can exist in a single species community. Whether this protective effect can be conferred to naïve individuals of additional species remains unclear. We encourage additional experimentation investigating cross-species protection through immunization using the exposure method and study we have described.

To our knowledge, ours is the first study to evaluate whether vaccinated hatchery fish can be used to protect wild fish populations against disease spread. Previous modeling studies of disease dynamics have pointed to the importance of fish stocking and biosecurity measures in hatchery systems to disease persistence in fish populations, but we have not encountered studies that have explored stocking as a means to prevent further disease spread. Disease has been suggested to be an important component in some fish populations. As such, it is important to understand both the disease dynamics and the potential management actions that can prevent disease spread and lead to sustainable and healthy fish populations.

Through our spatially-explicit IBM, we demonstrated that a protective effect could be elicited in a large fish population through a vaccination program designed around the immunization and release of hatchery-propagated individuals, at least under certain conditions. The degree of protection stemming from the vaccination program depended on factors such as infection rate, relationship between viral exposure and mortality due to disease, assumptions regarding fish clustering, and whether recovered individuals could periodically renew shedding of virus particles. When renewed shedding of virus particles could occur in recovered SIs, decreases in infection rate from immunization ranged from 52% to 61% under conditions of high infection and high probability of mortality, regardless of clustering assumptions and the stocking level. An even larger decrease in infection rate (65% decrease in infection rate) resulted from immunization under a condition of low infection rate, high probability of mortality, and high clustering. For most other conditions examined, vaccination decreased infection rates by anywhere from 28% to 42% across the stocking levels when renewed shedding of the virus could

occur. Vaccination similarly resulted in the largest relative increases in abundance under conditions of high infection and high probability of mortality regardless of clustering assumptions and under a condition of low infection, high mortality, and high clustering.

While the release of vaccinated hatchery-propagated fish lowered infection rates and increased population abundance, only in scenarios where recovered individuals could not resume shedding of virus particles did we observe cases where infection rate dropped to 0% in the last 10 years. In some cases, infection rates dropped to 0% even in the absence of vaccinating hatchery fish when recovered individuals could not resume shedding. We draw two main conclusions from this modeling result. First, it suggests that renewed shedding of virus particles by recovered individuals can be a significant factor affecting persistence of diseases in endemic waterbodies. The second conclusion is that to elicit a full herd immunity response in a population such as Lake Michigan Chinook salmon against a virus exhibiting characteristics similar to what was considered herein would likely necessitate a major increase in stocking levels.

3) **Financial Report and Operating Statement:** Please see separate attachments.

4) **Attachments:**

a. Narrative: Pages 3-27 of this document.
b. Six manuscripts are attached:

Appendix I. Standish IF, Millard EV, Brenden TO, Faisal M. 2016. A DNA vaccine encoding the viral hemorrhagic septicemia virus genotype IVb glycoprotein confers protection in muskellunge (*Esox masquinongy*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and lake trout (*Salvelinus namaycush*). Virology Journal 13: 203 DOI: 10.1186/s12985-016-0662-8.

Appendix II. Standish IF, Brenden TO, Faisal M. 2016. Does herd immunity exist in aquatic animals? International Journal of Molecular Sciences 17: 1898. DOI: 10.3390/ijms17111898.

Appendix III. Millard EV, LaPatra SE, Brenden T, Bourke AM, Fitzgerald S, Faisal M. In Press. DNA vaccination partially protects muskellunge against viral hemorrhagic septicemia virus (VHSV-IVb). Journal of Aquatic Animal Health.

Appendix IV. Faisal M, Standish IF, Vogelbein MA, Millard EV, Kaattari SL. Accepted for publication. Production and characterization of a monoclonal antibody against muskellunge (*Esox masquinongy*) immunoglobulin and development of an indirect ELISA for detection of circulating antibodies against viral hemorrhagic septicemia virus genotype IVb. Fish & Shellfish Immunology.

Appendix V. Standish IF, Faisal M. In review. A recombinant viral hemorrhagic septicemia virus genotype IVb glycoprotein produced in cabbage looper larvae (*Trichoplusia n*i) elicits antibody response and protection in muskellunge (*Esox masquinongy*). Target Journal: Journal of Aquatic Animal Health. [PLEASE LIMIT ACCESS TO THIS MANUSCRIPT UNTIL THE REVIEW PROCESS HAS BEEN COMPLETED.]

Appendix VI. Ivan LN, Brenden TO, Standish IF, Faisal M. In preparation. Individual-based modeling of the effectiveness of using vaccinated hatchery fish to prevent disease spread in a wild fish population. Target Journal: Ecological Applications. [PLEASE LIMIT ACCESS TO THIS MANUSCRIPT UNTIL THE REVIEW PROCESS HAS BEEN COMPLETED.]