ELICITING PROTECTION AND HERD IMMUNITY IN FISH AGAINST VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

By

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ABSTRACT

ELICITING PROTECTION AND HERD IMMUNITY IN FISH AGAINST VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

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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 are still no approved preventative measures against this virus. This dissertation seeks to address these knowledge gaps and investigate protective measures for eliciting immunity against VHSV-IVb. My studies focused primarily on muskellunge (*Esox masquinongy*) due to their high susceptibility and vigorous immune response following VHSV-IVb exposure. To elucidate the role of the muskellunge humoral immune response against VHSV-IVb, I first developed a monoclonal antibody (mAb), designated 3B10, against muskellunge immunoglobulin (Ig). The development of the 3B10 mAb allowed for the creation of a muskellunge-specific indirect ELISA to detect anti-VHSV antibodies. I demonstrate the use of this indirect ELISA assay 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 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, I then compared the protective

efficacy of two plasmids, differing only in their promoter sequence. 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. However, I found that immunized-protected muskellunge could still harbor and shed VHSV following viral challenge. Building from these results, I 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, I examined whether recombinant technology could provide similar protection. I expressed and purified a eukaryotic VHSV glycoprotein in cabbage looper (*Trichoplusia ni*) larvae using a recombinant baculovirus. After confirming the correct three-dimensional shape of this recombinant glycoprotein, the protective efficacy was assessed in muskellunge and resulted in 80% mean RPS. The culmination of my studies used the successful vaccination regimen and assays that I developed to investigate whether immunized fish can provide indirect protection to naïve fish. To accomplish this, I 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. The end result of this dissertation is an increased knowledge of the immunity against VHSV-IVb, as well as repeatable methods for eliciting this immunity.

This dissertation is dedicated to my love of fishing, family, friends and my dog Fin.

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Introduction

Viral hemorrhagic septicemia virus (VHSV) was first isolated in the Great Lakes basin over a decade ago (Elsayed et al. 2006). The newly emerged strain represented a novel freshwater genotype (IVb) documented to affect 28 species (Animal & Plant Health Inspection Service 2009). The detrimental effects of VHSV-IVb became evident with increasing mass mortality events involving numerous species throughout the basin (Faisal et al. 2012). The drastic spread and unknown nature of this virus underscored the urgent need to investigate this novel genotype as well as spurred increased funding and research opportunities. Researchers investigated viral pathogenicity and host susceptibility, revealing the disease course and susceptibility varied between fish species (Al-Hussinee et al. 2010; Kim & Faisal 2010a; b; c; Al-Hussinee et al. 2011). Though, the exact mechanisms that cause variability in VHSV infection and the resulting host immune response to genotype IVb are not well understood. However, several advances have been made to elucidate the mechanisms of protection against VHSV. For example, following exposure, survivors do appear to develop a specific immune response that protects against subsequent exposures (Bernard et al. 1983; Kim & Faisal 2012; Millard 2013). This finding provided researchers with the goal of understanding the mechanisms and dynamic of the host immune response against VHSV as well as the possibility of artificially eliciting this response through vaccination.

The emergence of VHSV-IVb in the Great Lakes basin represents a substantial threat to Great Lakes fish, as numerous ecologically and economically important species are highly susceptible to genotype IVb, including muskellunge (*Esox masquinongy*), largemouth bass (*Micropterus salmoides)* and freshwater drum (*Aplodinotus grunniens*). Additionally, susceptible species are

propagated in numerous state, federal and tribal hatcheries located throughout the basin. To date, VHSV has not been found in a hatchery facility in the Great Lakes basin, though this remains an active threat and weighs heavily on the minds of managers and researchers. There are no proactive preventative measures currently approved for use, and prevention is limited to strict biosecurity measures. A vaccine preparation would prove highly beneficial for protecting the dense naïve populations found in culture facilities. Previous studies have already demonstrated that various vaccine preparations can indeed elicit a protective immune response against other VHSV genotypes (Lococq-Xhonneus et al. 1994; Lorenzen et al. 1998; Lorenzen et al. 2000; Chico et al. 2009; Einer-Jensen et al. 2009). Though, few studies have examined the host immune response or vaccine preparations against VHSV-IVb. To this end, this dissertation describes the development and use of several tools that will aid in the study of immunity and vaccinology against VHSV-IVb. First, in Chapter One, I present a review of literature representing the extent of current knowledge on VHSV, associated immunity, and vaccination, which I use as the basis for the formulation of my study objectives. For example, rainbow trout (*Oncorhynchus mykiss*) is often the species of choice for many studies, though this species exhibits lower susceptibility to VHSV-IVb. Therefore, throughout this dissertation, muskellunge was selected as the primary species of study because this ecologically and economically keystone species exhibits high susceptibility to VHSV-IVb. Moreover, numerous studies have described the high susceptibility (Kim & Faisal 2010c), vigorous humoral immune response (Millard et al. 2012a; b; Millard 2013) as well as the viral shedding dynamics (Kim & Faisal 2012) associated with muskellunge and VHSV-IVb. Further, muskellunge populations have declined; resulting in initiation of hatchery propagation programs, providing a reliable fish source.

In Chapter Two, I describe the development of monoclonal antibody (mAb) against muskellunge immunoglobulin (Ig) which will prove valuable in the study of muskellunge immune response and assay development, namely an indirect ELISA for the detection of specific populations of muskellunge antibodies. I develop and optimize an indirect ELISA, which is then employed to examine anti-VHSV binding antibodies. I validate the assay in two ways: by detecting anti-VHSV antibodies in wild muskellunge from the Detroit River (Detroit, MI, USA) thereby indicating previous viral exposure and by measuring the primary and secondary immune response in individual muskellunge following immunization with a DNA vaccine.

Based on the results of Chapter Two, I compare in Chapter Three two DNA plasmids for their ability to elicit a protective response in muskellunge. Both plasmids contain the VHSV-IVb glycoprotein (G) gene but have different promoter sequences, either a carp β-actin or cytomegalovirus (CMV). The design of these plasmids is based on previous studies, which determined that the G gene represents the primary neutralizing VHSV surface antigen (Lorenzen et al. 1990; Jørgensen et al. 1995; Wagner & Rose 1996) and efficient transcriptional activity using these promoters in fish (Chico et al. 2009: Einer-Jensen et al. 2009). The immune response and efficacy associated with these preparations is first examined in muskellunge. Guided by the results of Chapter Three, I select the most efficacious plasmid preparation and determine whether the same plasmid can be used to elicit protection in three salmonid species. In Chapter Four, I examine the efficacy of a DNA plasmid preparation in three representative species: rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*). These species were selected to represent the range of salmonid genera regularly propagated throughout the Great Lakes basin.

In Chapter Five, I explore recombinant technology to develop a vaccine preparation, as the Food and Drug Administration (FDA) has not approved the use of DNA based vaccines for commercial aquaculture in the United States. Recombinant preparations, on the other hand, appear promising and comparatively innocuous. I produced and purified a eukaryotic recombinant VHSV glycoprotein preparation using a recombinant baculovirus vector containing the viral G gene to infect cabbage looper (*Trichoplusia ni*) larvae. After confirming glycoprotein is conformational correct, I assessed the efficacy of the protein in provoking a protective immune response in muskellunge.

The reagents, assays and preparations and vaccine regimens that I developed in the initial Chapters of this dissertation then allowed me to examine the concept of aquatic "herd immunity." In Chapter Six, I designed a novel study using multiple flow-through tanks to ensure equal viral exposures and examined whether protection is conferred to naïve individuals when co-mingled with immunized individuals. Naïve and immunize muskellunge were housed alone, co-mingled together or in tanks containing largemouth bass or Chinook salmon (*Oncorhynchus tshawytscha*), representing another susceptible species and a semi-resistant species, respectively (Kim & Faisal 2010b). Various response measures were compared between these different populations to determine if indirect protection is conferred from immunized to naïve individuals. The culmination of the tools, methodology and results of the following dissertation is a greater understanding of the host immune response against VHSV-IVb as well as several vaccine preparations and methodology that can now be used to proactively prevent VHSV infection.

Chapter One

Literature Review

1. VHSV-IVb characteristics and emergence

Viral hemorrhagic septicemia virus (VHSV) is a serious pathogen of finfish worldwide and a member of the genus *Novirhabdovirus*, family Rhabdoviridae in the order Mononegavirales. To date, VHSV has been documented to infect over 80 marine and freshwater species in the northern hemisphere and is a World Organization for Animal Health (OIE) reportable fish pathogen (OIE 2009). Initially, VHSV was recognized as only affecting freshwater species and primarily cultured rainbow trout. However, in 1979, VHSV was isolated from Atlantic Cod (*Gadus morhua*) from the coastal waters of Zealand, Denmark (Jensen et al.1979), which is recognized as the first isolation of VHSV in free-living fish in the marine environment. Isolations were restricted to Europe until 1988 when VHSV was detected in ovarian fluid from apparently healthy Chinook salmon (*Oncorhynchus tshawytscha*) returning to the Glenwood Springs Hatchery on the Orcas Island, Washington, U.S.A. (Hopper 1989) and coho salmon (*O. kisutch*) returning to the Makah National Fish Hatchery near Neah Bay, Washington, USA (Brunson et al. 1989). Over the next decade there were several isolations from wild fish in both brackish and marine environments along the Pacific coast including isolations from salmon in Washington (Eaton & Hulett 1991), Pacific cod (*Gadus macrocephalus*) and Pacific herring (*Clupea pallasii*) from Prince William Sound, Alaska (Meyers et al. 1992; 1994) and California (Cox & Hedrick 2001; Hedrick et al. 2003). In the years that followed, VHSV was isolated from 18 different fish species in Pacific waters (Kocan et al. 2001; Mork et al. 2004; Skall et al. 2005a). It soon became evident that VHSV was causing significant mortalities in free living fish

and possibly having population level effects (Marty et al.1998). For example, in 1993, two thirds of the 1988 year class (five year old fish) of Pacific herring from Prince William Sound and the Kodiak islands, Alaska, USA disappeared and 15-43% of fish exhibited external ulceration and hemorrhage (Meyers et al 1994). Numerous mortality events occurred along the Pacific coast and VHSV was isolated in all these incidents (Meyers & Winton 1995; Meyers et al. 1999).

This expansion of VHSV, led to a race to characterize the viral genome and compare geographically unique isolates. Comparison of the North American and European isolates using ribonucleic fingerprinting showed two geno-groups and indicated that the North American isolates did not originate from Europe (Oshima et al. 1993). Subsequent sequence analysis using restriction fragment length polymorphisms (RFLP) of polymerase chain reaction (PCR) products were able to accurately differentiate isolates into four major genotypes (I-IV) using three restriction endonucleases (Einer-Jensen et al. 2005) and further analysis showed that isolates differed along geographic trends (Bernard et al. 1992; Basurco et al. 1995; Benmansour et al. 1997; Stone et al. 1997; Snow et al. 1999; Thiery et al. 2002; Snow et al. 2004; Einer-Jensen et al. 2004; Einer-Jensen et al. 2005).

Genotype I isolates has been further subdivided into five sublineages designated (a through e). Genotype Ia is mainly associated with farmed rainbow trout throughout Europe but has also been associated with isolations in a few marine fish from the English Channel (Dixon et al. 1997). The remaining subtypes within the Genotype I group are found throughout Germany, Austria (Jonstrup et al. 2009), the Baltic Sea, Skagerrak, Katterat (Snow et al. 2004) and the Black Sea (Nishizawa et al. 2006). Genotype II was differentiated from Genotype I by N and G gene

sequence variation and isolates have been primarily found in Atlantic herring (*Clupea harengus*) in the Baltic including the Gulfs of Bothnia and Finland (Snow et al. 2004; Gadd et al. 2010). Genotype III includes marine isolates from farmed and wild fish from the North Atlantic, Flemish Cap (Lopez-Vaquez et al. 2006), Norwegian Coast (Dale et al. 2009), the North Sea and the waters surrounding the British Isles (Ross et al. 1994; Snow et al. 2004). Genotype IV includes all isolates from North America, Japan and Korea (Stone et al. 1997; Nishizawa et al. 2002; Kim et al. 2003). In Japan, VHSV genotype IV was first noted in 1999, when it was isolated from wild Japanese flounder (*Paralichthys olivaceus*) caught from the Wakasa Bay of Japan (Takano et al. 2000; Watanabe et al. 2002). Shortly thereafter the disease was identified as the causative agent of a viral disease observed in farmed Japanese flounder in Japan and Korea (Isshiki et al. 2001; Kim et al. 2003).

In 2003, a virus was isolated from spleen and kidney tissue homogenates of adult muskellunge (*Esox masquinongy*) collected from the Grosse Pointe Yacht Club, Lake St. Clair, Michigan, USA (Elsayed et al. 2006). Electron microscopy showed a virus with a bullet shaped morphology characteristic of rhabdoviruses. This was the first indication of VHSV in the Great Lakes basin. Phylogenetic analysis confirmed the isolate as VHSV and showed this Great Lakes strain was most closely related to the previously characterized North American and Asian genotype (IV) but was unique and thus designated as a novel genotype IVb (Elsayed et al. 2006). The spread of VHSV-IVb throughout the Great Lakes basin has been thoroughly documented and has been reviewed by Faisal et al. (2012; and references cited therein). As the causative agent of numerous mass mortalities, it is increasingly evident that VHSV represents a significant threat to global fisheries, wild and farmed alike. In addition to ecological impact, VHSV also causes serious

economic losses, yet details of the economic losses are not universally available. For example, VHSV caused an estimated 40 million lbs loss in European aquaculture in 1991 (Hill 1992) and an outbreak in 2000 on just two Danish rainbow trout farms resulted in approximately 50% mortality and a 211,000 € loss (Nylin & Olesen 2001).

In fish, VHSV causes the destruction of the endothelial lining of blood vessels (de Kinkelin 1979; Evensen et al. 1994) causing petechial to ecchymotic hemorrhages in the meninges, cutaneous and subcutaneous surfaces, muscles, internal organs and eyes leading to ascites, exophthalmia, and severe anemia and gill pallor (Skall et al. 2005a). The primary transmission route is horizontal waterborne transmission which has been demonstrated in with Pacific herring (Kocan et al. 1997; Hershberger et al. 2010; Hershberger et al. 2011a). Disease outbreaks typically occur at water temperatures from 3-12°C and are more severe at lower temperatures. VHSV-IVb also has a very large host range, with 28 Great Lakes fish species listed as susceptible (Animal & Plant Health Inspection Service 2009). Also, the ability of VHSV-IVb to persist in leeches (Faisal & Schulz 2009), *Diporeia* (Faisal & Winters 2011) and turtles (Goodwin & Merry 2011) may indicate an even larger host range than previously believed and/or the ability of numerous species to serve as vectors.

2. VHSV genetic structure

VHSV-IVb has a similar morphology to other rhabdoviruses. The VHSV virion is a bullet-to bacilliform-shaped capsid with a helical symmetry encased by an envelope. Electron microscopy shows that the virion has an average width of 65 nm and a length of 180 nm. However, the observed virion size is highly dependent on preparation procedures (Olberding & Frost 1975).

The genome of the Great Lakes index strain (MI03GL) is composed of a single stranded negative-sense ribonucleic acid (RNA) backbone spanning 11,184 nucleotides (GenBank GQ385941). The genome contains six open-reading frames (ORFs) in the order 3'- nucleocapsid (N) – phosphoprotein (P) – matrix protein (M) – glycoprotein (G) – nonstructural protein (NV) – RNA polymerase (L) - 5' (Schutze et al. 1999; Ammayappan & Vakharia 2009) (Figure 1.1).

The first ORF codes for the N gene and consists of 1215 nucleotides, coding for 405 amino acids and a final translated protein of 38-44 kDa (Wagner & Rose 1996; Byon et al. 2006). This nucleoprotein is tightly arranged around the entire negative-sense RNA genome and forms an N-RNA complex that serves to protect the genome from RNases as well as serving as the framework for both transcription and replication activity (Wagner & Rose 1996; Jayakar et al. 2004).

The second ORF encodes the P gene, which consists of 669 nucleotides, coding for 223 amino acids. This is translated to form a non-catalytic phosphoprotein cofactor with a molecular weight of approximately 24 kDa (Byon et al. 2006; Ammayappan & Vakharia 2009). The phosphoprotein is part of the two protein complex that forms the viral RNA-dependent RNA polymerase. The second part of this complex is encoded by the last and longest ORF of the VHSV genome, the L gene: consisting of 5955 nucleotides encoding 1985 amino acids and forming a 157-224 kDa protein (Emerson & Yu 1975; Byon et al. 2006; Ivanov et al. 2011). The phosphoprotein also acts as a chaperone of nascent RNA-free nucleoprotein by forming a nucleoprotein-phosphoprotein complex that prevents the nucleoprotein from binding to cellular RNA, thus maintaining the nucleoprotein in a soluble form (Jayakar et al. 2004; Ivanov et al

2011). Further, it has been hypothesized that the availability of the nucleoprotein to encapsulate nascent RNA modulates the switch from RNA transcription to RNA replication (Leppert et al. 1979).

The third ORF encodes the M gene and spans 606 nucleotides and is translated into the 19-22 kDa matrix protein which acts as a bridge between the viral envelope and the nucleocapsid in rhabdoviruses (Wagner & Rose 1996; Byon et al. 2006). The M protein is the most numerous viral protein and plays a regulatory role in viral transcription, replication, production, morphology and budding in rhabdoviruses (Finke & Conzelmann 2003; Jayakar et al. 2004). Additionally, in other rhabdoviruses the M protein shows direct inhibitory effects on host transcriptional machinery by inhibiting host polymerases and preventing export of host RNA from the nucleus (Jayakar et al.2004).

The fourth ORF encodes the G gene and is composed of 1524 nucleotides coding for a 65-80 kDa protein. This glycoprotein represents the major surface protein and forms trimeric spike protrusions 5-10 nm in length, which are made of non-covalently associated monomers located on the viral surface membrane. The association of these monomers-trimers is manipulated by changes in pH, temperature and detergents (Coll 1995). These glycoprotein trimers are also important for both virus antigenicity and attachment to susceptible cells (Jørgensen et al. 1995; Wagner & Rose 1996; Boyn et al. 2006). Additionally, the glycoprotein is the only VHSV protein that has been found to be glycosylated and is reduced to 55kDa by deglycosylation (Lenoir & De Kinkelin 1975; Thiry et al. 1990). Several of the potential glycosylation sites have been shown to be highly important in proper glycoprotein trimer assembly (Coll 1995). The

glycoprotein is also believed to be the primary neutralizing surface antigen for VHSV and other rhabdoviruses (Lorenzen et al. 1990; Jørgensen et al. 1995; Wagner & Rose 1996). Moreover, certain fragments and particularly conformationally-dependent epitopes of the VHSV glycoprotein play a variable role in the neutralizing antibody response (Rocha et al. 2002).

The fifth and smallest ORF covers 369 nucleotides and encodes NV gene and is translated into an approximately 13.6 kDa nonstructural protein, believed to be related to virulence (Ammayappan & Vakaria 2009; Ammayappan et al. 2011).

Following initial attachment to host cells, the virus enters host cells through endocytosis triggered by engagement of cell surface receptors with the viral G protein (Purcell et al. 2012). The viral and endosomal membranes fuse and the nucleocapsid is released into the cell cytoplasm. The RNA-dependent RNA polymerase produces new copies of the RNA genome, creating six monocistrontic mRNAs from which proteins are synthesized by host protein synthesis machinery. New virions are packaged when the N, L and P proteins are synthesized by free ribosomes and bind to the newly synthesized genome to form the ribonucleoprotein (RNP) core. This core then associates with the M protein to form an RNP-M complex. The glycoproteins are synthesized by endoplasmic reticulum-bound ribosomes and are glycosylated prior to transportation, insertion and trimeric association into the host cell plasma membrane. The complete viral assembly process and budding is driven by this glycoprotein assembly on the host cell membrane (Coll 1995). The RNP-M complex migrates to the areas of concentrated glycoprotein in the plasma membrane and ruptures the membrane during budding to form a fully mature virion (Purcell et al. 2012).

Rhabdoviruses have conserved untranslated regions between open reading frames for optimal translation of viral proteins (Schnell et al. 1996). These sequences consist of a putative transcription stop/ polyadneylation motif which generates the poly-A tail. Following the poly-A tail there is a di-nucleotide space (G/AC), which are transcribed and act as spacers between the two genes which the polymerase will skip before continuing transcription of the next gene (Ammayappan & Vakharia 2009). VHSV genes specifically are separated by conserved gene junctions that contain di-nucleotide gene spacers. The first 4 nucleotides at the termini of the VHSV genome are complementary and identical to other novirhabdoviruses genomic termini (Ammayappan & Vakharia 2009). The overall structure between different genotypes and isolates is conserved, however point mutations are common. For example, Maximum Likelihood and Bayesian analysis of the VHSV-IVb G gene sequence shows that the gene is changing at an estimated mutation rate of 2.58×10^{-4} substitutions per site per year (Stepien et al. 2015). Similarly, the N gene exhibited 4.26×10^{-4} substitutions per site per year. The NV gene, however, is the fastest evolving gene, evolving approximately seven times faster with 1.25×10^{-3} to 2×10^{-3} substitutions per site per year (Pierce & Stepien 2012; Stepien et al. 2015).

3. Fish immunity to viruses and host immune response to VHSV

Although teleost are considered an ancestral vertebrate, the teleost immune system has many potent attributes that can be compared with divergent vertebrate groups. Interestingly, many features of the mammalian immune system first appeared in jawed fishes; the mechanism of homolog V(D)J recombination (RAG expression), lymphocytes expressing major histocompatibility complex (MHC), immunoglobulin (Ig) and T cell receptors all arose in jawed

fishes (Rast & Litman 1998; Boschi et al. 2011). The emergence of these adaptive immune mechanisms in fish represents a substantial bridge between lower cephalochordates and higher teleost and has been review by Zhu et al. (2013). However, since fish are poikilothermic, immune response dynamics are highly environmentally dependent (Uribe et al. 2011).

It is evident that modern day teleosts still possess unique innate, humoral and cell-mediate immune mechanisms. For example, the teleost innate immune system has 17 toll-like receptors (TLRs) many of which are specific to teleosts and recognize conserved pathogen associate molecular patterns (PAMPs) (Palti 2011; Zhu et al. 2013). As in mammals, activation of these TLRs leads to cytokine production, a non-specific proliferation of B cells, and increased specific antibody production (Pasare & Medzhitov 2006; Uribe et al. 2011; Abos et al. 2013). Though, the exact function of some of these TLRs remains to be fully elucidated (Jault et al 2004; Liu et al. 2010; Avunje et al. 2011). Homologs of RIG-I-like receptors or RLRs and nucleotide-binding oligomerization domains (NLRs) have also been identified in teleosts and described to play a role in antiviral activities. These include RIG-I in grass carp and channel catfish (Rajendran et al. 2012, Yang et al. 2011a) and LGP2 and MDA5 in rainbow trout and grass carp respectively (Chang et al. 2011a, Su et al. 2010). Teleosts also have a fully developed classical complement system that assists in the opsonization and phagocytosis of pathogens (Dodds & Petry 1993; Zhu et al. 2013). Numerous interferon (IFN) genes have also been identified in teleosts. The classification of these interferons is inconclusive as most reports classify teleost IFNs as type I based on structural similarities while other authors suggest that teleost IFNs should be classified as type III IFN based on the genes being encoded by four introns and a receptor structure similar to type III IFN (Robertsen 2006; Zhu et al. 2013). The role of IFN in teleosts is integral in

modulating the protective antigen-specific immune response and antiviral activity (Chico et al. 2010).

Unlike higher vertebrates, teleosts lack bone marrow and the anterior kidney is believed to be the major hematopoietic organ (Zapata et al. 2006) as well as the primary location where B cells differentiate (Castro et al. 2013). The anterior kidney possesses a variety of blood cells that are maintained throughout the life of a fish. Following production in the anterior kidney, B lymphocytes can exit the tissue and traffic via the blood to the spleen or posterior kidney. There is evidence that it is in these two locations B lymphocytes are exposed to foreign antigens and then differentiate into plasmablasts (replicating, low antibody secretors, bearing minimal BCR) or into plasma cells (non-replicating, terminally differentiated, high antibody secreting cells, bearing no BCR). Plasma cells can be further broken down into two distinct populations: the short lived plasma cells (SLPC) and long-lived plasma cells (LLPC) (Ye et al. 2010). The anterior kidney harbors plasma cells but may not allow differentiation into plasmablasts, as LLPC can be found only in the anterior kidney of immunized fish and thus the primary source of prolonged humoral response in fish (Ye et al. 2010). Also, it would appear that a high titer humoral response is dependent on the continued activation of LLPC as the half-life of rainbow trout IgM can be as short as two days (Ye et al. 2010). One study indicated that a few naïve B cells may only differentiate into SLPC and these lower affinity B cells then undergo somatic hypermutation (Ye et al. 2011). The authors found a significant shift in affinity of circulating antibodies at a point that coincided with the development of LLPC within the anterior kidney (12-15 weeks following immunization). Overall, the highest affinity subpopulations do appear

much more persistent than the lower affinity subpopulations which occur earlier in the immune response (Ye et al. 2011).

The spleen acts as a secondary lymphoid organ and contains primarily resting B cells and is therefore an important site for both B and T cell activation and response (Zwollo et al. 2008; Salinas et al. 2011). The spleen is also the location of B cell clonal expansion (Weinstein et al. 2009). Circulating B cell are very numerous, in some cases accounting for up to 30-40% of the cells in peripheral blood of teleosts (Abos et al. 2013) and are easily detectable in the intestine (Rombout et al. 2011), skin (Wolfe et al. 2009) and gills (von Gersdorff Jørgensen et al. 2011).

On a genomic level, teleosts have limited somatic hypermutation and lack highly efficient affinity maturation (Zhu et al. 2013). Three heavy chain isotypes have been identified; with μ and δ corresponding to IgM and IgD and τ which codes for IgT and is unique to teleosts (Hansen et al. 2005; Danilova et al. 2005; Zhang et al. 2010). The location of these genes rules out class switching between constant regions, with the D and J segments restricted to the nearby C region and there is no Dτ or Jτ in IgM heavy chain cDNA (Hansen et al. 2005; Danilova et al. 2005). Therefore, IgM and IgT are never co-expressed by the same B cell as isotypic communication and class switching cannot occur (Schorpp et al. 2006). Current theories point to IgM being an integral systemic immunoglobulin, expressed as a tetramer or less commonly as a monomer (Chem 1971). The IgM+ B cells of teleost fish are also highly phagocytic and possess potent intracellular killing mechanisms (Li et al. 2006). The IgD immunoglobulin is produced by alternative splicing of mRNA (Edholm et al. 2011). In a recent study, Edholm et al. (2011) found that circulating IgD+ B cells lacked V regions, and activation occurs similarly to pattern

recognition molecules, indicating IgD as molecule involved in the innate immune response. Only recently, research and sequencing data from vertebrate and teleost genomes shed light on a new immunoglobulin isotype: IgT (Hansen et al. 2005) also designated IgZ in (Danilova et al. 2005). Fish lack IgA, so IgT appears to act primarily in association with mucosal defenses. Recent findings have demonstrated that IgT is integral to mucosal immunity and has a specific anti-pathogenic function in the gut similar to IgA in warm blooded animals and IgX in amphibians (Mussmann et al. 1996; Zhu et al 2013). IgT+ B cells constitute between 16-28% of trout B cells in the blood, spleen, head kidney and peritoneal cavity (Abos et al. 2013). In serum IgT is monomeric, while in the gut mucosa it was abundantly expressed in a polymeric form and associated by non-covalent interaction. Further, IgT has been found to play an integral role in the defense of pathogen invasion via gill tissue, as fish gill lamellae are considered a mucosal surface, constituting the main Ig isotype (51%) coating gill microbiota (Xu et al. 2016). Also, Zhang et al. (2010) provided evidence for a population of B cells that only express surface IgT and represent the primary population (54.3%) in gut associated lymphoid tissue. These IgT+ B cells however, lacked expression of T cell specific genes.

Numerous studies have examined the immune response against VHSV specifically. Following the initial exposure to VHSV in immersion challenges, viral replication was observed as early as 8 hrs at the base of fins (Harmache et al. 2006) and at similarly early time points in the gills (Brudeseth et al. 2002). From fin and gills, VHSV then spreads to internal organs (Harmache et al. 2006; Kurath 2008). Following an experimental VHSV challenge in olive flounder, there was an increase in TLR 2 and TLR 7, interleukin 8, and several interferon regulatory genes as soon as 3 hours post infection. By 12 hours post infection, viral concentrations had markedly increased

and remained high until 2 days post infection during which there was also an increase in the expression of IL 1β, IRF3, IRF7, and type I IFN genes (Avunje et al. 2011). After two days, the N gene mRNA was the most abundant viral transcript in infected tissues (Avunje et al. 2011) and up to 51.4-fold higher in fins than in an internal organ pool (Encinas et al. 2010). In another study examining experimentally infected and vaccinated fish, VHSV provoked an early up-regulation of IgM, IgT and CD3 + cells, and MHCII mRNA levels (Aquilino et al. 2014). When VHSV reaches a systemic viral infection in rainbow trout, IgM represents the main isotypic response in the spleen. IgD expression repertoires are also modified, but to a lower degree, suggested that IgD expressing B cells either lose their IgD expression or are relocated (Castro et al. 2013). VHSV is then shed through kidney and urinary tract and exits the host (Neukrich & Glass 1984). The idea that the kidneys are the major source of virus was corroborated, when tissue sections were examined using immunohistochemistry (IHC), revealing the presence of VHSV throughout the interstitium and tubular epithelia of the kidneys (Brudeseth et al. 2002; Al-Hussinee et al. 2010).

Innate and adaptive cell mediated immunity also represents a major protective mechanism against viral infections such as VHSV. Innate non-specific cell mediated killing has been described in several species (Secombes, 1996; Fischer et al. 2006).These cells are generally described as natural killer (NK) like cells or NCC (Ishimoto et al 2004; Shen et al. 2004; Cuesta et al. 2005; Sakata et al. 2005). Additionally, allospecific responses have been described in rainbow trout (Fischer et al. 2003) and ginbunas carp (*Carassius auratus langsforfi*) (Fischer et al. 1998; Hasegawa et al 1998). The adaptive cellular response appears to be similar to mammals where CD8+ T cells kill cells presenting viral peptides via the MHC class I (Pauly et al. 1995;

Parner et al. 1998; van Endert 1999). Though this exact mechanism has not been demonstrated in teleost, MHC class I (Fischer et al 2006; Klein & Sato 1998), TCR and CD8 (Fischer et al. 2006) homologs have been identified in fish. Functional studies suggest that expression, antigen presentation, and the mechanism of killing is similar to mammalian immunity (Dijkstra et al. 2003; Utke et al. 2007). Studies appear to differ in their findings of innate killing by NK-like cells of allogeneic (genetically dissimilar but derived from the same species) cells. For example, it has been demonstrated that NK-like cells from channel catfish (*Ictalurus puncatus*) can cause lysis allogeneic cells (Hogan et al. 1996) while NK-like cell from ginbuna crucian carp and rainbow trout cannot (Fischer et al. 1998; 2003; Utke et al. 2007). However, when rainbow trout are exposed to VHSV it does appear that peripheral lymphocytes are able to lyse VHSV infected allogeneic and xenogeneic (different species) cells (Utke et al. 2007). This is suggestive of involvement of both a cytotoxic T lymphocyte (CTL) and NK-like immune responses. Other studies similarly indicate a clonal expansion of specific cytotoxic T lymphocytes associated with rhabdovirus infection (Boudinot et al. 2001; Utke et al 2007). One study in particular demonstrated that peripheral lymphocytes isolated from rainbow trout vaccinated with a DNA vector containing the VHSV G gene were able to lyse both VHSV infected allogeneic and xenogeneic cells, while fish that received the VHSV N gene only developed an adaptive CTL response (Utke et al. 2008). These finding are indicative of the heavy involvement of a cell mediated response in providing protection.

4. Fish viral vaccines

RNA viruses and rhabdoviruses specifically are particularly troubling for fishery researchers and managers. In an effort to compact these pathogens scientists have been exploring vaccines for

decades. Various types of preparations and delivery methods have been examined. These have included using inactivated virus, prokaryotic or eukaryotic recombinant proteins, as well as DNA or other vectored delivery systems. For example, substantial efforts have been made to develop a vaccine against two widespread viruses, the Infectious pancreatic necrosis virus (IPNV) an *Aquabirnavirus* and Infectious salmon anemia virus (ISAV) an *Isavirus*. IPNV vaccine trials have included both DNA and recombinant baculovirus vectors, while trials have been conducted for ISAV have used inactivated virus and DNA plasmids (Gomez-Casado et al. 2011). Internationally, some of these preparations are already in use on a large scale. Norway, Canada and Chile have adopted commercially available IPN and ISA inactivated multivalent vaccines that are delivered through an intraperitoneal injection for use in aquaculture (Gomez-Casado et al. 2011). The use of these vaccines appears promising, however, the US Food and Drug Administration (FDA) has yet to approve the use of either DNA-based or attenuated preparations for commercial use in aquaculture or a field setting because of fear the virus may revert back to the wildtype or have unpredicted genetic consequences, such as incorporation in the host genome.

The first clinical VHSV vaccine trials tested using altered virus; either inactivated (killed) or attenuated (weakened). In 1995, de Kinkelin et al. surveyed VHSV vaccination protocols and showed that vaccination with inactivated virus conferred some protection to rainbow trout. Also, using an attenuated preparation and immersion delivery, vaccinated rainbow trout fry showed increased survival following VHSV challenge; however, commercial applications were limited as the virus maintained some of its' virulence (de Kinkelin et al. 1981).

Work towards a VHSV vaccine was advanced using monoclonal antibodies to identify and characterize viral determinants important for an antibody driven immune response, as early studies indicated that the production of neutralizing antibodies is important in the anti-VHSV immune response (Jørgensen 1971; Lorenzen et al. 1988). Proof of the importance of neutralizing antibodies came when Lorenzen et al. (1990) inoculated rainbow trout with neutralizing monoclonal antibodies for the glycoprotein gene (G) and showed their protective ability against a VHSV challenge. Moreover, fish immunized with donor sera from fish previously exposed to VHSV showed the presence of neutralizing antibodies and some passive protection (Hershberger et al. 2011b). Numerous studies have subsequently demonstrated the VHSV G protein to be the major target protein for neutralizing and protective antibodies (Bearzotti et al. 1995; Lorenzen et al. 1990; 1999; Einer-Jensen et al. 2004). Similarly, the G protein has been reported to be the neutralizing target protein for other rhabdoviruses (Huang et al. 1994; Kelley et al. 1972; Wiktor et al. 1973). In muskellunge specifically, following an experimental infection with VHSV-IVb, a neutralizing antibody response was first detectable by weeks 5-7 post challenge and led to a corresponding drop in viral titers in serum (Millard 2013). The immune response was more vigorous in fish that had been vaccinated with a DNA vaccine using VHSV-IVb G gene, detecting neutralizing antibodies after just four weeks (Millard 2013). Another study used pepscan mapping to show that two regions (amino acids 280 - 310 and 340 - 370) of the VHSV G gene had the ability to increase the type I IFN immune response (Chico et al. 2010). Thus the G gene has become the predominant target in the creation of subsequent vaccines.

Subsequent attempts to create a VHSV vaccine have used recombinant technology. Recombinant vaccines can be divided into two classes: 1) a recombinant or alteration of the original virus or 2) a subunit or single gene of the virus is amplified to produce a small fragment of the viral genome which is then cloned into another organism. The organism produces the encoded protein, which can then be purified and used as the inoculum. There have been numerous studies indicating success using recombinant vaccines for VHSV. Initially, Lorenzen et al. (1993) demonstrated that the VHSV G gene, cloned and expressed in *Escherichia coli* (*E. coli*) and then inoculated into rainbow trout induced antibody formation and minimal protection from viral challenge. Studies have repeated this work with comparable results when using both bacteria (*E. coli* and *Yersinia ruckeri*) and yeast (*Saccharomyces cerevisiae*). However, the efficacy of recombinant preparations may be limited and inconsistent because of improper protein folding which is attributed to a lack of post-translational alterations such as glycosylation and usually does not occur in prokaryotic expression systems such as (*E. coli* and *Yersinia ruckeri*) (Thiry et al. 1990; Estepa et al. 1994; Lecocq-Xhonneux et al. 1994; Lorenzen et al. 1999). In an attempt to subvert this improper protein folding, future recombinant vaccines will likely use eukaryotic protein expression systems. For example, in yeast (*Saccharomyces cerevisiae*), rhabdovirus G proteins are indeed glycosylated and associated with the plasma membrane (Klepfer et al. 1993). Work has also been conducted using a recombinant subunit vaccine produced in insect cells through the use of a baculovirus vector. The recombinant baculovirus containing the glycoprotein gene was used to infect insect cells. The infected cells induced the production of neutralizing antibodies and conferred immunity to rainbow trout fry during a VHSV challenge (Lecocq-Xhonneux et al. 1994). With another rhabdovirus, infectious hematopoietic necrosis virus (IHNV), a recombinant vaccine was capable of stimulating an

innate immune response mediated by increased leukocytes and pro-inflammatory cytokines production in rainbow trout immunized with a soluble non-glycosylated recombinant IHNV G gene vaccine (Verjan et al. 2008). Also a recombinant vaccine where the IHNV G protein was exchanged for the VHSV G protein was able to induce antibody formation similar to the VHSV wildtype virus (Romero et al. 2008). However, few studies have thoroughly investigated the efficacy of these preparations, particularly in combination with an adjuvant. Further, mechanisms of producing a large quantity of recombinant protein have received no attention, which is surprising as recombinant preparations appear rather innocuous and thus more likely to gain FDA approval.

The most promising trend in VHSV vaccination is the use of DNA plasmid vectors and akin to the findings on recombinant vaccines, studies indicate that DNA plasmids induce a proliferative specific and nonspecific immune response (Boudinot et al. 1998; Kim et al. 2000; Boudinot et al. 2004; Purcell et al. 2004; Purcell et al. 2006). Naked DNA plasmids containing an insert of the gene of interest and a general or specialized promoter are generally delivered intramuscularly into the host. The goal being that the antigen encoded on the plasmid will continue to be produced by cells within the immunized individual over time (Donnelly et al. 1997). In fish, this technology was first used by Anderson et al. (1996), where a DNA plasmid containing either the IHNV -N or G gene was used to immunized rainbow trout. Fish intramuscularly immunized with the plasmid containing the G gene insert developed an antibody response and protection against an IHNV challenge. An analogous study was conducted for a VHSV vaccine encoding either the N or G genes. The data correspondingly shows that only the G gene induces the production of neutralizing antibodies resulting and protection in rainbow

trout (Lorenzen et al. 1998). Furthermore, the G gene elicited the early production of Mx protein and results in an early non-specific antiviral response (Kim et al. 2000).

The use of specialized promoters contained within a plasmid has also been explored for maintaining gene expression in teleosts specifically. A plasmid containing the VHSV glycoprotein gene under the control of the carp β-actin promoter (1^{st} exon and 1^{st} intron) has shown similar transcription efficiency and protection when compared to the human cytomegalovirus (CMV) promoter (Chico et al. 2009). The carp β-actin promoter exhibited greater transcriptional levels than a CMV promoter and protection was similar following a VHSV challenge (Chico et al. 2009). In another study, fish developed a protective response following immunization with an *Edwardsiella tarda* mutant containing a plasmid with the VHSV G gene insert under the control of the carp β-actin promoter (Choi et al. 2012). The use of these promoters shows promise; however, one of the most hopeful results came from a dual DNA vaccination study using the G gene from both VHSV and IHNV. After a single intramuscular inoculated with a plasmid containing the VHSV G gene under control of a CMV promoter, fish where challenged and experienced 96% survival (Einer-Jensen et al. 2009).

Since environmental conditions heavily influence the immune response in fish, the incubation period for vaccination trials is described as degree days (° days) which are a function water temperature and the number of days. One, informative observation of the Einer-Jensen et al. (2009) study, is the authors allowed a longer incubation period before challenge (880° days) as well as a lower immersion challenge concentration [10^4 median lethal tissue culture dose ($TCID_{50}$)]. Other trials have allowed much fewer °days for the host to react to the antigen, with

as few as 300° days before challenge (Estepa et al. 1994). Further, other trials have used higher viral concentrations to challenge fish, with immersion challenge concentrations as high as $3x10^6$ $TCID_{50}\,mL^{-1}$ (Chico et al. 2009), possibly overwhelming the immune response of vaccinated individuals. Clearly the protective effects elicited by an antigen can be best observed when the host has sufficient time to react to the antigen. Additionally, the greatest measure of relative percent survival (RPS) will be achieved when fish are challenged with the minimum viral concentration that results in the complete mortality of control (mock vaccinated individuals). Another point that should be raised is that there is a huge lack of information regarding viral shedding in vaccinated survivors following challenge. Typically, all individuals are euthanized at the termination of the study, but this provides little information on the post-challenge efficacy of vaccine preparations.

Following the emergence of VHSV in the Great Lakes basin, one of the imperative questions was how to protect millions of reared fish in the many federal and state hatcheries. Broodstock reside in, and progeny are stocked into areas where this serious virus that is now widely distributed. Equally important is to fill the huge knowledge gap regarding the immune response of resident species against viral insults. For these reason, the development of tools to examine the immune response against VHSV will constitute the main thrust of this dissertation.

5. Does "herd immunity" exist in fish?

The transmission dynamics are largely uninvestigated for aquatic pathogens such as VHSV. Also, even though vaccination in aquaculture and specifically for salmonids is becoming commonplace, there have has been no attempts to control a viral pathogen in a wild aquatic

setting (McCallum et al. 2004; Gudding et al. 2014). There are numerous knowledge gaps that need to be examined if we are to entertain the idea of eliminating an aquatic pathogen such as VHSV. For example, numerous studies demonstrate that fish mucosa and its associated IgT molecules specifically are important barriers to pathogen invasion (Zhu et al 2013; Xu et al 2016); but it is unknown if IgT is passively shed into the water column. If this were the case, viral binding or even neutralization may even be occurring remotely from the host, within the water column, and resulting in indirect protection. Some fish species such as discus (*Symphysodon* spp.) larvae demonstrate feeding of paternal mucus which plays an important role in hormone and possible immune transfer – theorized to be similar to colostrum in mammals (Jakowska 1963; Chong et al. 2005). Few studies have examined herd immunity in an aquatic setting and it is still unclear whether a mechanism exists whereby immune individuals provide protection to naive individuals.

Human and veterinary medical practices have repeatedly demonstrated that the spread of contagious diseases can be disrupted when a number of individuals of a population are immune to a particular disease, a phenomenon known as "herd immunity". The proportion of immune individuals in a population above which a disease may no longer persist is known as "herd immunity threshold" or HIT (Plans-Rubio 2012a; b; Ramsay et al. 2003; Anderson & May 1985; Piedra et al. 2005; Fine 1993). Epidemics can occur when the proportion of susceptible individuals in a population is high such as the case when a new pathogen emerges in a new area. A high number of susceptible individuals leads to efficient transmission or a high contact rate known as the ($R_{\circ}$) value; derived from the number of secondary infections produced. Therefore, in systems where $R_{\circ} \geq 1$, transmission continues and pathogen is able to persist. If, however, $R_{\circ}$

≤ 1 the virus is no longer replacing itself in the population and pathogen prevalence decreases (Fine et al. 2011). Examining disease dynamics and accurately estimating transmission rates ($R_{\circ}$) in an aquatic system is not always feasible. Epidemics are often difficult to visualize in the field are only evident during large fish kills. Additionally, unlike studies of terrestrial ecosystems, the aquatic environment itself can often serve as a disease reservoir and aid in pathogen persistence.

Previous studies have demonstrated that the critical immunity threshold is variable for different diseases and situations. For example, during attempts to eradicate wild polioviruses, studies show that 100% herd immunity was accomplished with just 65 to 70% immunization coverage in North America (Nathanson 1982) while the same vaccine regimen in South America and India resulted in only an estimated 70% herd immunity (John & Samuel 2000). In some cases the threshold can be quite high for highly pathogenic diseases, up to 92 to 95% vaccination coverage for the elimination of measles (Anderson 1992). However, to understand how herd immunity is achieved in a population we must understand the transmission dynamics and the $R_{\circ}$value for the pathogen. We understand how VHSV is shed from infected individuals and horizontally transmitted to naïve individuals but researchers have not reliably estimated the $R_{\circ}$ value in a highly variable aquatic system. Since $R_{\circ}$ can be linked to a number of other factors including virulence, host susceptibility, passive immunity and contact rate, this value for VHSV remains unknown. As an initial step towards investigating this concept, this dissertation investigates whether cohabitation of immune and naïve individuals provides measurable protection to the naïve individuals.

6. Study Objectives

Since VHSV was detected in the Great Lakes Basin there has been a dramatic spread of the virus throughout the watershed, including detections in all five Great Lakes and numerous inland waterbodies. Researchers have made surprising discoveries on the biology, phylogeny, host range, disease course, and comparable susceptibilities. These findings and the accompanying development of highly sensitive molecular and serological diagnostic methods have helped initiate our understanding of the immunological defense against VHSV-IVb. However, there are still numerous knowledge gaps that need to be filled in order to effectively combat VHSV. Therefore the following study contains five objectives to build our understanding of this novel VHSV genotype in the Great Lakes. These objectives include the examination of the host immune response and associated antibody production kinetics as well as investigating protective effects and immune responses associated with vaccine preparations.

Throughout all experiments I attempt to overcome many obstacles. For example, it was often unfeasible to use the well-studied rainbow trout since this species is only mildly susceptible to genotype IVb, though this is the species of choice for most VHSV studies. This is disadvantageous, as the diagnostic, phylogenetic and commercially available biological resources available for this specific species are numerous. Further, some of the susceptible fish species in the basin cannot be kept in the lab for an extended period of time (e.g., freshwater drum (*Aplodinotus grunniens*), Lake Whitefish (*Coregonis clupeaformis*), etc.). Muskellunge, on the other hand, possess several attributes that makes it suitable for VHSV-research and therefore our predominant species of choice. Muskellunge are the most susceptible species to VHSV-IVb examined thus far (Kim & Faisal 2010a; b; c) and exhibited the greatest humoral

immune response against the VHSV in previous studies (Millard 2013; Millard et al. 2014). Muskellunge can also be maintained in captivity for extended periods of time and this species is cultured in numerous facilities both within and outside of the basin so specific-pathogen free stocks are also available. To this end, this study was designed with the following objectives:

Objective 1: Development of monoclonal antibody against muskellunge immunoglobulin and its used in the development of an indirect-ELISA to detect anti-VHSV antibodies in muskellunge

Two serological assays have been developed to detect anti-VHSV-IVb antibodies: the 50% plaque reduction test (virus neutralization assay) and a competitive-ELISA (Millard et al. 2014). However, the PNT is limited to neutralizing antibodies and does not detect binding antibodies. Additionally, the competitive-ELISA is not specific to immunoglobulin and may detect other humoral lectin proteins. Through a collaborative effort, a monoclonal antibody (mAb) against muskellunge IgM was developed (designated 3B10). Using this mAb, an indirect ELISA protocol was designed and optimized for the specific examination of anti-VHSV antibodies in muskellunge. The assay was utilized to gain an understanding of the antibody production dynamics following exposure to either VHSV or a DNA plasmid containing the viral G gene under control of a cytomegalovirus promoter (designated pVHSivb-G). The plasmid was delivered intramuscularly and the associated primary and secondary antibody production dynamics were examined, following an initial and second administration of the vector respectively. Antibody levels were also assessed following a VHSV challenge to fully elucidate the secondary humoral response of immunized individuals

Objective 2: Comparison of the protection against VHSV-IVb following immunization with two

DNA vectors containing the viral glycoprotein gene

Two vectors were utilized. The first vector was identical to that described above and has been used previously in a VHSV-IVb vaccination trial with muskellunge (Millard 2013). The second vector (pAE6) was specifically designed for enhanced gene expression in teleosts. The pAE6 vector (kindly provided by Dr. Chen, University of Connecticut) is based on a *Rous sarcoma* virus backbone and contains a carp β-actin enhancer region (Gene bank accession number M24113) for recognition and expression by fish cells. This system demonstrated previous success with VHSV (Chico et al. 2009). Both vectors contained an insert of the VHSV-IVb G gene and served as the primary antigen for immunization in muskellunge. The vectors were delivered via intramuscular injection and fish were allowed to react to the antigen. The number of doses and time muskellunge have to react to the antigen were also examined. The relative percent survival (RPS) was determined by comparing the cumulative mortality of vaccinated and mock vaccinated individuals (Amend 1981).

Objective 3: Assessment protection against VHSV-IVb following immunization with a DNA vector in representative salmonid species

Guided by the results of objective two, the DNA vector and that elicited the greatest RPS was then used to determine if protection is conferred less susceptible salmonids. Numerous studies have demonstrated protection against other VHSV genotypes in rainbow trout (Lorenzen et al. 1998; Einer-Jensen et al. 2009). Therefore, in this objective we assess protection in three representative species from hatchery propagated genera: rainbow trout (*Onchorhynchus mykiss*), brown trout (*Salmo trutta*), and lake trout (*Salvelinus namaycush*). The vector was again delivered intramuscularly and the vaccine efficacy of either one or two administrations was

determined. Additionally, different challenge routes such as intraperitoneal challenges (IP) as well as variable viral challenge concentrations were examined, as conventional immersion challenges proved ineffective in these less susceptible species (Kim & Faisal 2010c).

Objective 4: Production of eukaryotic recombinant VHSV glycoprotein and evaluation of protection in muskellunge

Studies have demonstrated success using recombinant VHSV proteins to elicit a protective immune response (Lorenzen et al. 1993; Estepa et al. 1994; Novoa et al. 2006; Romero et al. 2008). Though demonstrations of lasting protection are lacking and it is apparent that the application of recombinant proteins produced in prokaryotes is limited because of improper protein folding (Thiry et al. 1990; Estepa et al. 1994; Lorenzen et al. 1999). Promising research used a recombinant baculovirus containing the VHSV (genotype Ia) glycoprotein gene to infect insect cells (Lecocq-Xhonneux et al. 1994). I based my research along this production method; however, I also embed a 5' poly-histidine tag into the G gene sequence to facilitate protein purification. The recombinant baculovirus containing the complete G gene is used to infect whole cabbage looper (*Trichoplusia ni*) larvae which then produce glycoprotein. Following purification, the glycoprotein was then assessed for its ability to provoke a protective immune response in muskellunge upon VHSV challenge.

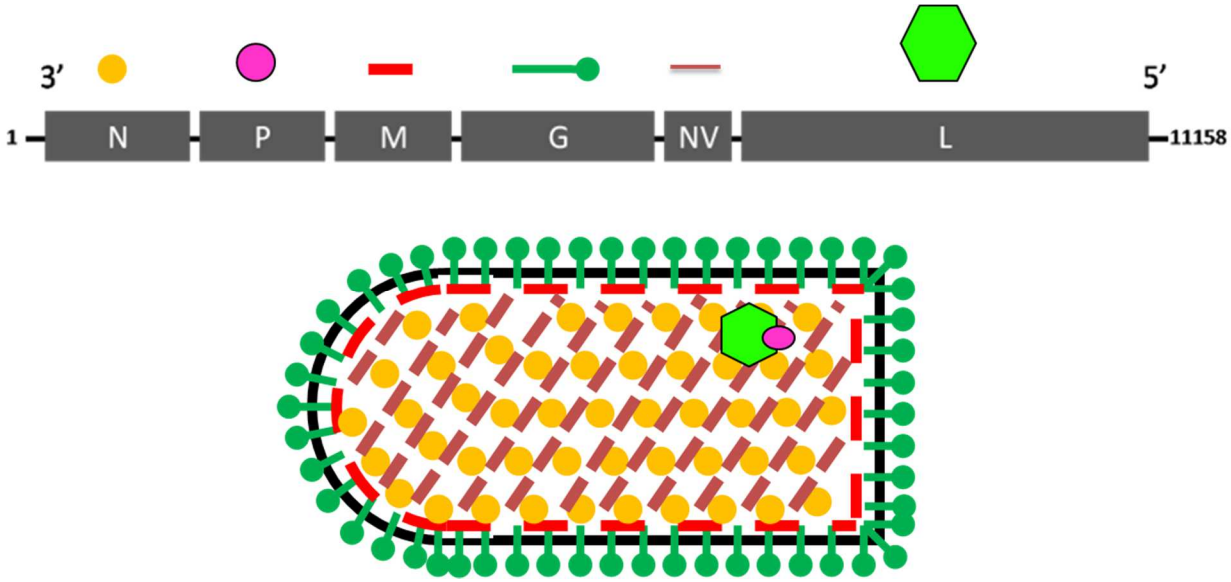
Objective 5: Determining if cohabitation with immunized fish lowers mortality and morbidity in naïve fish.

Using the findings from the previous objectives, the vaccine preparation that resulted in the greatest protection was used to immunize fish for cohabitation trials. Muskellunge were

immunized to artificially simulate immune individuals within the population. A novel experimental design included an infectious tank where virus was naturally shed from infected muskellunge and distributed equally to downstream tanks containing different ratios of immunized or naïve individuals. Initially, a pool of muskellunge was infected with a low IP dose of VHSV to produce chronically infected survivors that will shed virus into the water column. This strategy more closely mimics natural variability in exposure concentrations. Indirect protection was assessed using several response measures including viral shedding rates, cumulative mortality, anti-VHSV antibody levels and VHSV concentrations in tissues.

APPENDIX

Figure 1.1. The VHSV genome contains six open reading frames in the order 3'- nucleocapsid (N) – phosphoprotein (P) – matrix protein (M) – glycoprotein (G) – nonstructural protein (NV) – RNA polymerase (L) - 5'. The six proteins form the bullet shaped VHSV virion.



Chapter Two

Production and characterization of a monoclonal antibody against muskellunge (*Esox masquinongy*) IgM and development of an indirect ELISA for detection of seqrum antibodies against viral hemorrhagic septicemia virus genotype IVb.

Abstract

This study reports the development of a monoclonal antibody (designated 3B10) against the immunoglobulin (Ig) of muskellunge (*Esox masquinongy*), a large North American predatory fish species that is highly susceptible to the emerging freshwater strain of the viral hemorrhagic septicemia virus (VHSV), genotype IVb. The 3B10 monoclonal antibody (mAb), produced against concentrated muskellunge IgM belongs to the IgG3 *kappa* isotype and exhibits moderate but consistent average affinity (log aK = 5.96-6.25). Western blotting demonstrated that 3B10 mAb reacted primarily to the heavy chain of muskellunge IgM. 3B10 also reacted strongly with the IgM heavy chain of other esocids including the northern pike (*Esox lucius*), tiger muskellunge (*E. masquinongy* x *E. lucius*), and to a much lesser extent, chain pickerel (*E. niger*). While the 3B10 mAb did not bind to immunoglobulins from 11 other fish species resident in the Great Lakes basin, though, it reacted strongly to the common carp (*Cyprinus carpio*) Ig. The 3B10 mAb was used to develop and optimize an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-VHSV-IVb antibodies. ELISA conditions were optimal using 0.1 μg well^{-1} of coating antigen, using a 1:10 serum dilution, and 3.3 ng well^{-1} and 37.5 ng well^{-1} for the 3B10 and goat anti-mouse secondary antibodies respectively. Using the indirect ELISA, anti-VHSV-IVb antibodies were detected in sera from juvenile muskellunge vaccinated against the VHSV-IVb glycoprotein and from wild muskellunge sampled from the Detroit River,

Michigan, a VHSV-IVb endemic waterbody. This 3B10 mAb and ELISA will enable us to better understand the immune response of teleosts to VHSV-IVb.

1. Introduction

The Laurentian Great Lakes are home to numerous fish species, whose host defense mechanisms against pathogens remains largely unknown. One of the major problems in examining host immune responses is the absence of biological reagents that allow elucidation of immune mechanisms of indigenous fish species. For example, the lack of species-specific monoclonal antibodies (mAbs) has hampered the development of diagnostic immunoassays. This limitation dictated the use of more laborious neutralization tests and competitive/blocking enzyme-linked immunosorbent assays (ELISA) to detect and measure antibody responses against the emerging novel sublineage (IVb) of viral hemorrhagic septicemia virus (VHSV) (Millard & Faisal 2012a; b; Millard 2013; Millard et al. 2014; Wilson et al. 2014; Wilson-Rothering et al. 2015). While these assays have already proven beneficial in the detection of VHSV-IVb humoral immune responses, other non-antibody molecules in fish sera may contribute to measured endpoints (Nakao et al. 2006; Vasta et al. 2011).

The first isolation of VHSV-IVb was from muskellunge (*Esox masquinongy*) (Elsayed et al. 2006), a species that was later experimentally shown to exhibit higher susceptibility to the virus than other fish species in the Great Lakes basin (Kim and Faisal 2010a; b; c). Moreover, previous studies have demonstrated that muskellunge mount a more robust antibody response against VHSV-IVb as compared to other fish species collected from VHSV-IVb endemic waterbodies (Millard & Faisal 2010b). This species is long-lived and widely distributed in the Laurentian

Great Lakes basin (Coon 1999). These attributes, in addition to the ability to rear muskellunge in the laboratory for extended periods of time, make muskellunge an ideal target species for use in non-lethal virus surveillance and serosurveys.

Regulatory agencies have increasingly relied on serological assays for the indirect diagnosis of viruses and delineation of infected zones such as in the case of Avian Influenza Virus (Comin et al. 2013) and Measles Virus (Ratnam et al. 2000). To this end, we report on the development of a mAb specific to the heavy chain of muskellunge IgM. This mAb was used to develop an indirect ELISA for the detection of muskellunge serum antibodies against VHSV-IVb. The indirect ELISA helped elucidate kinetics of the primary and secondary immune response following DNA immunization against the VHSV-IVb glycoprotein (G) gene. Antibodies were also detected in wild mature muskellunge from the Detroit River, MI, a VHSV-IVb positive waterbody. The indirect ELISA will be a powerful tool in ongoing serosurveillance, and in examination of the muskellunge humoral response and assessment of vaccine efficacy against VHSV and other pathogens.

2. Materials and methods

2.1. Development of monoclonal antibody (mAb) against muskellunge IgM

2.1a. Administration of antigen to muskellunge

Muskellunge (4 months post-hatch), certified free of VHSV and other reportable disease, were obtained from Harrison Fishery Inc. (Hurdland, MO) and were acclimated in an aerated flow through 500L tank at 11°C. Fish were housed and all experiments were conducted in the University Research Containment Facility URCF (Michigan State University, East Lansing, MI).

Fish were fed live fathead minnows obtained from Anderson Farms Inc. (Lonoke, AR), certified as free of important disease according to World Organization for Animal Health (OIE). Fish were individually marked with fin clips or 9 mm passive integrated transponder (PIT) tags (Biomark© Inc., Boise, ID). Following pre-immune blood collection, each of nine fish was immunized intraperitoneally (IP) with 100 μg of trinitrophenylated- keyhole limpet hemocyanin (TNP –KLH) in sterile phosphate buffered saline (PBS; Sigma, St. Louis, MO), prepared as previously described (Bromage et al. 2006) and emulsified 1:1 with Freund's Complete Adjuvant (FCA; Sigma). Thereafter, blood was collected once monthly from each muskellunge to obtain high titer muskellunge sera. Blood samples were also collected from another group of eight muskellunge as a source of naïve serum for saturated ammonium sulfate (SAS) precipitation. All blood samples were collected by caudal venipuncture from fish anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222, Western Chemical, Ferndale, WA) buffered with 0.3 g L^{-1} sodium bicarbonate. Blood was kept at 4°C for 2 hours and then centrifuged at 2700*g* for 10 min at 4°C. Serum was then aliquoted and stored at -80°C. All animal protocols were approved by the Institutional Animal Care and Use Committee of Michigan State University (MSU).

2.1b. Production of mAb

Initially, attempts were made to immunopurify and generate clones from high-titer anti-TNP sera, but resultant clones did not exhibit sufficient antibody production. Thereafter, pooled serum from eight naïve muskellunge (~150 μL $fish^{-1}$) was concentrated using SAS precipitation. A SAS solution (pH 7.0, Fisher Scientific, Waltham, MA) was slowly added to the supernatant to achieve a final concentration of 20% (v/v) SAS. The reaction was incubated overnight at 4°C then centrifuged for 30 min at 3000*g.* The supernatant (representing the "20% cut") was

removed, aliquoted and stored at 4ºC until use. The process was then repeated on the remaining supernatant using 30%, and 40% concentrations of SAS, with a 2 hr incubation at 4°C for each concentration. Each of these two cuts was centrifuged at 3000*g* for 30 min and the precipitate was dissolved in solution composed of 500 µL PBS and 500 µL distilled H_2O. SAS precipitated "cuts" were analyzed by running 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (Sigma). Molecular weights were compared to the standards included in the EZ-Run™ Prestained *Rec* Protein Ladder (Fisher Scientific).

Following collection of pre-immune blood samples, two 17-week old Balb/c pristane-primed male mice (The Jackson Laboratory, Bar Harbor, ME, USA) were immunized IP with 50 µg of the 20% SAS precipitation of purified muskellunge IgM in 500 µl sterile PBS and emulsified 1:1 in FCA. Approximately four weeks following immunization, the surviving mouse was administered 10 µg of purified IgM in 50 µL of HyClone™ RPMI-1640 (hereafter referred to as RPMI; Hyclone Laboratories Inc., Logan, UT) via tail vein injection. Three days later, the mouse was euthanized, blood collected, and spleen aseptically removed. A single splenocyte suspension was prepared by puncturing the organ in several areas with a 22 gauge needle (Becton, Dickinson and Company, Franklin Lakes, NJ) attached to a 3 mL syringe (Becton, Dickinson and Company) and gently injecting RPMI into the organ, allowing splenocytes to spill out through the perforations made by the needle. Remaining splenocytes were released by gently drawing the spleen through the syringe (without the needle attached) as many times as necessary to break up the remaining tissue and dispense the cells into the petri dish of RPMI. Splenocytes were then drawn into a 50 mL tube, washed twice with 25 mL of RPMI, and centrifuged at 200*g*

at room temperature for 10 min. After the first wash and centrifugation, the supernatant was drawn off and 2 mLs of Red Blood Cell Lysing Buffer (Sigma) was added to the cell pellet swirling gently for 2 min, followed by the addition of 23 mLs of RPMI for the second wash. After the third wash, the splenocytes were combined with RPMI washed SP2/0 myeloma cells (American Type Culture Collection (ATCC), Manassas, VA) at a 5:1 ratio and fused in the presence of 1 mL of 50% (w/v) polyethylene glycol-1,450 Hybri-Max™ (PEG; Sigma) as previously described (Harlow & Lane 1998). For selection of myeloma-lymphocyte hybrids, RPMI medium was supplemented with 16.5% fetal bovine serum (Hyclone Laboratories Inc.) and 1xL-glutamine (Hyclone Laboratories Inc.). This medium was then mixed 1:1 with 1x hypoxanthine-aminopterin-thymidine (HAT) medium (Sigma), 1x oxaloacetate-pyruvate-insulin (OPI) media supplement (Sigma) and 26% conditioned RPMI medium (i.e. supernatant from SP2/0 myeloma cells). Cells were then dispensed into five 96 well Costar® cell culture plates (Corning, Corning, NY) at a volume of 150 μL $well^{-1}$ and the plates were incubated at 37°C and 5% CO_2.

2.2. Selection and characterization of the mAb

After colony formation, antibody-producing hybridomas were screened against muskellunge precipitated immunoglobulin using an endpoint ELISA as described by Shapiro et al. (1996). First, the 30% SAS precipitated cut of the naïve muskellunge sera was adjusted to 5 μg mL^{-1} in PBS (pH 7.4) and coated onto an EIA high affinity microplate (Corning) at100 μL $well^{-1}$ and incubated for 60 min at room temperature. The plates were then blocked for 1 hr at room temperature with 200 μL $well^{-1}$ of a solution composed of Tris buffered Tween-20 (TTBS, Sigma) containing 0.5% bovine serum albumin (BSA, Sigma). Next, 100 μL $well^{-1}$ of

supernatant from the hybridoma clones, diluted 1:10 in TTBS, were added. Plates were incubated for 1.5 hrs and washed 3 times with TTBS. Finally, a goat anti-mouse horseradish peroxidase (HRP) secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Gove, PA), 1:4000 in PBS was added to each well (100 µl $well^{-1}$) and incubated at room temperature for 1 hr. The reaction was visualized by the addition of 100 µL $well^{-1}$ of a solution composed of 7.7 µmol of 2,2-azino-bis (3-ethylbenzothiazoline -6-sulfonic acid, Sigma) and 10 µl of 30% hydrogen peroxide H_2O_2 in 10 mL citrate buffer (10 mM citric acid, pH 4.0, Sigma) (Shapiro et al. 1996). The optical density (OD) was read at 405_{nm} after 15 min on a Thermo Multi Scan MCC (Fisher Scientific) using DeltaSoft JV Data Analysis Software (BioMetallics Inc., Princeton, NJ). Based on this screening, positive hybridoma cells (3B10) was selected, plated using limiting dilutions, and then cloned three times using the limiting dilution method (Harlow & Lane, 1988).

2.2a. Western blotting

Following cloning of the 3B10 cells, 14 healthy colonies were screened for anti-muskellunge Ig activity using western blot. Briefly, 10 µg of the previously described 30% SAS-precipitated muskellunge Ig was added to an equal volume of 2x reducing sample buffer [RSB; 4XRSB with 30% sucrose (Sigma), 8% SDS (Fisher Scientific), 10% β-mercaptoethanol (Sigma), 0.25 M Tris-HCl (Fisher Scientific), a few granules of bromophenol blue (Sigma), pH 6.8]. Samples were boiled for 8 min and cooled prior to loading into a 10% SDS-PAGE gel. The denatured Ig was electrophoresed at 100 V alongside the EZ-Run™ Prestained *Rec* Protein Ladder. Muskellunge Ig was transferred overnight at 4°C and 30 V to a methanol-activated Immobilon-P polyvinylidene difluoride - FL membrane (PVDF-FL; Millipore, Billerica, MA) following the

manufacturer's protocol. The membrane was subsequently washed twice for 15 min each with 0.1% Tween-PBS (Sigma) and blocked with TTBS for 40 min. The membrane was then placed into a mini-protean II multi-screen slot blotter manifold (Bio-Rad, Hercules, CA) and 500 µL of 3B10 cone supernatant, diluted 1:25 in a 1:1 solution of RPMI (Hyclone Laboratories Inc.) and PBS (pH 7.4), was added to each lane. After rocking for 1.5 hrs at room temperature, supernatant was removed and each slot washed with 1 mL PBS. Hereafter, all incubations and washings were facilitated using an automated rocker unless otherwise noted. Following four, 4 min washes with 20 mL of 0.1% Tween-PBS, the membrane was incubated 60 min with 20 mL of 1:8000 dilution of a goat anti-mouse IgG secondary antibody Alexa Fluor 680 conjugate (Invitrogen, Eugene, OR) in PBS. After another four, 4 min washes, the membrane was air dried in the dark and scanned using a Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE).

Three clones that exhibited the strongest anti-muskellunge Ig activity were allowed to grow out to 80% confluency in 75 cm^2 tissue culture flasks (Corning). Hybridoma cells from the three clones were then injected IP into 8, 7 and 3 pristane-primed Balb/c mice for each of these clones respectively. Mice were monitored for the development of ascites for 14 days. Ascites fluid from a single individual was collected and purified using SAS precipitation and analyzed for purity with 10% SDS-PAGE as previously described. The SAS-purified 3B10 mAb was then diluted to 1 mg mL^{-1} in PBS with 50% glycerol (Sigma) and stored at -20°C until use.

2.2b. Immunoadsorption and SAS purification of high-titer muskellunge anti-TNP antibodies

To obtain a pool of highly pure anti-TNP sera and to investigate the titers of TNP-KLH vaccinated muskellunge, a kinetic ELISA was conducted employing the 3B10 mAb. Briefly, TNP (Sigma) was conjugated to a borate buffered BSA solution with addition of picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid, Sigma) to achieve an average haptenation ratio of TNP_8-BSA. TNP_8-BSA was diluted to a concentration of 10 μg mL^{-1}, coated onto EIA plates (Corning) at 100 μL $well^{-1}$, and incubated for 1hr at room temperature. Plates were blocked for 1 hr at room temperature with 200 μL $well^{-1}$ of a solution composed of 4% casein (Sigma), 150 nM NaCl (Sigma), 50 mM Tris Base (Sigma), 1 mmol L^{-1} EDTA (Sigma) and adjusted to pH 7.6. Immunized muskellunge sera were added to wells beginning at a 1:50 dilution and serial 1:3 dilutions were added down the remaining wells of the plate. The 3B10 mAb was diluted 1:250 in PBS and 100 μL $well^{-1}$ was added and incubated for 1 hr at room temperature. A goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Gove, PA), 1:2000 in PBS, was added to each well and incubated at room temperature for 1 hr. Substrate was then added to the plate as previously described. The developing plate was immediately placed on a plate reader and OD read at 405_{nm}, taking kinetic measurements every min for 10 mins to determine the maximum OD rate. A unit of antibody activity was defined as the volume of sample required to produce an OD rate of half the maximum rate (Shapiro et al. 1996).

Immunized muskellunge sera showing the highest anti-TNP activities were pooled and divided into aliquots; 250 μL for immunoadsorption and 400 μL for SAS precipitation. Immunoadsorption was performed using a haptenation ratio of TNP_{13}-BSA linked to cyanogen

bromide-activated sepharose beads (Sigma), per the manufacturer's instructions. Pooled muskellunge serum was incubated with a limiting quantity of TNP_{13}-BSA conjugated sepharose beads (approximately 4-fold the binding capacity of the bead aliquot), then incubated overnight at 4°C in a Bio-Spin chromatography column (Bio-Rad) using a rotator. Following washing of the column with ten bed-volumes of PBS, adsorbed antibodies were eluted from the immunosorbant with TNP-lysine (ICN Biochemicals, Cleveland, OH) in PBS. Eluates were dialyzed against PBS to remove hapten and concentrated with PEG 20,000 (Sigma) (Shapiro et al 1996). Meanwhile, SAS precipitation of the pooled serum was conducted as previously described. Fractions were analyzed for purity by running 10% SDS-PAGE under reducing conditions as previously described.

2.2c. Assessment of 3B10 mAb affinity to muskellunge Ig

To determine the affinity of the purified 3B10 monoclonal antibodies, the muskellunge anti-TNP preparations were used in an ELISA affinity-partitioning method as per the protocols outlined in Neito et al. (1984) and Shapiro et al. (1996). Incubations were carried out for 1.5 hrs at room temperature and plates were washed three times with TTBS unless otherwise noted. Briefly, each row of a 96-well EIA microplate was coated with 1:3 serial dilutions of 100 μL $well^{-1}$ TNP-BSA, beginning with a concentration of 10 μg mL^{-1}. The plate was incubated at 37°C for 1 hr then washed three times with TTBS. Plates were then blocked for 1 hr at room temperature with 200 μL $well^{-1}$ of the blocking solution described in section 2.2a. A serial 1:5 dilution series of 10^{-3} M TNP-lysine (Sigma) was prepared and 50 μL $well^{-1}$ added across plate columns to determine the 50% inhibitory concentration (average affinity = log aK). Two units of muskellunge anti-TNP pooled sera suspended in 50 μL of PBS were then added directly to the TNP-lysine containing

wells. Plates were incubated, washed and 50 µL of 3B10 mAb (1:2500 in PBS) was added to each well. Following incubation and washing, TTBS 100 µL well^{-1} goat anti-mouse HRP secondary antibody (1:2000) was added. Following 1 hr incubation, plates were washed three times with TTBS. Development and reading steps took place as described in section 2.2b. Antibody affinity was determined by the equation: log aK = $(1/[H_{50}]_i)$, where $[H_{50}]$ is the concentration of TNP-lysine required for a 50% reduction in the maximum O.D. rate for the coating solution "i" (Shapiro et al. 1996).

2.2d. Determination of 3B10 mAb species and chain specificity

The species and Ig chain specificity of the 3B10 mAb was assessed by conducting immunoblots against sera from 15 additional Great Lakes fish species selected to represent a range of teleost genre –freshwater drum (*Aplodinotus grunniens*), channel catfish (*Ictalurus punctatus*), northern pike (*Esox lucius*), chain pickerel (*Esox niger*), common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), tiger muskellunge (*Esox masquinongy* x *lucius*), walleye (*Sander vitreus*), Chinook salmon (*Oncorhynchus tshawytscha*), brown trout (*Salmo trutta*), lake whitefish (*Coregonus clupeaformis*), brook trout (*Salvelinus fontinalis*), smallmouth bass (*Micropterus dolomieu*), lake sturgeon (*Acipenser fulvescens*), and rockbass (*Ambloplites rupestris*). Each blot also contained a raw serum and SAS precipitated IgM sample from naïve muskellunge. Sera was added to 2x RSB, boiled for 8 min and cooled prior to loading into a 10% SDS-PAGE gel and run alongside the EZ-Run™ Prestained *Rec* Protein Ladder. The SDS and immunoblot then proceeded as previously stated in section 2.2a until the blocking step. The blot was washed 2x 10 min in TTBS, and blocked for 1 hr in 4% casein solution containing 5% sucrose (Sigma), 150 nM NaCl, 50 mM Tris Base and 1 mmolL^{-1} EDTA, (Sigma), pH of 7.6,

followed by four washes as previously stated. The blot was then incubated for 1.5 hrs in 20 mLs of the 3B10 mAb (1:2000 dilution in PBS), followed by the four washes and addition of the AlexFluor 680 as previously stated in section 2.2a.

2.2e. 3B10 mAb isotype

The specific IgG isotype of the final selected mAb was verified using the Mouse Monoclonal Sub-Isotyping Kit (American Qualex International, San Clemente, CA) following the manufacturer's instructions.

2.3. Development and application of an indirect ELISA using 3B10 mAb for anti-VHSV-IVb antibody detection

2.3a. Preparation of VHSV-IVb for coating

The VHSV strain used throughout the study was the Great Lakes index strain MI03GL (Gene bank accession number GQ385941), which was initially isolated in muskellunge in 2003 (Elsayed et al. 2006). The isolate has been maintained by continuous subculture in the e*pithelioma papulosum cyprini* (EPC) cell line (Winton et al. 2010). The EPC cell line was maintained and subcultured in 150 cm^2 tissue culture flasks (Corning) at 25°C using a basal media of Earle's salt-based minimal essential medium (EMEM) (Invitrogen, Carlsbad, CA) and supplemented with 10% tryptose phosphate broth (BD Biosciences, San Jose, CA), 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU mL^{-1}) (Invitrogen), streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), and sodium bicarbonate (7.5% w/v) (Sigma). The virus titer was determined using plaque assay on PEG-

treated EPC cells and a methylcellulose overlay (Batts & Winton 1989; Batts et al. 1991). Virus aliquots were stored at -80°C until needed.

VHSV purification was performed as described by Millard et al. (2014). Briefly, viral suspensions were overlaid on 10 to 60% discontinuous sucrose gradient column and ultracentrifuged at 80,000*g* for 2.5 hrs at 4°C. The fraction containing the purified virus was given a final wash by pelleting through TE buffer (20 mM Tris-HCl, pH7.5 and 1mM EDTA. pH 8.0; Sigma) at 80,000*g* for 1.5 hrs at 4°C.The resulting pellet was then suspended in PBS (pH 7.4), aliquoted and stored at -80°C until use. Protein concentration of the final preparation was determined using a Qubit® Fluorometer with the Qubit® Protein Assay Kit (Life Technologies, Carlsbad, CA).

2.3b. Production of control sera

Positive control sera consisted of adult muskellunge that had survived multiple experimental exposures to VHSV-IVb (Kim and Faisal 2012). Survivors (n = 8) were given two IP booster doses of 10^5 pfu VHSV-IVb in 100 µL of sterile PBS. Fish were subsequently maintained for three years as a source of positive hyper-immune sera. When needed, blood was collected non-lethally from individual fish, processed, and stored as previously described.

The ELISA negative control was a pooled serum from 77 naïve, juvenile muskellunge. Muskellunge [average 14.2 cm (SD = 1.4) mean fork length, 11.9 g (SD = 3.8)] were obtained from the Chautauqua State Fish Hatchery (Chautauqua, New York) at 14 weeks post-hatch, certified to be VHSV-free, and maintained at MSU-URCF as previously described. Individual

sera aliquots from each of the 77 fish were used for establishment of an indirect-ELISA positive/negative threshold cutoff value as described below. All sera were stored at -80°C until use.

2.3c. Indirect ELISA procedure

All muskellunge serum samples were stored at -80°C until use. Prior to ELISA, sera were heated at 45°C for 30 min to inactivate complement, vortexed, centrifuged at 2700*g* at 4°C for 10 min, and diluted 1:10 in PBS containing 1% nonfat dried milk (PBS-1% NFDM, Sigma). Polystyrene microplates (96-well, Microlon®600 with chimney wells; Greiner Bio-One, Monroe, NC) were sealed during all incubations (SealPlate®; Sigma). Plates were washed five times following each incubation period unless otherwise stated using PBS containing 0.05% TTBS in an automated microplate washer (BioTek, 4Lx405™ plate washer; Winooski, VT).

Microtiter assay plates were coated with 100 µL well^{-1} of purified VHSV-IVb and incubated overnight (14-16 hrs) at 4 °C in a humid chamber. Plates were blocked with the addition for 430 µL well $^{-1}$ of PBS containing 5% NFDM (PBS-5%NFDM; Sigma) and incubated at 37 °C for 1hr. Heat inactivated and diluted test and control muskellunge sera were then added to duplicate wells (100 µL well^{-1}) and an equal volume of diluent was added to the top and bottom rows for diluent control determination. After incubating at 25 °C for 1 hr, 100 µL of the 3B10 mAb was added to all wells and incubation was repeated (25 °C for 1 hr). Next, 100 µL of a HRP-conjugated goat anti-mouse monoclonal IgG secondary antibody (Invitrogen) was added to each well and incubated at 25°C for 1 hr. Plates were developed in the dark for 30 min at 25°C following the addition of 100 µL well^{-1} of 0.4 mg mL^{-1} *o*-phenylenediamine (Sigma) in

phosphate citrate buffer (Sigma) containing 3mM hydrogen peroxide (Avantor Performance Materials Inc., Center Valley, PA). The reaction was stopped with the addition of 50 mL of 3 M sulfuric acid (H_2SO_4; Avantor Performance Materials Inc.). Optical density (OD) was read at 490_{nm} on a BioTek, ELx808™ plate reader (BioTek) and analyzed using the Gen5 software (BioTek). The average value of blank wells was subtracted from test and control wells prior to analysis.

2.3d. ELISA optimization

Initial optimization of reagents was conducted using the protocol described above using standard checkerboard titration (Crowther 2000). The coating antigen (purified VHSV-IVb) was titrated using serial dilutions ranging from 8μg mL^{-1} to 0.25 μg mL^{-1}. Similarly, different sera dilutions ranging from 1:5 to 1:80 were examined. Serial dilutions of the newly developed 3B10 mAb (1:2,500 to 1:240,000) and HRP-conjugated goat anti-mouse IgG (1:2,000 to 1:64,000) were also titrated. Initial optimization was conducted using sera from individual wild muskellunge that were identified as positive for anti-VHSV-IVb activity using competitive ELISA as described in Millard et al. (2014). Subsequent optimization utilized the pooled VHSV-IVb hyperimmune serum from experimentally-infected muskellunge described above. Conditions that resulted in the highest OD_{490} ratio between positive and negative serum (P/N) OD values while maintaining a maximum OD_{490} value near 1.0 were considered optimal and selected for use in subsequent assays.

2.4. Indirect ELISA validation

Five positive individual samples, tested in triplicate, were used to examine intra and inter-assay variability. The detection limit of the assay was determined by measuring the maximum dilution of a 1:4 dilution series of the positive control that remained above the established positive/ negative threshold.

The cross-reactivity of muskellunge anti-VHSV-IVb antibodies was tested by indirect ELISA utilizing plates coated with VHSV-IVb (MI03) and five other pathogenic fish viruses. Viral isolates included a VHSV-IVa isolate (Makah), from coho salmon (*Oncorhynchus kisutch*) in the Pacific Northwest region of North America (Brunson et al. 1989) and a VHSV-IVc isolate (2000-149), from mummichog (*Fundulus heteroclitus*) in the Ruisseau George-Collete, Canada (Gagne et al. 2007). We also examined an infectious hematopoietic necrosis virus (IHNV) isolate (220-90, genogroup M), from rainbow trout in the Hagerman Valley, ID (LaPatra et al. 1991); an aquatic birnavirus, the infectious pancreatic necrosis, IPNV, isolated in our laboratory from brook trout (*Salvelinus fontinalis*) in a private aquaculture facility in 2013; and an iridovirus, largemouth bass virus (LMBV), also isolated in our laboratory from smallmouth bass from Lake St. Clair, MI in 2013. To standardize viral concentrations, the median tissue culture infectious dose $(TCID)_{50}$ of all viral stocks was first determined (Reed & Muench 1938). After determining the $(TCID)_{50}$, viral stocks were standardized to 10^3 $TCID_{50}$ $well^{-1}$ in cold PBS and compared to the purified VHSV-IVb coating preparation (0.1 μg $well^{-1}$). ELISA was otherwise conducted as previously described and mean OD values of duplicate wells of muskellunge sera were compared between different viral coating solutions. Five serum samples were tested with these viruses; two samples from naïve individuals, and three pooled sera from muskellunge immunized with a

pVHSivb-G vector containing the glycoprotein gene of VHSV-IVb (pVHSivb-G, described below).

2.5. Detection of circulating anti-VHSV-IVb antibodies in muskellunge sera

We used the indirect ELISA to test for anti-VHSV antibodies in two groups of muskellunge. The first group consisted of captive immunized muskellunge and samples were tested to elucidate the humoral immune response following vaccination. Samples from wild-caught, mature muskellunge from the Detroit River were also tested to determine previous VHSV-IVb exposure.

Captive muskellunge were composed of a group of pit-tagged individual muskellunge that were tracked following the intramuscular administration of a DNA vector containing the VHSV-IVb glycoprotein (G) gene. This pcDNAvector construct containing VHSV-IVb glycoprotein gene (hereafter referred to as pVHSivb-G) has been detailed elsewhere (Millard 2013). The basic construct design was modeled after successful DNA vaccines against VHSV genotype I (Lorenzen et al 1998, Heppell et al. 1998) and IHNV (Anderson et al 1996). Briefly, a sequence consisting of an *Eco*RI restriction site (G/AATTC), followed by a kozak consensus sequence (Kozak 1987) terminating with the first amino acid of the complete MI03GL VHSV-IVb isolate glycoprotein gene (1524 bp) was synthesized. An *Xba*I restriction site (T/CTAGA) was then added following the 3' termination codon. The assembled fragment was then digested using the described endonucleases and sub-cloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen) containing an immediate-early cytomegalovirus (CMV) promoter. The plasmid was transformed and propagated into K12 *Escherichia coli*. Sequencing confirmed the correct

glycoprotein gene sequence and orientation. The final vector, designated pVHSivb-G was suspended in sterile PBS and stored at -80°C until use.

The pVHSivb-G vaccine construct was then used to immunize muskellunge. For the duration of the experiment, muskellunge were housed in a circular 500-L tank containing supplemental aeration at 12°C and fed live fathead minnows. Two weeks prior to immunization, 32 juvenile muskellunge from the Chautauqua State Fish Hatchery cohort were intramuscularly pit-tagged with 9mm tags (Biomark© Inc.) so that individual muskellunge could be tracked over time. At this time, sera were also non-lethally collected to establish an OD baseline for individual muskellunge. Serum samples were collected, processed and stored as previously described. For immunization, fish were anesthetized as previously described and 10 µg pVHSivb-G was administered by IM injection into the left epaxial muscles slightly posterior to the pectoral fins. Sera were collected after 28 days, then at 14-day intervals for 70 days following administration. After 70 days, fish were intramuscularly administered a second of 10 µg of pVHSivb-G. Sera were collected again 35, 56 and 70 days following the second administration. Paired *t*-tests were then performed to examine significant changes in the OD values of the population over the study period.

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).

3. Results

3.1. Production of 3B10 mouse anti-muskellunge IgM mAb

The 20% SAS cut of the precipitated muskellunge IgM, appeared to be have the greatest purity, exhibiting intensely stained bands at the molecular weights ~73 kDa and ~26kDa, corresponding to the heavy and light chains of the muskellunge IgM molecule respectively (Figure 2.1). Following splenocyte collection, myeloma fusion and colony formation, the analysis of the endpoint ELISA led to the selection of the hybridoma clone (3B10). 3B10 was then cloned thrice using the limiting dilution method. Of the grown 3B10 clones, the supernatant of 14 were examined by western blotting to determine anti-muskellunge IgM antibody production. All clones exhibited strong bands at ~73 kDa corresponding with muskellunge heavy chain (Figure 2.2). Three clones were selected (depicted in *lanes* 12, 14, and 18 in Figure 2.2). The ascites fluid collected from a single mouse then underwent SAS precipitation, the 20% SAS cut demonstrated the highest purity (data not shown), and was selected for use in indirect ELISA.

3.2 Characterization of the 3B10 mAb

The isotype of the 3B10 mAb was determined to be IgG3-*kappa*. Based on the results of a kinetic ELISA performed on sera collect from TNP-KLH immunized muskellunge, serum from a single individual that exhibited the highest circulating anti-TNP antibody titers (>100,000 U mL^{-1}) was selected for Ig concentration and used to assess 3B10 mAb affinity and chain specificity

(data not shown). The average affinity (log aK) of the 3B10 mAb was calculated against chromatography purified anti-TNP antibodies using an affinity-partitioning ELISA. The 3B10 mAb affinity (log aK) values against two chromatography elutions averaged 6.08 and 5.96 respectively. While the average affinity against a 35% SAS precipitation of the same sera pool was 6.25. These consistent values indicate the mAb has a rather constant, but moderate affinity profile against muskellunge IgM.

The species and chain specificity of the 3B10 mAb was assessed using western blot. The Odyssey Program (Li-Cor) was used to estimate the molecular weight; showing that 3B10 is highly specific to the heavy chain of Ig from both esocids muskellunge and northern pike as well as to their hybrid, tiger muskellunge (Figure 2.3). Though, we note that the Ig heavy chain of both the northern pike and tiger muskellunge appears to be slightly larger than the muskellunge Ig heavy chain ~73-75 kDa. Minimal reactivity was also observed with the Ig of chain pickerel, another esocid species. The 3B10 mAb did however appear to react to common carp Ig heavy chain which also appeared to have a greater molecular weight then muskellunge Ig heavy chain. Interestingly, 3B10 mAb does not react another cyprinid; the fathead minnow Ig (Figure 2.3). No reactivity was observed with the Ig or sera from any of the other species that were examined.

3.3. Establishment of indirect ELISA

The VHSV coating concentration, serum dilution, 3B10 mAb concentrations as well as the conjugated goat anti-mouse HRP antibody concentrations were optimized using standard checkerboard titration protocols as mentioned above. The optimal concentration of purified VHSV-IVb and serum dilution were determined to be 0.1µg well^{-1} and 1:10 respectively (Table

2.1). The optimal concentrations of the 3B10 mAb and the goat anti-mouse HRP secondary antibody were determined to be 1:30,000 (3.3 ng well^{-1}) and 1:4000 respectively (37.5 ng well^{-1}) (Table 2.2). Conditions were optimized when coating took place overnight at 4°C, and all other incubation were conducted for 1 hr at room temperature, with the exception of blocking that was conducted at 37°C.

Following condition optimization, the positive/negative threshold was determined using the individual OD values from 77 naïve muskellunge which ranged from 0.025 to 0.15, with a mean of 0.025. Using the common method of threshold determination, i.e., the mean + 3 standard deviations [0.025 + 3(0.033)], the positive/negative threshold was determined to be 0.124. However, we also applied the more stringent approach of calculating the 95% confidence limit of the 95th quartile in SAS 9.2 (SAS Institute 2010) as previously described (Millard et al. 2014). The threshold calculated using this second method was 0.163, which we adopted as the final threshold.

3.4. Assay validation

The reproducibility of the indirect ELISA was examined using triplicates of five samples (Table 2.3). The intra-assay coefficient of variation (CV) ranged from 1.56% to 6.54% with a mean of 2.85%. The inter-assay CV ranged from 1.50% to 5.06% and a mean of 3.23 %. These values indicate low variation and strong reproducibility both within and between plates. The sera minimum detection limit determined by the maximum dilution of positive sera that remained above the established threshold (0.163) was 1:1024 (data not shown).

The viral specificity of the assay was determined by coating plates with standardized concentration (10^3 $TCID^{50}$ $well^{-1}$) of other pathogenic fish viruses and comparing the OD values. Throughout this analysis, the OD values for the blank well and negative muskellunge sera were all less than 0.089 indicting minimal non-specific binding (Table 2.4). In pVHSivb-G immunized muskellunge, reactivity was only observed against the two VHSV-IVb preparations. The OD values for VHSV-IVa and IVc ranged from 0.001 to 0.015. Similarly, no reactivity was observed against the other viral coating solutions, with OD values ranging from 0.000 to 0.018. In immunized muskellunge, the highest OD values were observed in the pool of sera collected 5 weeks following the second administration, where mean OD values were 1.017 and 0.854 for purified and standardized VHSV-IVb respectively.

3.5. Indirect ELISA detects anti-VHSV-IVb antibodies in muskellunge

Analysis was performed on two groups of muskellunge. In captive muskellunge prior to immunization with the pVHSivb-G (n =32), the mean OD value was 0.010 (SD = 0.008) (Figure 2.4). 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 an adaptive response and possible B cell proliferation.

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 (Figure 2.5). 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).

4. Discussion

The emergence of VHSV-IVb in the Great Lakes basin necessitated the development of novel biological reagents for surveillance efforts (Faisal et al. 2012). 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 (Kim & Faisal 2010c). 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 immuopurified 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 Ig and led to the successful development of 3B10 hybridomas that exhibited strong anti-muskellunge Ig antibody production. Additional biochemical analysis demonstrated that this IgG3 *kappa* isotype 3B10 mAb has a moderate but consistent affinity against either SAS precipitated or chromatography purified muskellunge Ig. In mice, the IgG3 isotype is associated the effector function of an early T-cell independent immune response, binding solely to the FcγRI receptor on macrophages (Gavin et al 1998). IgG3 molecules are chiefly specific to carbohydrates and repeating epitope antigens and self-association aggregation (Grey et al. 1971; Gavin et al 1998). Identification of the mAb as the IgG3 isotype is important as it informs the subsequent applications and specifications of the mAb, such as what secondary antibody is required for assay development.

The western blot using purified muskellunge Ig and 3B10 demonstrated that the generated mAb primarily recognizes the Ig heavy chain. Most of the other fish anti-immunoglobulin mAb that have been developed also target the Ig heavy chain (Romestand et al. 1995; Al-Harbi et al. 2000; Beelen, et al. 2004; Miyada et al. 2004; Rathore et al 2008; Sood et al. 2011), although a few mAbs have also targeted the Ig light chain (Scapigliati et al. 1999; Jang et al. 2004). The

molecular weight of the muskellunge Ig heavy chain is ~73 kDa, which is within the range known for teleost Ig heavy chain (Romestand et al. 1995; Bryant et al 1999; Beelen et al. 2004; Jang et al. 2004; Shin et al. 2006; Rathore et al 2008; Bag et al. 2009; Sood et al. 2012). 3B10 specificity does not appear to be limited to muskellunge as the mAb also reacts with northern pike and their hybrid: the tiger muskellunge. Bands produced with *Esox niger* serum were much fainter although the same amount of proteins were loaded to each lane, denoting that 3B10 exhibits lower reactivity to this species. Further, we found that 3B10 selectively reacted with cyprinid Ig; exhibiting strong activity against common carp Ig heavy chain, but at a greater molecular weight then the 70 kDa reported in previous studies (Rombout et al 1993; Koumans-van Diepen et al. 1995; Vesely et al. 2006) and closer to the 88kDa reported for other cyprinid species (Bag et al. 2009; Das et al. 2014). Though, it has also been demonstrated that the common carp possess several distinct heavy chain (constant region) gene sequences (Nakao et al. 1998) which appear to share determinants with surface T-like cells and are heterogeneous in sera (Egberts et al 1983). The antigenic cross reactivity of teleost immunoglobulin molecules have been reported before from fish species within same genus (Miyadai et al. 2004; Sood et al. 2012; G.W. Warr, Medical University of South Carolina, personal communication) and in some cases, across different genera and families (Rathore et al. 2008; Vesely et al. 2006). For example, a mAb against common carp Ig heavy chain was shown to react to Ig from a species of a different order, sheatfish (*Siluralus glanis*) (Vesely et al. 2006). 3B10 reacted with Ig molecules of muskellunge, northern pike, tiger muskellunge, and common carp, but not those of other fish species. The fact that 3B10 reacts strongly, not only with muskellunge but also with northern pike and common carp, 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 (Waterstrat et al. 1989; Shoemaker et al. 2003; Encinas et al. 2011; Kim et al. 2015). 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. There was a dramatic increase in OD values through the first six weeks and a peaked OD values were observed 8 weeks post-vaccination, indicates 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 vigorous primary and secondary humoral immune responses elicited by pVHSivb-G vaccination appears to mimic the natural response following rhabdovirus exposure (Lorenzen et al. 1998; LaPatra et al 2001; Millard 2013). The indirect ELISA was also capable of detecting

antibodies in 129 of 134 of muskellunge sampled from Detroit River and Lake St Clair, part of a known VHSV enzootic zone. This finding 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.

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.

APPENDIX

Table 2.1. Checkerboard titration for determination of VHSV-IVb coating concentrations and positive and negative muskellunge sera dilutions. Data is presented as optical density values and optimal conditions are in bold.

Sera Dilutions	OD_{490} values of VSHV-IVb coating concentrations ($well^{-1}$)					
	0.8 µg	0.4 µg	0.2 µg	0.1 µg	0.05 µg	0.025 µg
1:5 (+)	2.971	2.632	1.758	1.363	0.902	0.433
1:5 (-)	0.852	0.637	0.401	0.295	0.194	0.094
1:10 (+)	2.751	2.522	1.743	**1.327**	0.800	0.379
1:10 (-)	0.862	0.672	0.348	**0.268**	0.157	0.087
1:20 (+)	1.097	0.921	0.541	0.391	0.213	0.133
1:20 (-)	0.268	0.202	0.121	0.089	0.069	0.048
1:40 (+)	0.651	0.544	0.317	0.235	0.133	0.077
1:40 (-)	0.157	0.127	0.088	0.070	0.060	0.043
1:80 (+)	0.315	0.250	0.129	0.107	0.071	0.052
1:80 (-)	0.081	0.063	0.059	0.055	0.041	0.039

Table 2.2. Checkerboard titration for determination of 3B10 mAb and secondary antibody concentrations using positive and negative muskellunge sera. Data is presented as optical density values and optimal conditions are in bold.

Goat anti-mouse HRP dilutions	OD_{490} values of 3B10 mAb dilutions						
	1:2500	1:5000	1:15000	1:30000	1:60000	1:120000	1:240000
1:2000 (+)	3.787	3.596	2.996	1.804	1.338	0.846	0.493
1:2000 (-)	1.908	1.488	0.732	0.342	0.274	0.150	0.091
1:4000 (+)	3.461	3.038	2.042	**1.089**	0.628	0.377	0.226
1:4000 (-)	1.261	0.920	0.421	**0.200**	0.143	0.099	0.072
1:8000 (+)	2.704	2.197	1.288	0.728	0.418	0.239	0.139
1:8000 (-)	0.800	0.559	0.256	0.127	0.107	0.068	0.056
1:16000 (+)	1.811	1.420	0.790	0.432	0.258	0.161	0.096
1:16000 (-)	0.422	0.307	0.152	0.089	0.068	0.056	0.049
1:32000 (+)	1.083	0.869	0.437	0.236	0.144	0.091	0.072
1:32000 (-)	0.219	0.178	0.098	0.061	0.055	0.055	0.047
1:64000 (+)	0.704	0.530	0.266	0.146	0.097	0.062	0.050
1:64000 (-)	0.135	0.120	0.071	0.051	0.047	0.036	0.038

Table 2.3. Reproducibility and repeatability of indirect ELISA. Data is presented as optical density values. The standard deviation (SD) and percent coefficient of variation (CV%) are expressed for each sample.

Sample No.	Intra-assay variation (OD_{490} value)						Inter-assay variation (OD_{490} value)					
	1	2	3	Mean	SD	CV%	1	2	3	Mean	SD	CV%
1	3.660	3.800	3.710	3.723	0.058	1.56%	3.381	3.368	3.750	3.500	0.177	5.06%
2	2.120	2.251	2.176	2.182	0.054	2.46%	2.158	2.105	2.360	2.208	0.110	4.98%
3	1.146	1.174	1.112	1.144	0.025	2.22%	1.121	1.083	1.159	1.121	0.031	2.77%
4	0.939	0.974	0.954	0.956	0.014	1.50%	0.939	0.974	0.954	0.956	0.014	1.50%
5	0.848	0.980	0.975	0.934	0.061	6.54%	0.924	0.944	0.902	0.923	0.017	1.86%

Table 2.4. Viral specificity of muskellunge sera as depicted by mean OD_{490} values of pVHSivb-G vaccinated fish and two negative control (NC) muskellunge against different viral coating preparations. Three pools of sera collected from pVHSivb-G vaccinated muskellunge at 5, 8, and 10 weeks following a booster dose administration (week post booster = wpb) were also examined. Coating preparations included 0.1 µg $well^{-1}$ of purified VHSV-IVb (MI03) and 10^3 $TCID_{50}$ of the following: VHSV-IVb (MI03),VHSV-IVa (Makah), VHSV-IVc (2000-149) , IHNV (220-90), IPNV and LMBV.

Coating preparation	mean muskellunge sera OD_{490} values				
	vaccinated			negative control	
	5 wpb	8 wpb	10 wpb		
Purified VHSV-IVb	1.017	0.429	0.424	0.003	0.062
VHSV-IVb	0.854	0.375	0.345	0.065	0.011
VHSV-IVa	0.015	0.001	0.001	0.07	0.001
VHSV-IVc	0.014	0.004	0.001	0.011	0.001
IHNV	0.001	0.016	0.018	0.089	0.001
IPNV	0.004	0.000	0.004	0.044	0.011
LMBV	0.034	0.005	0.003	0.033	0.011
blank	0.008	0.005	0.002	0.001	0.006

Figure 2.1. A 10% SDS-PAGE followed by silver staining. Gel contains saturated ammonium sulfate (SAS) precipitation of the pooled naive muskellunge serum to be used for mice vaccination. *Lane 1* 3 µl of EZ-Run™ Prestained *Rec* Protein Ladder standards; *Lane 2* 2 µg of the 20% SAS; *Lane 3* 2 µg of the 30% SAS; *Lane 4* 2 µg of the 40% SAS; *Lane 5* 2 µg of the remaining SAS supernatant. Note the band at ~73 kDa (heavy chain) and ~26 kDa (light chain).

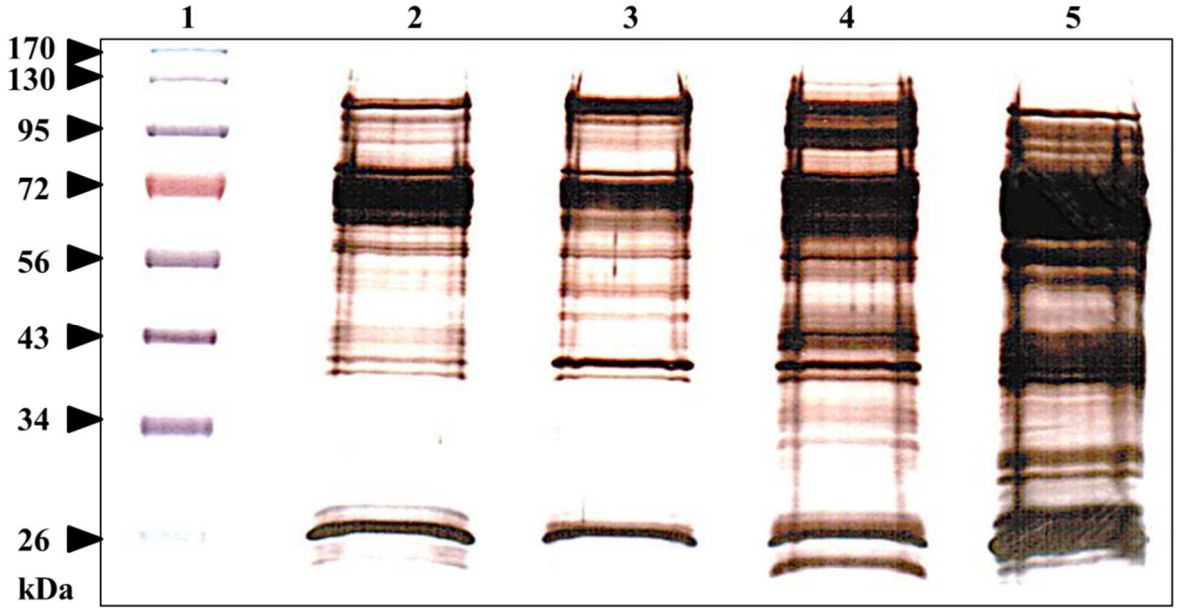


Figure 2.2. Immunoblot of 10% SDS-PAGE against SAS purified muskellunge Ig examining mAb production in 3B10 clones. *Lanes 1* RPMI media (negative control), *Lane 2* 3 µl of EZ-Run™ Prestained *Rec* Protein Ladder; *Lane 3 & 4* negative controls (10 µg lane^{-1} of muskellunge Ig followed by RPMI); *Lanes5-18* 10 µg lane^{-1} of the purified muskellunge Ig followed by 1:25 dilution of supernatant from 14 individual colonies; *Lanes -12, 14 & 18* selected clones. Note the band at ~73 kDa depicting mAb activity against muskellunge Ig (heavy chain).

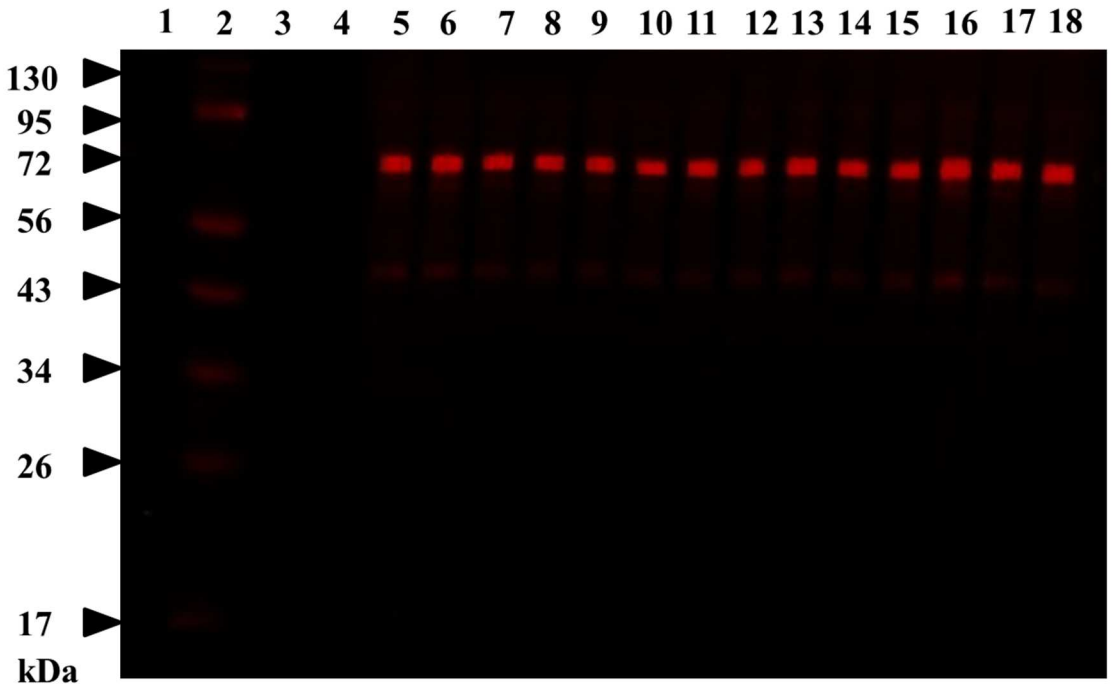


Figure 2.3. Two immunoblots of 10% SDS-PAGE gels examining 3B10 mAb against different fish species Ig. *Lanes 1a,5b* 3 µl of EZ-Run™ Prestained *Rec* Protein; *Lane 2a* 0.75 µg lane^{-1} of the SAS precipitated muskellunge Ig. Other *Lanes* are each loaded with 0.75 µg lane^{-1} of unpurified sera from different species unless otherwise noted. *Lane 3a* northern pike; *Lane 4a* 0.5 µg tiger muskellunge; *Lane 5a* chain pickerel. In the second immunoblot, *Lane 1b* fathead minnow; *Lane 2b* common carp; *Lane 3b* empty; *Lane 4b* SAS precipitated muskellunge Ig.

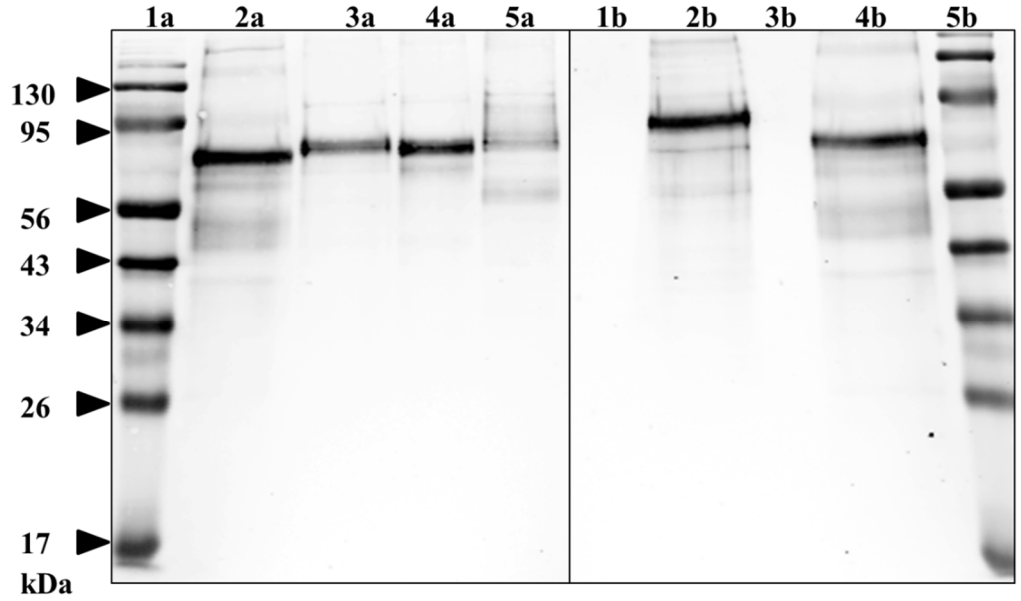


Figure 2.4. Levels of circulating anti-VHSV antibodies in 32 individual juvenile muskellunge, vaccinated with the pVHSivb-G vector on two occasions as indicated by OD_{490} values. Antibodies were assessed by the newly developed and optimized indirect ELISA using the 3B10 monoclonal antibody developed against the heavy chain of muskellunge immunoglobulin. The cut off value was estimated to be 0.163, above which a sample was considered positive.

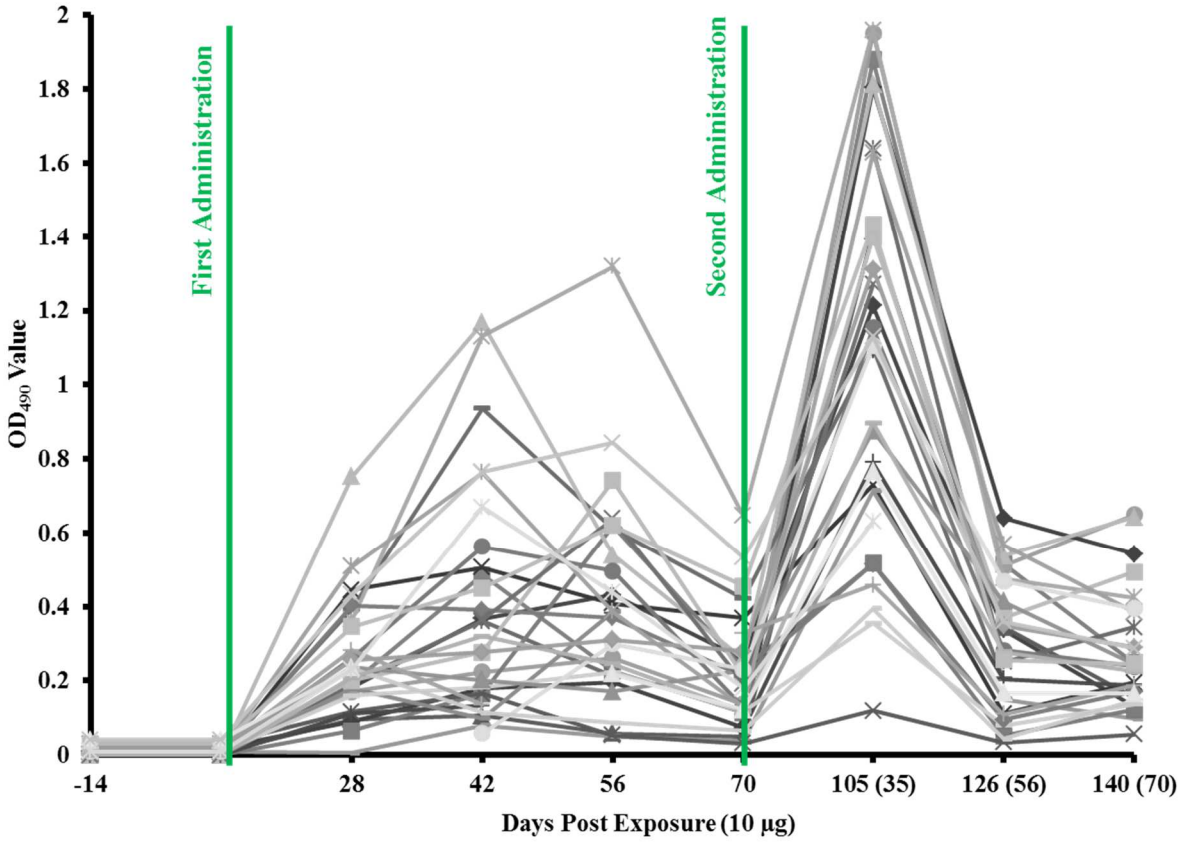
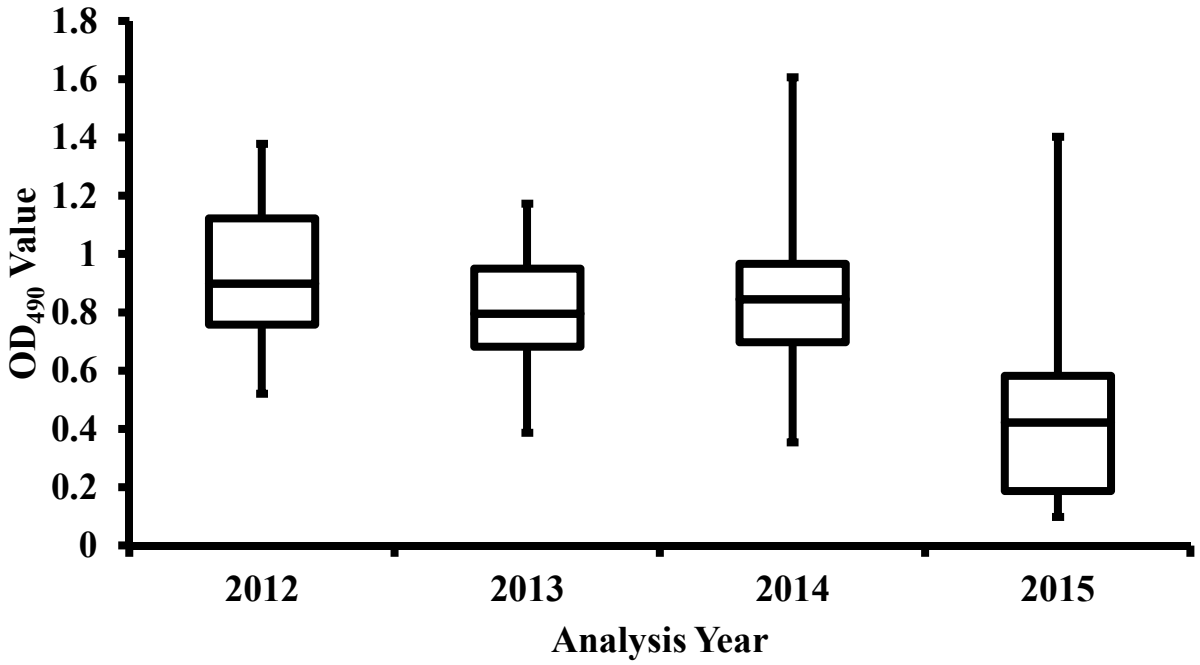


Figure 2.5. Levels of circulating anti-VHSV antibodies in Detroit River muskellunge sera collected 2012-2015 as assessed by the newly developed and optimized ELISA using the 3B10 monoclonal antibody developed against the heavy chain of muskellunge immunoglobulin. Data are presented as box plots of OD_{490} value displaying the mean and upper/lower quartiles; whiskers indicate the maximum and minimum observed values obtained for the respective populations. Cut off value was estimated to be 0.163, above which a sample was considered positive.



Chapter Three

A DNA vaccine encoding for the viral hemorrhagic septicemia virus genotype IVb glycoprotein confers protection in muskellunge (*Esox masquinongy*)

Abstract

This study builds upon a previous study performed in our laboratory that demonstrated that a DNA plasmid (pcDNA), containing a cytomegalovirus (CMV) immediate-early promoter and a viral hemorrhagic septicemia virus (VHSV) genotype IVb glycoprotein (G) gene insert (designated pVHSivb-G) conferred some protection in muskellunge (*Esox masquinongy*), a highly susceptible species. However, in the present study, muskellunge were given longer, up to 2400° days to mount an immune response before VHSV challenged by immersion. In four trials, we compared the protective response following vaccination with pVHSivb-G and pβ-VHSivb-G , the later containing a carp β-actin promoter, specifically designed and previously demonstrated to facility efficient gene transcription in fish cells. Following viral challenge, chronic shedding was also assessed in immunized, surviving muskellunge. Moreover, although DNA vaccines are typically reliant on a single IM administration, we investigated the effects of a booster dose of each plasmid. Under the outlined conditions, a single administration of the pVHSivb-G plasmid resulted in 95% RPS after an 1880° day incubation, while a booster administration after 940° day resulted in 100% RPS. Conversely, administration of the pβ-VHSivb-G resulted at best in a 25% RPS. Administration of the pVHSivb-G plasmid was also associated with the development of significant levels of circulating VHSV-binding antibodies, which reached peak levels 6-7 weeks post-vaccination, as determined by indirect ELISA. Low level transient shedding was detected in vaccinated survivors for up to 20 weeks post-challenge. Our data provides evidence that

muskellunge can be highly protected given sufficient optimization of a species-specific and virus-specific vaccination regimen. Additionally, we also shed light on several understudied aspects of VHSV DNA vaccination efficacy, including the duration of the secondary immune response, and viral shedding by immunized individuals following challenged.

1. Introduction

The emergence of a novel *Novirhabdovirus*, viral hemorrhagic septicemia virus (VHSV) genotype (IVb) in the Laurentian Great Lakes basin of North America alarmed fishery managers and researchers to the consequences its spread could bring to cultured facilities and wild fish populations (Elsayed et al. 2006; Faisal et al. 2012). This VHSV sublineage has an unusually wide host range, infecting 28 fish species with muskellunge (*Esox masquinongy*) being the most susceptible species documented to date (Animal & Plant Health Inspection Service 2009; Kim & Faisal 2010a; b; c). In the Great Lakes basin, numerous state and federal fish hatcheries are involved in propagation and stocking of a variety of fish species whose populations have been drastically suppressed from historical levels and in some cases are at risk of being endangered (Miller 1972). In most of these rehabilitation programs, efforts rely upon the collection of gametes from wild brood stock and raising offspring to life stages that will have high survival post-release (Dexter & O'Neal 2004). These culture practices engender risks of VHSV-IVb introduction into culture facilities and previously unexposed systems (Faisal et al. 2013) and emphasize the urgent need to develop an efficacious VHSV vaccine.

Two decades of research have demonstrated that DNA-based vaccines can be highly effective for conferring protection against European VHSV lineages in rainbow trout (*Oncorhynchus mykiss*),

a heavily cultured species due to economic importance (Kurath 2005). Indeed, most vaccine efficacy experiments to date have focused on this species and European VHSV genotype Ia (de Kinkelin & Bearzotti 1981; Boudinot et al. 1998; Lorenzen et al. 1998; Chico et al. 2009; Einer-Jensen et al. 2009). DNA preparations primarily incorporate the VHSV glycoprotein (G) gene under the control of a human cytomegalovirus (CMV) promoter (Lorenzen et al. 1990; 1998; 1999; 2001; Chico et al. 2009; Einer-Jensen et al. 2009; Hart et al. 2012). The G gene is targeted as the encoded protein is necessity for VHSV attachment, cellular internalization, and development of a neutralizing antibody response (Bearzotti et al. 1995; Lorenzen et al. 1990; Lorenzen & LaPatra 1999; Einer-Jensen et al. 2004). Studies show that intramuscular (IM) administrations of doses ranging from 1 μg (Chico et al. 2009; Einer-Jensen et al. 2009) to 10 μg (Lorenzen et al. 1998) of a plasmid containing the VHSV G gene, followed by an incubation period of 400-880° days conferred 83-96% relative percent survival (RPS) in rainbow trout, when immersion challenged with 10^4 to 3 x 10^6 median tissue culture infectious dose ($TCID_{50}$) mL^{-1} (Lorenzen et al. 1998; Chico et al. 2009; Einer-Jensen et al. 2009). A similar vaccine regimen was used for initial DNA vaccine trials against VHSV-IVb in muskellunge (Millard 2013). In that study, 10 μg of the pVHSivb-G plasmid, containing the IVb G gene and a CMV promoter was administered IM to muskellunge and after 539° days fish were VHSV immersion challenged with 10^5 plaque forming units (pfu) mL^{-1}, a dose that resulted in approximately 80% mortality in mock vaccinated fish. The G gene vaccination elicited the development of neutralizing antibodies, significant protection following challenge, and significantly lower infection prevalence in surviving fish. However, only 45% RPS was achieved, which is suboptimal especially when compared to what is achieved using conventional VHSV DNA vaccine in salmonids

The lower protection observed in muskellunge could be a result of numerous factors, foremost of which appears to be the low prevalence of a neutralizing antibody response at 539° days when fish were challenged. Additionally, the susceptibility of muskellunge to VHSV-IVb, with an intraperitoneal (IP) LD_{50} of only 2.2 pfu (Kim & Faisal 2010c) is comparatively much higher than that of rainbow trout to VHSV-Ia (Emmenegger et al. 2013). Since VHSV-IVb represents a novel freshwater genotype of VHSV, immune response kinetics and specifically the muskellunge immune competency against DNA vaccines are largely unknown. It is unclear if an initial administration of an antigen is sufficient to prime a protective response. On this basis, increasing the time fish had to mount an immune response, incorporating a booster dose and decreasing the viral challenge concentration will result in a more accurate determination of RPS. Research has shown that vaccination regimens and different plasmid promoter/enhancer sequences can also be important factors influencing the protection afforded to individual species (Heppell et al. 1998; Chico et al. 2009). For example another plasmid, pAE6, which contains a carp β-actin promoter (1^{st} exon and 1^{st} intron) has been used previously and demonstrated efficient transcription in fish (Brocal 2006; Chico et al. 2009; Choi et al. 2012).

In this present study, we compared two promoters contained in the pAE6 and pcDNA plasmids for their ability to deliver the VHSV-IVb G gene. We continued to utilize intramuscular administrations of 10 μg of plasmid as this was demonstrated by Millard (2013) to elicit a protective response in muskellunge. Building from the Millard (2013) study, we attempted to maximize vaccine efficacy in three ways: 1) by administering a second dose of each plasmid; 2) by increasing the time muskellunge have to mount an immune response; 3) by decreasing the

VHSV-IVb immersion challenge concentrations. Moreover, whereas earlier studies have examined the protective mechanisms and immune responses associated with rhabdoviral DNA-based preparations in rainbow trout (LaPatra et al. 2001; Lorenzen et al. 2001) and Pacific herring, *Clupea pallasii* (Hart et al 2012), little work has been conducted in other species such as muskellunge. We therefore assessed several unexplored aspects of vaccine efficacy. For example, we determined whether DNA vaccination could elicit the development of circulating anti-VHSV antibodies, specifically the kinetics associated with the primary and secondary antibody response using the indirect ELISA developed in Chapter Two of this dissertation. Finally, in another novel investigation we investigated the frequency and duration of viral shedding by immunized individuals following challenge.

2. Materials and methods

2.1. Fish

Two groups of muskellunge were used in this study. The first group of muskellunge, used in the first three vaccine trials, was obtained at 16 weeks post-hatch [average 14.2 cm (SD =1.4) fork length, 11.9 g (SD = 3.8)] from Chautauqua State Fish Hatchery (New York Department of Environmental Conservation, Chautauqua, NY). The second group of muskellunge, used in the fourth vaccine trial, was obtained at 14 weeks post hatch [average 15.6 cm (SD = 0.8) fork length, 16.0 g (SD = 1.1)] from Wolf Lake State Fish Hatchery (Michigan Department of Natural Resources, Mattawan, MI). Both groups of fish were certified to be free of important diseases according to World Organization for Animal Health (OIE) testing guidelines (2009). Fish were acclimated in 500-L circular fiberglass tanks in a continuous flow-through system with facility-chilled well water (11°C) and supplemental aeration. Vaccination and experimental challenges

were performed at the Michigan State University Research Containment Facility (East Lansing, MI). Muskellunge were fed live fathead minnows (*Pimephales promelas*) obtained from Anderson Farms Inc. (Lonoke, AR) that were certified free of important diseases according to OIE guidelines. An additional 60 fathead minnows were necropsied and underwent additional testing according to the American Fisheries Society guidelines (AFS-FHS 2012). Muskellunge were fed *ad libitum* throughout the study except for the 1st week post viral challenge when minnows were withheld. Two weeks prior to immunization, randomly selected fish were transferred and acclimated to 72-L polyethylene flow-through tanks (Pentair Aquatic Eco-Systems, Apopka, FL) with supplemental aeration. Up to ten fish were distributed to each tank, resulting in maximum density of 1 fish 7.2 L^{-1}.

2.2. Virus and cell culture

VHSV genotype IVb isolate used throughout the duration of the study was the Great Lakes index strain MI03 (Elsayed et al. 2006). The isolate has been maintained by continuous subculture in the cell line *epithelioma papulosum cyprini* (EPC). The EPC cell line was maintained and subcultured in 150-cm^2 tissue culture flasks (Corning Inc., Corning, NY) at 25°C using a basal media of Earle's salt-based minimal essential medium (MEM) (Invitrogen Corp., Carlsbad, CA) and supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU mL^{-1}) (Invitrogen), streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), and sodium bicarbonate (7.5% w/v) (Sigma, St. Louis, MO). Active viral concentrations were determined using plaque assay on EPC cell line using 7% (w/v) polyethylene glycol 15-20,000 MW (Fisher Scientific, Waltham, MA) and a 0.65%

methylcellulose (Sigma) overlay (Batts & Winton 1989; Batts et al. 1991). Virus was then aliquoted into cryogenic vials (Corning) for one time use and stored at -80°C.

2.3. Construction of pβ-VHSivb-G plasmid

The pAE6 vector (T. Chen, University of Connecticut, USA) contains a 2577 base pairs (bp) regulatory sequence of the carp β-actin gene, specifically the promoter sequence, 1st exon, and 1st intron of the gene (Liu et al. 1990). All polymerase chain reactions (PCR) were optimized and conducted on an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and visualized using gel electrophoresis. Briefly, viral RNA was extracted with the QIAamp Viral RNA (Qiagen Inc. Valencia, CA) kit following the manufacturer's instructions. The complete open reading frame (1524 bps) of the G gene (Gene Bank accession ADB93792), was reverse transcribed and amplified using conventional polymerase chain reaction (PCR). Reverse transcription was performed using the Affinity Script Multiple Temperature Reverse Transcriptase™ (Agilent Technologies Inc., Santa Clara, CA) following the manufacturer's instructions and the specific glycoprotein forward primer (GF1) 5'-ATG GAA TGG AAT ACT TTT-3' (Table 3.1). PCR was conducted in duplicate reactions using 5 µL of viral cDNA, 12.5 µL of 2X GoTaq Green MasterMix (Promega, Madison, WI), 0.8 µM of GF1 and the specific Greverse primer (GR1) 5'-TCA GAC CAT CGG GTT TCT-3'. DNase-free water was added to bring the final reaction volume to 25 µL. The reaction was incubated at 94°C for 5 min followed by 35 cycles of PCR (denaturing at 94°C for 30 sec, annealing at 60 °C for 30 sec and elongation at 72°C for 90 sec). The resulting amplicon was then used as template for a second PCR reaction for the addition of sub-cloning restriction sites. The glycoprotein forward primer 2 (GF2) 5'-CCA ACT CGA GAT GGA ATG GAA TAC TTT T-3' contained an *Xho*I restriction site

(CTCGA/G) and protective bases (CCAA) and the glycoprotein reverse primer 2 (GR2) 5'- CCA ATC TAG ATC AGA CCA TCG GGT TTC T-3' contained an *Xba*I restriction site (TCTAG/A) and protective bases. The PCR was conducted using the same reagent concentrations and cycle parameters described above. Products were excised and purified using Wizard SV Gel and PCR Clean-Up System (Promega). The purified PCR product was then cloned into the pCR®2.1-TOPO® vector (Invitrogen) and transformed into *dam-/dcm-* competent *E. coli* (New England BioLabs Inc., Ipswich, MA) following the manufacturer's protocol. Simultaneously, the pAE6 vector was transformed into the *dam-/dcm-* cells using the same protocol. These methyltransferase deficient cells were selected as methylation interfered with subsequent restriction digestion.

Transformed *E. coli* were plated on Luria-Bertani (LB) agar plates (Fisher Scientific Inc., Pittsburgh, PA) containing 50 μg mL^{-1} ampicillin (Roche, Indianapolis, IN) and incubated overnight at 37°C. Colonies were picked and analyzed using M13 primer set (forward: 5'-GTA AAA CGA CGG CCA G-3' and reverse: 5'-CAG GAA ACA GCT ATG AC-3') and the TOPO TA Cloning® (Invitrogen) instructions. To confirm the presence of the pAE6 plasmid, internal primers, pAE6 forward (pAE6F) 5'- TAA CGC AGG AAA GAA CAT-3' and pAE6 reverse (pAE6R) 5'- AAC GAC CTA CAC CGA ACT-3', were designed to create a 381 bp amplicon. The PCR was then conducted using 1 μL of LB containing *E. coli*, 12.5 μL of 2X GoTaq Green MasterMix (Promega), 0.4 μM of each primer, and DNase-free water to bring the final reaction volume to 25 μL. The reaction was incubated at 94°C for 5 min followed by 30 cycles of PCR (denaturing at 94°C for 30 sec, annealing at 49°C for 30 sec and elongation at 72°C for 30 sec). G gene amplicons were visualized using gel electrophoresis, purified and sequenced to confirm

identity and orientation. Positive colonies were inoculated into 50 mL LB broth cultures containing 100µg mL^{-1} ampicillin and incubated overnight at 37°C on a plate rocker. Both plasmids were purified using the Qiagen® Plasmid Midi Kit (Qiagen) following the manufacturer's instructions and suspended in DNase free water. Plasmid preparations were then quantified using the Qubit™ dsDNA BR kit and a Qubit™ Fluorometer (Invitrogen).

Purified pCR®2.1 vector containing the glycoprotein gene was digested using both *Xho*I and *Xba*I endonucleases (New England BioLabs) simultaneously. Duplicate reactions, each containing 1µg of purified plasmid, 1.5 U of both *Xba*I and *Xho*I, 5 µL of 10X Cutsmart Buffer (New England Biolabs) and DNase free water to a final volume of 50 µL. Reactions were incubated at 37°C for 90 min then inactivated at 65°C for 20 min. The pAE6 vector was linearized under the same conditions. The digested glycoprotein gene and linearized vector were then purified using Wizard SV Gel and PCR Clean-Up System (Promega).

The glycoprotein gene was ligated into the pAE6 vector using T4 DNA Ligase using the Rapid DNA Ligation Kit (Thermo Scientific, Waltham, MA) and manufacturer's instructions. The ligated plasmid was then transformed into One Shot® Top10 chemically competent cells (Invitrogen). The transformed cells were plated and grown overnight on LB agar plates described previously. Clones were picked and inserts were confirmed using a set of primers flanking the plasmid polylinker, the pAE6 cloning forward primer (pAE6CF) 5'- ATT CCA CTG CTC CCA TTC -3' and pAE6 cloning reverse primer (pAE6CR) 5'-ACT CCA CAT GGT CAC ATG CT-3', which amplify a 322 bp portion of the pAE6 plasmid. The PCR reaction consisted of 12.5 µL of 2X GoTaq Green MasterMix, 0.4 µM of each primer, 5 µL of template, and DNase-free water

(25 µL final reaction volume). The reaction was incubated for 35 cycles consisting of 94°C for 30 sec, 54°C for 30 sec and 72°C for 110 sec. Inserts were confirmed with gel electrophoresis, the reaction produced a 322 bp fragment in the empty undigested pAE6 vector and an 1836 bp amplicon in clones containing the complete VHSV-IVb glycoprotein gene sequence. Positive clones were sequenced to confirm sequence identity and orientation. Following confirmation, clones were inoculated in quadruplicate 450 mL LB broth cultures containing 50 µg mL^{-1} ampicillin and grown at 37°C overnight in a rocking incubator. Plasmids were purified using the Qiagen® Plasmid Maxi Kit (Qiagen Inc.) following manufacturer's instructions. Plasmids were quantified and suspended in sterile phosphate buffered saline (PBS; Sigma) at 1 mg mL^{-1} and stored at -80°C until use. The final products were designated pβ-VHSivb-G and pAE6 (mock).

2.4. Construction of pVHSivb-G plasmid

The pcDNA_3.1 (+) is a commercially available vector containing the human cytomegalovirus (CMV) immediate-early promoter. The DNA vaccine construct containing the VHSV-IVb glycoprotein gene (pVHSivb-G, Millard 2013) was modeled after successful DNA vaccines against VHSV genotype I (Lorenzen et al 1998, Heppell et al. 1998) and IHNV (Anderson et al 1996). The construction and production of this plasmid were outsourced to Life Technologies (Carlsbad, CA). In brief, an *Eco*RI restriction site (G/AATTC) followed by a kozak consensus sequence terminating with the first amino acid of the complete MI03GL VHSV-IVb isolate glycoprotein gene (1524 bp) was synthesized; an *Xba*I restriction site (T/CTAGA) was then added following the 3' termination codon. The assembled fragment was then digested using the described endonucleases and sub-cloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen). The plasmid was transformed and propagated into K12 *Escherichia coli*.

Sequencing confirmed the correct glycoprotein gene sequence and orientation. The pcDNA 3.1(+) vector without the glycoprotein gene was similarly propagated and used as a negative (mock) control. Both plasmids were suspended in sterile PBS and stored at -80°C until use. The final products were designated pVHSivb-G and pcDNA (mock).

2.5. Vaccination

Immediately prior to vaccination, all plasmid vectors were thawed and diluted to 10 μg in 100 μL sterile PBS. Randomly selected fish were anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222) (Western Chemical Inc., Ferndale, WA) and buffered with 0.3 g L^{-1} sodium bicarbonate. Muskellunge were administered 10 μg intramuscularly in left epaxial musculature. Four total trials took place utilizing a varied number of °days – which is a function of the water temperature times the number of incubation days – (from 540 to 2400° days) within which fish were allowed to mount immune responses prior to challenge. Additionally, booster doses were applied following 940° days in trials 3 and 4. All trials used 60 min immersion challenges with challenge dose varying among trials (see below). Viral challenges were conducted in glass aquaria containing 32 L of chilled well water and supplemental external aeration. Fish were challenged via immersion in static water containing infectious virus for 60 min. All negative challenge controls were similarly challenged with minimal essential media. After 60 min, fish were returned to their respective tanks and monitored daily for morbidity and mortality for 28 days.

2.5a. Trial 1

Trial 1 initially investigated the protection conferred by the pβ-VHSivb-G vector. Muskellunge were initially vaccinated with 10 µg of either the pβ-VHSivb-G and or pAE6 (mock) plasmids. A total of 29 pβ-VHSivb-G and 30 pAE6 muskellunge were divided into three tanks containing 9 to 10 fish each for both treatments respectively. An additional tank containing 10 muskellunge for each treatment was maintained as non-infected controls. Muskellunge were allowed to build an immune response for 540° days (approximately 7 weeks in 11°C water). Fish were then immersion challenged with 3 x 10^4 pfu mL^{-1} VHSV-IVb, returned to their respective tanks and monitored for 28 days. All mortalities were necropsied and kidneys, spleen and heart samples were aseptically collected and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., York, UK) on high speed for 2 min. Homogenates were diluted 1:4 (w/v) with MEM supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}) and Amphotericin B (250 µg mL^{-1}, Invitrogen). Samples were centrifuged at 2000*g* for 30 min at 4°C and inoculated onto EPC monolayers. After 14 days supernatant was removed, frozen at -80°C, thawed and centrifuged at 2000*g* for 15 min at 4°C. Supernatant was then re-infected onto fresh EPC monolayer and incubated for 14 days before being examined for viral cytopathic effect (CPE). RNA from suspicious samples was then extracted using the QIAamp Viral RNA kit and following the manufacturer's instructions. The presence of VHSV was confirmed using real-time reverse transcription polymerase chain reaction (rt-PCR) assay specific for VHSV (Jonstrup et al. 2013; Warg et al. 2014). The assay was conducted using the 900 nM of both the forward primer 5'-AAA CTC GCA GGA TGT GTG CGT CC-3' (rtPCRF) (Table 3.1), and reverse primer 5'-TCT GCG ATC TCA GTC AGG ATG AA-3' (rtPCRR) and 250 nM of probe 5'-/56-FAM/TA GAG GGC CTT GGT GAT CTT CTG /3BHQ_1/-3' (rtPCRP). Briefly, 25 µL

reactions contained 12.5μL of QuantiTect Probe RT-PCR kit (Qiagen) mastermix, 5 μL of RNA template and 0.25 μL of QuantiTect Probe RT/RNase enzyme. Reactions took place in an Eppendorf Realplex2 Mastercycler (Eppendorf) with 30 min at 50°C and 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 40 sec at 60°C and 20 sec at 72°C.

2.5b. Trial 2

In Trial 2, the other plasmid, pVHSivb-G was examined for its ability to elicit a protective response following a single administration and a longer period – an additional 1340 ° days compared to trial 1 – to react to the plasmid. Following a single administration of 10 μg of either pVHSivb-G or pcDNA (mock) plasmids, fish were allowed to mount an immune response for 1880° days (24 weeks at 11°C). Fish were divided into 2 tanks containing 10 fish each per preparation. An additional tank containing 10 muskellunge for each treatment was maintained throughout the study as non-infected controls. Experimental tanks were also immersion challenged with at a lower concentration of 2×10^3 pfu mL^{-1}. Samples were collected and viral re-isolation took place as previously described.

2.5c. Trial 3

Trial 3 compared the efficacy of the pβ-VHSivb-G and pVHSivb-G vectors. Twenty fish each were intramuscularly vaccinated with 10 μg of pβ-VHSivb-G, pAE6, pVHSivb-G and pcDNA. Fish were divided into 2 tanks containing 10 fish each per treatment. Again, an additional tank containing 10 muskellunge for each treatment was maintained throughout the study as non-infected controls. The fish were allowed to build an immune response for the same duration as Trial 2 (1880° days). However, after 940° days (12 weeks in 11°C water), a booster dose of 10

μg of their respective inoculum was administered to each fish. Following a second period of 940° days, fish were immersion challenged at a viral concentration of 2 x 10^3 pfu mL^{-1}. After 28 days, samples were collected and evaluated as previously described.

2.5d. Trial 4

In Trial 4, only the pVHSivb-G vector was examined. Twenty fish each were intramuscularly vaccinated with 10 μg of pVHSivb-G and pcDNA. Fish were divided into 2 tanks containing 10 fish each per treatment. With respect to conditions, Trial 4 was nearly identical to that of Trial 3 except that fish were given 1460° days (an additional 520° days) following booster administration before they were challenged. Fish had a total of 2400° days to mount an immune response. Experimental tanks were challenged with 2 x 10^3 pfu mL^{-1} and monitored for mortalities for 28 days. Samples were collected and evaluated as previously described.

2.6. Detection of anti-VHSV antibodies in muskellunge sera using an indirect ELISA procedure

Blood samples were collected non-lethal using a caudal venipuncture from 27 anesthetized muskellunge from Chautauqua State Fish Hatchery two weeks prior to immunization. Following collection, blood was stored at 4°C for 2 hours and centrifuged at 2700*g* for 10 min at 4°C. Serum was then aliquoted and stored at -80°C until analysis. Muskellunge then received an intramuscular vaccination of 10 μg of the pVHSivb-G preparation. Sera was collected and processed as described above every two weeks until 10 weeks (770° days) post vaccination. Half of the fish (n =15) were then immersion challenged with 2 x 10^3 pfu mL^{-1} as previously described. Sera were then collected from muskellunge 6, 12, 15, 18, 20, 30 and 34 weeks post

challenge. All sera samples were analyzed using a muskellunge specific indirect ELISA to assess circulating anti-VHSV antibodies as described in Chapter Two of this dissertation.

Prior to ELISA, serum was heat inactivated at 45°C for 30 min. Serum was centrifuged at 2700*g* for 10 min. at 4°C immediately prior to dilution in a solution of 1% nonfat dried milk in PBS (dilution of PBS-5% NFDM, Sigma). Indirect ELISA took place in polystyrene microplates (96-well, Microlon®600 with chimney wells; Greiner Bio-One, Monroe, NC). Plates were sealed during all incubation periods (SealPlate®; Sigma) and washed 5 times following each incubation period unless otherwise stated using PBS containing 0.05% Tween 20 (PBS-T20; Sigma) in an automated microplate washer (BioTek, 4Lx405™ plate washer; Winooski, VT).

Briefly, microtiter assay plates were coated with 100 µL well^{-1} of purified VHSV-IVb at 1 µg mL^{-1} and incubated overnight (14-16 hrs) at 4°C in a humid chamber. After the overnight incubation, plates were washed and unbound sites were blocked with the addition for 430 µL well $^{-1}$ of PBS containing 5% NFDM (PBS-5%; Sigma) and incubation at 37°C for 1 hr. Heat inactivated and diluted test and control muskellunge sera was then added to duplicate wells at 100 µL well^{-1}. After incubating at 25°C for 1 hr, plates were washed and 100 µL of 1:30,000 dilution of the 3B10 mouse anti-muskellunge-IgM mAb was added to all wells and again incubated at 25°C for 1 hr. Plates were washed and 100 µL of 1:4,000 dilution of a commercially available goat anti-mouse secondary horseradish peroxidase (HRP) conjugated antibody (Invitrogen) was added to each well and incubated at 25°C for 1 hr. Plates were developed by the addition of 100 µL of 0.4 mg mL^{-1} *o*-phenylenediamine (Sigma) in phosphate citrate buffer (Sigma) containing 3mM hydrogen peroxide (Avantor Performance Materials Inc., Center

Valley, PA). The reaction proceeded for 30 min at 25°C in the dark and was stopped with the addition of 50 L of 3 M sulfuric acid (H_2SO_4; Avantor Performance Materials Inc.). The optical density (OD) was read at 490 $_{nm}$ using a BioTek, ELx808™ plate reader (BioTek) and the Gen5 software (BioTek). The average value of blank wells was subtracted from test and control wells prior to analysis. The threshold established in Chapter Two of this dissertation, 0.163, was used for distinguishing between presence/absence of circulating antibodies.

2.7. Assessment of VHSV shedding by vaccinated and challenged muskellunge

Surviving muskellunge vaccinated with the pVHSivb-G vector from Trials 3 and 4 were used to examine for the presence of VHSV shedding. Samples were collected beginning four weeks post challenge and every four weeks for 28 weeks. Shedding analysis was conducted using a slightly modified protocol of that described by Kim & Faisal (2012). Briefly, individual surviving fish were transferred to glass aquaria containing 4 L of static facility chilled well water containing supplemental aeration. After 120 min, water was mixed and a 50 mL water sample was taken and fish were placed back into their original tanks. Samples were stored at 4°C until processing within 24 hrs. For processing, samples were vortexed and centrifuged at 2700*g*, at 4°C for 10 min. After centrifugation, a viral plaque assay (VPA) was conducted as previously described. After 6 days, cell monolayers were stained with crystal violet (Sigma) and 18% formaldehyde (Avantor Performance Materials Inc.).

2.8. Statistical analysis

For all evaluated trials, Kaplan-Meier survival estimates and mean time to death accounting for right censoring of survival data (i.e., mortality did not occur during the 28 day monitoring

period) were calculated for each tank of fish using PROC LIFETEST in SAS (SAS Institute 2010). 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 (Amend 1981).

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

Significant differences in cumulative mortality between or among treatments within a trial were tested using either *t*-tests (Trials 1, 2, and 4) or ANOVA followed by linear contrasts of factor level means of interest (Trial 3). T-tests were conducted using PROC TTEST whereas the ANOVA tests were conducted using PROC GLM in SAS.

The humoral response in pVHSivb-G vaccinated and challenged were initial examined using a paired *t*-test and subsequently analyzed using quantile regression, which allowed us to more fully examine the conditional relationship of the humoral response with respect to time. Separate regression models were estimated for post vaccination and post challenge responses. Conditional 0.50, 0.75, and 0.90 quantile regressions were fit to each set of data. For model fitting, OD values were $\log_e$ transformed with time (i.e., post vaccination or post challenge times) up to its third power used as explanatory variables. The significance of explanatory variables (i.e., time, square of time, cube of time) were tested using likelihood ratio tests. If an explanatory variable was not statistically significant, it was excluded as an explanatory variable and the model was refit. 95% confidence intervals for parameter estimates from the quantile regression models were obtained by resampling with the number of resampling iterations set at 1,000. Quantile regression models were fit in SAS using PROC QUANTREG.

3. Results

3.1. Vaccine efficacy

Throughout all trials, no mortalities occurred in the non-infected control tanks of any treatment (results not shown). In Trial 1, all three of the pβ-VHSivb-G replicates experienced 100% mortality (Figure 3.1), while the mean cumulative mortality for the pAE6 replicates was 96.7%. The mean day to death for the pβ-VHSivb-G replicates ranged from 8.0 (SE = 0.3) to 9.3 (SE = 0.6) days (Table 3.2) while the mean day to death of the pAE6 replicates ranged from 8.7 (SE = 0.4) to 10.6 (SE = 1.0) days. No significant difference was detected in the Cox proportional hazard ratio (1.55, 95% confidence limits 0.84-2.88) or cumulative mortality (*t*-test: $t = 1.00$, df = 2, $p = 0.422$) between pβ-VHSivb-G and pAE6 treatments. During necropsy, muskellunge exhibited characteristic clinical signs of acute VHSV infection including extensive petechial to ecchymotic hemorrhage throughout the musculature, liver, swim bladder and renal mesentery. Numerous fish exhibited severely pale gills, liver, and heart. VHSV was re-isolated from all of the mortalities on the EPC cell line and its identity confirmed by rt-PCR (data not shown).

For Trial 2, the two replicates receiving the pVHSivb-G plasmid experienced 0 and 10% cumulative mortality whereas the mock vaccinated replicates both experienced 100% mortality (Figure 3.1), resulting in a mean RPS of 95%. The mean day of death for the mock treatments was 11 (SE = 1.0) and 12.4 (SE = 1.3) whereas the mean day of death for the mock treatment was 16.0 (SE = NA) (mean day to death was only calculable for one tank and standard errors could not be calculated) (Table 3.2). The hazard ratio comparing the pVHSivb-G and mock treatments estimated from the frailty model was 0.016 with 95% confidence limits of 0.002 and 0.124 suggesting the pVHSivb-G treatment significantly reduced the hazard rate for muskellunge

(Table 3.3). There was a statistically significant difference in cumulative mortality between the pVHSivb-G and mock treatments (*t*-test: $t = -19.00$, df = 1, $p = 0.034$). All mortalities exhibited clinical signs of VHSV and rt-PCR confirmed VHSV-IVb as the cause of death of all mortalities.

In Trial 3, pβ-VHSivb-G replicates experienced cumulative mortality of 60 and 90% (mean = 75%) (Figure 3.1); with mean day of death of 7.7 (SE = 0.5) and 12.6 (SE=1.0) days (Table 3.2). Conversely, the pAE6 mock replicates experienced 100% cumulative mortalities with mean day of death of 8.7 (SE = 0.5) and 8.9 (SE = 1.1) days. Thus, the RPS for the pβ-VHSivb-G treatment was 25%. The hazard ratio comparing the pβ-VHSivb-G and pAE6 mock treatments was 0.561 with 95% confidence limits of 0.160 and 1.975 suggesting there was no significant difference in hazard between the treatments (Table 3.3). There was an overall statistically significant difference in cumulative mortality among all four treatment included in Trial 3 (ANOVA: $F = 14.30$, df = 3,4, $p = 0.013$). However, the linear contrast comparing the cumulative mortalities between the pβ-VHSivb-G and pAE6 mock treatments was not statistically significant ($F = 2.78$, df = 1,4, $p = 0.171$). The rt-PCR assay confirmed VHSV-IVb as the cause of death of all mortalities.

The pVHSivb-G replicates in Trial 3 experienced 0 and 30% mortality (Figure 3.1) with mean day of death of 10.6 (SE = 0.4) days (mean day of death could not be calculated for the other replicate since no mortalities occurred). Conversely, the pcDNA mock replicates experienced 100% mortality with mean day of death of 9.3 (SE = 0.9) to 10.4 (SE = 0.9) days resulting in an RPS of 85%. The hazard ratio comparing the pVHSivb-G and pcDNA mock treatments estimated was 0.068 with 95% confidence limits of 0.013 and 0.345 suggesting the pVHSivb-G

treatment significantly reduced the hazard rate for muskellunge. Cumulative mortality between the pVHSivb-G and pcDNA treatments was significantly different ($F = 32.11$, df = 1,4, $p = 0.005$). While the pVHSivb-G vector conveyed significant protection against infection. During necropsy, mortalities again exhibited distinct signs of acute VHSV infection.

With respect to comparisons between the pβ-VHSivb-G and pVHSivb-G treatments from Trial 3, the estimated hazard ratio for the treatments from the Cox proportional hazards frailty model was 10.94 with 95% confidence limits of 2.12 and 56.56 indicate that muskellunge vaccinated with the pβ-VHSivb-G vector had a much greater hazard than fish vaccinated with the pVHSivb-G vector (Table 3.3). The cumulative mortality of muskellunge vaccinated with the pVHSivb-G vector was also found to be significantly different than the cumulative mortality of fish vaccinated with the pβ-VHSivb-G vector based on linear contrasts of the factor-level means ($F = 16.00$, df = 1,4, $p = 0.016$).

In Trial 4, the pVHSivb-G replicates experienced 0% mortality while the pcDNA mock replicates experienced 100% mortality resulting in a RPS of 100% (Figure 3.1). The mean day of death of the pcDNA replicates were 12.2 (SE = 1.7) to 13.4 (SE =1.7) days (Table 3.2). This was clearly a significant level of protection. However, the hazard ratio between the treatments could not be calculated because of the lack of variability in treatment results. Similarly, the lack of variability in the treatment results resulted in questionable t-test results with regards to differences in cumulative mortality (t-test: $t = \infty$, df = 2, $p < 0.0001$).

3.2. Development of circulating anti-VHSV antibody response

Prior to vaccination the OD levels in the 27 naïve muskellunge were 0.008 (SD = 0.008). Anti-VHSV antibodies did not appear to significantly increased until measured 28 days following vaccine dose [0.248 (SD = 0.184)] (paired *t*-test; $p < 0.0001$, $t = 6.18$, df = 23). Anti-VHSV antibody levels appeared to peak between 42 days [0.315 (SD = 0.313)] and 56 days [(0.3.72 (SD = 0.256)] post exposure. Though, to precisely predict peak antibody levels and the duration of the secondary anti-VHSV antibody response following challenge, quantile regression analysis was utilized.

For all evaluated quantiles, the cube of time post-vaccination was not statistically significant by likelihood ratio testing and was dropped from the models (0.50: $x^2 = 3.30$, df = 1, $p = 0.069$; 0.75: $x^2 = 1.47$, df = 1, $p = 0.226$; 0.90: $x^2 = 3.80$, df = 1, $p = 0.051$). However, the square of time post-vaccination was statistically significant ($p < 0.0001$) for all models. The functional form of the quantile regression fitted relationships for the OD values was similar across the quantiles, and could perhaps best be characterized as bell-shaped (Figure 3.2). The model corresponding to a 0.90 quantile had a somewhat steeper relationship then the other models as a consequence of a few outlying OD values at between 4 and 10 weeks post-challenge (Table 3.4). Prior to administration of the pVHSivb-G vaccine preparation OD values were low [0.049 (SD = 0.017)]. Based on predicted relationships from the fitted quantile regression models, OD values would increase to a value 0.163 by 3 to 4.25 weeks depending on the quantile (Figure 4.2). Peak OD values would occur at around 7 weeks post vaccination, and would then decline back to 0.163 at around 10 to 11 weeks post vaccinations (Figure 3.2).

For the quantile regression models fit to the post challenge data, the cube of time post-challenge was not statistically significant (0.50: $x^2 = 2.31$, df = 1, $p = 0.128$; 0.75: $x^2 = 0.85$, df = 1, $p = 0.156$; 0.90: $x^2 = 0.57$, df = 1, $p = 0.449$). Similarly, the square of time post-challenge was not statistically significant (0.50: $x^2 = 2.09$, df = 1, $p = 0.148$; 0.75: $x^2 = 2.17$, df = 1, $p = 0.141$; 0.90: $x^2 = 1.44$, df = 1, $p = 0.231$). Consequently, both terms were dropped from the models. Time post-challenge was statistically significant ($p < 0.005$) for all models. The fitted relationships for each of the quantile regression models was suggestive of an exponential decline in OD values with respect to time (Figure 3.3, Table 3.4), however, this was likely influenced by the lack of observations for the 4 weeks immediately following challenge. Based on the predicted relationships from the regression models, OD values would not be expected to decline to less than the establish positive/negative threshold (0.163) until between weeks 45 and 65 post challenge (Figure 3.3).

3.3. Viral shedding in pVHSivb-G vaccinated survivors

Shedding was assessed in pVHSivb-G vaccinated survivors from trial 3 beginning 16 weeks post challenge and trial 4 beginning 4 weeks post challenge using a quantitative viral plaque assay. Low level of shedding was detected from a few survivors, with one fish at 4 and 20 weeks post challenge, and two fish 8 weeks post challenge shedding 10^4 to 2.0 x 10^4 pfu hour^{-1} (Figure 3.4). Two fish exhibited the maximum shedding concentration (10^5 pfu hour^{-1}) 16 weeks post challenge. No shedding was detected after 20 weeks post challenge from any individual. Overall, viral shedding from vaccinated survivors does appear minimal over a short term.

4. Discussion

This study reinforces the findings of Millard (2013), that a DNA containing the VHSV-IVb G gene downstream of a CMV promoter can confer protection in muskellunge. However, it also indicates that decreasing the viral challenge concentration to 2 x 10^3 pfu $mL^{-}1$ and increasing the number of degree days that vaccinated muskellunge have to mount an immune response prior to challenge can result in greater survivorship than the 45% RPS previously demonstrated (Millard 2013). Indeed, the time following exposure to an antigen and water temperature have been demonstrated to have a significant effect on host immune responses in fish (Le Morvan et al. 1998). 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. These levels of protection are more comparable to those previously achieved with DNA preparations against VHSV-I and a similar novirhabdovirus, IHNV in rainbow trout (Lorenzen et al. 1998; Einer-Jensen et al. 2009).

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. Previous studies have indicated efficient transcription by fish cells using the carp β-actin promoter (Brocal 2006; Choi et al. 2012) and one study by Chico et al. (2009) demonstrated nearly identical protection (83% RPS) when compared in the same study to CMV promoter. It is unclear why there was such low protection in muskellunge in the current study. Insufficient transcription by muskellunge is possible, though the carp β-actin promoter (Liu et al. 1990) has been successfully used in Ayu (*Plecoglossus altivelis*) (Cheng et al. 2002) which is a more derived species (Near et al. 2012) as well as rainbow trout (Chico et al. 2009) which is more ancestral compared to

muskellunge. However, transcription of this particular construct has not been studied. While it is possible that muskellunge could have been overwhelmed when challenged with 3 x 10^4 pfu mL^{-1} or given insufficient time to react to the antigen, this is unlikely as under the same conditions, muskellunge responded appropriately when administered the pVHSivb-G plasmid containing the CMV promoter.

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 7 weeks post vaccination. Though, in muskellunge from the previous study by Millard (2013), 7 weeks post vaccination corresponded with the initial development of a neutralizing antibody response, with only 1 of 12 vaccinated muskellunge exhibiting neutralizing anti-VHSV antibodies; though, four weeks later 60% of muskellunge exhibited neutralizing antibodies (Millard 2013). This finding emphasizes the importance a neutralizing anti-VHSV antibody response rather than just VHSV-binding antibodies in providing protection. 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 (Ye et al. 2010). Moreover, in rainbow trout, a period 12-15 weeks following immunization with a non-microbial antigen was associated with an increase antibody affinity (Ye et al. 2011) and may account for the higher survivorship in muskellunge. Further, in vaccinated muskellunge, following challenge, we were able to detect a vigorous secondary response, which we were able to predict would remain at detectable levels

for upwards of 40-50 weeks. 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 relative 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 (Bain et al. 2010). In general, VHSV shedding in fish surviving experimental challenge is of a transient nature and found only in a minority of fish (Kim & Faisal 2012). The results of this study similarly reflects these trends, however, determination of the kinetics of shedding by vaccinated and challenged fish should be ascertained. Unfortunately, other studies that described vaccine development against VHSV never addressed shedding as a measure of post-challenge vaccine efficacy (Boudinot et al. 1998; Chico et al. 2009; Einer-Jensen et al. 2009; Millard 2013)

Herein, 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 ant-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 (Tonheim et al 2008). 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.

APPENDIX

Table 3.1. Primer sequences.

Primer:	Sequence:
pAE6CR	5'-ACT CCA CAT GGT CAC ATG CT-3'
pAE6CF	5'-ATT CCA CTG CTC CCA TTC-3'
M13F	5’-GTA AAA CGA CGG CCA G-3’
M13R	5’-CAG GAA ACA GCT ATG AC-3’
pAE6F	5’- TAA CGC AGG AAA GAA CAT-3’
pAE6R	5’- AAC GAC CTA CAC CGA ACT-3’
GF1	5’-ATG GAA TGG AAT ACT TTT-3’
GR1	5’-TCA GAC CAT CGG GTT TCT-3’
GF2	5’-CCA ACT CGA GAT GGA ATG GAA TAC TTT T-3’
GR2	5’-CCA ATC TAG ATC AGA CCA TCG GGT TTC T-3’
rtPCRF	5'-AAA CTC GCA GGA TGT GTG CGT CC-3'
rtPCRR	5'-TCT GCG ATC TCA GTC AGG ATG AA-3'
rtPCRP	5'-/56-FAM/TA GAG GGC CTT GGT GAT CTT CTG /3BHQ_1/-3'

Table 3.2. Survivorship of individual tanks from each trial, number of fish in each tank, cumulative survival and mean day to death calculated using PROC LIFETEST in SAS.

	Fish	Cumulative mortality	Mean day to death ± SE
Trial 1			
pβ-VHSivb-G	10	100%	9.3 ± 0.6
pβ-VHSivb-G	10	100%	9.0 ± 0.3
pβ-VHSivb-G	9	100%	8.0 ± 0.3
pAE6	10	100%	10.4 ± 1.1
pAE6	10	90%	10.6 ± 1.0
pAE6	10	100%	8.7 ± 0.4
Trial 2			
pVHSivb-G	10	0%	NA
pVHSivb-G	10	10%	16
pcDNA	10	100%	11 ± 1.0
pcDNA	10	100%	12.4 ± 1.3
Trial 3			
pβ-VHSivb-G	10	90%	7.7 ± 0.5
pβ-VHSivb-G	10	60%	12.6 ± 1.0
pAE6	10	100%	8.7 ± 0.5
pAE6	10	100%	8.9 ± 1.1
pVHSivb-G	10	30%	10.6 ± 0.4
pVHSivb-G	10	0%	NA
pcDNA	10	100%	10.4 ± 0.9
pcDNA	10	100%	9.3 ± 0.9
Trial 4			
pVHSivb-G	10	0%	NA
pVHSivb-G	10	0%	NA
pcDNA	10	100%	12.2 ± 1.7
pcDNA	10	100%	13.4 ± 1.7

Table 3.3. Summary of the four trials conducted using muskellunge. Total number of fish challenged in each trial, mean cumulative % mortality, and mean days to death of each treatment. The table also includes mean day to death calculated using PROC LIFETEST in SAS. A Cox proportional hazard model was also fitted using SAS to examine the hazard ratio (HR) between glycoprotein (G) gene and mock vaccinated counterparts. The relative percent survival (RPS) was calculated between treatments as previously described (Amend 1981) and the associated *p*-values were calculated using ANOVA in SAS.

	Fish	**Mean Cumulative % Mortality**	**Mean day 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

Table 3.4. Parameter estimates from 0.50, 0.75, and 0.90 quantile regression models fit to $\log_e$ OD values post pVHSivb-G immunization and post VHSV challenge. The fitted regression models for post-vaccination included weeks post-vaccination (Time) and the square of weeks post-vaccination (Time×Time) as explanatory variables. The fitted regression models for post-challenge included weeks post-challenge (Time) as an explanatory variable.

Quantile	Model output	Intercept	Time	Time×Time
		Post-Vaccination		
0.5	estimate	-6.037	1.427	-0.102
	standard error	0.532	0.193	0.015
	95% confidence limits	-2.107	1.045 - 1.809	-0.059
0.75	estimate	-5.34	1.357	-0.097
	standard error	0.434	0.165	0.013
	95% confidence limits	-1.719	1.030 - 1.683	-0.052
0.9	estimate	-5.82	1.749	-0.129
	standard error	0.845	0.323	0.025
	95% confidence limits	-3.349	1.109 – 2.389	-0.1
		Post-Challenge		
0.5	estimate	1.304	-0.069	NA
	standard error	0.187	0.103	NA
	95% confidence limits	0.931 - 1.678	-0.041	NA
0.75	estimate	1.343	-0.048	NA
	standard error	0.224	0.016	NA
	95% confidence limits	0.896 - 1.791	-0.063	NA
0.9	estimate	1.573	-0.055	NA
	standard error	0.317	0.021	NA
	95% confidence limits	0.938 – 2.208	-0.085	NA

Figure 3.1. Cumulative mortality following VHSV-IVb immersion challenge of muskellunge by tank for the trials. Muskellunge were IM-vaccinated with 10 µg of ■ = pβ-VHSivb-G, ● = pAE6 (mock), ♦ = pVHSivb-G, ▲= pcDNA (mock). Time of challenge post-initial vaccination, +/-booster administration, and virus dose are as follows: Trial 1 (Top Left): 540° days, 3 x 10^4 pfu mL^{-1}; Trial 2 (Top Right): 1880° days, 2 x 10^3 pfu mL^{-1}; Trial 3 (Bottom Left): 1880° days, booster of 10 µg after 940° days, 2 x 10^3 pfu mL^{-1}; Trial 4 (Bottom Right): 2400° days, booster of 10 µg after 1460° days, 2 x 10^3 pfu mL^{-1}.

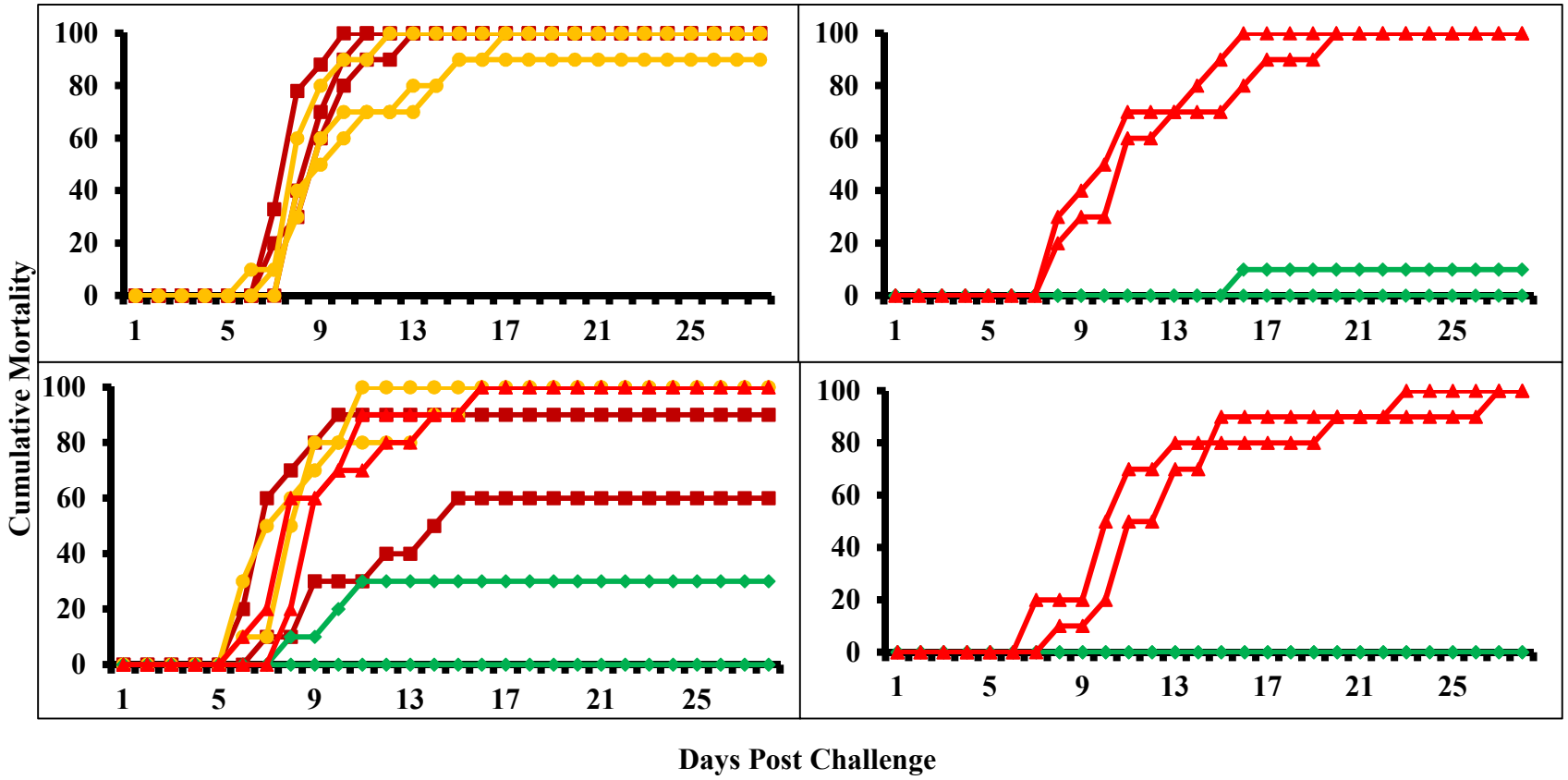


Figure 3.2. Levels of circulating anti-VHSV antibodies of 27 muskellunge for 10 weeks vaccinated with 10 µg of the pVHSivb-G indicated by indirect ELISA optical density (OD_{490}) values. The predicted relationships from 0.50, 0.75, and 0.90 quantile regression models was fit to the OD_{490} values as a function of time since vaccination. The dashed lines represent ±1 SE of the predicted relationships. The cut off value was estimated to be 0.163, above which a sample was considered positive.

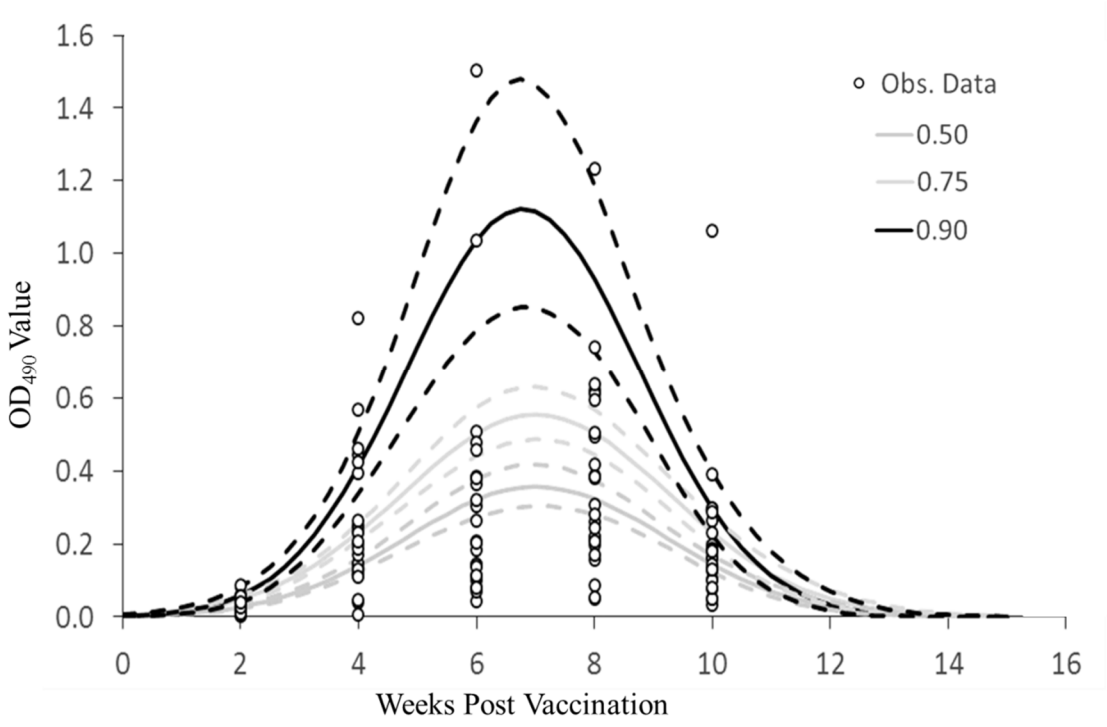


Figure 3.3. Levels of circulating anti-VHSV antibodies of 15 muskellunge following vaccinated with 10 µg of the pVHSivb-G and VHSV immersion challenged [(2 x 10^3 pfu mL^{-1}) for 60 min] indicated by indirect ELISA optical density (OD_{490}) values. Predicted relationships from 0.50, 0.75, and 0.90 quantile regression models fit to the OD values as a function of time since vaccination. The dashed lines represent ±1 SE of the predicted relationships. The cut off value was estimated to be 0.163, above which a sample was considered positive.

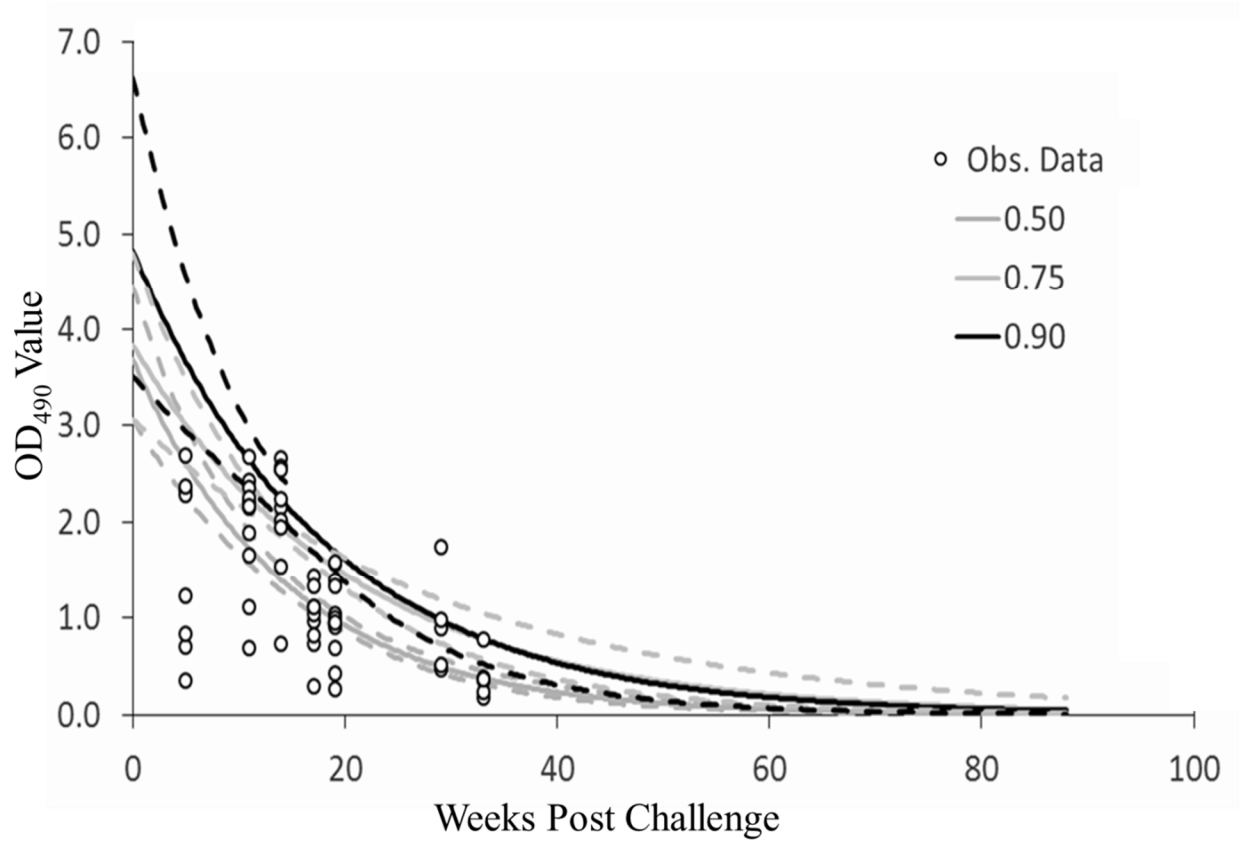
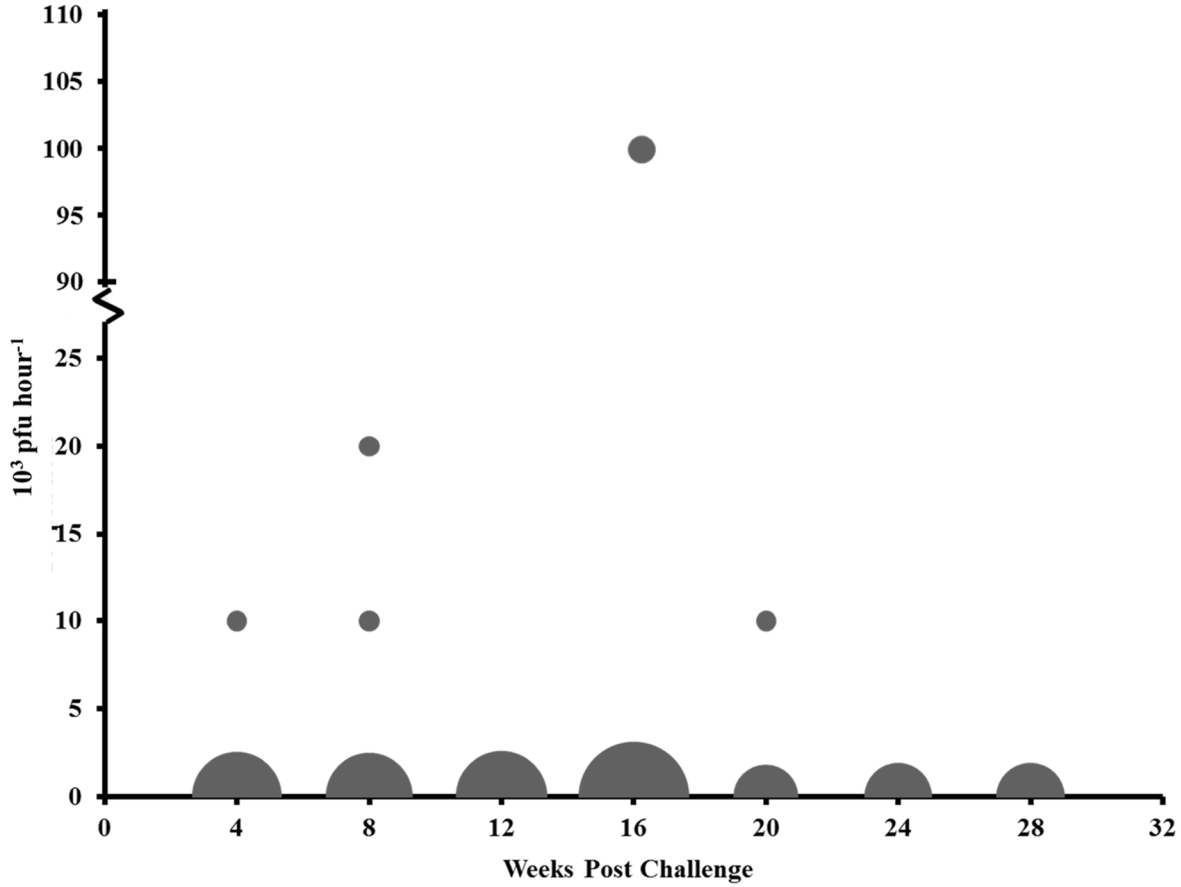


Figure 3.4. Weighted bubble graph displaying the analysis of shedding in pVHSivb-G vaccinated survivors (*n*=39) following an immersion challenge (2 x 10^3 pfu mL^{-1} for 60 min). Viral plaque assays (VPA) were conducted on water samples collected from individual surviving muskellunge every four weeks until 28 weeks post challenge.



Chapter Four

Efficacy of a DNA vaccine containing the viral hemorrhagic septicemia virus (IVb) glycoprotein gene in three salmonid species

Abstract

Previously we demonstrated that a DNA plasmid (pVHSivb-G) containing the Viral hemorrhagic septicemia virus (VHSV) genotype IVb glycoprotein (G) gene under the control of a cytomegalovirus (CMV) promoter can confer protection in muskellunge (*Esox masquinongy*), a highly susceptible species, when challenged with wildtype VHSV-IVb. In the present study we demonstrate that the pVHSivb-G plasmid can also confer protection in three salmonid species. Rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*) were selected for this study as representatives of three different genera that are common in the Laurentian Great Lakes and are heavily propagated in aquaculture as well as federal and state hatcheries. Our findings show that that an intramuscular administration of 10 μg of the pVHSivb-G plasmid results in significant protection against experimental challenge with VHSV-IVb. Up to 100% relative percent survival (RPS) was achieved with lake trout where not a single mortality was observed in immunized fish following an intraperitoneal VHSV-IVb challenge. Based on these findings, this DNA vaccine can protect numerous species and holds promise for protection of hatchery-propagated salmonids in general and captive broodstock in particular.

1. Introduction

A novel sublineage (IVb) of the highly pathogenic aquatic novirhabdovirus viral hemorrhagic septicemia (VHSV) emerged in the Great Lakes basin over a decade ago (Elsayed et al. 2006). The range of VHSV expanded nearly basin wide and caused numerous fish kills that involved multiple fish species (Faisal et al. 2012). More troubling, studies have confirmed the extraordinary wide host range of this novel VHSV lineage in freshwater fish (Lumsden et al. 2007; Kim & Faisal 2010a; b; c). However, the pathogenicity of genotype IVb also appears highly variable between species, with salmonids ranging from semi-resistance to susceptible (Kim & Faisal 2010b). These findings have left researchers and mangers to puzzle over ways to prevent the spread of VHSV and strategies to halt the possibility of viral dissemination into cultured fish populations. In the Great Lakes basin an extensive network of state, federal and tribal hatcheries collaborate in fish stock rehabilitation and enhancement programs. Of these fish stocks, millions of salmonid fry and fingerlings are annually propagated and released throughout the basin to ensure the perpetuity of a profitable recreational and commercial fishery. Some of these programs rely on the collection of gametes from fish returning to spawn in inland [e.g., Chinook salmon (*Oncorhynchus tshawytscha*), and coho salmon (*Oncorhynchus kisutch*)] while other programs raise captive broodstocks [e.g., rainbow trout, (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*)] (Dexter & O'Neal 2004). While field observation and experimental studies indicate that VHSV-IVb is not highly pathogenic to these species (Kim & Faisal 2010a; b); there are still concerns that exposed individuals may serve as viral reservoirs based on VHSV-IVb isolations from salmonids (Faisal et al. 2012). This is particularly true since VHSV-IVb has been previously detected in apparently health salmonid species and their sexual products (reviewed in Faisal et al. 2012). To date, stringent biosecurity

measures have successfully prevented VHSV-IVb from infecting hatchery populations. However, the potential risk of VHSV introduction into densely populated hatcheries underscores the need for a more concerted effort to heighten resistance to this deadly virus.

Previous studies have demonstrated that salmonid species can be protected against VHSV through the use of DNA-based vaccines (reviewed in Kurath 2005). Studies have extensively focused on rainbow trout because of its importance to aquaculture worldwide, and the VHSV genotype I sublineage a as it is the most prevalent in European aquaculture (Lorenzen et al. 1998; 1999; Einer-Jensen et al. 2009; Chico et al. 2009). VHSV vaccine preparations were designed to primarily target viral glycoprotein (G) gene encoded proteins since they are necessary for viral attachment to susceptible fish cells (Bearzotti et al. 1995; Lorenzen et al. 1990; Einer-Jensen et al. 2004). Studies performed in our laboratory (Millard 2013) as well as in Chapter Three of this dissertation have demonstrated that a DNA vector containing VHSV-IVb (G) gene (designated pVHSivb-G) can indeed confer protection in muskellunge (*Esox masquinongy*), a highly susceptible species to VHSV-IVb, following experimental viral challenge. Vaccination resulted in 95% relative percent survival (RPS) with a single administration or 100% RPS with two administrations.

To this end, the objective of this study was to determine if this same DNA vector preparation, containing the VHSV-IVb G gene can confer protection in salmonid species as we have demonstrated in muskellunge against this virus. Three species, rainbow trout, brown trout, and lake trout were selected to represent the three genera of trout and salmon found in the Great Lakes basin. Further, captive broodstocks of these species are maintained in hatcheries and in

some cases exposed to untreated surface water. These broodstock therefore experience a substantial viral threat and serve as the probable candidates for vaccination. The efficacy of this preparation will be assessed by determining the RPS following either one or two intramuscular administrations of a DNA vector containing the VHSV-IVb G gene under the control of a CMV promoter.

2. Materials and methods

2.1. Fish

Juvenile land-locked Eagle Lake morphotype of rainbow trout (*Oncorhynchus mykiss*) were obtained from the Oden State Fish Hatchery (MDNR, Alanson, MI). The rainbow trout were obtained at 12 weeks post-hatch [average of 3.6 cm (SD = 0.4) fork length, 0.7 g (SD = 0.3)]. Fingerlings of Gilchrest morphotype brown trout (*Salmo trutta*) were obtained at 16 weeks post-hatch [average 7.5 cm (SD = 1.1) fork length, 4.2 g (SD = 1.7)] from the same hatchery. Finally, juvenile Lake Superior strain of lake trout (*Salvelinus namaycush*) were obtained at 18 weeks post-hatch [average 17.6 cm (SD = 1.4) fork length, 24.1 g (SD = 6.5)] from Marquette State Fish Hatchery (MDNR, Marquette, MI).

All fish were certified to be free of important diseases according to World Organization for Animal Health (OIE) testing guidelines (2009). Fish were acclimated in 500-L circular fiberglass tanks in a continuous flow-through system with facility-chilled well water (11°C) and supplemental aeration. Vaccination and experimental challenges were performed at the Michigan State University Research Containment Facility (East Lansing, MI). All fish were fed *ad libitum* throughout the study; rainbow trout and lake trout were fed Skretting dry pellets (Skretting USA;

Tooele, UT), while the brown trout were fed Nelson's Silver Cup trout feed (Harrietta Hills, Harrietta, MI). Two weeks prior to immunization, fish were randomly distributed between 72-L polyethylene flow-through tanks (Pentair Aquatic Eco-Systems, Apopka, FL) with supplemental aeration.

2.2. Virus and cell culture

The VHSV genotype IVb isolate used throughout the duration of the study was the Great Lakes index strain MI03 (Elsayed et al. 2006). The isolate has been maintained by continuous subculture in the cell line *epithelioma papulosum cyprini* (EPC). The EPC cell line was maintained and subcultured in 150-cm^2 tissue culture flasks (Corning Inc., Corning, NY) at 25°C using a basal media of Earle's salt-based minimal essential medium (MEM) (Invitrogen Corp., Carlsbad, CA) and supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU mL^{-1}) (Invitrogen), streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), and sodium bicarbonate (7.5% w/v) (Sigma, St. Louis, MO). Active viral concentrations were determined using plaque assay on EPC cell line using 7% (w/v) polyethylene glycol 15-20,000 MW (Fisher Scientific, Waltham, MA) and a 0.65% methylcellulose (Sigma) overlay (Batts & Winton 1989; Batts et al. 1991). Virus was then aliquoted into cryogenic vials (Corning) for one time use and stored at -80°C.

2.3. Construction of pVHSivb-G plasmid

The DNA vector was identical to that used and described by Millard (2013) as well as in Chapters Two and Three of this dissertation. The construction and production of this plasmid were outsourced to Life Technologies (Carlsbad, CA). In brief, an *Eco*RI restriction site

(G/AATTC) followed by a kozak consensus sequence (Kozak 1987) terminating with the first amino acid of the complete MI03GL VHSV-IVb isolate glycoprotein gene (1524 bp) was synthesized; an *Xba*I restriction site (T/CTAGA) was then added following the 3' termination codon. The assembled fragment was then digested using the described endonucleases and sub-cloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen). The plasmid was transformed and propagated into K12 *Escherichia coli*. Sequencing confirmed the correct glycoprotein gene sequence and orientation. The pcDNA 3.1(+) vector without the glycoprotein gene was similarly propagated and used as a negative (mock) control. Both plasmids were suspended in sterile PBS and stored at -80°C until use. The final products were designated pVHSivb-G and pcDNA (mock).

2.4. Vaccination and challenge

Immediately prior to vaccination, the plasmids were thawed and diluted in sterile phosphate buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO). Randomly selected fish were anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222) (Western Chemical, Ferndale, WA) and buffered with 0.3 g L^{-1} sodium bicarbonate. All fish were immunized IM with 10 µg of plasmid DNA in the left epaxial musculature. Two trials took place for each species and in each trial after 546° days a subset of the vaccinated individuals was administered a second dose of the pVHSivb-G plasmid. For all three species a group of negative control fish remained unexposed to VHVS and was maintained throughout the study period. All fish were allows a total of 1001° days to react to the antigen prior to either intraperitoneal or immersion challenge. The number of ° days is a function of water temperature multiplied by the number of days trout had to mount an immune response. VHSV immersion challenges were conducted in glass aquaria containing 32 L

of static chilled well water containing 9.5 x10^4 pfu $mL^{-1}$for 60 min. All negative challenge controls were similarly challenged with MEM. After 60 min, fish were returned to their respective tanks and held at 11°C for 28 days during which they were monitored for morbidity and mortality. Cumulative mortality was used to determine the RPS (Amend 1981).

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

The statistical significance was analyzed using a conventional two-tailed χ^2-test.

All mortalities were necropsied and kidneys, spleen and heart samples were aseptically collected and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., York, UK) on high speed for 2 min. Homogenates were diluted 1:4 (w/v) with MEM supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}) and Amphotericin B (250 µg mL^{-1}, Invitrogen). Samples were centrifuged at 2000*g* for 30 min at 4°C and inoculated onto EPC monolayers. After 14 days supernatant was removed, frozen at -80°C, thawed and centrifuged at 2700*g* for 15 min at 4°C. Supernatant was then re-infected onto fresh EPC monolayer and incubated for 14 days before being examined for viral cytopathic effect (CPE). If CPE was not observed RNA from cell culture supernatant was then extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The presence of VHSV was confirmed using real-time reverse transcription polymerase chain reaction (rt-PCR) assay specific for VHSV (Jonstrup et al. 2013; Warg et al. 2014). The assay was conducted using the 900 nM of both the forward primer 5'-AAA CTC GCA GGA TGT GTG CGT CC-3' (rtPCRF), and reverse primer 5'-TCT GCG ATC TCA GTC AGG ATG AA-3' (rtPCRR) and 250 nM of probe 5'-/56-FAM/TA GAG GGC CTT GGT GAT CTT CTG /3BHQ_1/-3' (rtPCRP). Briefly, 25 µL reactions contained 12.5µL of QuantiTect Probe RT-PCR kit (Qiagen) mastermix,

5 µL of RNA template and 0.25 µL of QuantiTect Probe RT/RNase enzyme. Reactions took place in an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) with 30 min at 50°C and 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 40 sec at 60°C and 20 sec at 72°C.

2.4a. Rainbow trout trials

Rainbow trout trials (RBT-1 and RBT-2) differed in conditions only in the VHSV challenge route. In both trials, trout were immunized IM with 10 µg of plasmid DNA suspended in 100 µL of sterile PBS, n = 39, 29 for pVHSivb-G or pcDNA in RBT-1 and n = 48, 35 respectively for RBT-2. Trout from each treatment were returned to separate 72-L tanks and maintained throughout the study. After 546° days (6 weeks in 13°C water), a portion of the pVHSivb-G vaccinated fish were administered a 10 µg booster dose (n = 13, 31 for RBT-1, 2 respectively). Rainbow trout were then allowed to build an immune response for an additional 455° days prior to challenge (1001° days or 11 weeks at 13°C post initial vaccination). Fish from RBT-1 were anesthetized and challenged by IP injection with 9.5 x 10^5 pfu in 100 µL of sterile PBS. The negative control tank received sterile PBS. Fish from RBT-2 were challenged by immersion with 9.5 x10^4 pfu mL^{-1}.

2.4b. Brown trout trials

Brown trout trials (BNT-1 and BNT-2) were the same as rainbow trout trials in respect to conditions and viral challenge. Brown trout were immunized IM with 10 µg of pVHSivb-G or pcDNA plasmids (n = 56, 26 respectively for BNT-1 and n = 50, 28 respectively for BNT-2).

After 546° days, (*n*=18, 25 for BNT-1, 2 respectively), of the pVHSivb-G immunized brown trout received a 10 µg booster vaccine dose and were challenged 455° days later.

2.4c. Lake trout trials

For the purpose of consistency, the vaccination regimen in lake trout trials (LAT-1 and LAT-2) was identical to the rainbow and brown trout trials. However, in lake trout trials the immersion challenge route was not performed, instead we used two IP challenge concentrations. Lake trout were immunized IM with the pVHSivb-G or pcDNA plasmids (*n* = 61, 26 respectively for both LAT-1, 2). In both LAT-1 and LAT-2, After 546° days, 27 of the pVHSivb immunized lake trout received a 10 µg booster dose. Fish were challenged 455° days later. Fish in LAT-1 were IP challenged with 9.5 x 10^5 pfu in 100 µL PBS while lake trout in LAT-2 were IP challenged with a five-fold greater VHSV concentration, 4.75 x 10^6 pfu in 500 µL of PBS.

3. Results

Throughout this study, we are able to demonstrate significant protection in at least one trial of each of the three salmonid species. However, vaccine efficacy varied based on species, challenge route and the number of pVHSivb-G plasmid administrations. Throughout the study none of the unchallenged fish experienced mortality.

3.1a. Rainbow trout

Mock vaccinated rainbow trout in RBT-1 experienced moderate mortality following IP challenge, with 62.1% of individuals succumbing (Figure 4.1). The mortality was lower in both the G gene vaccination, with 26.9% cumulative mortality following a single administration and

15.4% mortality with two administrations. Significant protection was observed in G gene treatments, with 56.7% RPS ($\chi^2 = 6.83$, df = 1, $p = 0.009$) and 75.2% RPS ($\chi^2 = 7.84$, df = 1, $p = 0.005$) following one or two vector administration respectively (Table 4.1). During necropsy, all mortalities exhibited signs of VHSV infection including extensive petechial to ecchymotic hemorrhage throughout the musculature, liver, swim bladder and renal mesentery. VHSV was reisolated from individual tissues collected from all rainbow trout mortalities, including mock (18/18) and G gene (9/9) treatments. In RBT-2, only 3 mortalities in the 35 mock vaccinated fish occurred following immersion challenge. On the contrary, none of the vaccinated fish died and in both vaccination treatments (Figure 4.1, Table 4.1). But with a low mortality in the mock vaccinated fish, protection was not significant. Moreover, only two of the three mock vaccinated mortalities exhibited the clinical signs of VHSV described above and VHSV was not confirmed in this third individual.

3.1b. Brown trout

In BNT-1, mock vaccinated individuals experience 23.1% cumulative mortality (Figure 4.1) following IP challenge. Meanwhile, the vaccinated treatments experienced 5.3% with a single administration and 16.7% mortality following two administrations resulting in 77.2% and 27.8% RPS respectively (Table 4.1). Protection was only significant in the treatment that received a single administration of the pVHSivb-G plasmid ($\chi^2 = 4.48$, df = 1, $p = 0.034$). All brown trout mortalities exhibited the characteristic signs of VHSV infection previously described and VHSV was isolated from all tissues collected from mock (6/6) and G gene (5/5) mortalities. In BNT-2, mock vaccinated individuals experienced 10.7% cumulative mortality following immersion challenge (Figure 4.1). While the cumulative mortality of both the vaccinated treatments was

4%, resulting from only a single individuals dying in both the treatments, resulting in an identical 62.7% RPS (Table 4.1). However, mortalities were again too low to determine significant protection. We noted that the mortalities exhibited milder clinical signs of VHSV infection including less petechial hemorrhage throughout the liver, swim bladder and renal mesentery. Nevertheless, VHSV was isolated from tissue samples collected from all mock (3/3) and G gene (2/2) mortalities.

3.1c. Lake trout

In the first lake trout trial (LAT-1), fish were IP challenged using the moderate viral concentration used in previous trials. The mock vaccinated individuals experienced 30.7% cumulative mortality (Figure 4.1). Meanwhile, not a single vaccinated individual from pVHSivb-G treatments succumbed. Both treatments thus experienced significant protection (Table 4.1) with 100% RPS in both G gene treatments ($\chi^2 = 12.07$, df = 1, $p = 0.0005$ with a single administration) and ($\chi^2 = 9.79$, df = 1, $p = 0.0018$ with two administrations). Lake trout mortalities exhibited the clinical signs of VHSV infection as previously described and VHVS was isolated from all mock mortalities (8/8). In LAT-2, when IP challenged with a five-fold higher viral concentration, the mock vaccinated treatment experienced 34.6% cumulative mortality (Figure 4.1). As in the previous trial, not a single G gene vaccinated individual died. Both G gene treatments again experienced significant protection (Table 1.1) with 100% RPS ($\chi^2 = 13.85$, df = 1, $p = 0.0002$ with a single administration) and ($\chi^2 = 11.26$, df = 1, $p = 0.0008$ with two administrations). In both trials, lake trout mortalities exhibited the clinical signs of VHSV infection as previously described and VHVS was isolated from all mock mortalities (17/17).

4. Discussion

Despite their relatively low susceptibility (Kim & Faisal 2010b), field studies demonstrated that salmonids may contract VHSV-IVb infection (Faisal et al. 2012). Salmonids experience moderate mortality and morbidity upon exposure to VHSV-IVb (Kim & Faisal 2010b) and can act as a vehicle for viral dissemination, evidenced by frequently isolated from apparently healthy fish (reviewed in Faisal et al. 2012). 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 (Kim & Faisal 2010c) 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 (Subasinighe et al. 2001).

Studies describing vaccine development for fish pathogens used immersion in addition to intraperitoneal or intramuscular injections as administration routes as well as for experimental challenges (Estepa et al. 1994; Lococq-Xhonneux et al. 1994; Lorenzen et al. 1998; Chico et al. 2009). Unfortunately, immersion challenges did not result in sufficient cumulative mortality to accurately demonstrate significant protection and IP challenges were therefore utilized for viral exposure. While it may be argued that IP challenge does not mimic nature and is invasive in nature, the integrity of fish barriers are often compromised in high density aquaculture facilities due to handling and sorting, high density, social hierarchy conflicts, and imbalanced diet (Gilmour et al. 2005; Grobler et al. 2013).

Like other fish vaccines, the DNA vaccine was administered intramuscularly (Lorenzen et al. 1998; Chico et al. 2009; Einer-Jensen et al. 2009; Hart et al. 2012) and resulted in the in significant protection in the three salmonid species representatives following a single administration, a matter that corroborate well with the previous study using muskellunge in Chapter Three. 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 administration of pVHSivb-G plasmid died following challenge. This resulted in 100% RPS of all pVHSivb-G treatments and is indicative of significant protection. This protection was likely due in part to the long period (1001° days) provided to the host to develop an immune response against the viral antigen, which is substantially longer than the incubation reported (520-880° days) in other successful studies performed on rainbow trout using another VHSV genotype (Lorenzen et al. 1998; Einer-Jensen et al. 2009). The 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. Overall, a second administration of the vector appears unnecessary, though, the significant effects of second administration could be better evaluated using larger sample sizes.

In summary, we can now recommend 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 (Faisal et al. 2012). Though salmonids are less susceptible to genotype IVb, obtaining a protective vaccine will provide researchers and managers with a potent tool that can

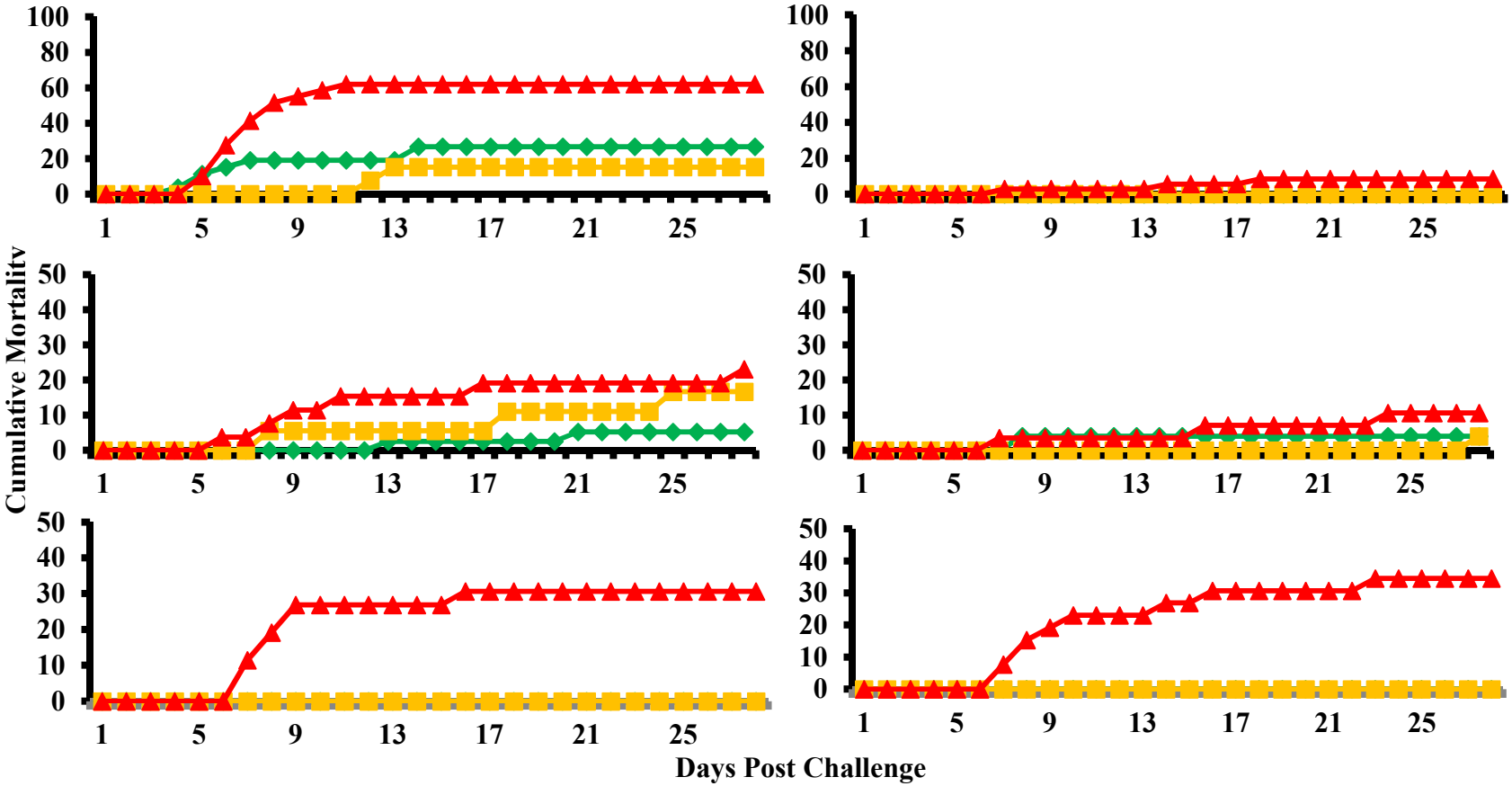
be used to limit transmission of VHSV-IVb. By vaccinating the millions of propagated salmonid fry and fingerlings prior to stocking, the Great Lakes hatchery system could be used to elicit a large-scale "herd immunity" response. With an overall decrease in the number of naïve-susceptible individuals being stocked, there would be a corresponding decreased viral transmission and an indirect protective effect to other naïve individuals. Moreover, the vaccine preparation may be used to protect of valuable captive broodstock. Broodstock populations in facilities using a surface water source are under constant threat of viral invasion and this vaccine preparation could be used to mitigate this peril. Certainly, there needs to be a more thorough examination of post vaccine efficacy and additional vector design before this preparation can be widely used. Regardless, the results we have obtained and the vaccination model we have developed can be used to examine the protection in other species and serve as a standard by which we can compare other vaccine preparations.

APPENDIX

Table 4.1. Results of vaccination trials in three salmonid species: rainbow trout (RBT-1 and RBT-2), brown trout (BNT-1 and BNT-2) and lake trout (LAT-1 and LAT-2). Table includes the number of fish in each treatment, the cumulative mortality and the relative percent survival (RPS) and the associated *p*-value calculated using a two-tailed χ^2-test (df = 1).

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

Figure 4.1. Cumulative mortality of trout following VHSV-IVb challenge after 1001° days. Trout were IM-vaccinated with 10 µg of pVHSivb-G (♦) or pcDNA (mock, ▲). Some trout were also administered a 10 µg booster dose of the VHSivb-G (■). Species and viral challenge dose and route were as follows: RBT-1 (Top left) rainbow trout, 9.5 x 10^5 pfu IP; RBT-2 (Top right) rainbow trout, 9.5 x 10^4 pfu mL^{-1} immersion; BNT-1 (Middle left) brown trout, 9.5 x 10^5 pfu IP; BNT-2 (Middle right) brown trout, 9.5 x 10^4 pfu mL^{-1} immersion; LAT-1 (Bottom left), lake trout, 9.5 x 10^5 pfu IP; LAT-2 (Bottom right) lake trout, 4.75 x 10^6 pfu IP.



Chapter Five

Production and application of a recombinant viral hemorrhagic septicemia virus genotype IVb glycoprotein

Abstract

The emerging *Novirhabdovirus* sublineage of the viral hemorrhagic septicemia virus (VHSV) genotype IVb has caused serious fish kills and become endemic throughout the Great Lakes basin of North America. This is troublesome since, until now, there are no protective vaccines currently approved against this deadly disease. Most recently, we demonstrated that significant protection can be elicited in a number of fish species using a DNA vaccine preparation containing the VHSV-IVb glycoprotein (G) gene. However, no DNA vaccine has been licensed in the USA for use in food animals, while the use of recombinant technology is becoming increasingly common. On this basis, we examine production of a recombinant glycoprotein for use as safe and effective subunit vaccine. A recombinant baculovirus containing a 5' polyhistidine tag embedded in VHSV-IVb G gene was used to infect cabbage looper (*Trichoplusia ni*) larvae. SDS-PAGE of nickel resin purified VHSV-IVb glycoprotein (rG) showed the protein remained glycosylated exhibiting a molecular weight of ~65kDa. In an indirect ELISA, muskellunge anti-VHSV-IVb antibodies were unable to distinguish between wells coated with the rG or purified whole VHSV-IVb, demonstrating that epitopes on the rG are probably identical to those found on VHSV envelope. We further utilized the rG as a vaccine antigen to elicit a protective response in naïve muskellunge (*Esox masquinongy*) against a VHSV-IVb immersion challenge. Muskellunge immunized with the rG were indeed protected, exhibiting 80% relative percent survival (RPS). Our results demonstrated that the cabbage looper

larvae infected with a recombinant baculovirus containing the VHSV-G gene serve as an excellent mechanism to produce viral glycoprotein for use as a vaccine against this deadly virus.

1. Introduction

A novel sublineage (genotype IVb) of the *Novirhabdovirus* viral hemorrhagic septicemia virus (VHSV) was first detected in the Laurentian Great Lakes basin in 2003 (Elsayed et al. 2006). In the years that followed, this new freshwater lineage spread throughout the basin, causing numerous fish kills (Faisal et al. 2012). This was worrisome as the Great Lakes fishery remains an important ecological resources and economic hotspot for both commercial and recreational fishing. The Great Lakes fisheries are extensively managed and reliant on stocking of hatchery propagation of numerous fish species that were found susceptible to this emerging VHSV strain (Kim & Faisal 2010a; b; c). Stocking however, results in the addition of naïve susceptible individuals and there are no preventative measures against VSHV-IVb currently approved by the American Food and Drug Administration (FDA). Recent advances using DNA vaccine technology and rhabdoviral glycoprotein gene (G) inserts have demonstrated significant protection against both VHSV and infectious hematopoietic necrosis virus (IHNV) (Lorenzen et al. 1998; Einer-Jensen et al. 2009). Canada has approved the use of a DNA vaccine against IHNV (Alonso & Leong 2013); while USA has not approved its use yet. Therefore, there is a need to examine other technology available to develop an efficacious vaccine against fish pathogenic rhabdoviruses.

There have been numerous studies indicating success using recombinant vaccines for VHSV. Initially, Lorenzen et al. (1993) demonstrated that the VHSV G gene cloned and expressed in

Escherichia coli (*E. coli*) and then inoculated into rainbow trout (*Oncorhynchus mykiss*) induced anti-VHSV antibody response, though, protection was not examined. In another study, a soluble non-glycosylated recombinant IHNV G protein preparation was able to induce an innate immune response and pro-inflammatory cytokines such as IFN-1 and IFN-γ in rainbow trout fry and some protection when challenged 3 days post immunization (Verjan et al. 2008). However, studies demonstrating an adaptive immune response that results in protection are lacking. Reports indicate that recombinant viral proteins produced in prokaryotes are limited for use in vaccine preparation due to improper protein folding which is attributed to a lack of post-translational alterations such as N-linked glycosylation (Thiry et al. 1990; Estepa et al. 1994; Lorenzen et al. 1999). This is important since VHSV glycoprotein epitopes are particularly dependent on their three dimensional conformation in order to elicit an effective neutralizing antibody response (Rocha et al. 2002). This led to efforts to produce protein using a eukaryotic system, such as insect cells infected with a recombinant baculovirus containing the VHSV (genotype Ia) glycoprotein gene. When recombinant baculovirus infected Sf9 (*Spodoptera frugiperda*) insect cells were intraperitoneally administered to rainbow trout fry, the glycoprotein elicited potent neutralizing antibodies response and resulted in 80.2% relative percent survival (RPS) (Lecocq-Xhonneux et al. 1994). Moreover, the combination of a baculovirus vector and Sf9 cells has also been shown to maintain native glycoprotein conformation and elicit a protective response against rabies virus in mice (Ramya et al. 2011). For the purpose of producing a subunit vaccine, prokaryotes appear inadequate because of the small scale and lack of glycosylation, while production using eukaryotes can circumvent both these shortcomings.

The production of recombinant glycoprotein in eukaryotes has demonstrated some success and is feasible for small scale production (Klepfer et al. 1993), however, insect larvae can be used to scale-up production and obtain larger quantities of protein (Kulakosky et al. 1998; Platteborze & Bloomfield 2000; Greenblatt et al. 2012). For example, whole cabbage looper (*Trichoplusia ni*) larvae, which are considered a pest species throughout North America, have been used with recombinant baculoviruses as a low cost protein production system (Greenblatt et al. 2012). Production in larvae appears highly efficient, with mg of recombinant protein g^{-1} of larvae tissue (Kovaleva and Davis 2016). One study found a single larva produced the equivalent amount of bioactive protein as 100 mL of an insect cell culture (Platteborze & Bloomfield 2000). Additionally, baculovirus infected cabbage loopers have already proven highly beneficial in advancing vaccine development. This system was employed to produce a highly efficacious subunit vaccine against both rabbit hemorrhagic disease virus (RHDV) (Pérez-Filgueira et al. 2007) and Human papillomavirus (Millán et al. 2010).

In this study, I describe the use of recombinant baculovirus containing the VHSV-IVb G gene to infect cabbage looper larvae and obtain purified, conformationally correct recombinant glycoprotein (rG). The rG protein was then examined for the ability to serve as alternative to whole virus in the development of VHSV-IVb specific diagnostic assays using the indirect ELISA developed in Chapter Two of this dissertation. The inability of muskellunge *Esox masquinongy* anti-VHSV-IVb antibodies to discriminate wells coated with the rG and purified whole VHSV-IVb would provide evidence that the rG is glycosylated. The rG is then assessed for its ability to elicit a protective immune response upon lethal VHSV-IVb immersion challenge. The indirect ELISA was again utilized to examine anti-VHSV antibodies levels

following either the administration of the rG and VHSV challenge. The overall efficacy of the rG was examined by determining RPS and assessment of viral shedding following challenge.

2. Materials and methods

2.1. Viral hemorrhagic septicemia virus

The viral hemorrhagic septicemia virus (VHSV) genotype IVb isolate used throughout the duration of the study was the Great Lakes index strain MI03 (Elsayed et al. 2006). The isolate has been maintained by continuous subculture in the cell line *epithelioma papulosum cyprini* (EPC). The EPC cell line was maintained and subcultured in 150 cm^2 tissue culture flasks (Corning) at 25°C using a basal media of Earle's salt-based minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) and supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU mL^{-1}) (Invitrogen), streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (FBS, Gemini Bio Products, West Sacramento, CA), and sodium bicarbonate (7.5% w/v) (Sigma, St. Louis, MO). Active viral concentrations were determined using plaque assay on EPC cell line using 7% (w/v) polyethylene glycol 15-20,000 MW (Fisher Scientific, Waltham, MA) and a 0.65% methylcellulose (Sigma) overlay (Batts & Winton 1989; Batts et al. 1991). Virus was then aliquoted into cryogenic vials (Corning Inc., Corning, NY) for one time use and stored at -80°C until needed.

2.2. Recombinant baculovirus

The assembly of the recombinant baculovirus was conducted by GenScript® (Piscataway, NJ). The 1524 bps of the complete VHSV-IVb glycoprotein gene were synthesized from the (GenBank accession number DQ401193) and a 5' 6X poly-histidine (HIS) tag was embedded

into the G gene sequence immediately following the native glycoprotein start codon (ATG). The amplicon was then subcloned into an *Autographa californica* nuclear polyhedrosis virus (AcNPV) using *Eco*R I and *Hind* III restriction sites. A suspension culture of the Sf9 cell line (*Spondoptera frugiperda*) was used to propagate the recombinant baculovirus prior to shipment to Michigan State University (MSU). The recombinant baculovirus stock was then stored at 4°C until further use.

Once at MSU, the Sf21 (*Spondoptera frugiperda)* cell line was used to re-propagate the recombinant baculovirus. The Sf21 cell line was kindly provided by Dr. Thiem (Entomology Department, MSU, East Lansing, MI). The cell line was maintained and subcultured in 75 cm^2 tissue culture flasks (Corning) at 28 °C using a basal media of Grace's Insect medium w/ L-glutamine and sodium bicarbonate (Sigma) and supplemented with penicillin (100 IU mL^{-1}), streptomycin (0.1 mg mL^{-1}), amphotericin B (0.25 µg mL^{-1}) and 10% FBS, pH 6.2. To produce a stock of the recombinant baculovirus briefly, 75 cm^2 tissue culture flasks of Sf21 cells in exponential growth phase were infected at a multiplicity of infection, MOI = 2, of the recombinant baculovirus stock. After 72 hrs all cells and media were harvested and centrifuged at 150*g*, and the supernatant was stored at 4°C until larvae infection.

2.3. Infection of cabbage looper (*Trichoplusia ni*) larvae

Since the cabbage looper is considered a pest species, larval infection was conducted by Scientific Service Inc. (Newark, DE). Briefly, cabbage looper larvae at the early 5^{th} instar were infected with 1 µL $larvae^{-1}$of the high-titer recombinant baculovirus stock. Larvae were incubated for a total of 75 hrs with hrs 0-46.5 at 27.5 °C, hrs 46.5-70.5 at 25°C, and 27.5 °C for

the remainder of the incubation. After 75 hours, all infected larvae were moribund; with a mean harvest weight of 331.5 mg larvae^{-1}. The larvae were then euthanized by freezing and stored at -80°C until shipment back to our laboratory on dry ice for protein purification. Upon receipt, larvae were stored at -80°C until processed.

2.4. Purification and analysis of recombinant glycoprotein

Larvae were thawed, homogenized using a mortar and pestle and purified using the His60 Ni Superflow Resin & Gravity Purification Kit (Clontech Laboratories Inc., Mountain View, CA) following the manufacturer's instructions. Final eluted fractions were quantified using the Qubit™ Protein Assay kit and a Qubit™ Fluorometer (Invitrogen). The protein concentrations were adjusted to 1 mg mL^{-1} using sterile PBS and designated rG.

A 10% SDS-PAGE was used to analyze the purity protein preparation. Initial analysis of fractions was conducted under denaturing conditions. Briefly, 5 µg of the purified rG protein was combined 5:1 with sample loading buffer containing 2% SDS (Sigma), 5% β-mercaptoethanol (Sigma) and 0.05% bromophenol blue (Sigma) and boiled for 5 min. A Precision Plus Protein™ Kaleidoscope™ (Bio-Rad Laboratories Inc., Hercules, CA) protein standard was loading alongside protein preparations into 1.0 mm gels, cast as described by Brunelle & Green (2014). Gels were run in a Mini-PROTEAN electrophoresis system (Bio-Rad Laboratories Inc.) at 150V for 60 min. Proteins were then visualized using Denville Blue™ Protein Stain (Denville Scientific Inc., Metuchen, NJ) following the manufacturer's instructions.

2.5. Recombinant glycoprotein epitope confirmation

The epitope conformation and binding capabilities of the rG produced in the cabbage looper was compared to a purified preparation of whole VHSV using the indirect ELISA developed in Chapter Two of this dissertation. The individual muskellunge sera used in this assay were obtained from either naive muskellunge or muskellunge surviving multiple IP infections with 10^5 pfu of VHSV-IVb. Serum aliquots from four positive sera samples (PC1-PC4), two negatives (NC1, NC2) and a rainbow trout (RBT) non-species control were first identified using the indirect ELISA. Prior to ELISA, sera were heat inactivated at 45°C for 30 min, vortexed, centrifuged at 2700*g* at 4°C for 10 min, and diluted 1:10 in PBS containing 1% nonfat dried milk (PBS-1% NFDM, Sigma).

The conditions of the indirect ELISA were previously optimized for coating with purified VHSV-IVb overnight at 4°C, and all other incubations were conducted for 1 hr at room temperature, with the exception of blocking that was conducted at 37°C. Under these conditions, the optimal concentration of purified VHSV-IVb and serum dilution were determined to be 0.1µg well^{-1} and 1:10 respectively. While, the optimal concentrations of the primary 3B10 mouse anti-muskellunge mAb and the goat anti-mouse horseradish peroxidase (HRP) secondary antibody were determined to be 1:30,000 (3.3 ng well^{-1}) and 1:4000 respectively (37.5 ng well^{-1}).

Four fractions from two separate larvae purifications exhibiting high concentrations of rG, and whole VHSV were first adjusted to a concentration of 1 µg mL^{-1} in PBS (pH 7.4), to mirror the optimal concentration determined for purified VHSV. Microtiter assay plates (96-well, Microlon®600 with chimney wells; Greiner Bio-One, Monroe, NC) were then coated with

100µL well^{-1} of each of these five preparations. Coated plates were then incubated overnight at 4°C in a humid chamber. Plates were washed and unbound sites were blocked with the addition for 430 µL well $^{-1}$ of PBS containing 5% NFDM (PBS-5%; Sigma Aldrich) at 37°C for 1hr. Heat inactivated and diluted sera was then added to duplicate wells at 100 µL well^{-1}. Thereafter the ELISA proceeded as previously described in Chapters Two and Three. Optical density (OD) was read at 490 $_{nm}$ on a BioTek, ELx808™ plate reader (BioTek, Winooski, VT) and the Gen5 software (BioTek).

2.6. Vaccination and challenge

Juvenile muskellunge were obtained at 14 weeks post-hatch [average 17.6 cm (SD = 1.4) fork length, 24.1 g (SD = 6.5)] from Wolf Lake State Fish Hatchery (Michigan Department of Natural Resources, Mattawan, MI) and certified free of important disease according to the guidelines of the World Organization for Animal Health (OIE 2009). Fish were acclimated to 12°C and held in a 500-L circular fiberglass tank in a continuous flow-through system with facility-chilled well water and supplemental aeration. Fish were housed and all experiments were conducted in the University Research Containment Facility (URCF, MSU, East Lansing, MI). Muskellunge were fed live fathead minnows (*Pimephales promelas*) obtained from Anderson Farms Inc. (Lonoke, AR) and similarly certified as free of important disease (OIE 2009). Muskellunge were fed *ad libitum* throughout the duration of the study except for the 1st week post viral challenge when minnows were withheld. Two weeks prior to immunization, randomly selected fish were acclimated to two 72-L polyethylene flow-through tanks (Pentair Aquatic Eco-Systems, Apopka, FL) containing supplemental aeration. Ten fish were distributed to two tanks treamtment^{-1} at a maximum density of 1 fish 7.2 L^{-1}.

For administration of vaccine, muskellunge were first anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222) (Western Chemical, Ferndale, WA) buffered with 0.3 g L^{-1} sodium bicarbonate (Sigma). Muskellunge (n = 20) were intramuscularly administered 10 µg of rG suspended in100 µL of sterile PBS in the left epaxial muscle slightly posterior to the pectoral fins. Mock vaccinated muskellunge (n = 20) similarly received 100 µL PBS. Muskellunge were allowed to react to the protein for 588° day (7 weeks at 12° C) before being VHSV-IVb immersion challenged. Immersion challenges were conducted in separate glass aquaria containing static water with VHSV-IVb at a concentration of 2 x 10^3 pfu mL^{-1} and supplemental aeration for 60 min. Fish were then move back into their original tanks and monitored for morbidity and mortality daily for 39 days at which point mortalities had ceased. The cumulative mortalities were then used to determine the mean relative percent survival (RPS) for the vaccinated treatment (Amend 1981).

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

The statistical significance of survivorship was calculated using a conventional two-tailed χ^2-test. To confirm the cause of death, all mortalities were necropsied and kidneys, spleen and heart samples were aseptically collected and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., York, UK) on high speed for 2 min. Homogenates were diluted 1:4 (w/v) with MEM supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}) and Amphotericin B (250 µg mL^{-1}, Invitrogen). Samples were centrifuged at 2700*g* for 30 min at 4°C and inoculated onto EPC monolayers. After 14 days supernatant was removed, frozen at -80°C, thawed and centrifuged at 2700*g* for 15 min at 4°C. Supernatant was then re-infected onto fresh EPC monolayer and incubated for 14 days before being examined for

viral cytopathic effect (CPE). If CPE was not observed RNA from cell culture supernatant was then extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The presence of VHSV was confirmed using real-time reverse transcription polymerase chain reaction (rt-PCR) assay specific for VHSV (Jonstrup et al. 2013; Warg et al. 2014). The assay was conducted using the 900 nM of both the forward primer 5'-AAA CTC GCA GGA TGT GTG CGT CC-3' (rtPCRF), and reverse primer 5'-TCT GCG ATC TCA GTC AGG ATG AA-3' (rtPCRR) and 250 nM of probe 5'-/56-FAM/TA GAG GGC CTT GGT GAT CTT CTG /3BHQ_1/-3' (rtPCRP). Briefly, 25 µL reactions contained 12.5µL of QuantiTect Probe RT-PCR kit (Qiagen) mastermix, 5 µL of RNA template and 0.25 µL of QuantiTect Probe RT/RNase enzyme. Reactions took place in an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) with 30 min at 50°C and 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 40 sec at 60°C and 20 sec at 72°C.

2.7. Post-challenge analysis of anti-VHSV antibodies and viral shedding

The rG vaccinated survivors were assessed for the presence of both circulating anti-VHSV antibodies and viral shedding. To detect anti-VHSV antibodies, blood was collected non-lethally from ten recombinant vaccinated survivors using a caudal venipuncture at 8 weeks post challenge. Following collection, blood was stored at 4°C for 2 hours then centrifuged at 2700*g* for 10 min at 4°C. Serum was then aliquoted and stored at -80°C until analysis. Serum was analyzed using the indirect ELISA as previously described.

Viral shedding of was assessed in ten individual rG vaccinated survivors at 8 and 10 weeks following challenge. These time points were selected based on the frequency and timing of

VSHV shedding that occurred in DNA vaccinated muskellunge surviving infection in Chapter Three. Analysis was conducted using a slightly modified protocol of that described by Kim & Faisal (2012). Briefly, individual surviving fish were transferred to glass aquaria containing 4 L of static facility chilled well water containing supplemental aeration. After 120 min, water was mixed and a 50 mL water sample was taken and fish were placed back into their original tanks. Samples were stored at 4°C until processing within 24 hrs. For processing, samples were vortexed and centrifuged at 2700*g*, at 4°C for 10 min. After centrifugation, a viral plaque assay (VPA) was conducted as previously described. After 6 days, cell monolayers were stained with crystal violet (Sigma) and 18% formaldehyde (Avantor Performance Materials Inc., Center Valley, PA).

3. Results

3.1. Recombinant protein production in larvae

Following purification of recombinant protein from several infected cabbage looper larvae, quantification indicated that yields of the purified glycoprotein (rG) varied considerably between eluted fractions; ranging from 117 to 420 μg mL^{-1}. SDS-PAGE analysis of fractions exhibiting the greatest protein concentration demonstrated high rG purity with a clear band at ~65 kDa (Figure 5.1). Purified fractions with highest rG protein concentration and purity were selected for additional analysis.

3.2. Epitope recognition using positive muskellunge sera in an indirect ELISA

Various sera were analyzed for their ability to bind to four fractions of the eluted r-ivb-G and compared to the whole VHSV virion using indirect ELISA. Optical density (OD) values for rainbow trout (RBT) were consistently below 0.010 (Figure 5.2). Furthermore, the two negative

control (NC) muskellunge sera also exhibited low OD values, all below 0.065. However, it was surprising how consistent the OD values of the positive controls (PCs) remained across the different coating solutions. PC1 exhibited the highest OD value of 3.133 in wells coated with whole purified VHSV as well as a low standard deviation (SD = 0.083) across all the coating reagents. PC2 and 3 exhibited similar reactivity to the rG, with OD values ranging from 0.614 to 0.865 and 1.500 to 1.681 respectively. Both PC2 and PC3 also exhibited low standard deviation between coating solutions, SDs = 0.092, 0.069 respectively. PC4 exhibited the greatest variation in OD values to different coating agents with a standard deviation of 0.156 and OD values ranging from 0.044 against the rG to 0.44 in the well coated with whole purified VHSV coated wells. Overall, the OD values for the all the positive sera was consistently highest in the wells coated with whole purified VHSV.

3.3. Efficacy of rG in protecting muskellunge

The two mock vaccinated replicate tanks experienced 70 and 80% cumulative mortality (Figure 5.3). 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, $p < 0.0001$). During necropsy, all mortalities exhibited clinical signs of acute VHSV infection including moderate to severely pale gills, liver, and heart. There was also extensive petechial to ecchymotic hemorrhage throughout the musculature, liver, swim bladder and renal mesentery. VHSV was isolated from visceral tissues of all mortalities (18/18).

3.4. Post-challenge analysis of immunized muskellunge

Unfortunately, sera was only obtained and analyzed from seven rG vaccinated survivors. OD values ranged from 0.094-0.817 while the mean OD value was 0.57 (SD = 0.31). An initial

comparison can be to OD values of sera obtained at an identical time point from nine muskellunge that received a DNA vaccine (10 μg of the pVHSivb-G plasmid described in the previous Chapters) and survived infection with an identical virus concentration (Figure 5.4). The mean OD values of the rG immunized survivors was significantly lower than the pVHSivb-G vaccinated survivors, [1.31 (SD = 0.74)] (unpaired *t*-test, $t = 2.17$, df = 13, $p = 0.049$). Therefore, at this one time point – when the secondary humoral response should be at its peak – the amount of circulating antibodies elicited by the rG appears lower than the secondary response observed following DNA vaccination.

Shedding was assessed in ten rG immunized-challenged survivors at 8 and 10 weeks post challenge using a viral plaque assay. Only a single individual at a single time point was found to be actively shedding. At eight weeks post challenge this individual was shedding 5×10^4 pfu $hour^{-1}$, a comparable shedding rate to those found in muskellunge administered the pVHSivb-G plasmid in Chapter Three.

4. Discussion

In this Chapter I describe the successful production of 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 (Thiry et al. 1990). 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 (Lecocq-Xhonneux et al. 1994). This

finding also corroborates numerous studies demonstrating that the VHSV G protein to be the major target protein for neutralizing and protective antibodies (Bearzotti et al. 1995; Lorenzen et al. 1990; 1999; Einer-Jensen et al. 2004) since the protein is integral for the attachment to susceptible cells (Jørgensen et al. 1995; Wagner & Rose 1996).

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. For example, in a previous study the protection of a prokaryotic recombinant glycoprotein was directly compared to a DNA vector containing the VHSV glycoprotein gene in Japanese flounder (*Paralichthys olivaceus*). Flounder vaccinated with the recombinant protein experienced 84-96% cumulative mortality compared to 4% cumulative mortality in the DNA vaccinated individuals (Byon et al. 2006). The use of a recombinant baculoviruses to infect larvae rather than insect cells would also allow for larger scale more efficient protein production (Platteborze & Bloomfield 2000; Greenblatt et al. 2012; Kovaleva et al. 2016). Although we have used cabbage looper larvae, the recombinant baculovirus could also be used to explore and optimize protein production in larvae from a wide range of insect species (Kulakosky et al. 1998).

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.

The recombinant protein vaccination regimen may require additional optimization in order to achieve equivalent protection as DNA-based vaccines. For example, allowing additional time before challenge may result in a more efficient neutralizing antibody response, similar to the results we demonstrated in Chapter Three. Where following single intramuscular administration of 10 μg of the pVHSivb-G plasmid in muskellunge, fish were given 1880° day before challenge, resulting in 95% RPS. However, in an initial comparison between the rG and pVHSivb-G preparations, muskellunge immunized with the rG protein seemingly exhibited a less vigorous secondary humoral response, evidenced by the lower levels of circulating anti-VHSV antibodies. In subsequent trials, the RPS of the rG protein could also be increased by use of a booster dose, adjuvant (Petrovsky & Aguilar 2004; Pérez-Filgueira et al. 2007; Nasicimento et al. 2012), or incorporation in a liposomal-based delivery system (Schmidt et al. 2016). Although, similar to our findings using the pVHSivb-G plasmid, immunized and protected individuals that survive infection do appear capable of disseminating the virus as we did note an instance of viral shedding. Viral shedding from immunized individuals, albeit at low levels and ephemeral, is often not investigated and our results demonstrate greater consideration of post challenge efficacy is needed.

In summary, we illustrate the successful use the use of a recombinant baculovirus in a eukaryotic expression system to obtain purified VHSV-IVb glycoprotein. We then demonstrated that this recombinant eukaryotic glycoprotein has numerous applications, namely as a subunit vaccine antigen with the ability to elicit a protective response in muskellunge. Recombinant technology holds clear promise for use as potential vaccine preparation that may be more innocuous than DNA-based preparations. Though these results are preliminary, and additional trials are necessary the use of recombinant technology certainly has promise and warrants further investigation.

APPENDIX

Figure 5.1. 10% SDS-PAGE of an eluted recombinant glycoprotein fraction. *Lane 1* 3 µL Precision Plus Protein™ Kaleidoscope™ protein standard. *Lane2* 5 µg of rG. Note the band at 65kDa corresponding with the predicated molecular weight of the glycosylated glycoprotein.

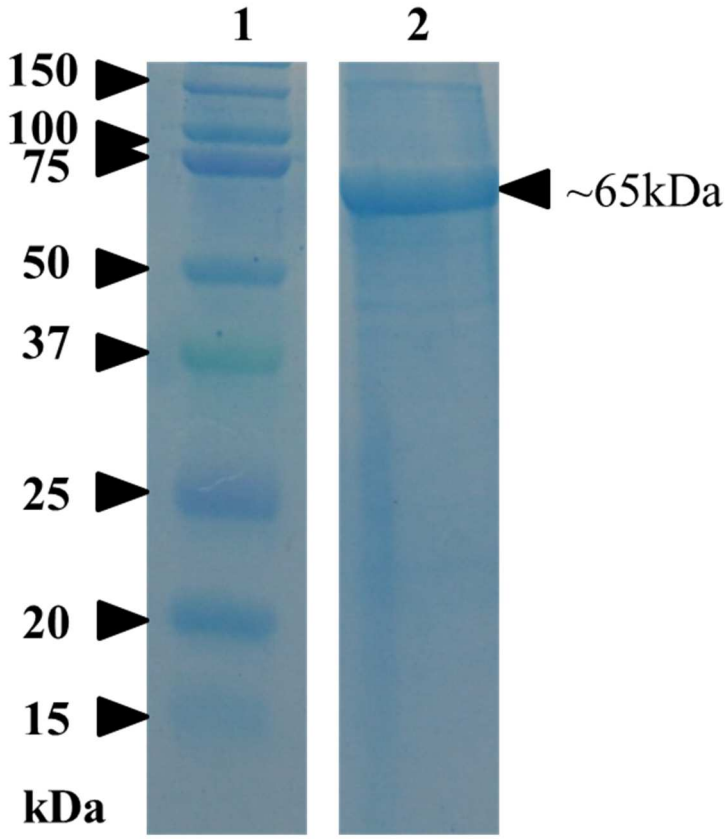


Figure 5.2. Anti-VHSV antibody binding to 0.1 μg well^{-1} of either purified recombinant VHSV-IVb glycoprotein (rG) or whole purified VHSV-IVb determined using indirect ELISA. Fractions A and B represent the 4th mL and 5th mL eluted from a single Cabbage Looper larva following Ni-resin purification. 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.

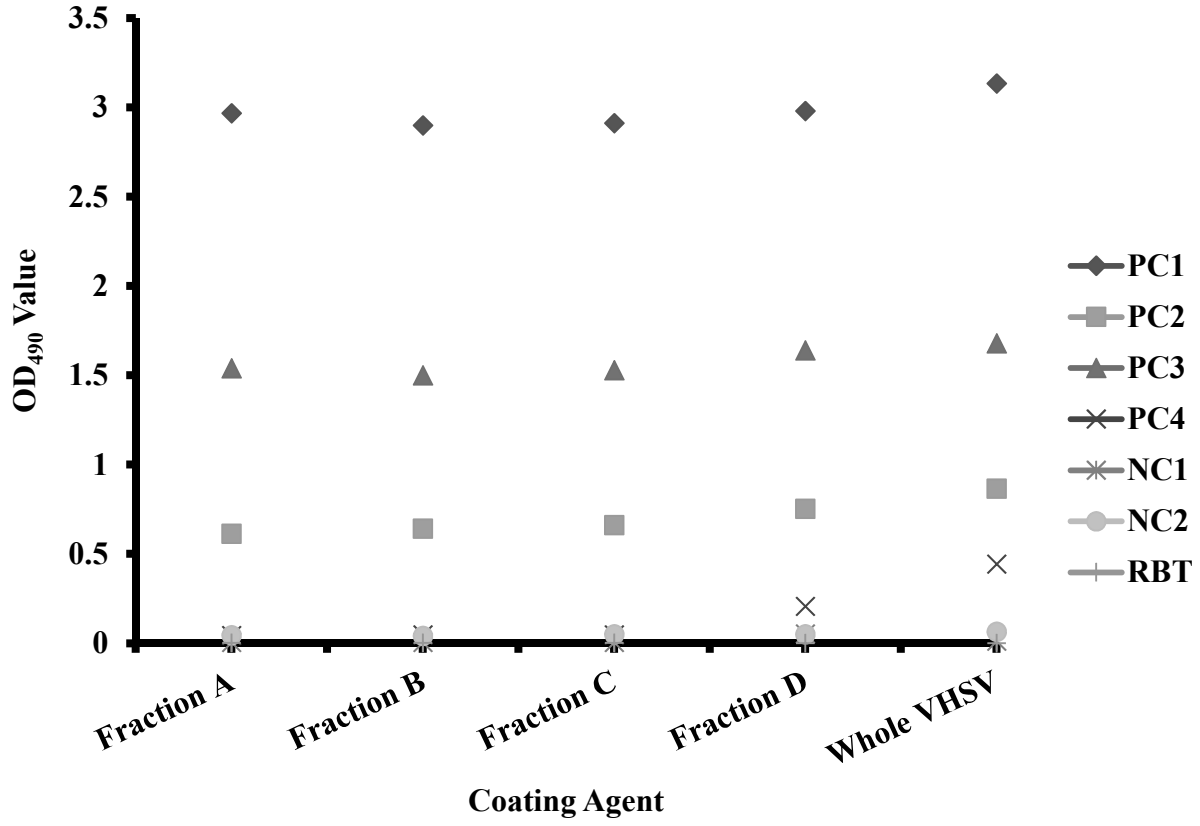


Figure 5.3. Cumulative mortality of muskellunge receiving 10 µg of rG (■) while mock individuals received PBS (●). Replicate tanks of each treatment (10 fish tank^{-1}) were VHSV-IVb immersion challenged (2 x 10^3 pfu mL^{-1}) and monitored for 39 days.

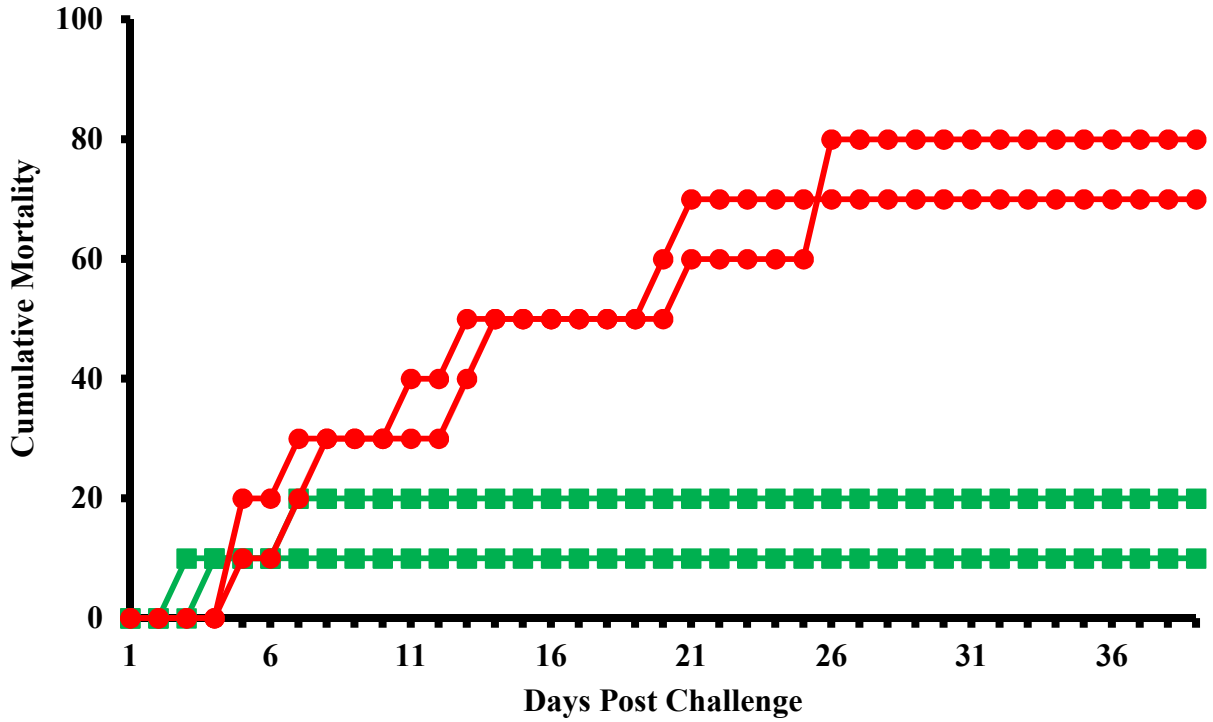
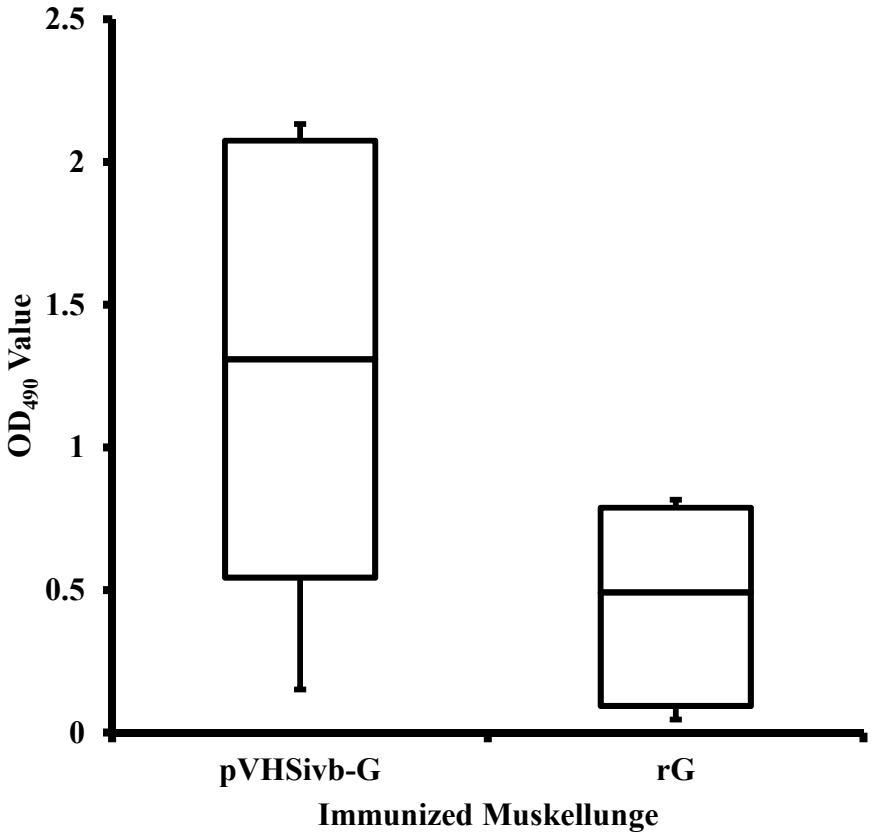


Figure 5.4. Box plot of indirect ELISA OD values indicating anti-VHSV-IVb antibodies in sera of rG (n = 7) or pVHSivb-G (n = 9) immunize muskellunge eight weeks post VHSV-IVb immersion challenge (2 x 10^3 pfu mL^{-1}).



Chapter Six

Investigation of aquatic "herd immunity" against the viral hemorrhagic septicemia virus genotype IVb using a DNA vaccine to elicit protection in muskellunge (*Esox masquinongy*)

Abstract

Viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb) is presently found throughout the Laurentian Great Lakes region of North America. In previous Chapters, I demonstrated that a vaccine consisting of a DNA plasmid containing the VHSV-IVb glycoprotein gene with a CMV promoter was developed that was highly efficacious in protecting muskellunge (*Esox masquinongy*) and three salmonid species. This study was conducted to determine whether, as in terrestrial animals, cohabitation of VHSV-IVb immunized fishes could confer protection to naïve fish, which is a necessity for herd immunity to result from an immunization program and for such a program to be effective in protecting wild populations. 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*). Mortality of naïve muskellunge was significantly lower when co-mingled with immunized muskellunge than when housed alone. Largemouth bass mortality was also lower when fish were co-mingled with immunized muskellunge versus when housed separately. No protective effect was observed when naïve muskellunge co-mingled with semi-resistant Chinook salmon, implicating the importance of acquired immunity in conveying protection. We attribute the protective mechanism of cohabitation to decreased shedding frequency as well as a lower mean shedding

rate resulting in higher survival of naïve fish, although other contributing factors are likely. Given that vaccinated muskellunge co-occurring with naïve muskellunge had significantly greater anti-VHSV antibody levels compared to vaccinated muskellunge housed alone, our results suggest there is a stimulatory effect on the humoral response of immunized muskellunge from cohabitation with susceptible individuals. This research provides the first evidence of the occurrence of herd immunity against fish pathogens.

1. Introduction

Hedrich (1933) introduced the concept of "herd immunity" following research involving measles outbreaks in humans. As part of that research, it was determined that epidemics declined when 68% of children under 15 years old developed immunity against measles (Hedrich 1933). Since this initial research, herd immunity and the associated critical vaccination threshold necessary to elicit this immunity has been investigated for both human and veterinary practices. The herd immunity threshold is dependent on the number of secondary infections (R_o), which is variable for each pathogen and environment. For example, during attempts to eradicate wild polioviruses, 100% herd immunity was accomplished with just 65 to 70% immunization coverage in North America (Nathanson 1982), while the same vaccine regimen in South America and India resulted in only an estimated 70% herd immunity (John & Samuel 2000). For highly pathogenic viruses, a high R_o results in a greater critical immunization threshold; in the case of measles, upwards of 92 to 95% vaccination coverage was predicted as being necessary for eradication of the disease (Anderson 1992). These thresholds have been investigated for numerous terrestrial pathogens; however, little work has been conducted on aquatic pathogens and whether the concept of herd immunity is even applicable in an aquatic setting is not presently known.

Given that many vaccine preparations have been developed against aquatic pathogens, it is somewhat surprising that aquatic herd immunity has received relatively little attention. Internationally, vaccines are already in use in commercial aquaculture. Norway, Canada and Chile have approved inactivated multivalent vaccines for Infectious pancreatic necrosis virus (IPNV) and Infectious salmon anemia virus (ISAV) (Gomez-Casado et al. 2011). Recently, Canada approved a DNA vaccine against Infectious hematopoietic necrosis virus (IHNV) (Alonso & Leong 2013). Similar, DNA vaccines encoding the glycoprotein (G) gene of VHSV genotype I were found to be efficacious in conferring protection to salmonids following virus challenge (Lorenzen et al. 1998, 2000; Einer-Jensen et al. 2009). Numerous studies have subsequently demonstrated the VHSV G protein to be the major target protein for neutralizing and protective antibodies (Bearzotti et al. 1995; Lorenzen et al. 1990; 1999; Einer-Jensen et al. 2004). It is unclear though, whether this immune response confers any protection to neighboring individuals.

The opportunity to investigate herd immunity came with the emergence of a novel genotype (IVb) of viral hemorrhagic septicemia (VHSV-IVb) virus when it was isolated in the Laurentian Great Lakes region of North American and found to be highly pathogenic to numerous Great Lakes fish species (Kim & Faisal 2010a; b; c). Since its first detection (Elsayed et al. 2006), it has subsequently spread to each of the Great Lakes and inland systems in the region and resulted in multiple mass mortality events of fish populations (Faisal et al. 2012). Presently, 28 Great Lakes species are listed as VHSV-IVb susceptible (Animal & Plant Health Inspection Service 2009), with reports documenting variability in the disease course and susceptibility among species (Kim & Faisal 2010a; b; c). In an effort to protect aquaculture facilities against spread of

the virus, but also with a goal towards development of a vaccination program that might be used to protect wild populations of fish, a vaccine consisting of a DNA plasmid containing the VHSV-IVb glycoprotein gene under the control of a cytomegalovirus (CMV) promoter was recently developed. This vaccine was found to be efficacious in eliciting protection in muskellunge (*Esox masquinongy*) (Millard 2013). In Chapters Three and Four of this dissertation, significant protection was observed in several species following an intramuscular administration of this plasmid. In muskellunge specifically, 95% relative percent survival (RPS) was achieved with a single administration, whereas a two vaccination regimen resulted in 100% RPS. Despite the efficacy of the VHSV-IVb vaccine, the lack of understanding as to whether a herd immunity response can be elicited in aquatic population leads to some uncertainty as to the potential usefulness of this vaccine in combatting outbreaks of the disease in wild populations. A successful demonstration of the concept of aquatic herd immunity would present the possibility of using the extensive hatchery system within the Great Lakes to eliminate pathogens. Hatchery propagated individuals could be immunized prior to stocking to supplement the herd immunity and establish a critical immunization threshold.

The goal of this study was to examine whether aquatic herd immunity against VHSV IVb could be elicited in a laboratory setting. The main focus of our efforts was on muskellunge, given that we have generated susceptibility, survivorship and vaccination regimens for this species (Kim & Faisal 2010c; Kim & Faisal 2012; Millard & Faisal 2012; Millard 2013). Vaccinated and naïve muskellunge were housed separately and together and exposed to VHSV-IVb, so that 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 could

be compared between fish co-mingled together or housed alone. We additionally comingle muskellunge with largemouth bass (*Micropterus salmoides*), a species that is also highly susceptible to VHSV IVb, and Chinook salmon (*Oncorhynchus tshawytscha*), a species that is resistant to VHSV IVb. These species were house both separately and in combination with vaccinated or naïve muskellunge and the cumulative mortality was compared between these treatments. Viral concentrations in the water as well as the terminal viral concentrations in the posterior kidney of survivors were assessed to determine whether immunization could confer herd immunity across species (co-mingled largemouth bass treatments). This will also show whether there is truly a protective effect from the immune individuals or simply due to a decrease in the number of susceptible individuals (co-mingled Chinook salmon treatments).

2. Materials and methods

2.1. Experimental fish and care

All fish used in the studies including were certified disease-free in accordance to World Organization for Animal Health (OIE) testing guidelines prior to use in studies. Two groups of juvenile muskellunge were used throughout the study. The first group was used for the first four trials and was obtained 14 weeks post-hatch [average 14.2 cm (SD = 1.4) fork length, 11.9 g (SD = 3.8)] from the Chautauqua State Fish Hatchery (New York Department of Environmental Conservation, Chautauqua, NY). The second group of juvenile muskellunge was used in the remaining trails and was obtained at 16 weeks post-hatch [average 12.7 cm (SD = 0.9) fork length, 16.1 g (SD = 3.7)] from the Wolf Lake Fish Hatchery (Michigan Department of Natural Resources, Mattawan, MI). All muskellunge were fed live fathead minnows (*Pimephales promelas*) obtained from Anderson Farms Inc. (Lonoke, AR) and certified as free of important

disease. An additional 60 minnows were necropsied and underwent additional testing according to the American Fisheries Society (AFS-FHS blue book 2012). Largemouth bass [average 6.8 cm (SD = 0.6) fork length, 4.9 g (SD = 1.0)] were obtained from a contained population at the La Crosse Fish Health Center, US Fish and Wildlife Service (Onalaska, WI) and were certified fee of OIE reportable diseases. Largemouth bass were fed Skretting Fry pellets (Skretting, USA, Tooele, UT). Chinook salmon [average 13.1cm (SD = 1.5) fork length, 21.4 g (SD = 5.9)] were obtained from the Wolf Lake Fish Hatchery (Michigan Department of Natural Resources, Mattawan, MI) and were fed 2-mm Skretting sinking pellets (Skretting).

All experimental fish were acclimated in a 500-L circular fiberglass tank in a continuous flow-through system with facility-chilled well water and supplemental aeration. Fish were housed and all experiments were conducted in the University Containment facility (Michigan State University, East Lansing, MI). Fish were fed ad libitum throughout the duration of the study, except for the 1st week post viral challenge when food was withheld. Two weeks prior to immunization, randomly selected fish were transferred and acclimated to 72-L polyethylene flow-through tanks (Pentair Aquatic Eco-Systems, Apopka, FL) containing supplemental aeration.

2.2.Virus and cell culture

The viral hemorrhagic septicemia virus (VHSV) genotype IVb isolate used throughout the duration of the study is the Great Lakes index strain MI03 (Gene bank accession GQ385941), originating from the initial isolation of VHSV in muskellunge in 2003 (Elsayed et al. 2006). The isolate has been maintained by continuous subculture in the cell line *epithelioma papulosum*

cyprini (EPC). The EPC cell line was maintained and subcultured in 150 cm^2 tissue culture flasks (Corning) at 25°C using a basal media of Earle's salt-based minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) and supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU mL^{-1}) (Invitrogen), streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Gemini, West Sacramento, CA), and sodium bicarbonate (7.5% w/v) (Sigma-Aldrich, St. Louis, MO). Active viral concentrations were determined using plaque assay on EPC cell line using polyethylene glycol and a methylcellulose overlay (Batts & Winton 1989; Batts et al. 1991). Virus was then aliquoted into cryogenic vials (Corning Inc., Tewksbury, MA, USA) for one time use and stored at -80°C until needed.

2.3. Construction of pVHSivb-G plasmid

The pcDNA_3.1 (+) is a commercially available vector containing the human cytomegalovirus (CMV) immediate-early promoter. The DNA vaccine construct containing the VHSV-IVb glycoprotein gene (pVHSivb-G, Millard 2013) was modeled after successful DNA vaccines against VHSV genotype I (Lorenzen et al 1998, Heppell et al. 1998) and IHNV (Anderson et al 1996). The construction and production of this plasmid were outsourced to Life Technologies (Carlsbad, CA). In brief, an *Eco*RI restriction site (G/AATTC) followed by a kozak consensus sequences terminating with the first amino acid of the complete MI03GL VHSV-IVb isolate glycoprotein gene (1524 bp) was synthesized. An *Xba*I restriction site (T/CTAGA) was then added following the 3' termination codon. The assembled fragment was then digested using the described endonucleases and sub-cloned into the eukaryotic expression vector pcDNA 3.1(+) (Invitrogen). The plasmid was transformed and propagated into K12 *Escherichia coli*. Sequencing confirmed the correct glycoprotein gene sequence and orientation. The vector,

designated pVHSivb-G, was diluted to 1 mg mL^{-1} in sterile phosphate buffered saline (PBS) and stored at -80°C.

2.4. Experimental design

As previously described, this experiment involved housing naïve muskellunge, largemouth bass, and Chinook salmon separately or in combination with vaccinated muskellunge. Vaccinated muskellunge were inoculated according to a regimen developed in Chapter Three that would result in an average of 95% RPS. Immediately prior to vaccination, the pVHSivb-G plasmid was thawed and diluted to a final concentration of 10 µg in 100 µL of sterile PBS. Randomly selected fish were anesthetized with 0.1 g L^{-1} of tricaine methanesulfonate (MS-222) (Western Chemical, Ferndale, WA), buffered with 0.3 g L^{-1} sodium bicarbonate. Muskellunge were vaccinated with 10 µg of the pVHSivb-G plasmid intramuscularly in the left epaxial muscle, slightly posterior to the pectoral fins. Vaccinated individuals that would be stocked in tanks with naïve individuals were intramuscularly passive integrated transponder (PIT)-tagged with 9 mm tags (Biomark© Inc., Boise, ID) so that naïve and vaccinated fish could be identified. A pool of each treatment was vaccinated at six week intervals and maintained 72-L polyethylene flow-through tanks (Pentair Aquatic Eco-Systems, Apopka, FL). Following vaccination fish were allowed to react to the antigen for 1880° days or 20 weeks at 13°C.

The experimental layout for this research was novel and consisted of multiple 72-L polyethylene flow-through tanks containing supplemental aeration that received water flow from a common source (Figure 6.1). Water initially flowed into a tank containing infected muskellunge. Naïve muskellunge obtained in the original batch of muskellunge from Chautauqua State Fish Hatchery

were used as shedders throughout all trials. Muskellunge were intraperitoneally (IP) infected with a low dose of VHSV that previous studies indicated would elicit shedding (Kim & Faisal 2010; Kim & Faisal 2012). Briefly, immediately prior to infection, muskellunge were anesthetized as previously described. Thawed VHSV was diluted to a concentration of 1.98 pfu $100\mu L^{-1}$ with sterile PBS and administered IP. Following infection, muskellunge were held in a separate 72-L polyethylene flow-through tank for up to seven days until each trial was initiated. In each trial the number of initial infected muskellunge placed into the shedder tank was equal to the number of downstream tanks i.e., four infected muskellunge for four downstream tanks. Once an infected individual succumbed to VHSV, that individual was removed and not replaced. Additionally, if all the infected individuals succumbed, water continued to flow through the empty shedder tank.

The water from the tank containing infected individuals was then equally distributed to tanks that containing different combinations of naïve and vaccinated fish. A total of 8 trials were conducted. All trials included tanks consisting solely of naïve muskellunge and vaccinated muskellunge and a tank where naïve and vaccinated muskellunge were co-mingled together. Some of the trials included tanks containing different combinations of vaccinated or naïve muskellunge, Chinook salmon or largemouth bass (Table 6.1). Initial densities within tanks were limited to be between 10 and 14 fish so as to limit any density effects on survival. One week prior to the initiation of each trial, randomly selected fish from each treatment and species were moved into their population tanks and acclimated prior to the introduction of virus into the system. Water temperatures were maintained at 11±1°C throughout all replicates through the use of a chiller system. Following the introduction of virus into the system, the experiment was run

for 28 days during which each population was monitored for morbidity and mortality. The only exception to this was trial 4, which was run for 60 days due to low mortality rates in any of the co-mingled combinations. Moribund fish were allowed to actively shed and infect other individuals rather than being removed from their respective tanks.

2.5. Response measures

2.5a. Mortalities

All mortalities were necropsied and kidneys, spleen and heart samples were aseptically collected and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., York, UK) on high speed for 2 min. Homogenates were diluted 1:4 (w/v) with MEM supplemented with 12 mM tris buffer (Sigma), penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}) and Amphotericin B (250 μg mL^{-1}, Invitrogen). Samples were centrifuged at 2700*g* for 30 min at 4°C and inoculated onto EPC monolayers. After 14 days supernatant was removed, frozen at -80°C, thawed and centrifuged at 2700*g* for 15 min at 4°C. Supernatant was then re-infected onto fresh EPC monolayer and incubated for 14 days before being examined for viral cytopathic effect (CPE). If CPE was not observed RNA from cell culture supernatant was then extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The presence of VHSV was confirmed using real-time reverse transcription polymerase chain reaction (rt-PCR) assay specific for VHSV (Jonstrup et al. 2013; Warg et al. 2014). The assay was conducted using the 900 nM of both the forward primer 5'-AAA CTC GCA GGA TGT GTG CGT CC-3' (rtPCRF), and reverse primer 5'-TCT GCG ATC TCA GTC AGG ATG AA-3' (rtPCRR) and 250 nM of probe 5'-/56-FAM/TA GAG GGC CTT GGT GAT CTT CTG /3BHQ_1/-3' (rtPCRP). Briefly, 25 μL reactions contained 12.5μL of QuantiTect Probe RT-PCR kit (Qiagen) mastermix,

5 µL of RNA template and 0.25 µL of QuantiTect Probe RT/RNase enzyme. Reactions took place in an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) with 30 min at 50°C and 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 40 sec at 60°C and 20 sec at 72°C.

2.5b.VHSV-shedding

Viral shedding was assessed once a week while individuals were alive in all infected muskellunge tanks during each trial, as this was used as a covariate for explaining variability in results among the trials. Additionally, shedding analysis was conducted once a week on all population tanks during trials 5- 8. Shedding was assessed using a modified protocol of that described by Kim & Faisal (2012). First, the entire flow though system was turned off and fish remained in their respective tanks with supplemental aeration. After 90 min, water was mixed and a 50 mL water sample was taken from each tank and the flow was resumed. Water samples were stored at 4°C until processing within 24 hrs. For processing, samples were vortexed and centrifuged at 2700*g*, at 4°C for 10 min. After centrifugation, a viral plaque assay (VPA) was conducted as previously described. After 6 days, cell monolayers were stained with crystal violet (Sigma) and 18% formaldehyde (Avantor Performance Materials Inc.). Viral plaques were counted and the viral concentrations and theoretical shedding rate (pfu $hour^{-1}$) for each tank was determined.

2.5c. Circulating anti-VHSV antibodies

The circulating anti-VHSV antibodies in the surviving muskellunge from trials 4, 5, 6 and 8 were examined using the indirect ELISA described in Chapter Two of this dissertation. At the

termination of the 28 day period of observation, surviving muskellunge were euthanized with 0.3 g L^{-1} of tricaine methanesulfonate buffered with 1.0 g L^{-1} of sodium bicarbonate. Blood was collected using a caudal venipuncture, stored at 4°C for 2 hours and centrifuged at 2700*g* for 10 min. at 4°C. The serum was then aliquoted and stored at -80°C until analysis.

The indirect ELISA was conducted as previously described. Briefly, all sera was heat inactivated at 45°C for 30 min. Serum was centrifuged at 2700*g*, 4°C, for 10 min. immediately prior to diluting in a solution of 1% nonfat dried milk in PBS (dilution of PBS-5% NFDM, Sigma). Solid phase ELISA took place in polystyrene microplates (96-well, Microlon®600 with chimney wells; Greiner Bio-One, Monroe, NC). Plates were sealed during all incubation periods (SealPlate®; Sigma) and washed 5 times following each incubation period unless otherwise stated using PBS containing 0.05% Tween 20 (PBS-T20; Sigma) in an automated microplate washer (BioTek, 4Lx405™ plate washer; Winooski, VT).

Microtiter assay plates were coated with 100 µL $well^{-1}$ of purified VHSV-IVb at 1 µg mL^{-1} in PBS (pH 7.4) and incubated overnight (14-16 hrs) at 4°C in a humid chamber. After the overnight incubation, plates were washed and unbound sites were blocked with the addition for 430 µL $well^{-1}$ of PBS containing 5% NFDM (PBS-5%; Sigma) and incubation at 37°C for 1 hr. Heat inactivated and diluted test and control muskellunge sera was then added to duplicate wells at 100 µL $well^{-1}$. After incubating at 25°C for 1 hr, plates were washed and 100 µL $well^{-1}$ of 1:30,000 dilution of a mouse anti-muskellunge mAb was added and incubated at 25°C for 1 hr. Plates were washed and 100 µL of 1:4,000 dilution of a commercially available goat anti-mouse horseradish peroxidase (HRP) secondary antibody (Invitrogen; Carlsbad, CA) was added to each

well and incubated at 25°C for 1 hr. Plates were developed by the addition of 100 µL of 0.4 mg mL^{-1} *o*-phenylenediamine (Sigma) in phosphate citrate buffer (Sigma) containing 3mM hydrogen peroxide (Avantor Performance Materials Inc.). The reaction proceeded for 30 min at 25°C in the dark. Without washing, the reaction was stopped with the addition of 50 mL of 3 M sulfuric acid (H_2SO_4;, Avantor Performance Materials Inc.). The optical density (OD) was read at 490 nm using a 630 nm reference wavelength on a BioTek, ELx808™ plate reader (BioTek) and the Gen5 software (BioTek). The average value of blank wells was subtracted from test and control wells prior to analysis.

2.5d. Viable VHSV concentrations in survivors from each population

Viable viral concentrations were determined in the survivors of all species in trials 4, 5, 6 and 8. At the termination of each trial all survivors were euthanized as previously described. A sample of the posterior kidneys was collected aseptically from each individual and stored at 4°C until processed individually within 24 hrs. The tissue was homogenized and diluted 1:10 (w/v) with MEM as previously described. Samples were vortexed and centrifuged at 2700*g* for 30 min at 4°C and supernatant was used to conduct a VPA as previously described. The number of viral plaques was used to determine active viral concentration (pfu mg^{-1}) within the posterior kidney.

2.6. Data analysis

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. Although the some shedding rates were repeat measures from a single tank of infected individuals, this aspect was not incorporate, as the analysis as the number of observations within each trial only

ranged from two to four. Each of the response measures from the experimental fish described in Section 2.5 were analyzed using generalized linear mixed-effect models. For cumulative mortality, a binomial distribution was assumed, whereas for the other response measures a Normal distribution was assumed following either $\log_e$ (circulating antibodies) or $\log_e + 1$ (viral shedding and viral concentration) transformation. Each of the mixed-effect models included as fixed-effect variables the housing combination×organism of interest interaction and $\log_e+1$ transformation of viral shedding of infected muskellunge in their respective trials as fixed-effect variables. The mixed effect models also included trial and trial×housing combination×organism of interest interaction as random effects. If an overall significant difference among the housing combination×organism of interest levels was detected for an individual response measure, than pre-planned comparisons of particular levels of interest were conducted (e.g., survival of naïve muskellunge when housed alone versus when co-mingled with vaccinated muskellunge). Denominator degrees of freedom for the tests of overall differences among treatments and the pre-planned pairwise comparisons were set using the Satterthwaite approximation. All analyses were conducted using PROC GLIMMIX in SAS (SAS Institute 2010).

3. Results

Each trial was initiated by the addition of IP infected muskellunge into the top flow-through tank. In nearly all trials, positive shedding by IP infected muskellunge was detected the within the first week of the trials, and then in most cases decreased to near zero or below detectable limits by the third and fourth week, at which point all infected muskellunge had succumbed. The only exceptions to this observed shedding dynamic was the sixth trial where no shedding was detected during any of the sampling events and the third trial where shedding increased from

week 1 to week 2. During the first two weeks of each trial, IP infected muskellunge also showed signs of acute VHSV infection, including extensive petechial hemorrhage along the dorsal surface and mortalities exhibited extensive hemorrhage throughout the musculature, liver, swim bladder and renal mesentery.

In all trials, it was evident that viral shedding by IP infected muskellunge was indeed resulting in the exposure and infection of muskellunge held in the downstream tanks. Within two weeks following the initiation of each trial, signs of VHSV infection were observed in muskellunge held in the downstream tanks, particularly in muskellunge from the all naïve treatments. Numerous fish exhibited severe petechial hemorrhage, erratic swimming, pale gills, and a few moribund muskellunge (<5) exhibited fungal infections of the gills and eyes. In all trials the observation of these morbid individuals was followed by a steep increase in mortalities, with the exception of trial 4, where muskellunge in all tanks remained moribund and did not succumb. Through all trials, VHSV-IVb was re-isolated from all but two mortalities: one naïve muskellunge co-mingled with Chinook salmon in trial 2 and one vaccinated muskellunge housed with largemouth bass in trial 7 (Table 6.2). Overall, statistical analysis showed that differences in shedding by infected muskellunge among the trials were not statistically significant ($F = 0.23$, df = 7,14, $p = 0.9706$). However, we still chose to include loge + 1 transformation of shedding of infected muskellunge in the trials as a covariate in the mixed-effect models.

3.1. Cumulative mortality

The highest 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% (Table 6.2). The mixed-effects logistic regression model fit to the mortality data from the experiment successfully converged on a solution. There was an overall significant difference among the housing combination and organism of interest levels (F = 5.27, df = 11,37.1, p <0.0001).The estimated coefficient for shedding of infected muskellunge was 0.0715 (SE = 0.1424), which was not significantly different from zero (F = 0.25, df = 1,5.05, p = 0.6369). The estimated variance for the trial effect was 1.177 (SE = 1.134), whereas the variance for the trial×housing combination×organism of interest interaction was 2.007 (SE = 0.852).

Based on the pairwise comparisons of mortality rates that were conducted, mortality was significantly greater in naïve muskellunge compared to vaccinated muskellunge when stocked separately (Table 6.3). Mortality was also significantly greater in naïve muskellunge when stocked alone compared to naïve muskellunge stocked with vaccinated muskellunge (Table 6.3). Average mortality of vaccinated muskellunge increased from 3.1% when stocked alone to 6.0% when stocked with naïve; however, these differences were not statistically significant (Table 6.3). Although the AM of largemouth bass when stocked with vaccinated muskellunge was 8.3% versus 39.6% and 53.8% when stocked alone or stocked with naïve muskellunge , the differences in mortality rates between these housing combinations was not statistically significantly different (Table 6.3), perhaps a consequence of limited number of trials that included some of these co-mingling combinations. Mortality of naïve muskellunge when stocked

with Chinook salmon was significantly greater than when naïve muskellunge were stocked with vaccinated muskellunge (Table 6.3), even though Chinook salmon are considered resistant to VHSV-IVb infection. There was no significant difference in mortality of Chinook salmon when stocked alone versus when stocked with naïve muskellunge (Table 6.3). In both cases, average mortality of Chinook salmon was 8.3%.

3.2.VHSV titers in tank water

The variances for the trial effect and trial×housing combination×organism of interest interaction for the analysis of VHSV-IVb titers in tanks could not be estimated (i.e., variance-covariance matrix for the random effects was not positive definite). As a result, the fitted model was an ANCOVA involving housing combination×organism of interest interaction and $\log_e$+1 shedding of infected muskellunge as explanatory variables. Based on this model, there was an overall significant difference among the housing combination×organism of interest interaction levels (F = 3.18, df = 6,72, p = 0.0080). The estimated coefficient for shedding of infected muskellunge was 0.049 (SE = 0.1224), which was not significantly different from zero (F = 0.16, df = 1,72, p = 0.6921).

The highest viral titers in tanks occurred when naïve muskellunge were comingled with Chinook salmon (Table 6.4). The next highest viral titers in tanks occurred when naïve muskellunge were housed separately, followed by when vaccinated muskellunge were stocked separately (Table 6.4). Pairwise comparisons between the housing combinations indicated that viral titers in tanks containing co-mingled naïve muskellunge and Chinook salmon were significantly greater than

all other co-mingled combinations (Table 6.4, Table 6.5). However, none of the other differences in viral titers between the cohabitation combinations were statistically significant.

3.3. Circulating anti-VHSV antibodies

The highest OD values were observed in vaccinated muskellunge when co-mingled with naïve muskellunge (mean = 1.04; SD = 0.64) (Table 6.6), whereas the lowest OD values were observed in naïve muskellunge when housed with vaccinated muskellunge (mean = 0.11; SD = 0.08). Mean OD values for the other housing combinations ranged from 0.21 for naïve muskellunge stocked along to 0.28 for vaccinated muskellunge held alone.

There was an overall significant difference in OD values among the housing combination×organism of interest interaction levels ($F = 8.41$, df = 4,9.96, $p = 0.031$). The estimated coefficient for shedding of infected muskellunge was 0.0715 (SE = 0.1424), which was not significantly different from zero ($F = 4.98$, df = 1,2.343, $p = 0.1365$). The estimated variance for the trial effect was 0.009 (SE = 0.116), whereas the variance for the trial×housing combination×organism of interest interaction was 0.177 (SE = 0.194). The OD values for vaccinated muskellunge co-mingled with naïve muskellunge were significantly greater than the values for all other co-mingled combinations (Table 6.7).

3.4. Viable VHSV concentrations in survivors

Viable VHSV-IVb concentrations in surviving fish were the greatest in naïve muskellunge housed alone. The average concentration was 16.8 pfu mg^{-1} with VHSV detected in 47.6% of surviving individuals (Table 6.8). Conversely, 60% of surviving naïve muskellunge when co-mingled with vaccinated muskellunge were found to be actively infected with VHSV-IVb, although in this case the average concentration was 3.0 pfu mg^{-1}. Active VHSV-IVb concentrations were detected in 52% of surviving vaccinated muskellunge co-mingled with naïve muskellunge with an overall average concentration of 0.7 pfu mg^{-1}. Meanwhile, active VHSV-IVb concentrations were detected in only 6% of surviving fish when housed with largemouth bass and 9% of surviving fish when stocked alone. VHSV-IVb concentrations in these cases were 0.24 pfu mg^{-1} (vaccinated muskellunge housed alone) and 0.43 pfu mg^{-1} (vaccinated muskellunge co-mingled with largemouth bass). In the case of Chinook salmon and largemouth bass, prevalence of active VHSV-IVb concentrations was low in surviving individuals and concentration levels themselves were also generally low. The only exception to this was active VHSV-IVb concentrations in Chinook salmon survivor when housed alone. The average VSHV-IVb concentration in this case was 4.13 pfu mg^{-1}.

There was no significant difference in active VHSV-IVb concentrations among the housing combination×organism of interest interaction levels ($F = 1.87$, df = 8,16.87, $p = 0.1325$). We attribute the lack of an overall difference in viral concentrations in survivors from the co-mingled combinations to the high number of zero concentrations that were observed. The estimated coefficient for shedding of infected muskellunge was -0.003 (SE = 0.056), which was not significantly different from zero ($F = 0.00$, df = 1,1.94, $p = 0.9558$). The estimated variance for

the trial effect was 0.156 (SE = 0.217), whereas the variance for the trial×housing combination×organism of interest interaction was 0.452 (SE = 0.056).

4. Discussion

In this study we used a novel study design to examine the concept of aquatic herd immunity against VHSV. We introduced virus into the system using shedding elicited following an IP infection of muskellunge, which we believe more closely mimics natural infection. Additionally this method of exposure allowed viral concentrations to naturally vary and by ensuring the equal distribution of water, tanks were exposed to an identical viral concentration. Using this study design we can discern several aspects of herd immunity. 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. For example, 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 then then those obtained from vaccinated muskellunge alone. This heightened immune response likely resulted from increased viral exposure from co-mingling with susceptible muskellunge. The data robustly demonstrates that herd immunity indeed exists in aquatic systems containing a single species.

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. This could be due to increased stress resulting from cohabitation with active Chinook salmon and is supported by the high number of viral detections and significantly higher viral concentrations that occurred in the water of comingled tanks. Overall, naive muskellunge housed with Chinook salmon did have a much higher OR (102.91) when compared to those housed with vaccinated muskellunge indicating that cohabitation with a semi-resistant did not positively affect survivorship.

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 additional of a semi-resistant species, indicating that protection may be more than just physical in nature and is reliant on immunity. 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.

Few researchers have examined the concept of herd immunity in aquatic system as aquatic epidemics can be difficult to visualize and quantify. Epidemics occur when a high number of susceptible individuals leads to efficient transmission or contact known as the ($R_{\circ}$) value; derived from the number of secondary infections a single individual produces. The $R_{\circ}$ value is linked to a critical immunity threshold which is the proportion of the population that must be immune or vaccinated in order to prevent further transmission of a pathogen. This value is known as the herd immunity threshold (HIT) or V_c and is calculated by $[1-(1/ R_{\circ})]$ (Fine et al. 2011). The $R_{\circ}$ and transmission dynamics are largely uninvestigated for aquatic pathogens such as VHSV. For example it is clear that fish mucosa and the associated IgT immunoglobulin specifically is an important barrier to pathogen invasion (Mussmann et al. 1996; Zhu et al 2013); but it unclear if IgT is passively shed into the water column. The $R_{\circ}$ is highly variable aquatic systems being influence by virulence, host susceptibility, host behavior, innate immunity and contact rate, environmental conditions, etc. This variability complicates the calculation of $R_{\circ}$, however, the results of this study can be used to forgo this calculation and inform an agent-based model which can be used to assess aquatic herd immunity on a much larger scale then possible in this study. Modeling will explore whether vaccination of hatchery propagated fish prior to stocking could be used to elicit a large-scale “herd immunity” response

In summary, we have demonstrated the existence of 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.

APPENDIX

Table 6.1. The number of fish and species housed in each tank of eight cohabitation trials i.e. NM_VM denoted a co-mingled tank containing naïve muskellunge with vaccinated muskellunge. Similarly LMB=largemouth bass, CHK=Chinook salmon.

Trial	Tank	ID	Naïve muskellunge	Vaccinated muskellunge	Largemouth bass	Chinook salmon
1	1	NM_VM	6	6	0	0
	2	VM	0	12	0	0
	3	NM_LMB	6	0	6	0
	4	NM	12	0	0	0
2	1	NM_VM	6	6	0	0
	2	VM	0	12	0	0
	3	NM_CHK	6	0	0	6
	4	NM	12	0	0	0
3	1	NM_VM	7	7	0	0
	2	VM	0	12	0	0
	3	NM_LMB	7	0	7	0
	4	NM	12	0	0	0
4	1	NM_VM	7	7	0	0
	2	VM	0	14	0	0
	3	NM	14	0	0	0
5	1	NM_VM	6	6	0	0
	2	VM	0	12	0	0
	3	VM_LMB	0	6	6	0
	4	LMB	0	0	12	0
	5	NM	12	0	0	0
	6	NM_CHK	6	0	0	6
6	1	NM_VM	6	6	0	0
	2	VM	0	12	0	0
	3	VM_LMB	0	6	6	0
	4	LMB	0	0	12	0
	5	NM	12	0	0	0
	6	NM_CHK	6	0	0	6
7	1	NM_VM	6	6	0	0
	2	VM	0	12	0	0
	3	LMB	0	0	12	0
	4	NM	12	0	0	0
	5	VM_LMB	0	6	6	0
8	1	NM_VM	6	6	0	0
	2	VM	0	10	0	0
	3	VM_LMB	0	6	6	0
	4	LMB	0	0	12	0
	5	NM	10	0	0	0
	6	NM_CHK	6	0	0	6
	7	CHK	0	0	0	12

Table 6.2. Results of eight cohabitation trials. Table describes the number of fish, cumulative mortality, viral re-isolations and relative percent survival (RPS) of different housing treatments for each trial.

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 w/ naïve muskellunge	66.7%	4/4	27.3%
Naïve muskellunge w/ largemouth	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	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%

Table 6.2. (cont'd)

	75.0%	9/9	10.0%
Largemouth bass			
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%

Table 6.3. Pairwise comparisons of survivorship between tank housing treatments. Survivorship was modeled using a mixed effect logistic model in SAS. Shedding values obtained during each trials were $\log_e$ transformed and used as a covariate. 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

Table 6.4. VHSV titers in water for trials 5, 6, 7, and 8. The table shows the number of water samples that were examined as well as the number of those samples that tested positive. The mean shedding rates are expressed in 10^4 pfu hour^{-1} and were calculated by averaging viral titer measures within individual housing treatments then averaging across replicates.

Housing Treatment	Samples	VHSV Positive	Mean Shedding Rate (10^4 pfu hr^{-1})
Naïve muskellunge	16	5	5.08 (SD = 6.69)
Vaccinated muskellunge	16	3	5.00 (SD = 7.71)
Naïve muskellunge w/ vaccinated muskellunge	16	2	1.56 (SD = 1.88)
Vaccinated muskellunge w/ largemouth bass	10	1	11.11 (SD = 19.24)
Naïve muskellunge w/ Chinook salmon	10	7	53.47 (SD = 22.87)
Largemouth bass	10	0	NA
Chinook salmon	4	0	NA

Table 6.5. Pre-planned comparisons of VHSV-IVb shedding rates in housing combinations. Shedding rates were modeled using a mixed effect normal model following a $\log_e +1$ transformation of the data. 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	df	*t*-statistic	*p*-value
Chinook	Chinook and naïve muskellunge	72	-2.85	0.006
Chinook and naïve muskellunge	naïve muskellunge	72	2.27	0.026
Chinook and naïve muskellunge	naïve muskellunge and vaccinated muskellunge	72	3.39	0.001
Largemouth	largemouth and vaccinated muskellunge	72	-0.65	0.515
Largemouth	naïve muskellunge and vaccinated muskellunge	72	-0.75	0.453
largemouth and vaccinated muskellunge	vaccinated muskellunge	72	-0.40	0.691
naïve muskellunge	naïve muskellunge and vaccinated muskellunge	72	1.28	0.205
naïve muskellunge	vaccinated muskellunge	72	0.77	0.442
naïve muskellunge and vaccinated muskellunge	vaccinated muskellunge	72	-0.51	0.614

Table 6.6. The OD_{490} value indicating the presence of anti-VHSV antibodies in muskellunge following VHSV exposure. Sera samples were collected from different populations of muskellunge at the termination of cohabitation trials 4, 5, 6 and 8. The mean OD values were determined using an indirect ELISA and were calculated by averaging over survivors within individual tanks and then averaging across tanks.

Post Exposure Survivors	Survivor Sera	Mean OD value
Naïve muskellunge	18	0.21 (SD = 0.16)
Vaccinated muskellunge	38	0.28 (SD = 0.17)
Naïve muskellunge w/ vaccinated muskellunge	19	0.11 (SD = 0.08)
Vaccinated muskellunge w/ naïve muskellunge	25	1.04 (SD = 0.64)
Vaccinated muskellunge w/ largemouth bass	15	0.36 (SD = 0.44)

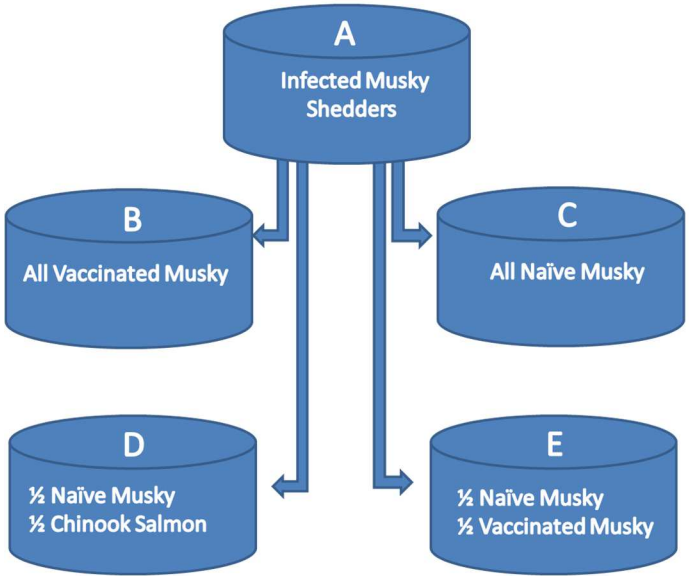
Table 6.7. Pairwise comparisons of circulating binding anti-VHSV antibody OD values in surviving muskellunge. OD_{490} values were modeled using a mixed effect model following $\log_e$ transformation of the data. The t-statistic, degrees of freedom, and *p*-value for each of the housing combinations 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

Table 6.8. Number of positive detections and mean VHSV concentrations in survivors from cohabitation trials 4, 5, 6 and 8. Plaques were enumerated using a VPA as previously described. The mean viral concentrations were calculated by titers in survivors from all of the cohabitation trials examined.

Housing Treatment	Samples	Positive	Mean Viral Concentration (pfu mg^{-1})
Naïve muskellunge	21	10	16.8 (SD = 27.0)
Vaccinated muskellunge	35	3	0.2 (SD = 0.4)
Naïve muskellunge w/ vaccinated muskellunge	20	12	3.0 (SD = 5.3)
Vaccinated muskellunge w/ naïve muskellunge	19	10	0.7 (SD = 0.3)
Largemouth bass w/ vaccinated muskellunge	12	0	0.0 (SD = NA)
Vaccinated muskellunge w/ largemouth bass	17	1	0.4 (SD = 0.7)
Chinook salmon w/ naïve muskellunge	10	0	0.0 (SD = 0.0)
Largemouth bass	15	2	0.6 (SD = 1.1)
Chinook salmon	10	2	4.1 (SD = NA)

Figure 6.1. Example of a trial of the herd immunity tank design and layout. The complete system is flow through, with water flowing into Tank A containing muskellunge intraperitoneally infected with the Viral hemorrhagic septicemia virus $<LD_{50}$ (1.98 pfu). The water from this tank is then equally distributed to Tanks B, C, D and E. These tanks contained either vaccinated (B) or naïve (mock vaccinated) muskellunge (C). Some tanks would also contain a mix of vaccinate and naïve individuals (E) or Chinook salmon and naïve muskellunge (D). By comparing the mortality, morbidity and mean day to death in each of these tanks, we can examine the protective effect of cohabitation with vaccinated individuals.



Chapter Seven

Conclusions and future research

1. Conclusions

Herein I have described innovative work aimed at eliciting protective immunity in fish against an emerging sublineage of viral hemorrhagic septicemia virus (VHSV). My work has focused on the development of tools, diagnostic assays and vaccine preparations, with the goal of preventing the spread and deleterious effects of VHSV genotype IVb within the Great Lakes basin. Although VHSV has yet to be isolated from a state or federal hatchery, it may be only a matter of time, as the virus is now nearly ubiquitous throughout Great Lakes waters (Faisal et al. 2012; records of the Aquatic Animal Health Lab, Michigan State University). At the initiation of my work, the basin was faced with a novel virus, highly pathogenic to a wide range of fish and with neither proactive preventative measures nor viable treatment options. Management and research efforts were further hampered by a lack of diagnostic tools for the indirect detection of VHSV.

To effectively combat this novel VHSV genotype, it was necessary to develop biological reagents to aid in serosurveillance and provide evidence of previous viral exposure. This would allow researchers and managers to determine the precise occurrence of VHSV within the Great Lakes Basin. I selected muskellunge (*Esox masquinongy*) for most of my studies as the species is the ideal candidate for serosurveillance and laboratory investigation, due to their high susceptibility to VHSV-IVb and vigorous humoral response (Kim & Faisal 2010c; Millard 2013).

In Chapter Two, I describe the development of a monoclonal antibody (mAb) against muskellunge immunoglobulin (IgM). The mAb (designated 3B10) was shown to react to the muskellunge IgM heavy chain in addition to the IgM heavy chain from two other common Great Lakes species, northern pike (*Esox lucius*) and common carp (*Cyprinus carpio*). I demonstrated the application of this mAb in an indirect ELISA for the detection of binding anti-VHSV antibodies in muskellunge. With this indirect ELISA, I showed that a DNA vaccine containing the VHSV-IVb G gene and cytomegalovirus (CMV) promoter could elicit a robust humoral immune response by tracking anti-VHSV antibodies in tagged individuals over time. Muskellunge exhibited a more vigorous secondary response following the administration of a booster dose of the plasmid and ELISA optical density (OD) values peaked approximately seven weeks following each administration. Conversely, in a study conducted by Millard (2013) using the same plasmid, at seven weeks following administration fish exhibited a low prevalence of neutralizing anti-VHSV antibodies, resulting in 45% relative percent protection (RPS) when challenged. This finding indicates that peak binding anti-VHSV antibody levels do not necessarily correspond with peak neutralizing antibody levels or protection. Further, the indirect ELISA was also successfully used to detect anti-VHSV antibodies in wild muskellunge from the Detroit River, a body of water where VHSV-IVb is known to be endemic. The successful use of this indirect ELISA to detect anti-VHSV antibodies in both wild and immunized fish demonstrates only the initial application of the 3B10 mAb and indirect ELISA in the study of teleost immune response as well as serosurveillance efforts. The creation of this mAb and its use in determining anti-VHSV antibody production dynamics will also be of utmost importance in the evaluation of vaccine preparations and subsequent release strategies. Moreover, given that

the mAb also reacts with northern pike and common carp immunoglobulin, a similar ELISA could be optimized for assays involving these species.

In Chapters Three and Four, I determined that the pVHSivb-G plasmid could confer significant protection against viral challenge in muskellunge as well as in three representative salmonid species, rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and lake trout (*Salvelinus namaycush*). I demonstrated that a single administration of the pVHSivb-G plasmid could elicit a protective response in species exhibiting either a high or low susceptibility; with muskellunge and lake trout experiencing 95% and 100% mean RPS respectively. The results described in these studies indicate that the incubation period (number of °days) needed to mount a protective immune response varies between species. In the Millard (2013) study, fish were challenged (albeit at a higher concentration) 539° days following administration. By increasing this period to 1880° days, mean RPS increased to 95%. Again, anti-VHSV antibody levels following immunization were examined and statistical modeling confirmed that seven weeks post administration corresponded to peak anti-VHSV binding levels. Furthermore, following challenge, anti-VHSV antibody levels in immunized individuals were predicted to remain at detectable levels for upwards of 40-50 weeks.

Also in Chapter Three, I show that the plasmid promoter sequence of a DNA vector can have a profound influence on protection. Under identical conditions, 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%). Overall, the protection elicited by the pVHSivb-G plasmid described in both Chapters Three and Four corroborates the results of

numerous studies with other VHSV genotypes (Lorenzen et al. 1998; Einer-Jensen et al. 2009; Chico et al. 2009). Although protection is the usual standard by which vaccine efficacy is determined, the examination of viral shedding by immunized clinically protected individuals is a novel yet important concept. I show that, though viral shedding appears rare and ephemeral, these immunized and subsequently challenged individuals can nevertheless harbor VHSV and actively shed virus into the water column for up to 20 weeks following exposure. This finding demonstrates the urgent need to include these parameters when assessing post-vaccination efficacy as these survivors may act as viral reservoirs and actively disseminate VHSV.

In Chapter Five, I described my efforts to produce a eukaryotic recombinant VHSV-IVb glycoprotein for use as an innocuous vaccine antigen. Ultimately, success came through the use of a recombinant baculovirus vector to infect cabbage looper larvae (*Trichoplusia ni*), from which purified protein was obtained. I demonstrated that this system is highly efficient, in some cases >1 mg of highly purified recombinant protein (rG) was obtained from a single larva. Further, the rG appeared to be in the correct native conformation, evidenced by both the molecular weight and the inability of anti-VHSV binding antibodies to differentiate between the recombinant protein and whole purified VHSV when both were used as a coating agent in the indirect ELISA. Moreover in the one vaccination trial that was conducted, the recombinant protein did appear to elicit a protective response in muskellunge, resulting in 80% mean RPS. Though we observed significant protection, and anti-VHSV binding antibodies were detected following challenge, levels were comparatively lower than when muskellunge received the pVHSivb-G plasmid; although data from Chapter Three demonstrate that anti-VHSV binding antibody levels may not be directly correlated to peak protection. In a truncated analysis of viral

shedding, I confirm that recombinant-immunized fish likewise seem capable of disseminating the VHSV following challenge.

Chapter Six represents the culmination of the numerous tools and knowledge developed in the previous chapters, which I used to examine the concept of "herd immunity" in an aquatic system, a first of its kind. The examination of this complex concept necessitated a novel study design. First, I designed an interconnected system containing numerous flow-through tanks, where intraperitoneally infected individuals were placed in an upstream tank and delivered virus to naïve and immunized fish in downstream tanks via natural shedding. This method of viral delivery resulted in variable and transient viral exposure, which would more closely mimic the conditions of natural infection in the Great Lakes Basin. Based on the results of Chapter Three, the pVHSivb-G plasmid was used to simulate immune individuals in the population. The results from eight trails clearly show that cohabitation of immune muskellunge with naïve individuals indeed provides protection to the naïve cohort; with the average cumulative mortality of naïve muskellunge and largemouth bass decreasing from 80.2% to 36.5% and 39.5% to 8.3% for each species respectively. Additionally, no protection was observed when naïve muskellunge were housed with Chinook salmon, a semi-resistant species. This finding is indicative of a protective mechanism other than simply a decrease in viral transmission. Additionally, comingling appears to have a proliferative effect on the immune response of immunized individuals, exhibiting higher levels of anti-VHSV binding antibodies and lower mean terminal tissue titers when compared to immunized individuals housed alone.

The data illustrates that this "herd immunity" effect is due in part to decreased viral transmission as well as a proliferation in the immune response of immune individuals leading to viral neutralization. To date, there have been no studies investigating whether teleosts can actively shed binding or neutralizing antibodies into the water column, however numerous studies clearly demonstrate that the teleost mucosa along with the recently described closely associated IgT immunoglobulin, are integral in host defense. In an aquatic environment, antibodies are likely shed into the water column and may result in remote viral neutralization. This represents an innovative concept that is corroborated by our results and certainly warrants future investigation. The cumulative efforts of this dissertation show that numerous species can be protected either directly, using DNA-based or recombinant vaccine preparations, or indirectly via herd immunity.

2. Future research directions and applications

Herein I have described the development of several tools and their application in pioneering research conducted on the immune response against and prevention of VHSV. The methods I outlined have numerous applications in the study of VHSV, as well as in the study of other pathogenic viruses and their fish host species. For example, the anti-muskellunge Ig 3B10 mAb and indirect ELISA can now be applied to serosurveillance efforts, using muskellunge as a sentinel species to precisely determine the geographic range of VHSV within the Great Lakes basin. Additionally, this ELISA can be used to elucidate temporal tendencies in anti-VHSV antibodies similar to the negative trend I noted in the Detroit River muskellunge population. The mAb can be similarly used to study the muskellunge humoral immune response, antibody affinity maturation, IgM expression or to develop assays for the indirect detection of other emerging pathogens such as the fathead minnow nidovirus (FHMNV), or golden shiner reovirus

(GSRV, records of the Aquatic Animal Health Lab, Michigan State University). Additionally, the protocol we outline for the development of the anti-muskellunge mAb can be applied to develop mAb for other aquatic species; a highly beneficial tool that is lacking for nearly all teleosts.

The vaccine preparations we have developed and examined also have numerous applications. Of the preparations I have examined, the pVHSivb-G DNA plasmid was the most promising and elicited significant protection in all species that were examined. This represents the first preventative measure available to combat VHSV-IVb. The need for and application of this preparation are diverse. For example, we have already demonstrated the use of this plasmid in our study of the humoral response in muskellunge. However, the preparation could similarly be used to examine the cell-mediated response to VHSV-IVb, as well as the host immune response on a transcriptional level. Throughout this dissertation, I have evaluated only a portion of the immune response dynamics; a lack of genomic tools available for muskellunge hinders investigation of other protective mechanisms. The lower circulating anti-VHSV antibodies associated with the administration of the recombinant glycoprotein illustrate this, and are indicative of alternative protective mechanisms that warrant investigation. With increases in the availability and ease of genomic sequencing, the whole muskellunge genome should be sequenced and annotated. Obtaining genomic information on cytokines and regulatory factors would allow a more rigorous investigation of viral immune response and specifically the cellular immune responses against VHSV-IVb.

Since VHSV now appears nearly endemic throughout the Great Lakes basin, it is remarkable that VHSV has not found its way into any of the numerous hatchery facilities within the Great Lakes basin, which contain dense populations of naïve individuals. The preparation and vaccination regimens we have explored could be used for the protection of propagated species prior to stocking. By showing that a longer incubation prior to exposure results in the greatest protection, vaccination at an early life stage would be ideal. Immunization would act to prime the immune response of these individuals should they encounter VHSV and increase the likelihood of survival. More importantly and possibly more feasible, the vaccine preparations could be used in the protection of valuable captive broodstock and susceptible species found in zoos or aquariums. In an era of increasing globalization, there remains a threat of introduction of novel pathogens including other VHSV genotypes. The introduction and establishment of genotype IVa from the Pacific coast or genotypes I, II or III from Europe could have devastating consequences on the Great Lakes trout and salmon populations. I theorize that a multivalent VHSV preparation that elicits protection against numerous relevant genotypes will likely become standard practice in the future.

However, before these preparations can be widely utilized in wild populations additional analyses and modifications are necessary before they will be approved for commercial use. I demonstrate that future efforts should investigate post-challenge efficacy and the potential of vaccinated-survivors to shed viable virus, a parameter that is sorely lacking in most clinical studies in general. Additional clinical trials will require species-specific regimen optimization and assess the minimum vector concentration required to elicit a protective response. If preparations are to be used on a large scale such as in a hatchery setting, researchers must also

explore the efficacy of more feasible administration methods such as immersion, ultrasonic bombardment or oral delivery. Also, there is a widespread public distrust of DNA vaccines for fear of the unforeseen genetic consequences caused by the introduction of foreign genomic material into the environment, even though similar preparations have been commercialized and are currently in use internationally. Additionally, the antibiotic selection gene, which is currently contained on the DNA plasmid for propagation purposes, will need to be removed before the United States Food and Drug Administration (FDA) would approve its use. The limitations likely to be encountered during FDA approval were also an additional motivation for the development of the recombinant VHSV glycoprotein. As a single viral protein, this preparation is more innocuous and more likely to gain FDA approval then the DNA vaccine. The fact that a single purified viral protein (rG) can elicit a protective response warrants additional clinical trials, which can examine the use of an adjuvant to increase protection. Moreover, the method we described of producing the recombinant glycoprotein, i.e. using insect larvae, can be easily scaled up to produce large amounts of protein for wide-scale vaccination.

Possibly the most valuable scientific achievement produced from this body of work is the demonstration of indirect protection in an aquatic ecosystem. Our demonstration that immunized fish provide indirect protection to naïve individuals will prove invaluable to future research, although it also brings to light questions regarding the exact mechanism of this protection. Ensuing efforts must scrutinize whether antibodies are actively shed into the environment by immune individuals and can remotely bind or neutralize virus in the water column. This would be a groundbreaking finding and would have profound implications for aquatic research in general. The ultimate aim of this research is to determine if immunized individuals can be used

to establish a herd immunity threshold (HIT) in a large aquatic system. Specifically, whether hatchery propagated individuals, immunized prior to stocking, would result in a decrease in viral transmission/infection, constituting a herd effect. If this could be achieved, and the number of immune individuals in a population reaches a critical threshold, then the prevalence of aquatic pathogens such as VHSV would decrease and perhaps eventually be eliminated. This study demonstrates that immunized individual provide protection, but fails to estimate the viral transmission rate (R_o) which would allow determination of a critical threshold. Viral transmission is likely highly variable in an enormous system with the large number of species that occur in the Great Lakes. Subsequent studies thus face a daunting task in determining R_o.

In conclusion, the study design and methods we have described can be easily adapted and applied to study other aquatic pathogens. Further, the values we have obtained relating to herd effect (e.g., the survivorship in a co-comingled community, viral shedding, etc.) can be applied in the creation of an agent-based model for the examination of immunity on a much larger scale such as one of the Great Lakes. Simulations can also be varied under different scenarios and assumptions to determine effects of vaccination. The use of a model to aid in the calculation of the HIT can then be coupled with the results provided in this dissertation to guide and evaluate release strategies of immunized individuals. In summary, the results of this dissertation demonstrate that viral hemorrhagic septicemia virus can indeed be effectively combated, possibly in systems as large as the Great Lakes basin.

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