HUMORAL IMMUNE RESPONSE OF GREAT LAKES FISHES TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

By

Elena Virginia Millard

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ABSTRACT

HUMORAL IMMUNE RESPONSE OF GREAT LAKES FISHES TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB

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Viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb) is a recently emerged pathogen of Great Lakes fishes in North America. Following initial fish kills in Lake St. Clair, Michigan (MI) in 2006, VHSV-IVb went undetected for nearly three years there until another die-off ensued in the summer of 2009. This prompted questions regarding host immunity to the virus as well as the usefulness of the virus isolation assay as a surveillance tool. Herein, I describe evidence of humoral immunity to VHSV-IVb in several populations of wild fishes and the development of antibodies by muskellunge (*Esox masquinongy*) following experimental challenge and vaccination. Using a 50% plaque neutralization test (PNT) and a competitive enzyme-linked immunosorbent assay (cELISA), antibodies were detected in Lake St. Clair fish during all sampling years between 2004-2011 (six summers). In contrast, VHS virus was only detected during the summer of 2006 and 2009, and in both cases, fish were sampled during or immediately following mortality events. Neutralizing (PNT) and/or binding (cELISA) antibodies were detected in 5 of 13 fish species including muskellunge, northern pike (*E. lucius*), freshwater drum (*Aplodinotus grunniens*), smallmouth bass (*Micropterus dolomieu*) and channel catfish (*Ictalurus punctatus*). The greatest overall seroprevalence and antibody titers were detected in muskellunge. Antibodies were also detected by cELISA in

muskellunge from Lower Fox River/Green Bay, Wisconsin and from Thornapple Lake, Michigan.

Muskellunge experimentally infected with VHSV-IVb developed neutralizing antibodies by 5 – 7 weeks (385 – 539 degree days) post-challenge. The development of neutralizing antibodies corresponded to a decrease in virus presence and titers in sera, indicating their role in limiting VHSV-IVb infection. A reduced neutralizing antibody response was mounted by fish exposed with the lowest dose of the virus, suggesting a threshold level of infection is necessary for induction. In surviving fish, neutralizing antibodies were detectable for a longer period of time after infection then was the virus in tissues (assessed in a parallel study). To better understand host immune response in this species, a DNA vaccine encoding the glycoprotein (G) gene of VHSV-IVb was developed. DNA vaccination primed the adaptive immune response to respond to VHSV-IVb as evident by high levels of neutralizing antibodies in 100% of surviving muskellunge by only 4 weeks (310 degree days) after challenge. Compared to plasmid control fish, pVHSivb-G-vaccinated fish were significantly protected (relative percent survival = 45.2%) following lethal virus challenge 7 weeks (539 degree days) post-vaccination. Surviving fish from the pVHSivb-G vaccinated group also had significantly lower infection prevalence and tissue viral loads compared to control fish. The cELISA, developed as part of this dissertation, and the PNT are non-lethal immunological tools that will find multiple applications in future studies for assessment of adaptive immunity to VHSV-IVb.

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This dissertation is dedicated to my parents, Ronald and Eileen Millard.

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INTRODUCTION

Viral hemorrhagic septicemia (VHS) is an emerging disease of wild freshwater fish in the Great Lakes region of the United States and Canada. The hemorrhagic disease is caused by VHS virus genotype IVb (VHSV-IVb), an RNA virus within the family *Rhabdoviridae* and genus *Novirhabdovirus* (Dietzgen et al. 2012). Within the past decade, the virus caused large-scale mortality events as it spread throughout naïve populations of susceptible hosts (Kim & Faisal 2011a, Faisal et al. 2012). The virus was isolated from many ecologically and economically important fish species such as muskellunge (*Esox masquinongy*), freshwater drum (*Aplodinotus grunniens*) and smallmouth bass (*Micropterus dolomieu*) (Faisal et al. 2012). Following initial fish kills in Lake St. Clair in 2006, VHSV-IVb was not detected from fish in this lake during sampling efforts for nearly three years until another die-off ensued (Faisal et al. 2012). A similar trend was apparent in other water bodies. This raised questions both about the host immune response to this virus and the diagnostic power of the traditional virus isolation test.

Initial studies on VHSV-IVb were crucial for elucidating aspects of the disease course, pathogenesis, and host range of this emerging virus (Al-Hussinee et al. 2010, Kim & Faisal 2010a, b, Al-Hussinee et al. 2011, Goodwin & Merry 2011, Groocock et al. 2012). Experimental studies confirmed the wide host range of the virus and revealed a great diversity of susceptibilities among species and between individuals of the same species.

Such differences could reflect differences in host immune responses to the virus. Fish that do survive VHS are protected against subsequent exposures (Bernard et al. 1983, Kocan et al. 2001, Hershberger et al. 2010, Kim & Faisal 2012). If fish develop protective immunity to VHSV-IVb, it might be hypothesized as the reason that mortality events have generally diminished over time in wild populations where the virus is established.

The study of the immune response is also critical from a pathogen surveillance standpoint. Virus isolation is the currently accepted international standard for testing for VHS. Tissues (and/or ovarian fluids) are infected on susceptible cell lines and isolated viruses are confirmed, usually by molecular methods [World Organization for Animal Health (OIE), 2012]. The process takes up to 28 days and furthermore, the sensitivity of this method for detecting surviving or carrier fish is unknown (OIE 2012). It is known that VHS virus is isolatable for a shorter time after infection compared to the duration of the antibody response (Enzmann & Konrad 1993), and this is especially the case at warmer water temperatures (Jørgensen 1992, Goodwin & Merry 2011). As such, screening fish for virus-specific antibodies can increase the diagnostic ability for detecting rhabdoviral exposure in fish that survive the disease (LaPatra 1996, Schyth et al. 2012). Following outbreaks of VHS among cultured rainbow trout (*Oncorhynchus mykiss*) in Denmark, Schyth et al. (2012) found that while virus isolation remained superior during the outbreak, survivors were more likely to be detected as such based on the presence of

antibodies in the 4-5 month period that followed. Serological testing can also be done non-lethally which is ideal for valuable and long-lived species.

The aim of this project was to develop serological reagents and indirect screening assays for VHSV-IVb and use these tools to study the host immune response. In particular, studies focused on the adaptive, humoral immune response of muskellunge (*Esox masquinongy*). Muskellunge, a native Great Lakes species, are highly prized as a sport fish and serve an important ecological role as a top predator in ecosystems where they exist (Crossman 1986, Bozek et al. 1999). The species is considered vulnerable to overexploitation and habitat alterations (Scott & Crossman 1973, Crossman 1986) and the sustainability of some populations is reliant on continued human intervention through stocking (Battige 2011). Muskellunge were one of the species that died in initial die-offs due to VHSV-IVb in Lake St. Clair in 2006 (Elsayed et al. 2006) and Lake Ontario (Lumsden et al. 2007) and there were concerns regarding the long-term effects of VHSV-IVb on this species. Kim & Faisal (2010b,c) later confirmed experimentally that VHSV-IVb is indeed highly virulent to this species.

The overall project goal was to better understand the host immune response to VHSV genotype IVb. Chapter 1 presents a review of the literature on host immune response to VHS virus and vaccination. In Chapter 2, the kinetics of the neutralizing antibody response of experimentally exposed muskellunge was studied over the course of disease and recovery. In Chapter 3, neutralizing antibody responses among 13 fish

species residing in a VHSV-IVb enzootic lake, Lake St. Clair, Michigan were assessed over a multi-year period (2004-2010). Fish were sampled during, and in the months and years following, VHSV-IVb positive mortality events and in some cases, comparisons were made between serological and virus isolation results. Nine species from Lake St. Clair were also screened for evidence of an immune response using a competitive enzyme-linked immunosorbent assay (cELISA) (Chapter 4). The production of polyclonal antiserum to VHSV-IVb and the development of the cELISA assay are described in Chapter 5. Using muskellunge as an indicator for VHSV exposure based on the presence of antibodies by cELISA, fish were tested from five water systems in Michigan and Wisconsin (including VHSV negative and positive areas). As an additional tool to study host immune responses, a VHSV-IVb DNA vaccine was developed (Chapter 6). The vaccine was tested for its ability to induce innate and adaptive protective immunity in rainbow trout (*Oncorhynchus mykiss*) and muskellunge, respectively. Overall conclusions, areas of future research, and management applications are addressed in Chapter 7.

CHAPTER 1

REVIEW OF LITERATURE

Viral hemorrhagic septicemia virus (VHSV) causes VHS, an internationally reportable disease of fishes of coastal North America, Western Europe, Japan and Korea. VHSV has an exceptionally broad host range compared to other fish rhabdoviruses, and includes at least 80 species of cold, cool and warm-water fishes from fresh, brackish and marine ecosystems (OIE 2012). Some fish species can become infected and remain carriers of the virus without showing any clinical signs. The virus is highly virulent in other species causing severe hemorrhagic disease and death. Within the past decade (2003-2013), a novel sublineage of the North American genotype IV, designated type IVb, emerged in the Great Lakes region of the US and Canada. Large-scale fish kills marked the virus entry into new water systems, with a few known exceptions (e.g. Lake Superior, Baseline Lake). VHSV-IVb was found to be capable of naturally infecting many ecologically and economically important fish species and was quickly regarded as a threat to Great Lakes commercial and recreational fisheries. The many unknowns regarding potential effects of this disease on the health of wild and cultured Great Lakes fish populations prompted a large investigation, in which this dissertation is a part. The investigation aimed to a) compare the susceptibility of different fish species, b) identify potential species involved in virus transmission (carrier fish hosts, invertebrates), c) develop supplementary and non-lethal testing methods, and d) investigate host

immunity. In addition to providing information on the ability to predict what species of fish may be most affected by this disease in the short and long-term, this research will allow for more targeted surveillance efforts and provide a better understanding of how the virus is maintained in water systems from year to year. The collection of studies in this dissertation in particular focused on host immune responses to VHSV-IVb. Many studies focused on muskellunge, *Esox masquinongy* (Order Esociformes, family *Esocidae*), an important Great Lakes top predator, and also a prized sport fish, that was involved in initial mortality events and was later confirmed experimentally to be highly susceptible to the disease.

VIRAL HEMORRHAGIC SEPTICEMIA VIRUS

Structure and infection cycle. Viral hemorrhagic septicemia virus (VHSV) is a member of the family *Rhabdoviridae* (order Mononegavirales) and the genus *Novirhabdovirus* (Dietzgen et al. 2012). Rhabdoviruses are a diverse group of viruses that include important pathogens of vertebrates, invertebrates and plants (Dietzgen et al. 2012). All rhabdoviruses have a single molecule of linear, negative sense single-stranded RNA (ssRNA) that encodes five structural proteins. These proteins are designated L (RNA-dependent RNA polymerase), G (glycoprotein), N (nucleoprotein), P (phosphoprotein) and M (matrix protein) (Dietzgen et al. 2012). An additional non-structural (or non-virion) protein (NV) characterizes viruses of the genus Novirhabdovirus (Kurath & Leong 1985, Schütze et al. 1999). The genus

Novirhabdovirus contains viruses of fish including VHSV, infectious hematopoietic necrosis virus (IHNV) of salmonids, Hirame rhabdovirus of Japanese olive flounder (*Paralichthys olivaceus*) and Snakehead virus of warm-water species of Southeast Asia (Dietzgen et al. 2012). Both IHNV and VHSV are reportable pathogens to the World Organization for Animal Health (OIE).

Genes are arranged in the order 3'-N-P-M-G-NV-L-5' and non-coding leader and trailer regions are present at the 3' and 5' ends respectively (Dietzgen et al. 2012) (Figure 1.1). The N, P and L proteins together with the viral RNA form the nucleocapsid (Dietzgen et al. 2012). G proteins are spike-like homotrimeric proteins that span the lipid bilayer and are the only surface protein (Gaudin et al. 1992). Like other rhabdoviruses, the G proteins of VHSV are a key protein involved in virulence and tropism because of its role in initiating the infection cycle (Bearzotti et al. 1995). G proteins mediate virus attachment to host cellular membrane receptors during the first step of the infection cycle (Smail & Snow 2011). While the exact receptors used by VHSV and other fish novirhabdoviruses is not known, evidence suggests that the receptor complex contains fibronectin as a component (Bearzotti et al. 1999, Liu & Collodi 2002). The expression of the host cell receptors used by a particular virus thus influence host range as well as tissue tropism (Bearzotti et al. 1999). Adsorption is followed by receptor-mediated endocytosis, fusion with primary lysosomes, and release of the nucleocapsid into the host cell cytoplasm (Granzow et al. 1997). The virus uses its own RNA polymerase (L) to replicate in the cytoplasm and proteins are produced using the host cell's translational

machinery (Dietzgen et al. 2012). The virion is assembled at the inner surface of the cell membrane and the glycoprotein-studded envelope is acquired during budding from the cell. The function of the NV protein is not fully defined. The protein is expressed in infected cells but not present in mature virions of IHNV (Kurath & Leong 1985). The NV gene product of IHNV was found to be essential for viral replication and it believed to have a role in pathogenicity (Thoulouze et al. 2004).

Transmission and VHS disease. Transmission of VHSV is horizontal and spread by direct contact with infected fish or water. VHSV is shed in urine and reproductive fluids from infected fish (Neukirch 1985, OIE 2012). Transmission of the virus by ingestion of infected fish has also been demonstrated experimentally in rainbow trout (*Oncorhynchus mykiss*) (Schönherz et al. 2012). Unlike IHNV, there is no evidence that VHSV can be vertically transmitted. Reservoirs of VHSV include clinically infected fish as well as inapparently infected fish (covert carriers) (OIE 2012). VHSV-IVb has recently been isolated from several invertebrate species including amphipods [*Diporeia* spp. (Faisal & Winters 2011)] and leeches [*Myzobdella lugubris* (Faisal & Schulz 2009)]. Their role as potential reservoirs or vehicles for virus transmission is unclear at this time.

Epithelial tissues of gill and skin, particularly near the base of fins (Harmache et al. 2006), are important sites of early viral replication and considered primary routes of entry (Neukirch 1984, Yamamoto et al. 1992). Leukocytes and endothelial cells are

considered important cell types for virus replication (Wolf 1988, Yamamoto & Clermont 1990). The virus spreads in blood to early target organs including spleen and kidney where it causes marked necrosis of hematopoietic tissue of the head kidney (reviewed in Kim & Faisal 2011b, Smail & Snow 2011). As the kidney and spleen are main immune organs, damage likely impairs host response to this virus. The cause of death is blood extravasation leading to deprivation of vital tissues of oxygen. VHSV can cause disease in all life stages of susceptible species; however generally mortality is highest in younger fish and naïve adults (OIE 2012). Mortality caused by VHS (genotype I) in freshwater rainbow trout for example often reaches 80-100% in fry and fingerling rainbow trout (Olesen 1998) and typically less (30-70%) in adults (Skall et al. 2005). The temperature range of VHSV-I and VHSV-IVb are similar; the virus has an optimum of 9-12°C and an upper limit of 18-20°C (Goodwin & Merry 2011, OIE 2012).

Geographic distribution and genotypes. Prior to the 1980's, VHSV was thought to be strictly a disease of cultured rainbow trout in Western Europe, where it caused extensive economic losses for the aquaculture industry (Wolf 1988, Olesen 1998, Snow et al. 2004). This was challenged when VHSV was isolated from coho (*O. kisutch*) and chinook (*O. tshawytsha*) salmon returning to hatcheries in the Pacific Norwest region of the U.S. in 1988 (Brunson et al. 1989, Hopper 1989). Subsequent investigations revealed that the virus exists endemically among an extensive reservoir of free-ranging marine and anadromous fish in the North Pacific Ocean off the western coast North America (Meyers et al. 1992, Meyers & Winton 1995, Hedrick et al. 2003), the North

Atlantic Ocean, North and Baltic Seas around Europe (Dixon et al. 1997, Mortensen et al. 1999, Smail 2000), as well as coastal Japan (Takano et al. 2000). Based on historical evidence and genetic analysis of isolates, VHSV is considered to have originated from a marine source and adapted several times to freshwater, where it proved to be highly virulent to cultured rainbow trout in Europe (Einer-Jensen et al. 2004) and naïve populations of wild freshwater fish in the Great Lakes region of North America.

Based on phylogenetic analyses, four main genotypes (I-IV) are recognized that cluster based on geographic location (Snow et al. 1999, Einer-Jensen et al. 2004, Snow et al. 2004, Einer-Jensen et al. 2005, Pierce & Stepien 2012). Some genotypes are further divided into multiple sublineages (Ia-Ie, IVa-IVc). Genotypes I-III are predominantly isolates endemic among marine species around Western Europe, whereas genotype IV consists of North American isolates, as well as a few from East Asia. Genotype Ia contains the highly virulent freshwater isolates from cultured rainbow trout in western Europe. Genotype III has caused losses of cultured turbot (*Scophthalmus maximus*) in the British Isles. In North America, type IVa exists among marine species in the northeastern Pacific Ocean, particularly in herrings and sardines (Meyers & Winton 1995, Hedrick et al. 2003). Natural epizootics due to VHSV-IVa have been documented in small Pacific herring (*Clupea pallasi*) and several other marine fish species (Meyers et al. 1999, Traxler et al. 1999). VHSV-IVa proved to be highly virulent in juvenile Pacific herring under experimental conditions (Kocan et al. 1997). In older herring, the virus

establishes a chronic infection with mortality associated with stress (Meyers et al. 1994). Isolates of IVa are found in wild and farmed marine fish species in Japan (Takano et al. 2000, Isshik et al. 2001, Nishizawa et al. 2002) and Korea (Kim et al. 2003). Type IVb consists of freshwater isolates from the Great Lakes region of the U.S. and Canada. The VHSV strain isolated from fish off the Atlantic coast of Canada in 2000 (Gagne et al. 2007), was recently designated type IVc (Pierce & Stepien 2012).

VHSV-IVb in the Great Lakes. VHSV-type IVb emerged in the Great Lakes region of North America within the last decade. The earliest known occurrence of this sublineage is 2003 from muskellunge collected from Lake St. Clair, Michigan (Elsayed et al. 2006). In 2006-2007, the virus was isolated from large-scale die-offs of free-ranging fish in Lake Ontario (Groocock et al. 2007, Lumsden et al. 2007), the St. Lawrence River, (Groocock et al. 2007), Lake St. Clair and Lake Huron (Faisal et al. 2012). The virus then spread to all of the Great Lakes, to several inland lakes in Michigan, Wisconsin, and New York, as well as to at least one site in the Mississipi watershed in Ohio (reviewed in Kim & Faisal 2011a,b, Faisal et al. 2012). Analysis of partial G gene sequences of over 100 Great Lakes isolates from 2003-2009 by Thompson et al. (2011) revealed a low genetic diversity (maximum 1.05%), consistent with a recent introduction.

Species involved in wild fish die-offs, distribution and history of spread, pathogenicity, and host range has been discussed previously (Kim 2010, Kim & Faisal 2010b, Kim & Faisal 2011, Faisal et al. 2012). Currently, 28 fish are regulated under the VHS Federal

Order aimed at preventing spread of VHSV-IVb into aquaculture facilities (United States Department of Agriculture, Animal and Plant Health Inspection Service 2008). These fish were naturally infected with the virus, though some fish did not show clinical signs of disease. Experimental studies by Kim & Faisal (2010a, b, c) have identified two important determinants in the outcome of infection, which include the infective dose and fish species. The virus is highly virulent in naïve juvenile fish of the following species: Great Lakes muskellunge (family Esocidae) (Kim & Faisal 2010c), lake herring/cisco (family *Salmonidae*; *Coregonus artedii*) (Weeks et al. 2011), and largemouth bass (family *Centrarchidae*, *Micropterus salmoides*). The lethal dose 50 (LD_{50}) by the intraperitoneal (IP) injection route of infection was 2.2 plaque forming units (pfu) for musky and 1.5×10^2 pfu for largemouth bass. Yellow perch (family *Percidae, Perca flavescens*) are of moderate susceptibility (IP LD_{50} 2.5×10^5 pfu). Most salmonids (family *Salmonidae*), including rainbow trout, brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon, and coho salmon were relatively resistant (IP LD_{50}: 1.4×10^6 to $> 7 \times 10^7$ pfu) (Kim & Faisal 2010a). Survivors may play a role in maintaining the virus in the ecosystem (Kim and Faisal 2012, Faisal et al. 2012). Preliminary studies in our laboratory suggested that salmonids shed the virus for an extended period after infection, despite never displaying signs of disease (Shavalier & Faisal, pers. com.). Studies by Kim and Faisal (2012) demonstrated that muskellunge can indeed shed the virus for up to 15 weeks post-infection and after that, may resume shedding upon exposure to stressors (e.g. handling).

IMMUNE RESPONSE OF FISH

Lymphoid tissues. Fish have both innate (non-specific) and adaptive (specific) immune systems that are similar in form and in some case function to mammals, with a few key differences. The immune tissues of teleost fish differ from mammals in that they lack bone marrow, lymph nodes and germinal centers (Zapata & Amemiya 2000). The anterior (head) kidney is the primary lymphoid tissue, a main site of hematopoiesis, and the site of B lymphocyte development, whereas the thymus is the primary T lymphocyte tissue (Fange 1986, Rombout et al. 2005, Zapata et al. 2006, Todo et al. 2011, Roberts 2012). The spleen and kidney both have a reticuloendothelium system for filtering and trapping pathogens, and are both sites of lymphocyte activation of B and T cells (Zwollo et al. 2005, Solem & Stenvik 2006, Ye et al. 2011). Melanomacrophage centers throughout the reticuloendothelial tissue house many macrophages that clear material from circulation, including immune complexes. Teleosts have mucosa-associated lymphoid tissue (MALT) in the skin, gill, and gut, although organized germinal centers (in skin, or similar to Peyer's patches in mammalian gut) have not been identified (reviewed in Salinas et al. 2011). The gills contain phagocytic cells along brachial capillaries and lymphocytes (Roberts 2012). Mucus coating epithelial tissue of skin, gill, and gastrointestinal tract is a protective barrier for physically trapping and sloughing organisms to prevent colonization (Roberts 2012).

Innate immunity. The innate immune system is an ancient form of host defense against infection consisting of barriers, soluble (humoral) substances, and cells. Innate immunity in fish has been the subject of several thorough reviews (Ellis 2001, Magnadottir 2006, Whyte 2007, Gomez & Balcazar 2008). The innate response is based on a system of evolutionarily conserved host receptors expressed on cell surfaces, inside cellular compartments and on soluble molecules that distinguish microbes from host products (Medzhitov & Janeway 1997). Pattern recognition receptors (PRRs) bind molecules called pathogen-associated molecular patterns (PAMPs) present only on microbes, thus giving the host the ability to distinguish an invading pathogen from self molecules and cells. This system of recognition allows for a rapid "non-specific" response in the absence of prior exposure. Engaged PRRs activate innate pathways such as phagocytosis, complement, coagulation, inflammation and apoptosis (Medzhitov & Janeway 1997).

The skin and mucus layer contain numerous protective humoral substances that inhibit pathogen entry and replication (*e.g.* lysozyme, lectins, proteases, complement) and many of these substances are also present in serum and tissue fluids (Ellis 2001, Fast et al. 2002, Esteban 2012, Najafian & Babji 2012). In some cases, the repertoire of innate humoral substances of fish is more diverse than that of mammals (Vasta et al. 2011, Nakao et al. 2011, Roberts 2012). It is thought that such unique structural and functional diversity of the teleost innate humoral immunity could allow a broader recognition of pathogens during the innate response. In some cases, innate humoral

molecules may cause non-specific neutralization or binding to viruses in assays. Lectins are humoral molecules characterized by the presence of a carbohydrate recognition domain that binds sugar and glycoprotein PAMPS (Vasta et al. 2011). Ladderlectin, isolated from plasma of rainbow trout, was shown to bind directly to VHSV-IVb *in vitro* (Reid et al. 2011). Another lectin, the 6S inhibitor of infectious pancreatic necrosis virus (IPNV) is present in sera of some salmonids without prior exposure to IPNV (Jørgensen 1973, Dorson & de Kinkelin 1974, Park & Reno 2005), and this substance can result in neutralization of IPNV in cell culture by preventing viral adsorption (Kelly & Nielsen 1985, Park & Reno 2005).

Cells of innate immunity of teleosts include macrophages, granulocytes (neutrophils, basophils, eosinophils, eosinophilic granular cells), nonspecific cytotoxic cells, and dendritic-like cells. Macrophages and neutrophils are specialized phagocytic cells that kill pathogens directly, and are also important in initiating inflammation including releasing cytokines to increase mobilization of additional phagocytes (Roberts 2012). Cytokines involved in fish immunity have been recently reviewed (Alejo & Tafalla 2011). Macrophages can be found throughout the body but are concentrated in kidney, spleen and in some species, atrium of heart, as reticuloendothelial cells (Roberts 2012). Interestingly, some B cells of fish also have potent phagocytic activity suggesting their role in innate as well as adaptive responses (Li et al. 2006, Zhang et al. 2010). In mammals, natural killer (NK) cells are the effector cells of innate anti-viral immunity. NK-like cells that kill virus-infected and tumor cells have been found in several fish species

(Fischer et al. 2006, Nakanishi et al. 2011). Several other types of cells with non-specific cytotoxic activities have been identified in fish (reviewed in Nakanishi et al. 2011).

Adaptive (acquired) immunity. Fish are the most primitive vertebrates to have an adaptive immune system. The major components of adaptive immunity include lymphocytes (B and T cells), major histocompatibility complex (MHC) molecules, T and B cell receptors, and immunoglobulins (Iwana & Nakanishi 1996, Roberts 2012). Upon first encounter with an antigen, the adaptive response takes time to develop as antigen-specific cells are selected, proliferate, and undergo changes in affinity. In mammals, dendritic cells are professional antigen presenting cells (APCs) that have a primary role in antigen presentation to T cells, thereby linking innate and adaptive responses (Banchereau et al. 2000). T cells can be divided into two main functional groups. Cytotoxic (killer) T cells express CD8 co-receptor, interact with endogenous antigens (generated inside cells) presented on MHC Class I molecules, and kill abnormal cells by inducing apoptosis or releasing molecules. Helper T cells express co-receptor CD4, recognize exogenous antigens presented in the context of MHC Class II by APCs, and function to activate and coordinate other immune cells, including B cells, through cytokine production. B cells secrete antibodies (plasma B cells) or differentiate into long-term memory B cells. Antibodies function in direct neutralization of pathogens, or opsonize pathogens for removal by phagocytes. T cells can be further divided into several effector subsets based on cytokine production and cell surface markers. Functional and gene expression evidence to date suggest the presence of helper and

cytotoxic T cells in teleosts and similar activation pathways (Fischer et al. 2006, Toda et al. 2011). However, physical characterization of cells involved in the adaptive immune response of fish has been hindered compared to mammals due to lack of specific reagents (Nakanishi et al. 2002, Fischer et al. 2006, Nakanishi et al. 2011, Esteban et al. 2012, Verrier et al. 2011). Functionally analogous dendritic cells have been recently defined in teleosts (tDC) (Bassity & Clark 2012). In addition to being morphologically similar to mammalian DCs, this cell type induced a potent, proliferative mixed leukocyte response and was phagocytic and migratory suggesting its role as a specialized APC (Bassity & Clark 2012). Recent advances have characterized many T-cell associated proteins in multiple species of fish including surface co-receptors (CD8, CD4), proteins associated with signal transduction (e.g. CD3 complex), and many secreted cytokines (Laing & Hansen 2011). One study recently showed proliferation of CD4+ T cells in ginbuna crucian carp (*Carassius auratus* langsdorfii) after allogenic and antigen-specific stimulation (Toda et al. 2011), supporting that CD4+ T cells in fish are analogous to mammalian CD4 + helper T cells (Laing & Hansen 2011). The finding of sequence homologues for MHC Class I, TCR and CD8 coreceptor in several fish species suggests that antigen presentation to cytotoxic T cells is also similar to what occurs in higher vertebrates (Utke et al. 2007). More studies that link protein expression with function are needed.

The main humoral component of the adaptive immune response is immunoglobulin, which is secreted by B cells. In teleosts, immunoglobulin M (IgM) is the predominant

immunoglobulin type in plasma and the main one associated with systemic responses (Kaattari & Piganelli 1996). IgM has multiple effector functions including neutralization, precipitation and agglutination, opsonization and complement-mediated functions (Roberts 2012). Two additional immunoglobulin classes including IgD (Wilson et al. 1997, Edholm et al. 2010) and IgT (also called IgZ, and analogous to mammalian IgA) (Danilova et al. 2005, Hansen et al. 2005) have more recently been identified in several species of teleosts. Studies have shown IgT to be an important immunoglobulin in the gut mucosa (Zhang et al. 2010) and skin mucus of rainbow trout (Bordon 2013). The role of IgD is not yet elucidated (Castro et al. 2013). Multiple B cell subsets exist in fish including B cells expressing only IgM, IgD or IgT and one subset that expresses both IgM and IgD (Salinas et al. 2011). Recent studies using pyrosequencing and CDR3-length spectratyping have shown that VHSV infection significantly changes profiles of B cells in spleen, which is evidence of clonal expansion of B cells (Castro et al. 2013). Interestingly, both immunoglobulin M (IgM)+ and IgT+ B cells responded. This evidence suggests an initial relationship between IgT and the anti-viral response (Castro et al. 2013).

Immune memory occurs in teleosts but does not share all the features associated with immune memory in mammals (Kaattari 1992, Ma et al. 2013). Characteristics of fish memory response associated with subsequent exposures to an antigen include a more rapid antibody response, higher antibody titers, and increased sensitivity to antigens (reviewed in Kaattari 1992). The overall increase in magnitude of antibody titers

between the primary and secondary response is not as profound in teleosts as it is in mammals, though this can vary based on temperature (Roberts 2012). In teleosts, isotype-switching does not occur during affinity maturation as it does in mammals (Stavnezer et al. 2008). Instead, distinct B cell subsets produce the different isotypes (e.g. IgM, IgT) as discussed above. Affinity maturation of IgM antibody in rainbow trout involves a gradual, and slower switch from lower to higher affinity antibodies, which corresponds to a higher degree of disulfide polymerization of IgM (Ye et al. 2011). Ye et al. (2011) showed that low affinity, low titer antibody subpopulations are progressively replaced by higher affinity subpopulations that have higher titers and appear later. The highest affinity antibodies appeared 15 weeks after exposure to TNP-keyhole limpet hemocyanin and remained elevated through 27 weeks when the study period was terminated.

IMMUNE RESPONSE TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS

Innate immune response of fish to VHSV. VHSV activates a robust innate antiviral immune response followed by an adaptive response and induction of immunological memory. The ability of the host to prevent and limit virus infection initially by physical and chemical barriers is an important determinant in the outcome of disease (reviewed in Esteban 2012). The essential role of external barriers is evidenced by the fact that much higher doses of VHSV-IVb by immersion are required to produce disease and mortality compared to the intraperitoneal route (Encinas 2010, Kim & Faisal 2010 a,b,c).

Even between individuals of a susceptible species, huge variation exists in their ability to prevent the development of clinical infection. The genetic component of host resistance was recently studied using a collection of rainbow trout clones with differential susceptibility to VHSV by immersion (Verrier et al. 2012). The study revealed that mechanisms determining host resistance were indeed associated with innate/intrinsic mechanisms and not specific host immunity. In particular, the induction of interferon by some clones prevented early viral replication whereas this response was absent in cells from susceptible fish. Induction of a systemic antiviral state by cytokine release may occur following early VHSV replication in epidermal cells of fin tissues (Quillet et al. 2001, 2007).

The innate antiviral response of fish to VHSV is mediated primarily by cytokines, notably, the type I (α/β) interferon (IFN) system and its inducible proteins (Verrier et al. 2011, Purcell et al. 2011). VHSV stimulates a rapid and robust type I IFN response by a variety of host cells (Tafalla et al. 2008, Hansen et al. 2012, Lovy et al. 2013). The IFN system of fish has been recently reviewed (Zou & Secombes 2011). Double-stranded RNA intermediates produced during early viral replication (Smail & Snow 2011) and viral surface glycoproteins of VHSV and IHNV are both potent inducers of the IFN response in fish (Acosta et al. 2006, Verjan et al. 2008, Smail & Snow 2011). It is host Toll-like receptors or other PRRs that recognize these conserved viral patterns (Purcell et al. 2011). IFNs induce an antiviral state in neighboring cells through regulating the

transcription of numerous genes including proteins that inhibit viral replication (Ellis 2001, Murphy et al. 2008).

The IFN response and associated changes in host gene expression are studied by quantitiative PCR or subtractive suppressive hybridization (Verrier et al. 2011). Studies have demonstrated a "core" set of interferon-stimulated genes (ISGs) involved in the antiviral response to rhabdoviral infection or DNA vaccination, many of which are conserved among vertebrates (reviewed in (Verrier et al. 2011). VHSV is a potent inducer of the intracytoplasmic "VHSV and interferon-induced genes" vig1 (aka viperin) vig2 and ISG15/vig3 as well as Mx 1-3 genes. Mx increases in the liver after infection of yellow perch with VHSV-IVb and upregulation was correlated with increasing viral load (Olson et al. 2013). In mammals, Mx proteins have broad range anti-viral activities against RNA viruses (Leong et al. 1998) and direct antiviral activity of teleost Mx proteins have been confirmed by transfection experiments (Caipang et al. 2003). Virus-infected cells also produce secretable antiviral factors (e.g. chemoattractants Vig7, Vig8, and galectin 9). In response to DNA vaccination, the upregulation of these factors is similar, and timing of peak levels corresponds to the timing of the non-specific/cross-protective phase that occurs within a week after vaccination (Purcell et al. 2004, 2012).

The antibody response to VHSV. The specific antibody response to VHSV is best characterized in terms of the neutralizing antibody response. Early studies showed that rainbow trout exposed to low-virulence strain of VHS [F1, Denmark (Jensen 1965);

genotype Ia] showed enhanced resistance against subsequent challenge with a virulent strain (Jørgensen 1976). Early studies also demonstrated the presence of virus-neutralizing antibodies in serum of rainbow trout after experimental and natural infection (Jørgensen 1971, de Kinkelin et al. 1977, Dorson & Torchy 1979, Jørgensen 1982, Olesen & Jørgensen 1986). Neutralization is mediated by IgM antibodies based on studies that demonstrated rabbit antiserum against trout IgM inhibits the neutralization reaction (Olesen & Jørgensen 1986). The reaction of neutralizing antibodies against neutralizing epitopes of virus G proteins is measured by the 50% plaque neutralization test (PNT) and serum neutralization test (SNT). The *in vitro* neutralization reaction between VHSV-neutralizing antibodies and the virus is enhanced by the addition of complement, a heat-labile molecule, which is supplied by adding unheated, naïve rainbow trout sera into the reaction (Dorson & Torchy 1979). Serum being tested for antibodies is first heated to 45°C for 20-30 minutes in order to destroy any pre-existing complement that may be bound to antibodies in the serum (Dorson & Torchy 1979, Sakai 1981). The specific mechanism by which complement aids in neutralization still remains unclear (Purcell et al. 2012) but some results suggest that the classical complement activation pathway is involved (Lorenzen et al. 1999). The complement system is a collection of serum proteins that is a powerful innate defense (reviewed in Boshra et al. 2006). Complement may facilitate direct lysis of enveloped viruses (Lorenzen et al. 1999), aid neutralizing antibodies in the formation of immune complexes or inhibit virus attachment to cell receptors (Pier et al. 2004).

The protective nature of specific antibodies against VHSV has been demonstrated by passive transfer experiments. Rainbow trout immunized with neutralizing serum, or the purified immunoglobulin portion of serum from challenge survivors (Bernard et al. 1983), were protected against VHSV challenge (de Kinkelin et al. 1977, Lorenzen et al. 1999). Subsequent studies demonstrated that antibodies against the G proteins mediated protection (Lorenzen et al. 1990). Rainbow trout injected with monoclonal antibodies against N, M1 (now phosphoprotein), and M2 (now matrix) proteins in contrast, were not protected (Lorenzen et al. 1990). It is know well established that the G proteins of VHSV and IHNV are the major surface antigen that elicits the production of neutralizing antibodies (Lorenzen et al. 1990, Bearzotti et al. 1995). The time course of development of neutralizing antibodies after experimental infection has been studied primarily in rainbow trout after infection with VHSV-genotype I. Neutralizing antibodies begin to appear by 2-4 weeks after infection with peak responses occurring by 6-10 weeks after infection at 10°C (reviewed in Lorenzen & LaPatra 1999). Because the antibody response does take several weeks, the role of neutralizing antibodies in protecting naïve fish from a primary, acute infection is unlikely (Lorenzen & LaPatra 1999, Purcell et al. 2012). Neutralizing antibodies after VHSV infection have been reported to last at least a year in serum of survivors (Olesen & Jørgensen 1986).

Non-neutralizing antibodies, or binding antibodies, are also induced after rhabdoviral infection. Various assays have been developed for measuring non-neutralizing antibodies against VHSV including ELISA, western blot (Lorenzen et al. 1993),

immunofluorescence (Olesen et al. 1991, Jørgensen et al. 1991) and counter-current immunoelectrophoresis (Enzmann et al. 1993, Enzmann & Konrad 1990, 1993). ELISA assays used immunoglobulin-captured virus as the coating phase (Jørgensen et al. 1991, Olesen et al. 1991, Fregeneda-Grandes & Olesen 2007), viral-infected cell culture supernatants (Kim et al. 2008), or recombinant G protein fragments (Encinas et al. 2011). Detection of rainbow trout serum antibodies against VHSV were detected using anti-rainbow trout antibodies or antisera.

In several studies where PNT and ELISA were performed in parallel, more survivors were detected by ELISA-based techniques (Jørgensen et al. 1991, Olesen et al. 1991, Encinas et al. 2011). It was hypothesized by Olesen et al. (1991) that this is because with ELISA, more types of antibodies can be detected. PNT detects only neutralizing antibodies, or those reacting against neutralizing epitopes on the G proteins. Olesen et al. (1991) hypothesized that ELISAs (and immunofluorescence techniques) would measure additional populations of antibodies such as those against non-neutralizing areas of the G proteins, as well as against other antigens exposed on the plate: L (RNA-dependent RNA polymerase), G (glycoprotein), N (nucleoprotein), P (phosphoprotein, formerly M1) and M (matrix protein, formerly M2), however, this was not characterized in that study. From studies with IHNV, it seems that the most prevalent antibodies after rhabdovirus infection are against G and P proteins, in which G is embedded. Using western blot, Ristow (1993) characterized antibodies against all five structural proteins of IHNV in sera of rainbow trout after IHNV infection. Antibodies were detected against L

(RNA-dependent RNA polymerase), G (glycoprotein), N (nucleoprotein), M1 (now P, phosphoprotein) and M2 (now M, matrix protein). In fish that had only been exposed once to IHNV, the M1 (now P) protein was the most commonly detected protein by western blot, while in fish that had repeated exposures to IHNV, antibodies against the G protein were the most frequently produced.

Neutralizing antibodies appear to decline sooner in rainbow trout after VHSV (genotype I) infection than do non-neutralizing antibodies. For example, Olesen et al. (1991) experimentally infected rainbow trout with VHSV-I at 0 and 14 weeks. The highest titers of both PNT and ELISA (using Ig-captured VHSV infected-cell culture supernatants) were detected by 10 weeks post-infection. Between 18-23 weeks (the last sampling point), there was a decline in PNT titers (to 80) while ELISA titers increased. However, the fact that fish in this study received a second exposure at 14 weeks post-infection should be taken into account. Encinas et al. (2011) found a similar trend of PNT antibodies being shorter-lived using ELISA that employed purified fragment of recombinant G proteins of VHSV-genotype I as coating phase (Encinas et al. 2011). Between 4 and 10 weeks after infection, the percentage of fish detected as survivors based on ELISA increased to 100%. In contrast, in that same time period, the percentage positive decreased from 65% to 35%. Of sera testing positive by only one assay, 40% of sera were PNT negative, ELISA positive and only 3% were PNT positive, ELISA negative.

In general, less is known about protection induced by non-neutralizing antibodies against fish rhabdoviral infections. Some studies have shown that non-neutralizing antibodies can also be protective (Lorenzen et al. 1990). The immune response of other species of fish besides rainbow trout against VHSV is also in general less studied. Some species do not mount a neutralizing antibody response, at least not in levels detectable by standard PNT assays. Pacific herring surviving infection with VHSV-IVa are protected upon subsequent exposure, and passive transfer of sera produces protection, yet neutralizing antibodies are not detected by standard neutralization assays (Kocan et al. 2001, Hershberger et al. 2007, Hershberger et al. 2011). It is possible that the neutralizing antibody response in some species could be reduced or absent.

VHSV immune response and temperature. Immune responses of fish to infectious organisms are influenced by host factors (e.g. age, health status, genetic variability) and external factors such stress, light level, and temperature (Bly et al. 1997, Le Morvan et al. 1998, Kollner et al. 2002). Temperature in particular has been shown to affect the host response and disease course following VHSV infection. VHSV and other fish rhabdoviruses are generally considered “hit and run” viruses; that is, they cause severe, acute disease resulting in high mortality of their host (Purcell et al. 2012). However, VHSV can also establish a long-term, persistent disease state in asymptomatic, carrier fish, which is usually associated with low temperatures and virus infection of neural tissues (Wolf 1988, Smail & Snow 2011, Purcell et al. 2012). Persistent infections with

VHSV have been demonstrated up to 400 days in rainbow trout held at low water temperatures of 4°C (Jørgensen 1982, Neukirch 1984, 1985) or up to 200 days in Pacific herring infected with VHSV-IVa (Hershberger et al. 2010). The ability of VHSV to cause recurring outbreaks of disease may be associated with persistence of the virus in carrier fish (Purcell et al. 2011). At higher temperatures, the virus is cleared more rapidly (Jørgensen 1982, Goodwin & Merry 2011).

The immune system of teleosts functions through a range of permissible temperatures, with a species' optimal immune response occurring at that species' normal summer temperature (Ellis 1988, Bly & Clem 1992, Ellis 2001, Magnodittir 2006). Temperatures below a species' permissible range tend to have an immunosuppressive effect on the immune system by inhibiting or delaying responses, particularly, the development of adaptive immunity. The priming phase of the adaptive response is perhaps the most sensitive to non-permissible temperatures, due to slower primary T helper and CTL response, however once memory cells are generated, they are not impaired (reviewed in Iwama & Nakanishi 1996). At colder temperatures, innate responses are prolonged to compensate for the suppression of the adaptive response, however, antibodies are eventually produced even at colder temperatures (Bly & Clem 1992, Le Morvan et al. 1998). For example, in a study by Lorenzen et al. (2009), rainbow trout were vaccinated with the VHSV or IHNV DNA vaccine and held at 5°C, 10°C or 15°C for 40 days prior to VHSV challenge. Neutralizing antibodies were produced by a higher proportion of fish, and in higher titers, when fish were kept at their optimal temperature (15°C). In contrast

antibodies were completely absent by this time in fish held at 5°C. In this same study, fish vaccinated with IHNV- DNA vaccine were only cross-protected against VHSV challenge if they were acclimated to the colder temperatures (5-10°C). Since cross-protection is mediated by innate anti-viral immunity, these results suggest that at colder temperatures, innate responses lasted longer.

It has been suggested that at colder temperatures, the maintenance of the virus or antigen for a longer period of time, actually prolongs the induction of the adaptive response during the priming phase, leading to an even more robust response when it does develop (Jørgensen 1982, Lorenzen et al. 2009). Fish acclimated at 10°C to VHSV, followed by transferring of fish to a range of temperatures, found that antibody titers and protection were higher by 9 week PV in fish that were actually moved to colder temperatures. These studies suggest that vaccination against rhabdoviruses seems optimal in terms of long-term protection if fish are vaccinated and kept at the low end of permissible temperatures in order to prolong the period of time in which antigen is presented to the immune system (Lorenzen et al. 2009).

DNA VACCINATION AGAINST VHSV AND THE ANTIBODY RESPONSE

DNA vaccination technology. DNA vaccination is defined as the intentional transfer of genetic material to somatic cells for the purposes of influencing the immune system (Tonheim et al. 2008). Genetic material from a pathogen of interest is introduced by

means of recombinant DNA technology into an expression vector (plasmid). The plasmid is used to deliver the gene of interest to host cells. The plasmids used in DNA vaccines can be described as consisting of two units: the antigen expression unit and the production unit (Schirmbeck & Reimann 2001; Figure 1.1). The antigen expression unit consists of the promotor/enhancer, antigen sequence, and polyadenylation sequences needed for expression of the protein by the vaccinated host. The production unit consists of elements necessary for amplification of the plasmid in bacteria as well as selectable markers (antibiotic resistance genes). The plasmid is replicated in *Escherichia coli* (*E. coli*) to generate large amounts and then is subsequently purified back out for use as a DNA vaccine.

DNA vaccination against fish rhabdoviruses. DNA vaccinations have been investigated for many fish pathogenic viruses, bacteria, and parasites (reviewed in Tonheim et al. 2008). The most successful DNA vaccines for aquatic animals, and animals in general, are those against fish rhabdoviruses (Lorenzen & LaPatra 2005, Kurath et al. 2007). Anderson et al. (1996) first reported the efficacy of a DNA vaccine in fish. Rainbow trout vaccinated with a plasmid containing the glycoprotein (G) gene of IHNV produced antibodies and were significantly protected from lethal challenge with the virus. The basic vaccine construct consisted of the G gene of IHNV located downstream of the cytomegalovirus (CMV) promoter in the eukaryotic expression vector (pcDNA3, Invitrogen). Vaccines containing the same design but containing the G gene of VHSV genotype I were soon developed and proved highly efficacious in protecting

rainbow trout from lethal challenge with VHSV (Lorenzen et al. 1998, Heppell et al. 1998). A number of DNA vaccines based on this same construct design have since been developed for other strains of both IHNV and VHSV and their efficacy shown in other fish species besides rainbow trout including chinook, sockeye (*Oncorhynchus nerka*) and Atlantic (*Salmo salar*) salmon (reviewed in Kurath et al. 2007). Effective DNA vaccines for other fish rhabdoviruses have also been developed. These include vaccinations against the North American spring viremia of carp virus (SVCV) [isolate SVCVnc (Goodwin 2002)] in koi (*Cyprinus carpio koi*) (Emmenegger & Kurath 2008) and Hirame rhabdovirus in Japanese flounder (*Paralichthys olivaceus*) (Takano et al. 2004). Vaccine efficacy is commonly expressed in terms of relative percent survival (RPS) which is calculated according to the following formula: [1-(average CPM of vaccinates/average CPM of negative controls)] × 100 (Amend 1981).

In typical laboratory trials of the IHNV and VHSV DNA vaccines, duplicate or triplicate groups of fish are given a single injection (intramuscularly in skeletal muscle) of a small quantity of plasmid DNA (generally 0.1 to 1 μg) and challenged by immersion 4-10 weeks later. Virus doses used for challenge vary based on virus, virus strain, size and species of fish, however, doses consistently produce high cumulative percent mortality (75-100%) in control (empty plasmid)-vaccinated fish virus (Kurath et al. 2007). In salmonids, DNA vaccines are highly efficacious even under these lethal challenge circumstances, providing nearly 100% protection. In 2005, the first DNA vaccine, Apex®-IHN (Novartis), was approved for use in Canada for the prevention of IHN in

farmed Atlantic salmon (Salonius 2007). In Europe and in US, the commercialization of the Apex-IHN vaccine as well as other DNA vaccines in fish are restricted due to safety concerns (Alonso & Leong 2013).

Following intramuscular injection of IHNV/VHSV DNA vaccines, some pDNA is injected directly into cells (e.g. myocytes, antigen presenting cells) and some goes into the extracellular compartment between cells. Extracellular pDNA is taken up by surrounding cells and/or re-distributed from the site of administration. Myocytes in particular at the site of immunization are important for initial plasmid uptake and subsequent antigen expression (Boudinout et al. 1998, Garver et al. 2005, Tonheim et al. 2007). Host cells' use their own translational machinery to transcribe the pDNA into mRNA and then translate the mRNA into protein (reviewed in Tonheim et al. 2008). This *in vivo* expression of antigenic proteins that occurs following DNA vaccination ensures the vaccinated animal "sees" the antigen in its native conformation with all proper post-translational modifications (Schirmbeck & Reimann 2001). This aspect is important for maintaining epitope integrity and for generating neutralizing (protective) antibodies (Schirmbeck & Reimann 2001). Immediate and transitory detection of plasmid DNA in blood following injection suggests that redistribution of pDNA to other sites is likely by means of the circulatory system (Garver et al. 2005, Tonheim et al. 2007). Upregulation of specific receptors used by dendritic cells at the site of VHSV G injection, suggest recruitment of this cell type and their role as antigen-presenting cells of plasmid DNA (Ortega-Villaizan et al. 2009).

Three phases of antiviral immunity. Expression of the G protein by host cells induces early, non-specific immune responses that are later replaced by a long-lasting, specific immunity. The immune response of salmonids to IHNV-G and VHSV-G DNA vaccination has been described in terms of three sequential but interrelated phases of immunity (Lorenzen & LaPatra 2005, Kurath 2005, and as reviewed in Kurath et al. 2007). These phases are the early antiviral response (EAVR), the specific antiviral response (SAVR) and the long-term antiviral response (LAVR).

The EAVR phase is characterized by a rapid onset of protection and low specificity. In rainbow trout immunized with DNA vaccines encoding the G protein of IHNV and VHSV, protection is established within days after vaccination. Significant levels of protection compared to control fish occur as early as 4 days (60 degree days, 15°C) post-vaccination (PV) in rainbow trout immunized with the pIHN-G (1 μg) and subsequently challenged with IHNV. Similarly, a high level of protection occurred in VHSV-G vaccinated fish challenged only 8 days after vaccination (Lorenzen et al. 2000). Numerous studies have confirmed the ability of IHNV and VHSV-G DNA vaccines to also provide cross-protection at early time points against fish viruses other than those in which they were vaccinated against. For example, this type of early cross-protection was observed in rainbow trout vaccinated with pVHS-G and challenged with IHNV at 4,7 and 14 days (60, 105, 210 degree days) but not by 28 days PV (420 degree days) (LaPatra et al. 2001).

Early phase protection is mediated by non-specific antiviral immune mechanisms such as IFN production. The exact mechanisms of early protection and cross protection against fish rhabdoviruses following DNA vaccination are still being investigated but a characteristic transcriptional pattern is upregulated (Purcell et al. 2012). Transcriptional upregulation of Mx and IFN regulatory factors 1 and 2 (IRF-1 and IRF-2) prove that like VHS virus, IHN and or VHS DNA vaccination induce a type I interferon response (Boudinot et al. 1998, Kim et al. 2000, McLauchlan et al. 2003, Purcell et al. 2004). Recent analysis by cDNA microarray has demonstrated upregulation of other humoral defense genes (e.g. complement C3, complement regulatory proteins, MHC class II associated molecules) (Byon et al. 2006).

The specific antiviral response (SAVR) phase is characterized by high RPS against the homologous virus against which trout were vaccinated and the presence of neutralizing antibodies (Kurath et al. 2007). The shift from the EAVR to this specific phase of protection occurs between 2-4 weeks PV (Lorenzen et al. 2002a, LaPatra et al. 2001). Cross-protection with the IHNV and VHSV no longer occur in DNA-vaccinated rainbow trout challenged after 4 weeks post-vaccination (Kurath et al. 2007). While some neutralizing antibodies can be detected after rhabdovirus DNA vaccine as early as 23 days PV in a few fish (Boudinot et al. 1998), high seroprevalence can be expected between 6-12 weeks PV in sera of rainbow trout, chinook salmon and Atlantic (reviewed in Kurath et al. 2007). Challenged fish show enhanced neutralizing antibody response in

comparison to control fish in several studies suggesting that vaccination primes the neutralizing antibody response (Kurath et al. 2007). Though most studies test neutralizing antibodies, Byon et al. (2006) demonstrated ELISA binding IgM antibodies in sera of Japanese flounder after VHSV-I (isolate KRRV9822) DNA vaccination.

The kinetics of neutralizing antibody development and protection are dependent on temperature and vaccine dose. The dose-response to VHS DNA vaccine was reviewed in Lorenzen et al. (2000). Fish given higher (but still low) doses of the vaccine (1 or 0.1 μg) were significantly protected compared to fish given only 0.01 μg. Similarly, LaPatra et al. (2000) found that 1 μg of IHN DNA vaccine induced complete protection but a lesser dose (0.1 μg) did not protect 150 g fish. Some studies have found that higher doses of the vaccine result in higher seroprevalence and titers (Kurath et al. 2007). Furthermore the timing of the virus challenge in fish given a constant dose affected protection. Protection ranged from 80-97% relative percent survival (RPS) between 3 weeks and 4 months (at 11-14°C), but protection was reduced by 5.6 months PV (64% RPS). Interestingly, the level of protection in many studies is higher than the proportion of fish with detectable neutralizing antibodies (McLauchlan et al. 2003, reviewed in Purcell et al. 2012) or ELISA-antibodies (Byon et al. 2006). These findings lead authors to conclude that cell-mediated immunity is likely involved in protection as well, or that neutralizing/non-neutralizing antibodies below assay detection limits are still sufficient to provide protection. Indeed, passive transfer of neutralizing sera diluted to the point

where it is no longer neutralizing *in vitro*, still provides protection after challenge (LaPatra et al. 1994).

The long-term antiviral response (LAVR) is used to refer to a phase months to years after fish rhabdoviral vaccination. It is thought that DNA vaccines induce long-term immunological memory in fish and protection mediated during the LAVR is due to effector cells of adaptive immunity (Kurath et al. 2007). This phase is the least well characterized. Studies have reported that after VHSV DNA vaccination, neutralizing antibody levels begin to decline 3-6 months PV and are completely absent by 9 months PV (McLaughlin et al. 2003). Kurath et al. (2006) demonstrated significant protection (66% RPS) of rainbow trout as late as 2 years after IHNV vaccination. Similarly, rainbow trout vaccinated with the VHSV DNA vaccine were significantly protected (88% RPS) after challenge 9 months PV (McLauchlan et al. 2003). In both studies antibodies were no longer detectable at the time of challenge. After IHNV vaccinated fish were challenged at 2 years PV, a strong neutralizing antibody response was present by 28 days PC, which suggests an anamnestic neutralizing response was mounted.

RESEARCH QUESTIONS AND OBJECTIVES

The overall project goal was to better understand the host immune response to the Great Lakes strain of VHSV (type IVb). Four main research questions were addressed in this study. The research questions and related objectives are as follows:

1. **Do muskellunge surviving VHSV-IVb mount a neutralizing antibody response that can be demonstrated by an *in vitro* assay (Chapter 2)**? The objective of this study was to investigate the kinetics of neutralizing antibody production and virus levels in serum following experimental immersion exposure. An experimental infection of muskellunge using three different virus doses was carried out and blood was collected from fish at various time points over the course of 20 weeks. Sera were tested for neutralizing antibodies using a PNT assay. Virus levels in sera were also quantified, so that the relationship between the development of antibodies and viremia could be assessed. The PNT assay and its reagents were established for use in our laboratory based on previously published studies on VHSV- genotype I and IHNV.
2. **Can evidence of immunity to VHSV-IVb be detected among wild fish residing in an enzootic lake (Chapter 3, 5)?** The objectives of this study were to determine if fish from Lake St. Clair, Michigan have detectable levels of neutralizing antibodies in their sera, and if so, whether differences exist in terms of prevalence and titers between species. Sera from past fish mortality events and virus surveillance efforts (2004 to 2009) were tested, as well as samples collected for the purposes of this study in May 2010 and 2011. Testing of sera from multiple years was helpful to detect patterns of antibody levels in relationship to known outbreaks of the virus.
3. **Do fish infected with VHSV-IVb also produce binding antibodies (Chapter 4, 5)?** To explore this question, a competitive ELISA was developed for measuring binding antibodies to VHSV-IVb. Rabbit antiserum to VHSV-IVb was produced for

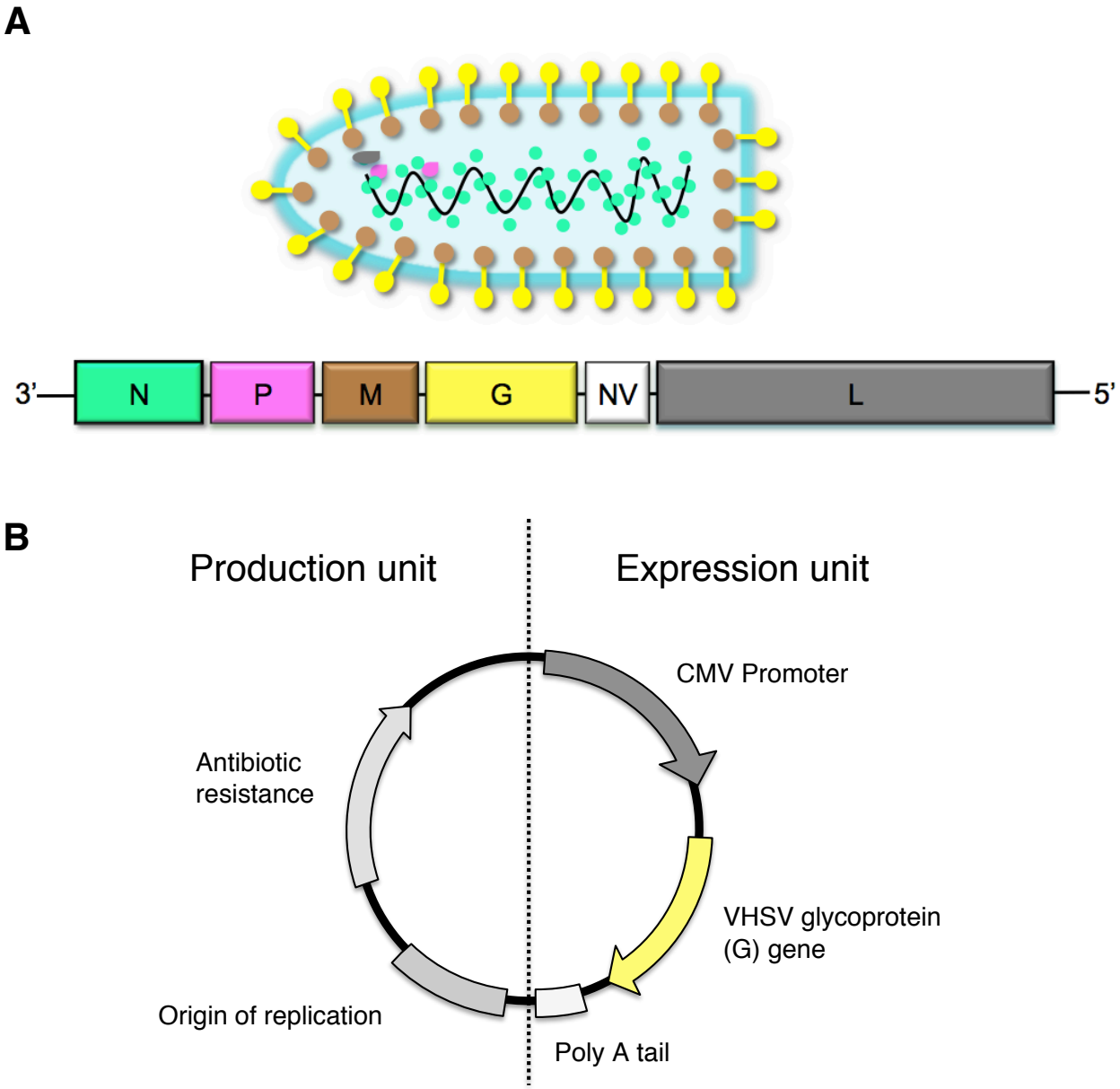
use as the competition antibody. As part of the assay development, a positive-negative threshold was established for muskellunge, and comparisons were made between antibody results obtained by cELISA and PNT for several groups of fish. Using muskellunge as an indicator species, I evaluated the use of the cELISA as a surveillance tool for detecting virus presence in five water systems in the Great Lakes (Chapter 5). Muskellunge were sampled between 2005 and 2012 from two enzootic areas (Lake St. Clair and Lower Fox River/Green Bay, Wisconsin) and from three inland lakes that were considered negative for VHSV-IVb (Lake Hudson and Thornapple Lake, MI, and Butternut Lake, WI). Virus exposure status, based on seroconversion, was compared to virus isolation results from samples collected from the same individuals or from fish during parallel sampling periods. In Chapter 4, I preliminarily investigated the use of the cELISA for detecting antibodies in other species sampled from Lake St. Clair.

4. **What is the efficacy of a DNA vaccine encoding the VHSV-IVb glycoprotein (Chapter 6)?** The objectives of this study were to 1) evaluate efficacy of a VHSV-IVb DNA vaccine in muskellunge based on induction of an adaptive immune response, and protection and virus clearance following lethal challenge with VHSV-IVb and 2) evaluate ability of vaccine to elicit an innate immune response as determined by cross-protection challenge against IHNV in rainbow trout. The vaccine containing the VHSV-IVb glycoprotein gene was developed as part of this study. The design was based on previously published VHSV-genotype I and IHNV constructs. The virus challenge time-point post-vaccination (7 weeks) was selected based on

experimental studies in Chapter 2.

APPENDIX

Figure 1.1. A) Schematic of the typical *Novirhabdovirus* virion (70 nm x 180 nm) and its linear, negative-sense single-stranded RNA molecule of approximately 11,000 nucleotides. The genome encodes 5 structural proteins [L (RNA-dependent RNA polymerase), G (glycoprotein), N (nucleoprotein), P (phosphoprotein) and M (matrix protein)] and one non-virion protein (NV). **B)** Diagram showing the general design of a DNA vaccine containing the G gene of viral hemorrhagic septicemia virus (VHSV) (modified from Schirmbeck and Reimann (2001) and Kurath et al. (2007)). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.



CHAPTER 2

DEVELOPMENT OF NEUTRALIZING ANTIBODY RESPONSES IN MUSKELLUNGE, *ESOX MASQUINONGY* (MITCHILL), EXPERIMENTALLY EXPOSED TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (GENOTYPE IVB)

Millard EV, Faisal M (2012) Development of neutralizing antibody responses in muskellunge, *Esox masquinongy* (Mitchill), experimentally exposed to viral haemorrhagic septicaemia virus (genotype IVb). J Fish Dis 35:11-18

ABSTRACT

A complement-dependent 50% plaque neutralization test was used to assess the neutralizing antibody response of muskellunge, *Esox masquinongy*, experimentally infected with viral hemorrhagic septicemia virus (VHSV, genotype IVb) by immersion. Groups of muskellunge were challenged with varying concentrations of VHSV-IVb: Group 1 with 10^2 plaque forming units (pfu) mL^{-1}, Group 2 with 4×10^3 pfu mL^{-1}, Group 3 with 10^5 pfu mL^{-1}, and Group 4 with 0 pfu mL^{-1}. The fish were held at a temperature of 11 ± 1°C and were sampled over a 20-week period. Neutralizing antibodies were not detected in sera of any of the negative control fish throughout the study. Low neutralizing titers were detected in Groups 1-3 by 6 days post-infection (PI). Neutralizing titers of ≥ 80 were not detected again until 3, 4 and 7 weeks PI for Groups 2, 3 and 1 respectively. Peak titers for those groups occurred 16, 11 and 17 weeks PI. VHSV-IVb was detected in sera up to 11 weeks PI. Results of this study show that survivors can be detected by a serological technique, despite being virus negative. This may benefit the investigation of VHSV-IVb distribution in the Great Lakes and the study of host immune responses to this emerging sublineage.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is an internationally reportable pathogen to the World Organisation for Animal Health (OIE). This fish rhabdovirus is a member of

the genus *Novirhabdovirus* (Walker et al. 2000). Since its initial isolation in Denmark in the 1960's (Jensen 1963), the virus has resulted in devastating losses of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), throughout Europe and is now known to occur endemically among marine fish of the Pacific and Atlantic Coast of North America, Europe and Japan (reviewed in Skall et al. 2005). A novel freshwater VHSV strain, genotype IVb, has been present in the Great Lakes ecosystem since at least 2003 (Elsayed et al. 2006) and has caused multiple wild fish die-off events particularly in 2005 and 2006 (reviewed in Bain et al. 2010, Kim & Faisal 2011a). To restrict the spread of VHSV-IVb into new geographic areas in North America, federal and state agencies limited the movement of live fish across the states, implemented strict biosecurity measures in gamete-collection facilities and hatcheries and began wild fish surveillance programs to trace the virus' distribution.

To date, the gold standard diagnostic test for VHSV in fish, as outlined by the U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section (USFWS and AFS-FHS 2010) and the OIE (2009), is virus isolation in cell culture from tissue samples and subsequent confirmation by the reverse-transcriptase polymerase chain reaction (RT-PCR). Although ideal for clinically infected fish, this testing method has some disadvantages in that it requires fish to be sampled lethally. It also relies on the virus being present at levels that are high enough at the time of tissue collection for isolation on susceptible cell lines, a condition that does not often exist in recovered, sub-clinically infected, and carrier fish. As a result, there is a need to use additional methods to

determine VHSV presence within a population or a waterbody.

Experimental studies have unraveled some aspects of the biology of this emerging VHSV sublineage and have focused mainly on differential host susceptibilities, disease course, and tissue changes (Al-Hussinee et al. 2010, Kim & Faisal 2010a, Al-Hussinee et a. 2011). When Kim and Faisal (2010c) infected juvenile muskellunge, the fish were found to be particularly susceptible to VHSV-IVb. Some individuals in the same tanks with fish experiencing high mortality however were able to survive. Furthermore, muskellunge that survived one virus challenge seemed resistant to re-infection following a second challenge suggesting that some protective benefit had been obtained (Kim & Faisal 2012). Observations such as these highlight how little is actually known about VHSV-IVb host interactions, particularly the host immune response.

From studies on other genotypes of VHSV and infectious hematopoietic necrosis virus (IHNV), it has been demonstrated that the viral surface glycoproteins (G) elicit the development of immune responses including antibody formation. For example, viral G proteins induce antiviral interferons (IFNs) (Acosta et al. 2006) and expression of immune related genes (Novoa et al. 2006, Tafalla et al. 2007). G proteins also induce the formation of neutralizing antibodies, which are an important part of the protective immune response (Engelking & Leong 1989, Lorenzen et al. 1990).

Unlike the relatively short-term presence of virus in infected fish tissues, neutralizing

antibodies last longer after infection and as such their presence has been used successfully to indicate past exposure of fish to rhabdoviruses (reviewed by LaPatra 1996). In a recent serological survey of a VHSV-IVb endemic waterbody, Lake St. Clair (Michigan, USA), neutralizing antibodies against the virus were detected in sera of several fish species, with muskellunge expressing the highest antibody titers (Millard & Faisal 2012b). The current study was designed to characterize the development of neutralizing antibodies in muskellunge following experimental infection.

MATERIALS AND METHODS

Cell culture and virus isolate. The *epithelioma papulosum cyprini* (EPC; Fijan et al. 1983) cell line was used throughout this study. The cell line was maintained at 25^{o}C in 150cm^{2} tissue culture flasks (Corning) in minimum essential medium with Earle's salts (EMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 10% tryptose phosphate broth (TPB; BD Biosciences), 2.0 mM L-glutamine (Invitrogen) and sodium bicarbonate (12 mM; Sigma-Aldrich).

The index VHSV-IVb strain, MI03, originally isolated from muskellunge from Lake St. Clair (Elsayed et al. 2006) was propagated on the EPC cell line at 15°C in EMEM with 2% FBS, 10% TPB, 2.0 mM L-glutamine, 12 mM sodium bicarbonate, penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}) and amphotericin B (2.5 µg mL^{-1})

(Invitrogen). When approximately 90% cytopathic effects (CPE) were present, media from infected cell cultures was clarified by centrifugation (2900 × *g* for 30 min at 4^{O}C). Supernatant was divided into aliquots and stored at -80^{O}C until immediately before use. The stock titer was determined by plaque assay on 7% polyethylene glycol (PEG)-treated EPC cells as described in Batts & Winton (1989).

Muskellunge. Certified juvenile muskellunge were obtained from the Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, Iowa, USA). Fish were acclimatized for 3 weeks in holding tanks in a continuous flow-through system at the Michigan State University Research Containment Facility (URCF; East Lansing, Michigan, USA). Muskellunge were fed live fathead minnows, *Pimephales promelas* (Rafinesque), obtained from Anderson Minnow Farm (Lonoke, Arkansas, USA). Fish were maintained according to protocols approved by Michigan State University's Institutional Animal Care and Use Committee.

Experimental infection. Acclimatized juvenile muskellunge (4 months old; 17.0 ± 5.0 g; total length 16.4 ± 1.2 cm) were divided into four groups for the immersion challenge. Groups 1-3 consisted of approximately 80 fish each. Fish were exposed to the following concentrations of VHSV: Group 1 with 10^2 plaque forming units (pfu) mL^{-1}, Group 2 with 4×10^3 pfu mL^{-1} and Group 3 with 10^5 pfu mL^{-1}. A fourth group (Group 4; 70 fish) served as the negative control. The doses were chosen to produce acute,

subacute, and chronic courses of infection as determined for juveniles of this species (Kim & Faisal 2010c). The experimental infection was performed in aquaria that contained static, aerated water to which the virus was added and the immersion challenge continued for 90 min. The negative control group received sterile cell culture media. Each challenge group was then distributed between two randomly assigned replicate tanks. Water temperature was maintained at 11 ± 1°C throughout the duration of the study. Fish were monitored on a daily basis and tissues including kidney, spleen and heart were collected from any fish that died.

Four fish per group (two per replicate; 16 total) were sampled at the following time points post-infection (PI): 0, 12, 24, and 36 hr; 2, 4, 6 and 8 days and then once weekly up to 9 weeks PI. The number of sampled fish was then reduced to two fish per challenge group in order to allow for a longer observation period. Two fish per group were sampled 11, 12, 13, 15, 16, 17, 18 and 20 weeks PI until all fish had been sampled. Moribund fish were selected for sampling first, and if no morbidity was observed, fish were selected randomly. Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories; 0.25 mg mL^{-1}) and blood samples were aseptically collected by puncture of the caudal vein. The blood samples were allowed to coagulate overnight at $4^{o}C$, centrifuged at 4500 × *g* for 20 min at $4^{o}C$, and sera were collected. Sera aliquots were stored at $-80^{o}C$ until assayed.

Assessment of VHSV in infected muskellunge serum samples. The EPC cell line was used for isolation of VHSV from sampled muskellunge serum samples and tissues from mortalities in accordance with suggested procedures (USFWS & AFS-FHS 2010). The amount of infectious virus in sera was quantified by the plaque assay. RT-PCR, using VHSV specific primers, was used to confirm the re-isolation of VHSV from samples that exhibited CPE after two successive passes. Cell culture negative sera were also tested by RT-PCR to detect levels of virus that may have been below the detection limit of the cell culture assays.

50% plaque neutralization test (50%PNT). Sera from disease-free lake trout, *Salvelinus namaycush* (Walbaum), was used as a source of fish complement. Lake trout had been reared in isolation at URCF. Fish were anesthetized using 0.1mg mL^{-1} MS-222 buffered with sodium bicarbonate and blood was collected non-lethally. Sera were separated as previously described and stored in single-use aliquots in liquid nitrogen until immediately before use. The naïve sera used as a source of complement the assay (1:30 dilution, unheated) was tested to be free of background neutralization activity prior to use.

The 50%PNT was performed based on procedures previously described for detecting neutralizing antibodies in rainbow trout sera against VHSV genotype I and IHNV (Olesen & Jørgensen 1986, LaPatra et al. 1993) with some modifications. Fish sera were heat inactivated for 30 min at 45^{o}C. Serial two fold dilutions of sera, starting at a

dilution of 1:20, were then mixed with an equal volume of virus containing 1.5 x 10^3 pfu VHSV-IVb, and incubated at 18^{o}C for 60 min with constant gentle agitation. Half way through the incubation, an equal volume of naïve lake trout complement was added to the reaction. Serum-virus-complement mixtures were then inoculated onto replicate wells of PEG pretreated EPC cells grown in 24 well tissue culture plates (Corning). Virus adsorption was allowed to take place for one hour at 15^{o}C before the inoculum was removed and cells overlaid with 1% methylcellulose. After 5-6 days of incubation at 15^{o}C, plates were fixed and stained with 0.5% crystal violet in 50% formalin and plaques enumerated. The neutralizing antibody titer is reported as the reciprocal value of the highest serum dilution causing a 50% reduction in the average number of plaques counted for the negative control. The negative control was naive muskellunge sera with complement and virus. Both a negative and positive control muskellunge sera were included each time the assay was performed.

RESULTS

Disease course and virus re-isolation. The experimental challenge caused morbidity and mortality in infected muskellunge that varied among the three challenge groups. Moribund and dead fish exhibited classical signs and lesions of VHSV such as severely anemic gills, external, and internal hemorrhages. Other than fish euthanized for sampling, Groups 1, 2 and 3 experienced 2.4%, 7.6% and 12.5% mortality respectively.

Most mortality occurred between 6 days and 4 weeks PI. VHSV was re-isolated from the internal organs of dead fish from Groups 1-3 and its identity confirmed by RT-PCR. No VHSV-related mortalities were observed in the negative control group.

VHSV was isolated from the sera of some of the sampled fish; however, the number of viremic fish varied between challenge groups (Table 2.1). In Group 1, none of the sampled fish tested positive by cell culture. In Group 2, 12.9% of sampled fish were positive. VHSV was first detected in sera 6 days p.i and last detected 6 weeks PI. In Group 3, 23.2% of sera were positive. The virus was detected as early as 2 days PI and as late as 11 week PI. For both groups, peak viral titers occurred 6 days PI. All virus positive cell culture samples were confirmed positive for VHSV-IVb by RT-PCR. Additionally, RT-PCR performed on samples not exhibiting cytopathic effects after the second passage revealed the presence of the virus in one fish from Group 1 sampled 15 days PI and one additional fish from Groups 2 and 3 sampled 6 and 4 weeks PI, respectively.

Neutralizing antibody response. Negative control muskellunge sampled between 0 and 9 weeks PI did not have any detectable neutralizing activity (titer <20). Some, but not all, of the serum samples from virus-challenged muskellunge were positive for neutralizing antibodies. Low titers (20-80) were detected in Groups 1-3 by 6 days PI (Figure 2.1). Titers of ≥ 80 were detected again 3, 4 and 7 weeks PI for Groups 2, 3, and 1, respectively. Overall, 9.0% (7/78) of samples were positive for neutralizing

activities from fish challenged with the lowest concentration of VHSV (10^2 pfu mL^{-1}). In the group challenged with 4×10^3 pfu mL^{-1} (Group 2), 29.0% (20/69) of fish developed neutralizing antibodies. A peak titer of 5120 for this Group was detected in a sample taken 16 weeks PI, which was the last sampling point. For Group 3 fish, that were challenged with the highest concentration of virus (10^5 pfu mL^{-1}), 20.0% (13/65) of the serum samples had neutralizing antibodies. The highest titers were detected between 9 weeks PI and the end of the sampling period (13 weeks PI). The highest titer (20,480) was detected 11 weeks PI.

DISCUSSION

When naïve muskellunge were exposed to VHSV-IVb, virus-neutralizing antibodies developed in sera of some individuals. This finding clearly demonstrates the ability of this susceptible species to mount a humoral immune response against the virus. The immune response, including the development of neutralizing antibodies, of rainbow trout to other genotypes of VHSV has been reviewed (Lorenzen & LaPatra 1999). Evidence of an adaptive immune response to VHSV, although not neutralizing antibodies, has been reported for Pacific herring, *Clupea pallasii* (Valenciennes), (Kocan et al. 2001, Hershberger et al. 2007) as well as wild brown trout (*Salmo trutta* L.), pike (*Esox lucius* L.) and whitefish (*Coregonus* sp.) (Enzmann et al.1993). This report is the first to characterize the development of neutralizing antibodies following experimental exposure

to VHSV-IVb; as well as the first to examine the immune response of experimentally infected muskellunge to a viral pathogen to the best of our knowledge.

In the present study, neutralizing antibodies were not detected in any of the negative control fish at the starting dilution of 1:20. Therefore a titer of <20 is defined as being negative for neutralizing antibodies to VHSV-IVb. Relatively low neutralizing titers up to 80 were detected in some experimentally infected muskellunge in the first 6 days after immersion challenge; with titers of 20 and 40 occurring as early as 36 hr PI. Similarly, low neutralization titers occurring the first week after infection were reported by LaPatra et al. (1993). The nature of this early neutralizing activity remains to be elucidated and could, partially or totally, be related to innate humoral factors that were expressed upon VHSV exposure. Specific antibody development takes 3-4 weeks in rhabdovirus immunized rainbow trout (LaPatra et al. 2001, Boudinot et al. 1998) and this response is influenced by many factors such as temperature (Le Morvan et al. 1998). In this context, anti-viral factors such as IFN-like substances (Pakingking et al. 2004) and Mx protein (Cuesta & Tafalla 2009) that are induced early following infection with VHSV could limit virus infectivity of cells.

Neutralizing antibody titers of ≥80 were not detected again until 3 weeks PI. In samples taken 6 weeks PI, 62.5% (5/8) of Groups 2 and 3 muskellunge had neutralizing antibodies. Titers increased throughout the sampling period with the maximum titers for the groups occurring 17 (1280), 16 (5120) and 11 weeks (20480) for Groups 1, 2 and 3

respectively. Such titers are comparable to those obtained from serum samples of rainbow trout experimentally infected with VHSV genotype I (Olesen & Jørgensen 1986, Olesen et al. 1991). In both studies, neutralizing antibodies were detected by 4 weeks PI and maximum titers of 5120-10,240 occurred 6-10 weeks PI. In this study, it is interesting to note that out of all the antibody positive fish, only 4 of these individuals also tested positive for the virus. Moreover, all of these double positive fish were sampled 6 weeks PI and earlier, meaning that later during the disease course, after mortalities have ceased, it seems that survivors are more likely to be detected based on the presence of virus-specific antibodies in their sera, versus the virus itself.

The total duration of neutralizing antibodies in sera of survivors of VHSV infection is currently unknown. Olesen et al. (1991) reported a decrease to a mean titer of 80 between 18 and 23 weeks PI, even following a second virus challenge 14 weeks PI. On the other hand, Olesen and Jørgensen (1986) reported some trout having VHSV neutralizing antibodies for over a year. In the present study, antibodies were detected as long as 17 weeks PI; however, the total duration was not determined due to the sampling design. In a concurrent study in our laboratory (data not shown), we found that neutralizing antibodies are still detectable in sera of several muskellunge that survived a double exposure to VHSV-IVb 90 weeks after their original exposure. Frequent non-lethal blood collection from this group is underway in order to determine the maximum duration of circulating neutralizing antibodies.

This study has also demonstrated that a number of infected muskellunge have not mounted a VHSV neutralizing antibody response. Fish exposed to the lowest dose (10^2 pfu mL^{-1}) had considerably more non-responders than Groups 2 and 3. While this finding may suggest an optimum virus concentration for neutralizing antibody development, the frequent sampling in the experiment does not allow confirmation of this observation. This variation in antibody responses and titers reflects the individual heterogeneity among experimental fish. It is also indicative of multiple pathways of defense that the muskellunge immune system utilizes toward the same inducing antigen.

From the present study, it was determined that muskellunge, an important Great Lakes species, can carry evidence of past exposures to the virus, even when negative for the virus itself. These results will have important implications in investigating the distribution and spread of VHSV in the Great Lakes as well as provide the background information necessary to assess the efficacy of potential vaccine preparations.

ACKNOWLEDGMENTS

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APPENDIX

Table 2.1. Virus isolation and titer range in plaque forming units (pfu) mL^{-1} of serum of sampled muskellunge, *Esox masquinongy*, experimentally infected with viral hemorrhagic septicemia virus (VHSV, genotype IVb). "VHSV Pos." column indicates number of VHSV-positive serum samples/ number of samples tested. Groups were challenged by immersion in the following concentrations of virus: 10^2 pfu mL^{-1} (Group 1), 4×10^3 pfu mL^{-1} (Group 2), and 10^5 pfu mL^{-1} (Group 3).

	Group 1	Group 2		Group 3	
Days PI	VHSV Pos.	VHSV Pos.	Titer range (pfu mL^{-1})	VHSV Pos.	Titer range (pfu mL^{-1})
0	0/4	0/3[a]	-	0/4	-
0.5	0/4	0/3[a]	-	0/3[a]	-
1	0/4	0/4	-	0/4	-
1.5	0/4	0/4	-	0/4	-
2	0/4	0/4	-	1/4	5.0×10^2
4	0/4	0/4	-	2/4	5.0×10^2-7.0×10^3
6	0/4	2/4	5.0×10^2-7.6×10^6	2/4	9.5×10^5
8	0/4	0/3[a]	-	2/4	8.5×10^4
Weeks PI					
2	0/4	1/4	2.3×10^5	3/4	5.0×10^2-3.6×10^5
3	0/4	1/4	5.0×10^2	0/4	-
4	0/4	3/4	2.3×10^4-4.2×10^6	1/4	6.0×10^3
5	0/4	0/4	-	0/4	-
6	0/4	2/4	1.0×10^3-8.0×10^4	0/4	-
7	0/4	0/4	-	1/4	5.0×10^2
8	0/4	0/4	-	1/4	1.0×10^3
9	0/4	0/4	-	2/4	1.0×10^4-3.6×10^4
11	0/2	0/2	-	1/2	3.0×10^3
12	0/2	0/2	-	0/2	-
13	0/2	0/2	-	0/2	-
15	0/2	0/2	-	-	-
16	0/2	0/1	-	-	-
17	0/2	-	-	-	-
18	0/2	-	-	-	-
20	0/2	-	-	-	-

[a] Although four fish were sampled, there was not enough serum from one fish for testing.

Figure 2.1. Development of neutralizing antibodies measured by the 50% plaque neutralization test (50%PNT) in three groups of muskellunge, *Esox masquinongy*, challenged by immersion with viral hemorrhagic septicemia virus (VHSV-IVb). An antibody titer of ≥20 is positive. Groups 1, 2 and 3 were exposed to 10^2, 4×10^3 and 10^5 pfu mL^{-1} respectively. The last sampling points were 20, 16 and 13 weeks for Groups 1, 2 and 3, respectively.

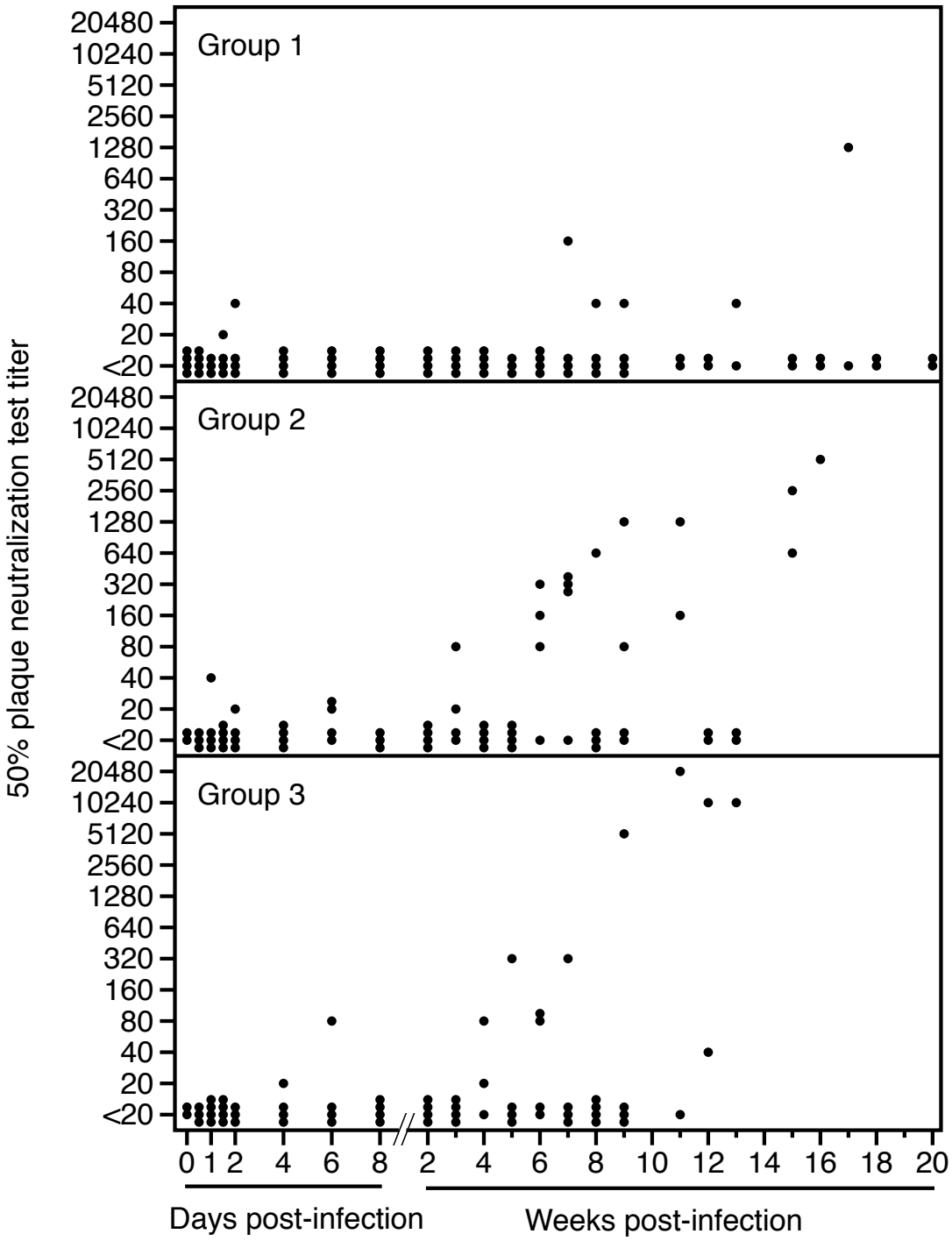

Figure 2.1. (cont'd)

[a] Several samples did not yield enough serum to be included for the 50%PNT test: Group 1 (one fish each at 24 hr and 5 weeks PI); Group 2 (two fish at 0 hr PI, one fish each at 12 hr and 8 days PI); and Group 3 (two fish at 0 hr PI, and one fish each at 12 hr, 2 days and 4 weeks PI).

CHAPTER 3

HETEROGENEITY IN LEVELS OF SERUM NEUTRALIZING ANTIBODIES AGAINST VIRAL HEMORRHAGIC SEPTICEMIA VIRUS GENOTYPE IVB AMONG FISH SPECIES IN LAKE ST. CLAIR, MICHIGAN, USA

Millard EV, Faisal M (2012) Heterogeneity in levels of serum neutralizing antibodies against viral hemorrhagic septicemia virus genotype IVb among fish species in Lake St. Clair, Michigan, USA. J Wildl Dis 48:405-415

ABSTRACT

The presence of neutralizing antibodies against viral hemorrhagic septicemia virus (VHSV-IVb) was investigated in sera of 13 fish species collected from Lake St. Clair, Michigan, USA, a VHSV-endemic water body. We tested 297 sera collected May 2004–June of 2010, using a complement-dependent 50% plaque neutralization test (50% PNT). Neutralizing antibodies were detected in 23% (67/297) of the samples. The highest overall antibody prevalence (85%, 34/40) and mean positive antibody titer (12,113; SD = 11,699) were detected in muskellunge (*Esox masquinongy*). Antibodies were also detected in 50% (15/30) of sampled northern pike (*E. lucius*), 25% (15/61) of freshwater drum (*Aplodinotus grunniens*), and 7% (3/41) of smallmouth bass (*Micropterus dolomieu*). All sera from channel catfish (*Ictalurus punctatus*), lake sturgeon (*Acipenser fulvescens*), quillback (*Carpiodes cyprinus*), rock bass (*Ambloplites rupestris*), shorthead redhorse (*Moxostoma macrolepidotum*), silver redhorse (*M. anisurum*), walleye (*Sander vitreus*), white perch (*Morone americana*), and yellow perch (*Perca flavescens*) were negative. Antibodies in one or more fish species were detected in all sampling years (2004, 2006, 2007, 2009, and 2010), whereas in parallel sampling periods, VHS virus was only detected in 2006 and 2009. Our results suggest the continued presence of VHSV-IVb in the Lake St. Clair ecosystem, and underscore the importance of assessing immune responses of fish populations to determine prior virus exposure.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is a pathogenic fish virus belonging to the family *Rhabdoviridae*, genus *Novirhabdovirus*. In spring-summer of 2005 and 2006, a novel North American VHSV sublineage (designated IVb) was implicated in fish die-off events in Lake St. Clair, Lake Erie, and Lake Ontario. Mortality episodes involved numerous fish species including freshwater drum (*Aplodinotus grunniens*; Lumsden et al. 2007), round gobies (*Neogobius melanostomus*; Groocock et al. 2007), muskellunge (*Esox masquinongy*), gizzard shad (*Dorosoma cepedianum*), yellow perch (*Perca flavescens*), and others (Winton et al. 2008, Kim & Faisal 2011). Archived samples date the presence of VHSV-IVb in the Lake St. Clair ecosystem to at least 2003 (Elsayed et al. 2006). The discovery of this reportable virus in the Great Lakes initiated fish movement restrictions and surveillance testing for purposes of zoning and monitoring the spread of the virus. Within the next 5 yr, VHSV-IVb was isolated from all five Great Lakes, the St. Lawrence River, and several inland lakes in Michigan, New York, Ohio, and Wisconsin, USA (Kim & Faisal 2011).

Fish are tested for VHSV following guidelines of the American Fisheries Society (Batts & Winton 2010) and the World Organization for Animal Health (OIE 2012). Laboratory detection of VHSV requires isolation in cell culture followed by confirmation of the isolated virus, most commonly by reverse transcriptase polymerase chain reaction (RT-PCR). Although this approach is currently considered the gold standard for VHS

diagnosis, populations that exhibit low infection prevalences or those that have recovered and cleared the virus, can be missed.

Fish surviving infection with VHSV (Olesen & Jørgensen 1986) and other pathogenic rhabdoviruses, including infectious hematopoietic necrosis virus (IHNV; Amend & Smith 1974, Jørgensen et al. 1991) and spring viremia of carp virus (SVCV; Ahne 1986), mount an adaptive immune response that includes the production of neutralizing antibodies. Survivors demonstrate enhanced resistance to disease upon subsequent exposures (LaPatra et al. 1993, Kocan et al. 2001, Ahne et al. 2002). Antibodies to VHSV remain in sera for an extended period after the virus is no longer detectable, and have been used in epizootiologic studies to identify fish populations that previously have been infected (LaPatra 1996).

In Lake St. Clair, following the initial large-scale mortality events in spring-summer 2006, and despite surveillance testing, the virus was not isolated again until a smallmouth bass (*Micropterus dolomieu*) die-off occurred in June 2009. One logical explanation for the absence of VHSV outbreaks in Lake St. Clair in 2007, 2008, and 2010, is that fish residing in this infected zone might have developed some immunity to the virus. We sought to determine if fish in Lake St. Clair have neutralizing antibodies against VHSV-IVb and compare the levels of these antibodies among resident fish species.

MATERIALS AND METHODS

Field serum samples. Most fish were captured using trap nets set in Anchor Bay, Lake St. Clair (Figure 3.1) by personnel of the Michigan Department of Natural Resources (MDNR) and the Aquatic Animal Health Laboratory (AAHL, Michigan State University, East Lansing, Michigan, USA). In May 2010, blood samples were collected by caudal venip uncture from channel catfish (*Ictalurus punctatus*, *n* = 25), freshwater drum (*n* = 10), muskellunge (*n* = 21), northern pike (*Esox lucius, n* = 16), quillback (*Carpiodes cyprinus*, *n* = 7), rock bass (*Ambloplites rupestris, n* = 24), shorthead redhorse (*Moxostoma macrolepidotum*, *n* = 6), silver redhorse (*M. anisurum*, *n* = 13), smallmouth bass (*n* = 25), walleye (*Sander vitreus*, *n* = 10), white perch (*Morone americana*, *n* = 12) and yellow perch (*n* = 3). Lake sturgeon (*Acipenser fulvescens*, *n* = 15) were sampled by the MDNR the first week of June 2010. Blood was sampled nonlethally from all 13 species and fish were released, with the exception that 11 of the 21 muskellunge were euthanized following blood collection to obtain tissues (kidney, spleen, and heart) for VHSV testing. Gametes were also collected from all mature muskellunge for VHSV testing. Water temperature in May 2010 was 9–15°C.

Archived sera from muskellunge (*n* = 6) and freshwater drum (*n* = 40) in May 2007 and muskellunge (*n* = 10) in 2009, originated from apparently healthy fish collected for VHSV surveillance. Serum samples from northern pike in May 2004 (*n* = 10), all species in May 2006 (*n* = 28), and smallmouth bass in June 2009 (*n* = 16), were collected as part of targeted sampling efforts during, or immediately following, mortality events in

Lake St. Clair. Most fish were captured alive and immediately transported on ice to the AAHL. Upon arrival, blood was collected and tissues (kidney and spleen) and reproductive fluids (from ripe fish) were sampled for VHSV testing.

For all captured muskellunge, weight and total length were recorded and a dorsal fin ray clip was taken for age estimation (Clark et al. 2004) by MDNR researchers. Blood samples from all fish were kept at 4°C overnight and then centrifuged (2,500 × *g*, 10 min, 4°C). Sera were aliquoted and stored at -80°C until testing.

Cell line and virus isolate. The *epithelioma papulosum cyprini* (EPC) cell line (Fijan et al. 1983) was cultured at 25°C in Earle's minimum essential medium (EMEM; Life Technologies) supplemented with 10% tryptose phosphate broth (TPB; Becton, Dickinson and Company), 10% fetal bovine serum (FBS; Hyclone), 2 mM L-glutamine (Life Technologies), and buffered with 7.5% sodium bicarbonate. The Great Lakes strain of VHSV-IVb (Elsayed et al. 2006) was propagated on the EPC cell line and aliquots of supernatant were stored at -80°C for use in the 50% plaque neutralization test (PNT). The virus titer was determined by plaque assay as described in Batts & Winton (1989).

Virus isolation. All serum samples, tissues, and reproductive fluids collected were tested for VHSV in accordance with suggested procedures (Batts and Winton, 2010). Serum samples were diluted 1:20 (v/v) in EMEM with 10% TPB, 14 mM tris buffer, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}; Life Technologies), gentamicin

sulfate (100 μg mL^{-1}; Sigma-Aldrich), and amphotericin B (2.5 μg mL^{-1}; Lonza). Tissues and reproductive fluids were diluted 1:4 (w/v), homogenized, and centrifuged (2,500 × *g*, 30 min, 4°C). Homogenate supernatants and serum samples were inoculated onto EPC cells grown to confluency in cell culture medium (EMEM with 10% TPB, 5% FBS, 2 mM L-glutamine, 14 mM tris, and 100 U penicillin, 100 μg streptomycin, 100 μg gentamicin sulfate, and 2.5 μg amphotericin B mL^{-1}). Plates were incubated at 15°C for two successive passages and any samples exhibiting cytopathic effects (CPE) were subject to confirmation using RT-PCR.

Neutralizing antibody detection. A complement-dependent 50% PNT (LaPatra et al. 1993) was used with some modifications to detect VHSV-IVb neutralizing antibodies in fish sera. Samples were heat inactivated for 30 min at 45°C and serial twofold dilutions, at a starting dilution of 1:20, were mixed with an equal volume of VHSV-IVb (2.0×10^{4} plaque-forming units mL^{-1}). Serum-virus mixtures were incubated for 30 min at 18°C with constant gentle motion. An equal volume of unheated naïve sera from lake trout (*Salvelinus namaycush*), diluted 1:30, was added as a source of complement and the incubation period was repeated. Mixtures were adsorbed for 1 hr at 15°C to 7% polyethylene glycol pretreated EPC cells in duplicate wells of 24-well tissue culture plates. Fluid was then removed and cells overlaid with 1% methylcellulose in cell culture medium (with 2X concentrated EMEM). After incubation for 5 days at 15°C, the cells were fixed and stained with 0.5% crystal violet in formalin. Titers are reported as the

reciprocal of the highest serum dilution that reduced the number of virus plaques by 50% compared to the negative control. Each time the assay was performed, positive and negative control sera were included.

Complement source. Naïve sera used in the 50% PNT were collected from adult lake trout that had been raised at the Michigan State University Research Containment Facility (MSU UCRF). Prior to blood collection, fish were anesthetized using 0.1mg mL^{-1} tricaine methanesulphonate (MS-222, Argent Chemical Laboratories) buffered with sodium bicarbonate. Blood was processed as previously described, and aliquots of pooled sera were stored in liquid nitrogen until immediately before use.

Naïve sera examination. Serum samples from three groups of naïve muskellunge, originating from hatcheries with no history of VHSV exposure, were tested with the 50% PNT to determine if innate neutralization activities against VHSV-IVb exist in sera of unexposed fish. Fish were maintained at 11±2°C in a flow-through system at the MSU UCRF and fed fathead minnows (*Pimephales promelas*) raised in a farm with no prior history of VHSV. Blood was collected weekly from Groups I ($n = 30$; ~4 mo old) and II ($n = 46$; ~6 mo old) for 9 wk. Fish from these groups were euthanized at the time of blood collection with an overdose of MS-222 (0.25 mg mL^{-1}) and organs tested in cell culture to confirm the absence of VHSV. Blood samples were collected nonlethally from Group III ($n = 7$, ~22 mo old) muskellunge every 6 wk for six sampling periods (total of 42 sera).

Specificity of VHSV-IVb neutralizing antibodies. To test the specificity of the serum neutralizing activities against VHSV-IVb, six Lake St. Clair muskellunge sera (titers of 20–20,480) and a single freshwater drum serum (titer of 40) were tested with IHNV (strain 220-90; LaPatra et al. 1994). Serum samples were tested in parallel with VHSV-IVb and IHNV using the 50% PNT conditions as described previously.

Statistical analysis. Differences in the occurrence of antibodies between species, and between mature versus immature muskellunge, were compared using a two-tailed Fisher's exact test. Pearson's correlation was used to determine if antibody titers in sera of muskellunge were associated with weight or length. Significant differences were accepted at $P < 0.05$.

RESULTS

Virus isolation. All serum samples from Lake St. Clair fish were negative for VHSV. Tissues collected in 2004, 2007, and 2010, and all reproductive fluids from muskellunge also were negative. VHSV was isolated in cell culture from tissues of 19 of 28 fish sampled in 2006. All muskellunge, northern pike, rock bass, and shorthead redhorse were VHSV-positive, as were 55% of freshwater drum and 20% of silver redhorse. Most of these fish exhibited external and internal petechial hemorrhages, exophthalmia, and congestion of internal organs. VHSV was also isolated from tissues of smallmouth bass

(25%; 4/16) collected during a mortality event in June 2009. The identity of VHSV was confirmed in all cell culture-positive samples by RT-PCR.

Innate neutralization of VHSV by sera of naïve muskellunge. Most (89%) naïve muskellunge sera did not neutralize VHSV-IVb at the starting serum dilution of 1:20 (titer <20; Table 3.1). Titers of 20–80 were observed in sera of 13 of 118 muskellunge (11%). Therefore, only neutralization titers >80 are considered antibody-positive for wild muskellunge and its congener, northern pike. A specific cutoff value for other fish species could not be ascertained because of the absence of samples collected from naïve fish. Results for these species are reported at titers of ≥20.

Neutralization specificity of fish sera. Neutralization titers ranging from 20 to 20,480 in muskellunge sera and 40 in a freshwater drum serum did not cross-react with IHNV at titers of ≥40 (Table 3.2).

Neutralizing antibodies against VHSV-IVb. Blood samples were collected from a total of 297 Lake St. Clair fish representing 13 species (Table 3.3). Neutralizing antibodies against VHSV-IVb were detected in 23% (67/297) of sera, and in all study years (2004, 2006, 2007, 2009, 2010). Four species, muskellunge, northern pike, freshwater drum, and smallmouth bass, were antibody-positive. The overall antibody prevalence in muskellunge (85%; 34/ 40) was significantly higher than northern pike (50%; 15/30; $P = 0.0032$), freshwater drum (25%; 15/61; $P < 0.0001$), and smallmouth

bass (7%; 3/41; $P < 0.0001$). Excluding archived sera, fish sampled in 2010 followed a similar trend; muskellunge exhibited the highest antibody prevalence (86%; 18/21), followed by northern pike (63%; 10/16), freshwater drum (20%; 2/10), and smallmouth bass (12%; 3/25). All sera from channel catfish, lake sturgeon, quillback, rock bass, shorthead and silver redhorses, walleye, white perch, and yellow perch were negative (titers <20).

Neutralization titers reached the highest levels in muskellunge; 91% (31/34) of antibody-positive individuals had titers of 2,560–40,960 (Figure 3.2). In comparison, maximum titers for other antibody-positive species did not exceed 1,280 (northern pike and freshwater drum) and 320 (smallmouth bass). Mean positive titers were higher for muskellunge (mean = 12,113; SD = 11,699) compared to northern pike (mean = 608; SD = 449), freshwater drum (mean = 201; SD = 339), and smallmouth bass (mean = 213; SD = 92). Among muskellunge, the prevalence of neutralizing antibodies was significantly higher in reproductively mature fish than immature fish (Table 3.4; $P < 0.0001$). Antibody titer in this species was not correlated with length ($n = 40$, $r = 0.24$, $P = 0.14$), or weight ($n = 39$, $r = 0.18$, $P = 0.27$).

For some species, lower antibody prevalences were detected during VHSV-positive sampling events. The lowest yearly antibody prevalence for muskellunge (33%; 1/3) was detected in 2006 when this species was VHSV-positive. In 2006, anti-VHSV antibody prevalence in freshwater drum (0%; 0/11) was also lower compared to 2007

(33%; 13/40) and 2010 (20%; 2/10). Similarly, none of the smallmouth bass from a VHSV-positive mortality event in 2009 were antibody-positive, whereas the following year, 12% (3/25) had antibodies.

DISCUSSION

Neutralizing antibodies are targeted against viral glycoproteins and are an essential component of the protective immune response of fish against rhabdoviruses (Engelking & Leong 1989, Lorenzen et al. 1990). Muskellunge that survive infection with VHSV-IVb develop neutralizing antibodies in their sera (Millard & Faisal 2012), and are partially resistant to disease, despite high levels of shed virus in surrounding water (Kim & Faisal 2012). In this context, fish residing in a VHSV-endemic water body, such as Lake St. Clair, could be intermittently re-exposed to VHSV, without experiencing disease outbreaks. In this study, we detected evidence of an acquired immune response in sera of four of the 13 fish species sampled, albeit at different prevalences and titers.

The majority of Lake St. Clair muskellunge (85%) and northern pike (50%) were positive for VHSV-IVb antibodies. Neutralizing antibodies were detected in fewer sera from freshwater drum (25%) and smallmouth bass (7%), and were completely absent in sera from channel catfish, lake sturgeon, quillback, rock bass, shorthead redhorse, silver redhorse, walleye, white perch, and yellow perch. Interspecific variation in antibody prevalence could be related to differences in species' ecologic niches, which would

influence the frequency of exposure to the virus. Esocids (muskellunge and northern pike) are top predators in the Lake St. Clair ecosystem. It is possible that their high trophic position provides more opportunities for virus exposure through ingestion of VHSV-infected prey fish. Neutralizing antibody prevalence also might be influenced by species' susceptibility to the virus, or differences in the immune mechanisms employed against VHSV-IVb. Of the 13 species sampled, seven (muskellunge, northern pike, freshwater drum, smallmouth bass, rock bass, and shorthead and silver redhorses) were positive for VHSV in this investigation, and the remaining species, with the exception of lake sturgeon and quillback, are also known to be naturally susceptible (USDA–APHIS 2008). The dose of VHSV-IVb necessary for infection and disease in these species, however, is variable. It is possible that a relatively low virus concentration in the water could infect and induce a neutralizing antibody response by species of high susceptibility (e.g., muskellunge; Kim & Faisal 2010c); whereas species that are less susceptible or resistant (e.g., lake sturgeon; M. Faisal, unpubl. data) might not become infected. However, because the experimental susceptibility among species included in this study is known only for muskellunge, yellow perch, and smallmouth bass (Kim & Faisal 2010a, b), a correlation between occurrence of antibodies and susceptibility cannot be made at the present time. The absence of antibodies detectable by the 50% PNT in the nine aforementioned antibody-negative species does not necessarily imply the lack of an immune response. VHSV induces both cell-mediated and humoral defenses, including non-neutralizing antibodies that would not be detectable by the 50% PNT (Lorenzen et al. 1999).

Antibody prevalence was lower in immature muskellunge (ages 2–3) than reproductively mature fish (ages 6–20). Juvenile muskellunge (4 mo old) produce neutralizing antibodies to VHSV-IVb under laboratory conditions (Millard & Faisal 2012). Thus, the absence of antibodies in immature Lake St. Clair muskellunge is not due to an inability to mount a neutralization response. Rather, it could be due to having fewer opportunities for virus exposure. Spawning congregations, for example, have been identified as a likely route for VHSV transmission, due to increased fish densities and increased virus concentration in the water from infected individuals (Hershberger et al. 2010, VHSV Expert Panel and Working Group 2010). The absence of serum antibodies in immature muskellunge, as well as the small sample size, could also suggest an increased mortality rate upon VHSV exposure of young muskellunge. Intraspecific variation in neutralization titers could also be attributed to exposure dose, as well as time post-exposure.

Antibody-positive status for muskellunge and northern pike was defined as having a neutralization titer of >80. This is because when we tested several groups of naïve juvenile muskellunge, with no history of VHSV-IVb exposure, a few individuals exhibited low levels of innate virus neutralization. The nature of the low level neutralization remains to be elucidated; however, some virus inhibitors have been reported in sera of unexposed rainbow trout (*Oncorhynchus mykiss*; Dorson & de Kinkelin 1974, Park & Reno 2005). The absence of neutralization activities in the majority of non-esocid fish

sera tested in this study led us to believe that such VHSV inhibitors might not be present in sera of these species. Thus, titers of ≥20 were considered positive. By setting the definition of antibody-positive slightly higher for esocid fish, the number of antibody-positive fish might be underestimated by seven individuals.

Evidence for the presence of immunoglobulins in several antibody-positive Lake St. Clair muskellunge was confirmed using an indirect ELISA that utilizes a monoclonal anti-muskellunge IgM antibody (Millard, Kaattari, and Faisal, data not shown). In our analysis, we also confirmed that virus neutralization activity in muskellunge sera was specific to VHSV, because no cross-reactivity with IHNV was observed at titers >20. Rainbow trout sera with antibodies against VHSV-I also do not cross-react with IHNV or SVCV (Olesen & Jørgensen 1986).

Virology results from this study, as well as additional testing of tissue samples of Lake St. Clair fish by the AAHL, confirm the inability to isolate VHSV during the summers of 2007 and 2010. Antibodies, however, were detected in all sampling years. The duration of virus in tissues, induction and duration of the immune response, and outcome of rhabdovirus infection are influenced by several factors, including virus dose, water temperature, and various properties of the host (LaPatra 1998, Lorenzen & LaPatra 1999). When Kim (2010) infected muskellunge held at 11°C with VHSV-IVb by immersion, virus titers peaked within the first 2 wk postinfection (pi). Most fish sampled later in the disease course had no, or substantially lower, virus titers in tissues,

however, virus was detectable in some fish up to 9 wk pi. At higher temperatures, VHSV is cleared from tissues more rapidly (Jørgensen 1982, Goodwin & Merry 2011). Antibody titers peak later in the disease course, by approximately 6 wk in rainbow trout infected with VHSV-I at 13°C (Olesen & Jørgensen 1986), and by 11–16 wk in muskellunge infected with VHSV-IVb at 11°C (Millard & Faisal 2012). The duration of VHSV-neutralizing antibodies is not fully understood, but antibodies remain detectable in some fish 1 yr pi (Olesen & Jørgensen 1986).

In 2006, when fish were collected immediately following a VHSV outbreak, the virus was readily isolated in cell culture. Antibodies were only detected in 14% of the serum samples collected from these fish, and all antibody-positive individuals were also virus-positive, indicating an early convalescent stage of infection. Neutralizing antibodies were detected in >85% of muskellunge sampled in May of 2007, 2009 and 2010, indicating that the majority of adult muskellunge have survived encounters with the virus. Given the high proportion of antibody-positive individuals, it seems likely that adult muskellunge have established immunity to the virus.

In the absence of acute disease outbreaks, serologic testing would aid in the identification of fish populations that previously have been infected with VHSV. Blood samples can be taken non-lethally, which avoids the unnecessary sacrifice of valuable and endangered fish stocks. Our results suggest that muskellunge is a good species to sample non-lethally as an indicator of past VHSV-IVb exposure. Assessing immunity

among Great Lakes fish stocks would provide a more accurate understanding of VHSV distribution, and could potentially be used to predict the vulnerability of a given population to VHSV outbreaks, both of which would provide valuable information for fisheries managers.

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APPENDIX

Table 3.1. Distribution of neutralizing titers against viral hemorrhagic septicemia virus in 118 serum samples from naïve juvenile muskellunge (*Esox masquinongy*). Group I (~ 4 mo.; 17 cm, 1 SD; 19 g, 4 SD), Group II (~ 6 mo.; 16 cm, 2 SD; 17 g, 5 SD), Group III (~ 22 mo.; 32 cm, 1 SD; 148 g, 21 SD).

Titer	Group I (*n* = 30)	Group II (*n* = 46)	Group III (*n* = 7) [a]	Overall
<20	30	40	35	105 (89.0%)
20	0	1	5	6 (5.1%)
40	0	4	2	6 (5.1%)
80	0	1	0	1 (0.9%)

[a] Blood was collected non-lethally at 6-wk intervals (6 time points; 42 total sera).

Table 3.2. Serum neutralization specificity of Lake St. Clair muskellunge (1-6; *Esox masquinongy*) and freshwater drum (7; *Aplodinotus grunniens*). Sera were tested in parallel with viral hemorrhagic septicemia virus (VHSV-IVb) and infectious hematopoietic necrosis virus (IHNV) using a 50% plaque neutralization test (50% PNT).

Serum no.	50% PNT titers to:	
	VHSV-IVb	IHNV
1	20	<20
2	320	<20
3	320	<20
4	1,280	20
5	10,240	<20
6	20,480	<20
7	40	<20

Table 3.3. Prevalence (%) of viral hemorrhagic septicemia virus (VHSV-IVb) neutralizing antibodies among Lake St. Clair fish species.

Species [a]	No. antibody-positive/No. tested (prevalence [%]) [b]						Mean positive titer [c]	SD [c]
	2004	2006	2007	2009	2010	Overall		
Muskellunge	—	1/3 (33)	6/6 (100)	9/10 (90)	18/21 (86)	34/40 (85) [d]	12,113	11,699
Northern pike	2/10 (20)	3/4 (75)	—	—	10/16 (63)	15/30 (50) [e]	608	449
Freshwater drum	—	0/11	13/40 (33)	—	2/10 (20)	15/61 (25) [f]	201	339
Smallmouth bass	—	—	—	0/16	3/25 (12)	3/41 (7)	213	92
Channel catfish	—	—	—	—	0/25	0/25	NA	NA
Lake sturgeon	—	—	—	—	0/15	0/15	NA	NA
Quillback	—	—	—	—	0/7	0/7	NA	NA
Rock bass	—	0/3	—	—	0/24	0/27	NA	NA
Shorthead redhorse	—	0/2	—	—	0/6	0/8	NA	NA
Silver redhorse	—	0/5	—	—	0/13	0/18	NA	NA
Walleye	—	—	—	—	0/10	0/10	NA	NA
White perch	—	—	—	—	0/12	0/12	NA	NA
Yellow perch	—	—	—	—	0/3	0/3	NA	NA
Overall	2/10 (20)	4/28 (14)	19/46 (41)	9/26 (35)	33/187 (18)	67/297 (23)	NA	NA

[a] Scientific names are provided in text.

[b] Dashes indicate no sera were collected.

Table 3.3 (cont'd)

[c] NA = Not applicable.

[d] Significantly higher antibody prevalence compared to northern pike ($P = 0.0032$), smallmouth bass, and freshwater drum ($P < 0.0001$).

[e] Significantly higher antibody prevalence compared to smallmouth bass ($P < 0.0001$) and freshwater drum ($P = 0.0192$).

[f] Significantly higher antibody prevalence compared to smallmouth bass ($P = 0.0332$).

Table 3.4. Maturity, sex, age, and size of Lake St. Clair muskellunge (*Esox masquinongy*) included in this study [a].

	Sex			Range (mean, SD)			No. antibody positive (>80)/ No. sampled
	M	F	ND	Age (years)	Length (cm)	Weight (g)	
Immature [b]	3	0	1	2–3 (3, 1)	45–73 (61, 12)	452–2200 (1370, 717)	0/4 [c]
Mature	23	11	0	6–20 (11, 3)	88–127 (107, 11)	4000–13,800 (8285, 3079)	34/34

[a] Table excludes two muskellunge (titers of <20) captured in 2006 due to incomplete measurements.

[b] Classification of immature fish was based upon the absence of ripe gametes and was confirmed by examination of reproductive organs. In one immature muskellunge, sex could not be determined (ND).

[c] Antibody prevalence of immature muskellunge was significantly less than that of mature muskellunge ($P < 0.0001$).

Figure 3.1. Lake St. Clair (Lake Erie watershed, USA) showing sampling sites. Most fish were captured using trap nets (●) positioned between 42° 39'N, 82° 46'W and 42° 37'N, 82° 46'W in Anchor Bay. Lake sturgeon (*Acipenser fulvescens*) were captured using survey setlines (★: 42° 37'N, 82° 37'W). Smallmouth bass **(***Micropterus dolomieu***)** from a mortality event in June of 2009 (▲: 42° 29'N, 82° 53'W), and a single muskellunge **(***Esox masquinongy***)** collected May 2006 (◆: 42° 21'N, 82° 54'W), were caught using scoop nets.

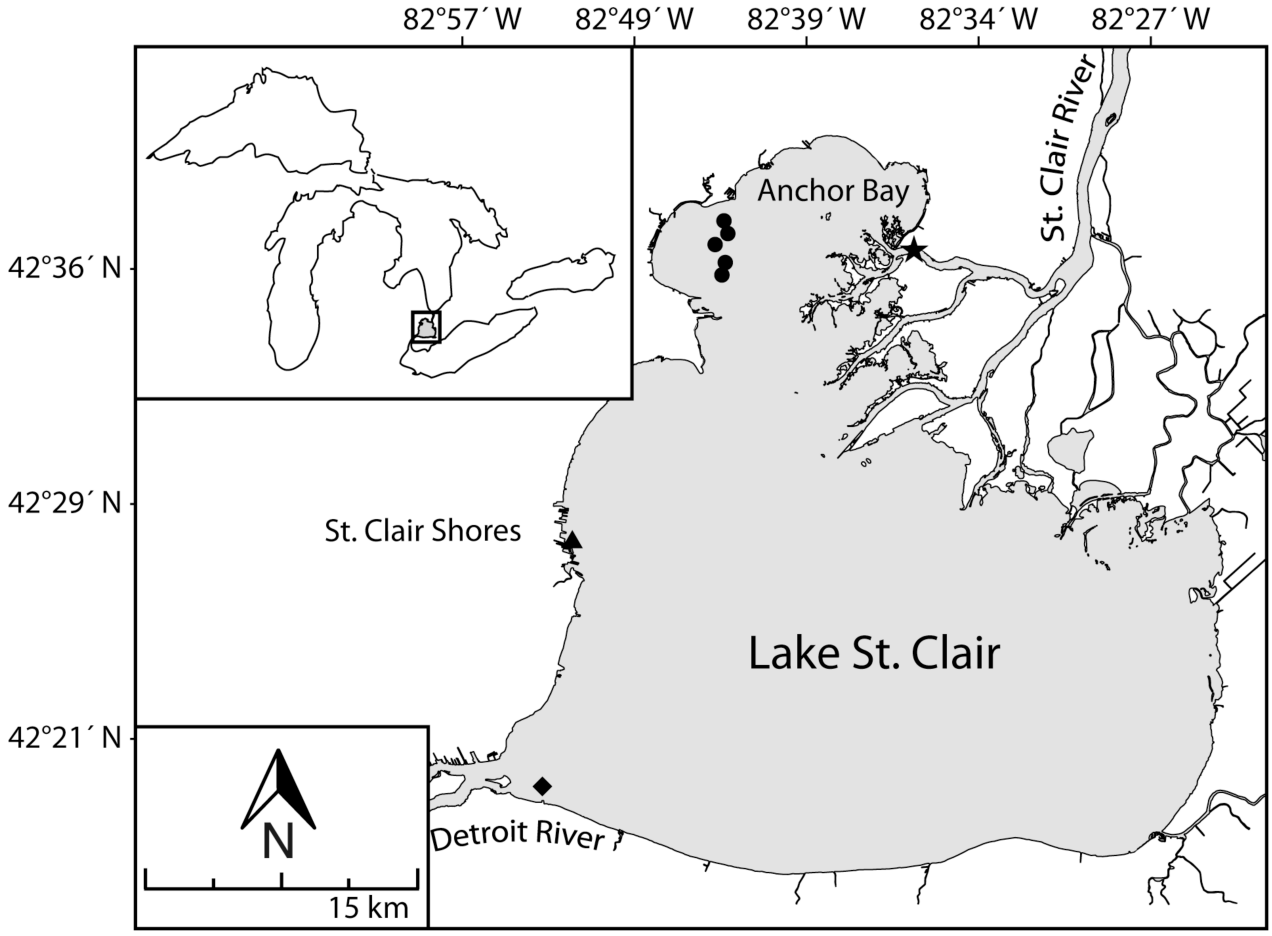
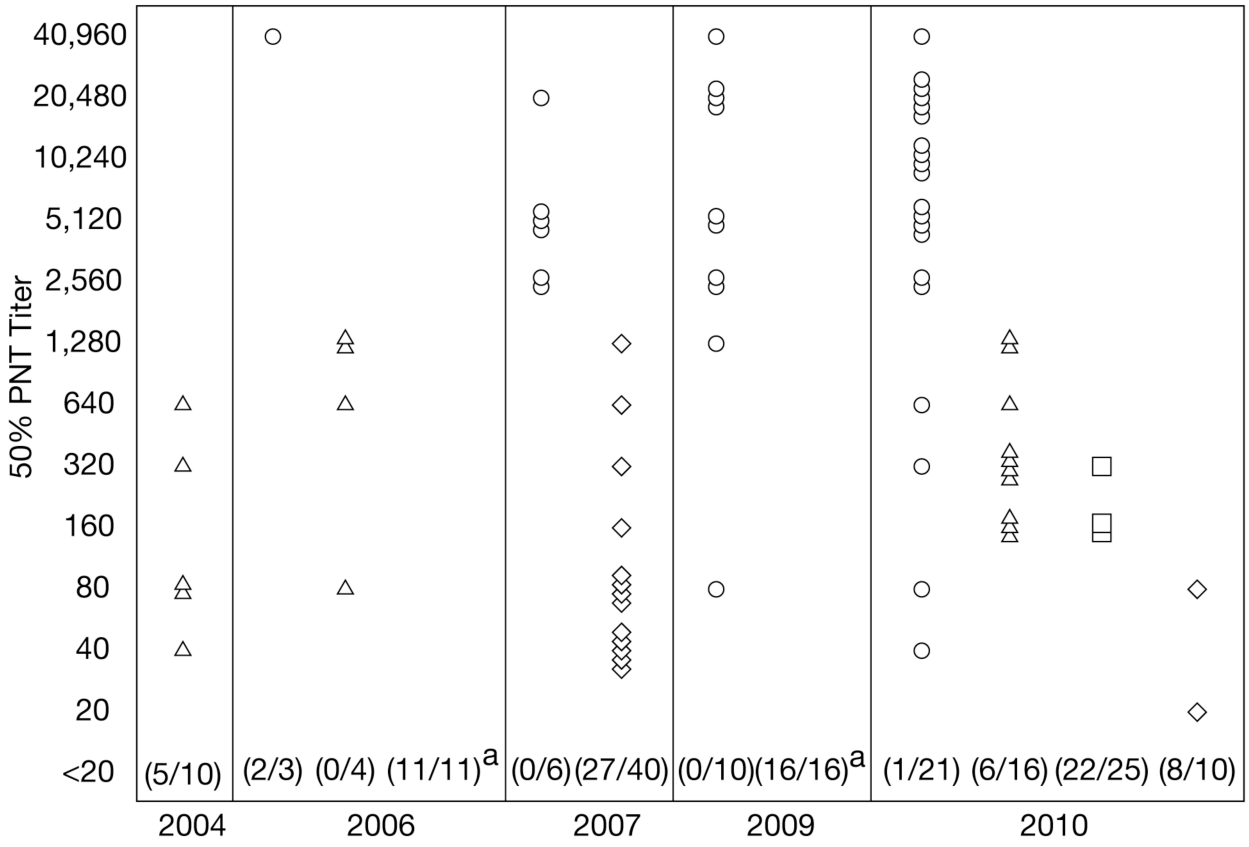


Figure 3.2. Antibody titers measured by a 50% plaque neutralization test (50% PNT) against viral hemorrhagic septicemia virus (IVb) from Lake St. Clair fish species that were antibody-positive: muskellunge (○ *Esox masquinongy*), northern pike (△ *E. lucius*), smallmouth bass (□ *Micropterus dolomieu*), freshwater drum (◇ *Aplodinotus grunniens*). Neutralization titers of >80 are considered positive for muskellunge and northern pike. The parenthesis denote the number of sera with titers of <20/number sampled.



[a] All freshwater drum in 2006 and smallmouth bass in 2009 had titers of <20.

CHAPTER 4

APPLICATION OF A COMPETITIVE ELISA FOR DETECTING CIRCULATING ANTIBODIES AGAINST VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (GENOTYPE IVB) IN REPRESENTATIVE FISH SPECIES IN LAKE ST. CLAIR, MICHIGAN, USA

ABSTRACT

Viral hemorrhagic septicemia virus genotype IVb (VHSV-IVb) is a serious novirhabdovirus of freshwater fish that has invaded the Laurentian Great Lakes region or North America within the last decade. We tested sera (n = 113) from nine fish species for the presence of antibodies against VHSV-IVb using a competitive enzyme-linked immunosorbent assay (cELISA). Fish were sampled in May 2010 from Lake St. Clair, a VHSV-IVb endemic lake in Michigan, USA. Inhibition of the virus-specific rabbit antiserum by sera of freshwater drum (*Aplodinotus grunniens*), smallmouth bass (*Micropterus dolomieu*), and channel catfish (*Ictalurus punctatus*) strongly suggests the presence of VHSV-IVb antibodies in sera of these species. This cELISA does not require species-specific reagents and is a rapid, efficient and non-lethal test for determining indirectly the presence of VHSV-IVb in fish populations.

BODY

Viral hemorrhagic septicemia virus (VHSV) (family *Rhabdoviridae*, genus *Novirhabdovirus*) is a serious viral pathogen of fishes worldwide (Wolf, 1988). The introduction of VHSV-genotype IVb (VHSV-IVb) in the Laurentian Great Lakes of North America has led to mass mortality events among naïve populations of wild, freshwater fish (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Faisal et al. 2012). VHSV-IVb has been isolated from at least 28 freshwater fish species (United States

Department of Agriculture, Animal and Plant Health Inspection Service, 2008). Existing surveillance programs are hampered by the inability to detect asymptomatically infected and recovered fishes using the standard method of virus isolation in cell culture. Additionally, for threatened and endangered fish species, a non-lethal test is desired. Serological assays, that detect virus-specific antibodies, are a viable alternative or supplementary tool to determine VHSV exposure in a fish population (Hattenberger-Baudouy et al. 1995, Millard & Faisal, 2012b, Schyth et al. 2012).

Recently, we used a serum neutralization assay [50% plaque neutralization test (PNT)] to assess the presence of neutralizing antibodies in wild and experimentally exposed muskellunge (*Esox masquinongy*) (Millard & Faisal 2012a, 2012b). Over a period of several years, antibodies were detected in a much greater proportion of wild-caught muskellunge from Lake St. Clair, Michigan than was the virus. While the PNT remains a valuable tool for measuring protective antibody levels, not all circulating antibodies have functional VHSV-neutralizing activity. Furthermore, the assay requires 5-6 days to perform and is not cost effective for large-scale serological surveys. For these reasons, indirect enzyme-linked immunosorbent assays (ELISA) have been developed and used to measure VHSV-genotype I antibodies in rainbow trout (*Oncorhynchus mykiss*) (Jørgensen et al. 1991, Olesen et al. 1991, Fernandez-Alonso et al. 1998, Encinas et al. 2011). The absence of commercially available anti-immunoglobulins for many Great Lakes species makes assessing VHSV-IVb antibodies by indirect ELISA not feasible. Conversely, competitive ELISAs (cELISAs) are well suited as antibody screening

assays for pathogens with wide host ranges. Antibody levels are measured according to the degree to which sera inhibit the binding of a virus-specific hyperimmune antiserum. cELISAs have previously been developed for several rhabdoviruses including spring viremia of carp virus (SVCV; Dixon et al. 1994), vesicular stomatitis virus (Alvarado et al. 2002) and rabies virus (Zhang et al. 2009).

In this study, we applied a cELISA to assess the presence of VHSV-IVb antibodies in 113 serum samples from nine fish species representing a range of trophic levels. Fish were caught on May 5 and 7, 2010 from the Anchor Bay area of Lake St. Clair, Michigan, a lake that is known to be enzootic for VHSV-IVb. The previous summer, VHSV-IVb was isolated from smallmouth bass (*Micropterus dolomieu*) that were a part of a mortality event that occurred near to the sampling site. Fish were caught using trap nets positioned between 42° 39'N, 82° 46'W and 42° 37'N, 82° 46'W. Tested species were channel catfish (*Ictalurus punctatus*), rock bass (*Ambloplites rupestris*), northern pike (*Esox lucius*), silver redhorse (*Moxostoma anisurum*), smallmouth bass, freshwater drum (*Aplodinotus grunniens*), white perch (*Morone Americana*), shorthead redhorse (*Moxostoma macrolepidotum*), and quillback (*Carpiodes cyprinus*). The number of fish tested ranged from 5 to 24 per species (Table 4.1). Blood samples were collected non-lethally. Sera were separated after centrifugation (2500 × g, 10 min, 4°C) and stored at -80°C.

Fish sera were heat-treated (45°C for 30 min) and incubated overnight in phosphate buffered saline (PBS) with 1% nonfat dried milk (PBS-1%NFDM, Sigma-Aldrich, St. Louis, Missouri, USA) prior to running the cELISA to reduce non-specific binding. Plates were washed five times with PBS containing 0.05% Tween 20 (Sigma-Aldrich) in an automated microplate washer following each step unless noted. Briefly, 96-well polystyrene microplates (Microlon®600, Greiner Bio-One, Monroe, North Carolina, USA) were coated with purified VHSV-IVb (1 $\mu g \cdot mL^{-1}$) overnight at 4°C. Unbound sites were blocked with 5% nonfat dried milk (PBS-5%NFDM, Sigma-Aldrich). Fish test sera and control sera, diluted 1:10 in PBS-1%NFDM, were added to duplicate wells and incubated prior to addition of rabbit hyperimmune serum to VHSV-IVb (1:512,000). A commercial anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich) was used as the detection antibody and a colorimetric substrate was used to develop the reaction. After 30 min and without washing, color development was stopped with 3 M sulfuric acid. Results were interpreted as the percent inhibition of the optical density (OD) of the rabbit hyperimmune serum according to the following formula: [1-(Average $OD_{405/490}$ fish serum/ Average $OD_{405/490}$ rabbit serum)] x 100

The rabbit hyperimmune serum to VHSV-IVb used in the cELISA as the competition antibody was produced by immunization of New Zealand white rabbits with purified VHSV-IVb (data not shown). Negative inhibition values were treated as zeros in all analyses. Data were analyzed using the MIXED procedure in SAS Version 9.2 (SAS

Institute, Inc. 2010). Mean percent inhibition for each species was compared to the overall mean percent inhibition. Differences in mean percent inhibition among species were tested using pair-wise comparisons of least-squares means. The type-I error rate was set at 0.05.

Some fish sera were able to successfully block the rabbit hyperimmune serum from binding to purified VHSV-IVb during the assay (Figure 4.1). The overall mean percent inhibition for all 113 fish tested was 8.8% (SD = 16.1%) and the overall median was 3.6%. Inhibition levels were the highest in freshwater drum. Percent inhibition values for this species ranged from 0.0 to 88.3% (mean = 29.4%, SD = 33.3%) (Table 4.1). Smallmouth bass percent inhibition ranged from 0.0 to 58.9% (mean = 20.0%, SD = 21.1%). The mean of both freshwater drum [$t = 4.62$, degrees of freedom (df) = 104, $p < 0.0001$] and smallmouth bass ($t = 2.76$, $df = 104$, $p = 0.0034$) was significantly greater than the overall mean percent inhibition calculated for all 113 sera. Inhibition was also detected in several sera of channel catfish (range = 0.0 to 63.9%; mean = 10.9%, SD = 14.7%). The mean of this species was, however, not significantly different than the overall mean ($t = 0.74$, $df = 104$, $p = 0.2311$). Mean percent inhibition levels of rock bass ($t = 1.95$, $df = 104$, $p = 0.9732$), northern pike ($t = 1.69$, $df = 104$, $p = 0.9533$), silver redhorse ($t = 1.51$, $df = 104$, $p = 0.9327$), white perch, ($t = 1.15$, $df = 104$, $p = 0.8732$), shorthead redhorse ($t = 1.18$, $df = 104$, $p = 0.8795$) and quillback ($t = 0.43$, $df = 104$, $p = 0.6670$) also did not differ from the overall mean. Percent inhibition of these species ranged from 0.0 to 18.4%.

Mean percent inhibition levels differed among the resident fish species, $F_{8,104} = 5.17$, $p < 0.0001$. Freshwater drum had significantly greater mean percent inhibition than channel catfish ($t = 3.48$, $df = 104$, $p = 0.0007$), rock bass ($t = 4.91$, $df = 104$, $p < 0.0001$), northern pike ($t = 4.62$, $df = 104$, $p < 0.0001$), silver redhorse ($t = 4.50$, $df = 104$, $p < 0.0001$), white perch ($t = 3.93$, $df = 104$, $p = 0.0002$), shorthead redhorse ($t = 3.63$, $df = 104$, $p = 0.0004$), and quillback ($t = 3.02$, $df = 104$, $p = 0.0032$). Smallmouth bass had a greater mean percent inhibition compared to rock bass ($t = 3.38$, $df = 104$, $p < 0.0010$), northern pike ($t = 3.18$, $df = 104$, $p = 0.0020$), silver redhorse ($t = 3.05$, $df = 104$, $p = 0.0029$), white perch ($t = 2.63$, $df = 104$, $p = 0.0097$) and shorthead redhorse ($t = 2.49$, $df = 104$, $p = 0.0145$). The mean percent inhibition of freshwater drum was not different than smallmouth bass ($t = 1.55$, $df = 104$, $p = 0.1243$), nor were any of the other pairwise comparisons between species (Table 4.2).

Without established positive-negative cELISA thresholds for the species tested in this research we were unable to estimate seroprevalence for each species, which hinders our ability to conclude what species were producing VHSV-IVb antibodies. However, the wide range in inhibition values for species such as smallmouth bass, freshwater drum, and channel catfish is suggestive that at least some individuals of these species were producing VHSV-IVb antibodies. For other species, such as silver redhorse, white perch, and quillback where maximum inhibition values ranged from 9.5 to 18.4%, VHSV-IVb antibody production remains less certain. Current studies are

directed toward determining positive-negative threshold(s) for the cELISA. These studies are challenged by the absence of reliable sources of fish for some species that we can be certain have not been exposed to VHSV-IVb.

The presence of high inhibitions in several fish species residing in Lake St. Clair is suggestive of current or previous infection with VHSV-IVb. Both smallmouth bass and freshwater drum have been involved in fish kills from which VHSV-IVb was isolated during the four-year period preceding collection (Faisal et al. 2012). Prior to collection in June 2009, VHSV-IVb was isolated from tissues of smallmouth bass involved in a mortality episode. Differences in the occurrence and levels of antibodies among species could be related to differences in susceptibility to VHSV-IVb, immune response to the virus or life history. A longer life span, piscivorous diet or engaging in certain behaviors such as spawning congregations would likely increase exposures to VHSV-IVb, and thereby the presence of antibodies in surviving fish.

Some sera of smallmouth bass, freshwater drum, and channel catfish produced considerable inhibition levels by cELISA, but were negative for neutralizing antibodies by PNT (titers <20) in our previous study (Millard & Faisal 2012b). This suggests the presence of non-neutralizing antibodies against the glycoprotein, or antibodies against other viral proteins exposed in the virus preparation (Olesen et al. 1991). Detection of antibodies in non-neutralizing sera by ELISA-based assays has been reported previously for rainbow trout (Olesen et al. 1991, Encinas et al. 2011) and common carp

(*Cyprinus carpio*) against VHSV-genotype I and SVCV, respectively (Dixon et al. 1994). Some studies have found that non-neutralizing antibodies last longer after infection compared to neutralizing antibodies (Olesen et al. 1991, Encinas et al. 2011). It is also possible that some species have a reduced or absent neutralizing antibody response to VHSV. Pacific herring (*Clupea pallasii*) for example do not mount a detectable neutralizing antibody response against VHSV-IVa, despite survivors being protected against subsequent viral challenges (Kocan et al. 2001, Hershberger et al. 2007).

Despite the limitations, the results of this study suggest that the developed cELISA can be applied to many different species without the need for custom immunoglobulins against each species tested. Furthermore, while not the intent of the present investigation, it appears that the cELISA is capable of detecting a broader range of VHSV-IVb antibodies compared to the PNT, and as such, may be more sensitive in detecting prior virus exposure for some species. Our results suggest that smallmouth bass and freshwater drum would be good species to target for future VHSV-IVb serological surveys. The VHSV-IVb cELISA is a promising tool for cost-effective and timely VHSV-IVb serological surveys.

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APPENDIX

Table 4.1. Viral hemorrhagic septicemia virus-genotype IVb competitive ELISA results of 113 fish of 9 species sampled from Lake St. Clair, MI, May 2010. Bold font indicates species for which the mean inhibition was significantly different than the overall mean. SD = standard deviation

Species	Scientific name	No. fish	Percent inhibition			
			Mean	SD	Median	Range
Channel catfish	*Ictalurus punctatus*	24	10.9	14.7	5.3	0 – 63.9
Rock bass	*Ambloplites rupestris*	21	2.8	2.5	1.9	0 – 6.9
Northern pike	*Esox lucius*	14	2.4	2.3	1.4	0 – 8.1
Silver redhorse	*Moxostoma anisurum*	14	3.1	4.1	1.7	0 – 14.5
Smallmouth bass	*Micropterus dolomieu*	12	20.0	21.1	7.4	0 – 58.9
Freshwater drum	*Aplodinotus grunniens*	10	29.4	33.3	9.4	0 – 88.3
White perch	*Morone Americana*	8	3.1	4.0	0.6	0 – 9.5
Shorthead redhorse	*Moxostoma macrolepidotum*	5	1.4	1.7	0.2	0 – 3.6
Quillback	*Carpiodes cyprinus*	5	6.1	8.6	0.1	0 – 18.4
Overall		113	8.8	16.1	3.6	0 – 88.3

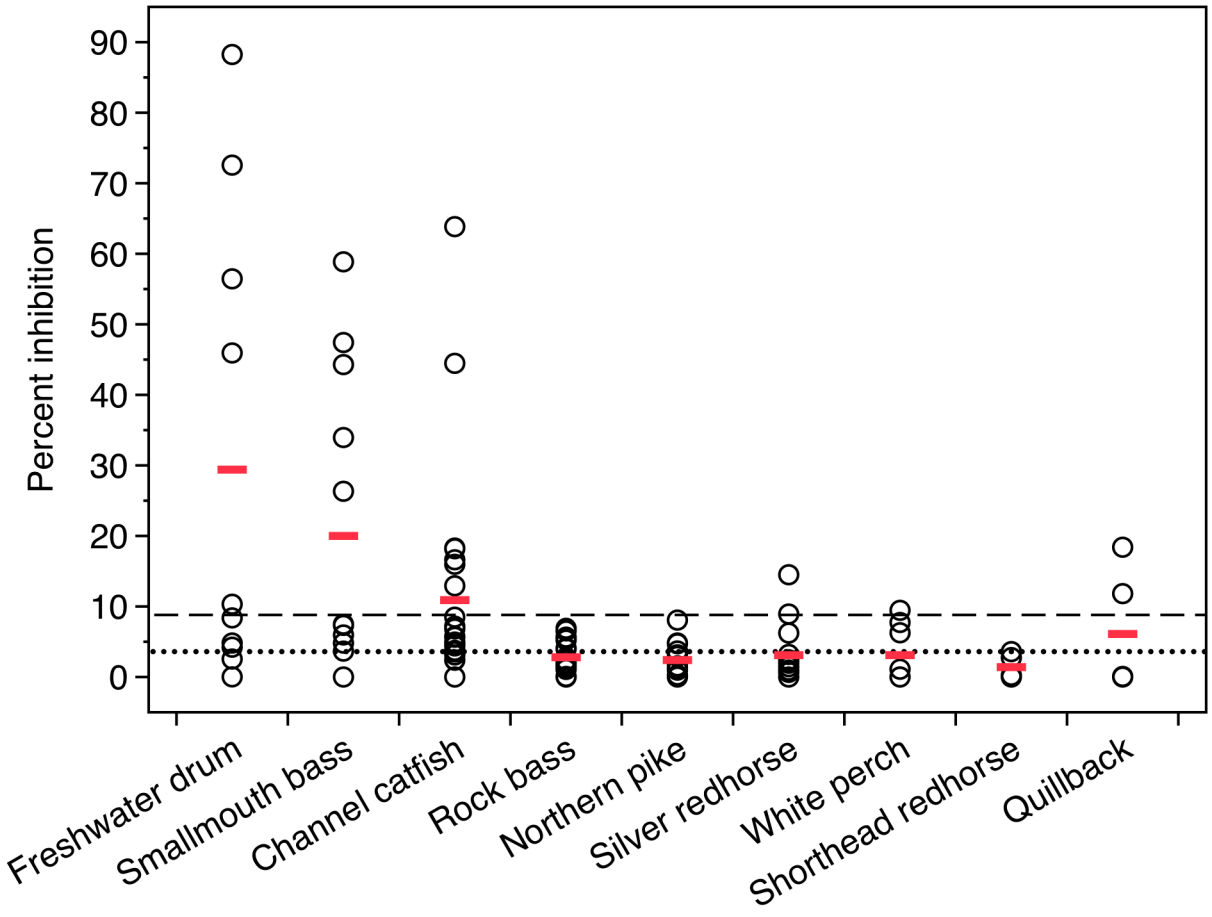
Table 4.2. Pairwise differences in least-squares means for percent inhibition values from 13 fish species from Lake St. Clair, Michigan. Blood samples were collected in May of 2010 and tested for VHSV-IVb antibodies by cELISA. Differences in least-squares means (Diff.), *t* and *p values* for comparisons between species are shown. Degrees of freedom = 104. The type-I error rate was set at 0.05. Bolded *p values* indicate statistically significant comparisons. Species abbreviations are as follows: CCF = channel catfish, ROB = rock bass, NOP = northern pike, SIR = silver redhorse, SMB = smallmouth bass, FRD = freshwater drum, WHP = white perch, SHR = shorthead redhorse, QUI = quillback.

		Mean percent inhibition		
Species	Species	Diff.	*t*	*p*
CCF	ROB	8.12	1.93	0.0564
CCF	NOP	8.50	1.79	0.0758
CCF	SIR	7.80	1.65	0.1027
CCF	SMB	-9.10	-1.83	0.0706
CCF	FRD	-18.44	-3.48	**0.0007**
CCF	WHP	7.84	1.36	0.1758
CCF	SHR	9.55	1.38	0.1707
CCF	QUI	4.85	0.70	0.4854
ROB	NOP	0.37	0.08	0.9388
ROB	SIR	-0.32	-0.07	0.9471
ROB	SMB	-17.22	-3.38	**0.0010**
ROB	FRD	-26.57	-4.91	**<.0001**
ROB	WHP	-0.28	-0.05	0.9614
ROB	SHR	1.43	0.20	0.8387
ROB	QUI	-3.27	-0.47	0.6415
NOP	SIR	-0.70	-0.13	0.8961
NOP	SMB	-17.60	-3.18	**0.0020**
NOP	FRD	-26.94	-4.62	**<.0001**
NOP	WHP	-0.66	-0.11	0.9163
NOP	SHR	1.06	0.14	0.8859
NOP	QUI	-3.65	-0.50	0.6202
SIR	SMB	-16.90	-3.05	**0.0029**
SIR	FRD	-26.24	-4.50	**<.0001**
SIR	WHP	0.04	0.01	0.9950
SIR	SHR	1.75	0.24	0.8117
SIR	QUI	-2.95	-0.40	0.6885
SMB	FRD	-9.35	-1.55	0.1243

Table 4.2 (cont'd)

SMB	WHP	16.94	2.63	**0.0097**
SMB	SHR	18.65	2.49	**0.0145**
SMB	QUI	13.95	1.86	0.0657
FRD	WHP	26.28	3.93	**0.0002**
FRD	SHR	28.00	3.63	**0.0004**
FRD	QUI	23.29	3.02	**0.0032**
WHP	SHR	1.71	0.21	0.8314
WHP	QUI	-2.99	-0.37	0.7104
QUI	QUI	-4.70	-0.53	0.5986

Figure 4.1. Distribution of percent inhibition values of sera from 113 fish of nine species from Lake St. Clair, Michigan, May 2010. Sera were tested for antibodies against viral hemorrhagic septicemia virus-genotype IVb using a competitive ELISA. The mean percent inhibition value of each species is indicated by a red dash. Dashed and dotted horizontal lines indicate the overall mean (8.8) and median (3.6) percent inhibition values.



CHAPTER 5

DETECTION OF ANTIBODIES TO VIRAL HEMORRHAGIC SEPTICEMIA VIRUS-IVB IN SERA OF MUSKELLUNGE (*ESOX MASQUINONGY*) USING A COMPETITIVE ELISA

ABSTRACT

A competitive enzyme-linked immunosorbent assay (cELISA) was developed for the detection of antibodies to viral hemorrhagic septicemia virus-genotype IVb (VHSV-IVb) in fish sera. Assay conditions were standardized using known negative and positive muskellunge (*Esox masquinongy*) sera. A positive-negative threshold of 14.6% inhibition was established based on analysis of sera of 60 muskellunge with no previous exposure to VHSV-IVb. The cELISA was then used to investigate immune responses of wild muskellunge sampled from five water bodies in Michigan and Wisconsin, USA between the years of 2005 and 2012. Antibodies were detected in fish from Lake St. Clair, Michigan and Lower Fox River/Green Bay, Wisconsin. Both water systems were considered enzootic for VHSV-IVb. Additionally, antibodies were detected in muskellunge from Thornapple Lake, a Michigan inland lake previously considered negative for VHSV-IVb based on virus isolation methods. Muskellunge populations from Lake Hudson, MI and Butternut Lake, Wisconsin lacked evidence of an immune response to VHSV-IVb. When results of the cELISA were compared to the 50% plaque neutralization test for several groups of fish, there was 78.4% agreement between the tests for antibody presence. The cELISA is a rapid and efficient test for the detection of binding antibodies to VHSV-IVb and will be a useful non-lethal tool for monitoring the spread of this serious pathogen.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) (family *Rhabdoviridae*, genus *Novirhabdovirus*) (Dietzgen 2012) is the causative agent of viral hemorrhagic septicemia (VHS). VHS is a World Organization for Animal Health (OIE) reportable disease of freshwater and marine fish. In the Great Lakes region of North America, a new sublineage of the virus, designated VHSV-IVb, has emerged within the last decade and caused large-scale mortality events in wild freshwater fish populations (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007, Faisal et al. 2012). VHSV-IVb has since been isolated from at least 28 fish species (United States Department of Agriculture Animal and Plant Health Inspection Service 2008) and concerns exist regarding its continued spread to naïve fish populations and potential introduction into aquaculture systems (VHSV Expert Panel and Working Group 2010). Ongoing VHSV-IVb surveillance efforts aim to determine virus distribution and delineate VHSV-IVb positive and negative zones in the Great Lakes basin. Testing for VHSV-IVb is commonly conducted according to the international standard, which is virus isolation from fish tissues in cell culture followed by confirmation of the isolated virus by reverse-transcriptase polymerase chain reaction (OIE 2012, United States Fish and Wildlife Service and American Fisheries Society-Fish Health Section 2010). During and immediately following VHS outbreaks, VHSV can be readily isolated in this manner (OIE 2012, Schyth et al. 2012). Detection of the virus from clinically healthy fish (carriers) is more problematic and dependent on many factors such as environmental temperature and time since exposure (OIE 2012).

In the absence of clinical VHS disease outbreaks, such as for surveillance purposes or surveying enzootic populations, the use of antibody-based detection assays can increase the capacity to screen populations for exposure to VHSV (Hattenberger-Baudouy 1995, LaPatra et al. 1996, Millard and Faisal 2012b, Schyth et al. 2012). After the onset of antibody production, circulating antibodies have been detected in the range of months to years after VHS infection in sera of surviving fish (Olesen & Jørgensen 1986, Olesen et al. 1991, Enzmann & Konrad 1993, Millard and Faisal 2012a). Another benefit of serological testing is that it can be done non-lethally, an attribute that is especially ideal for the study of threatened, valuable and long-lived species. It is important to consider that the presence of virus-specific antibody indicates seroconversion but does not necessarily indicate fish ever developed clinical VHS disease and does not distinguish between subclinically infected and recovered fish.

Neutralizing antibodies are directed against viral surface glycoproteins (G) and are an important component of the protective immune response of rainbow trout (*Oncorhynchus mykiss*) to VHSV (Lorenzen et al. 1990, Bearzotti et al. 1995, Lorenzen & LaPatra 1999). This set of antibodies is measured by neutralization tests such as the 50% plaque neutralization tests (PNT) that rely on an exogenous source of fish complement (Dorson & Torchy 1979). Rainbow trout surviving infection with VHS also produce binding antibodies and several indirect enzyme-linked immunosorbent assays (ELISA) have been developed for their detection (Jørgensen et al. 1991, Olesen et al.

1991, Fernandez-Alonso et al. 1998, Encinas et al. 2011). In some of these studies, binding antibodies were detected in a higher percentage of fish and lasted longer than did neutralizing antibodies. Compared to the PNT, ELISA was considered a more feasible test for surveillance studies given the capacity to screen a larger number of samples simultaneously, quicker test results, and reduced processing costs (Olesen et al. 1991, LaPatra 1996).

In this study we developed a competitive ELISA (cELISA) to detect antibodies to VHSV-IVb and applied its use to sera from wild muskellunge (*Esox masquinongy*) from systems where the virus is enzootic and in systems where the virus has never been detected. Competitive ELISAs, such as the one developed by Dixon et al. (1994) for detection of spring viremia of carp virus (SVCV) antibodies, utilize a virus-specific rabbit antiserum to compete with fish sera for virus binding sites. As such, the cELISA can be used on multiple fish species without the need to develop specific anti-immunoglobulin reagents for each species tested as would be necessary for indirect ELISAs. This is a more feasible approach given the wide range of fish hosts for VHSV-IVb and the absence of commercially available antisera to immunoglobulins for many of these species. We focused on muskellunge for this initial study due to the known susceptibility of this species to VHSV-IVb (Kim & Faisal 2010) and the availability of sera from unexposed, experimentally infected and naturally infected populations. Furthermore, muskellunge have been shown to be good indicators for VHSV-IVb presence in an

ecosystem based on the presence of neutralizing antibodies in a previous study (Millard & Faisal 2012b).

MATERIALS AND METHODS

Preparation of virus stock and purified virus. The MI03 index strain of VHSV-IVb (Elsayed et al. 2006) was used throughout this study. To produce purified virus for use as the cELISA coating antigen and for immunization of rabbits, VHSV-IVb was propagated in *Epithelioma papulosum cyprini* (EPC) cells (Winton et al. 2010) in minimum essential medium with Earle's salts (EMEM; Life Technologies) supplemented with 10% tryptose phosphate broth (BD Biosciences), 2.0 mM L-glutamine (Life Technologies) and 12 mM sodium bicarbonate (Sigma-Aldrich). After cells had lifted, flasks were briefly frozen at -20°C and cell debris were removed by centrifugation (5200 × *g*, 30 min, 4°C). Resulting supernatants were filtered using a Corning® bottle-top vacuum filter system and ultracentrifuged using a SW 32 Ti rotor (Beckman Coulter) for 80,000 × *g* for 2.5 hr at 4°C (Olesen et al. 1999). Concentrated virus pellets were resuspended in TE buffer (20 mM Tris-HCl, pH 7.5 and 1 mM EDTA, pH 8.0) and stored at -80°C.

For purification, virus suspensions were overlaid on 10-60% discontinuous sucrose gradient columns and ultracentrifuged (80,000 × *g*, 2.5 hr, 4°C). The fraction containing the purified virus was used immediately for immunization of rabbits. For use as the

cELISA coating antigen, this fraction was given a final wash by pelleting through TE buffer (80,000 × *g*, 150 min, 4°C). The resulting pellet of purified virus was resuspended in phosphate buffered saline [PBS (0.138 M NaCl; 0.0027 M KCl); pH 7.4; Sigma-Aldrich), divided into aliquots, and stored at -80°C until use. The protein concentration of the final virus preparation was determined using a Qubit® Fluorometer with the Qubit® Protein Assay Kit (Life Technologies).

The virus used in experimental infections and the PNT was propagated in EPC cells as previously described (Millard & Faisal 2012b). The titers of the virus stocks were determined by a virus plaque assay on EPC cells. Cells were pretreated with a 7% solution of polyethylene glycol (PEG; 20,000 MW; JT Baker) (Batts and Winton 1989).

Production of polyclonal antibody to VHSV-IVb. Three New Zealand white rabbits were given a series of two immunizations of purified VHSV-IVb over a 5-week period. Purified virus (0.5 mL, $10^{10\text{-}12}$ $TCID_{50}$) was emulsified 1:1 in Freund's complete adjuvant (Sigma-Aldrich) for the first immunization and in Freund's incomplete adjuvant (Sigma-Aldrich) for the second. The immunizations were administered subcutaneously over the dorsum (10 sites, 0.1 mL·site^{-1}). Serum was collected from each rabbit prior to immunization (pre-immune sera) and 3 weeks after the second immunization (hyperimmune serum). Sera were heat-treated for 45°C for 45 min, divided into aliquots, and stored at -80°C. Rabbits and fish were cared for in accordance with guidelines

defined by Michigan State University's (MSU) Institutional Animal Care and Use Committee (AUF 07/07-123-00, 09/10-140-00, 02/10-013-00).

Competitive ELISA procedure and control sera. The immunoassay was initially established as an indirect ELISA system following general principles described by Voller et al. (1979) and Crowther (2009). A variety of types of plates, blocking and diluent reagents, and incubation times and temperatures were tested in preliminary trials and titrations were carried out to determine several suitable reagent combinations. Purified virus (coating antigen) at a starting dilution of 16 $\mu g \cdot mL^{-1}$ and rabbit hyperimmune serum to VHSV-IVb at a starting dilution of 1:2000 were tested in 2-fold serial dilutions. The anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich) was tested at dilutions of 1:1000, 1:2500 and 1:5000. Final concentrations for the competitive immunoassay were then optimized using three fish sera controls. The positive control sera were from two adult muskellunge collected from a VHSV-IVb positive water body in Michigan that were positive for neutralizing antibodies by PNT (titers of 20,480 and 1280). The negative control consisted of a pooled serum sample from naïve hatchery-reared muskellunge from outside the Great Lakes basin that were negative by PNT. After standardization trials, fish sera controls were included on each assay plate and consisted of the high positive control (PNT titer of 20,480), a low positive control (1:80 dilution of the high positive), and the negative control serum.

Polystyrene microplates (96-well, Microlon®600 with chimney wells; Greiner Bio-One) were used as the solid phase for the cELISA. Plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T20; Sigma-Aldrich) in an automated microplate washer (BioTek, ELx405™) following each step, and were sealed for all incubations unless otherwise stated. Briefly, assay plates were coated with 100 $\mu l \cdot well^{-1}$ of purified VHSV-IVb in PBS at a concentration of 1 $\mu g \cdot mL^{-1}$ and incubated overnight (16-18 hr) at 4°C in a humid chamber. Prior to running the assay, plates were washed and unoccupied sites were blocked with the addition of 420 $\mu l \cdot well^{-1}$ of PBS containing 5% nonfat dried milk (PBS-5%NFDM; Sigma-Aldrich) for 1 hr at 37°C. Fish control and test sera, diluted 1:10 in PBS-1%NFDM (hereafter referred to as sample diluent) and pretreated as described in the following paragraph, were then added to duplicate wells (100 $\mu l \cdot well^{-1}$). At this time, sample diluent alone was added to duplicate diluent control wells and 12 wells reserved for the rabbit serum control. After incubating for 1 hr at 25°C, rabbit hyperimmune serum to VHSV-IVb (1:512,000 in sample diluent, 100 $\mu l \cdot well^{-1}$) was added to rabbit serum control wells and all wells that had received fish serum during the previous step. Sample diluent alone was again added to diluent control wells and plates were incubated for 1 hr at 25°C. A commercial anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich), 1:1000 in sample diluent, was then added to all wells (100 $\mu l \cdot well^{-1}$) and the incubation period of 1 hr at 25°C was repeated. Enzymatic development of the plates was started by the addition of substrate (0.4

$mg \cdot mL^{-1}$ o-Phenylenediamine in phosphate citrate buffer containing 3 mM hydrogen peroxide; 100 $\mu l \cdot well^{-1}$) and color development proceeded for 30 min at 25°C in the dark. Without washing, the reaction was stopped with 3 M sulfuric acid. An absorbance microplate reader (BioTek ELx405) was used to read dual wavelength optical density at 405 and 490 nm ($OD_{405/490}$). The average $OD_{405/490}$ of the diluent control wells was subtracted from each well. Results were interpreted as the percent inhibition of the average $OD_{405/490}$ of the rabbit serum control. The following formula was used to calculate percent inhibition: [1- (Average $OD_{405/490}$ fish serum / Average $OD_{405/490}$ rabbit serum control)] × 100. Negative inhibition values were treated as zeros in all analyses.

All fish control and test sera were heat-treated for 30 min at 45°C. The night prior to performing the assay, fish sera (22 μl each) were diluted 1:10 in sample diluent in 96-well dilution plates that had been pre-blocked with PBS-5%NFDM. Rabbit serum (1:1000) was also pre-incubated overnight prior to the assay in sample diluent.

Establishment of a positive-negative threshold for muskellunge. Muskellunge were obtained from Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, Iowa), which is located outside the Great Lakes basin (hereafter referred to as naïve muskellunge). Naïve muskellunge were kept in a flow-through system at the MSU Research Containment Facility and fed fathead minnows (*Pimephales promelas*)

purchased from Anderson Minnow Farm (Lonoke, Arkansas). Naïve muskellunge and minnows were certified to be free of VHSV and other reportable viruses. Blood was drawn from the caudal vein of 60 naïve muskellunge (average weight ~80 g) using a sterile syringe and needle. Blood samples were allowed to clot at 4°C overnight and then centrifuged (2500 × g, 10 min, 4°C). Sera were collected, divided into aliquots, and stored at -80°C. These sera were tested by the cELISA in order to determine a baseline level of inhibition present in sera of muskellunge that were not exposed to VHSV. The positive-negative threshold for the cELISA test was established by calculating the upper 95% confidence limit to the 95^{th} quantile of the inhibition values for the naïve muskellunge. We chose to use this quantile approach for determining thresholds rather than the more common "mean + 3 standard deviations (SD)" approach because its interpretation is not dependent on the underlying distribution of percent inhibition values of naïve fish sera. The approach is also more robust to decisions regarding how negative inhibitions are calculated and to potential anomalous observations. The quantiles and their upper 95% confidence limits were calculated in SAS 9.2 (SAS Institute, Inc. 2010). Confidence limits for the quantiles were calculated using the bootstrapping approach of He & Hu (2002). A total of 5,000 bootstrap samples were used to calculate the confidence limits.

Use of sera from fish surviving experimental VHSV-IVb infection to test assay. The cELISA was first tested using sera from two groups of muskellunge that were experimentally challenged with VHSV-IVb (MI03 strain) by immersion in previous

studies. Fish (n = 60-90 per dose group) were challenged by immersion in aerated water containing 4×10^3 or 10^5 plaque-forming units (pfu)·mL^{-1} VHSV-IVb (experimentally exposed fish) or an equal volume of tissue culture media (mock-challenged fish). Fish were sampled between 1 and 15 weeks (11 and 165 degree days) post-infection (PI). The first group of sera tested by the cELISA represents a subset of these samples and consisted of 27 sera from individual experimentally exposed fish and 16 mock-challenged fish (2-6 fish per time-point). Further details of this trial can be found in Millard and Faisal (2012a).

The second group of sera consisted of 42 sera collected from 22 individual muskellunge (4 months of age, 13 g) that were survivors of two previous VHSV-IVb immersion trials (Kim & Faisal 2012). Fish (n = 234) were initially exposed to a low dose of the virus (1.4×10^3 pfu·mL^{-1}) and survivors were re-challenged 22 weeks (1700 degree days) later with doses ranging from 10 to 10^6 pfu·mL^{-1}. The 22 fish that survived both challenges were later tagged with passive integrated transponder (PIT) tags and serial blood samples were collected from the same individuals 67 and 79 weeks (5200 and 6000 degree days) PI (45 and 57 weeks post-second exposure). Water temperature was maintained at approximately 11 ± 1°C for both studies.

Application of the assay to field-collected sera of muskellunge. A total of 200 blood samples were obtained from wild muskellunge sampled from five water bodies in

Michigan (MI) and Wisconsin (WI) (Table 5.1). Fish were sampled prior to or during spawning between the years of 2005 and 2012. Most fish were of reproductively mature age. Four locations were within the Great Lakes basin and included two areas considered enzootic for VHSV-IVb (Lake St. Clair, MI and Lower Fox River/Green Bay, WI) and two inland lakes (Lake Hudson and Thornapple Lake, MI) considered negative for VHSV-IVb based on past viral testing. The fifth water body (Butternut Lake, WI) was a VHSV-negative inland lake located outside the Great Lakes basin. Muskellunge from Lake St. Clair were sampled in spring 2010 and spring 2011 near Anchor Bay and the Detroit River (Figure 5.1). Lower Fox River muskellunge were collected in spring 2010, 2011 and 2012 near the mouth of the Fox River at Green Bay. Lower Fox River muskellunge are primarily residents of Green Bay waters of Lake Michigan except during spawning. The last isolations of VHSV-IVb from fish in Lake St. Clair and Green Bay (and connected waters) were in spring 2009. As part of routine monitoring efforts in the states of MI and WI, sera were collected from muskellunge from Butternut Lake in spring 2005 and 2007 and from Lake Hudson and Thornapple Lake in spring 2008 and 2010.

No fish were euthanized for the purposes of this study. Blood samples were collected non-lethally, or from fish euthanized for other purposes (health inspections or VHSV-IVb surveillance for state regulatory agencies). All samples from fish tested in this study were negative for VHSV and other viruses using virus isolation in cell culture as described in Millard and Faisal (2012b), Faisal et al. (2012) and WI DNR (unpublished).

Samples tested by virus isolation included internal tissues from all Butternut Lake fish (kidney, spleen) and most Lake St. Clair fish (kidney, spleen, heart), and non-lethal samples (reproductive fluids and/or sera) from all fish from Lakes St. Clair and Hudson, Lower Fox River and Thornapple Lake.

Comparison of cELISA and PNT results. Results obtained by cELISA and PNT were compared for several groups of muskellunge to assess the agreement between the two methods. The first group consisted of fish from the first experimental immersion trial (n = 27, sampled between 1 and 15 weeks PI). The second group consisted of the wild muskellunge from Lake St. Clair (n = 38) and Lower Fox River (n = 23). Muskellunge from Lower Fox River (all years) and muskellunge from Lake St. Clair (2011) were tested by PNT for this study. PNT results of remaining sera as well as details of the assay are as reported in Millard and Faisal (2012a, 2012b). Briefly, two-fold dilutions of heat-treated test sera and positive and negative controls were combined with an equal volume of pre-titrated VHSV-IVb. Mixtures were incubated for 30 min at 18°C. Sera from naïve lake trout (*Salvelinus namaycush*) was then added as a source of complement and the incubation was repeated. Samples were plaque assayed on EPC monolayers and fixed and stained with 0.5% crystal violet in 50% formalin after 6 days. The neutralization titer of each test sera was defined as being the reciprocal of the highest serum dilution that prevents the formation of 50% of the viral plaques in relation to the negative control sera. Cohen's kappa was used to assess agreement of the assays for classifying muskellunge as positive or negative for antibodies based on the respective

thresholds (PNT ≥160, cELISA ≥14.6%). Spearman rank correlation was used to assess correlation between PNT and cELISA titers.

RESULTS

Establishment of the cELISA. The individual rabbit hyperimmune serum that was selected for use in the assay neutralized VHSV-IVb *in vitro* and had a strong reactivity to the virus in preliminary indirect ELISA trials. Pre-immune serum from this rabbit was non-neutralizing and did not bind to purified virus in the ELISA. In preliminary trials, we determined that blocking and diluent reagents containing nonfat milk reduced background levels better than did reagents with bovine serum albumin or goat serum. Incubation periods were carried out at 25°C since temperature could be maintained consistently in an incubator compared to room temperature. Sufficient washing after each step with PBS-T20, as opposed to PBS alone, was critical for inhibition detection and assay reproducibility.

In a series of competitive ELISAs, we determined that inhibition was best detected at any given dilution of fish control sera when a) the virus was coated to plates under non-saturating conditions, b) the maximum absorbance of the rabbit serum control wells was approximately 1.0 ± 0.2 OD, and c) the conjugate was used at higher compared to lower concentrations. At the minimum conjugate dilution tested (1:1000), negligible background was present in diluent control wells and the reagent was still economical, so

this dilution was used for subsequent trials. The final concentrations of purified virus and rabbit hyperimmune serum for use in the cELISA were determined by titrations in the presence of 1:10 dilutions of negative and positive fish sera controls. The optimal concentration of virus was determined to be 1.0 $\mu g \cdot mL^{-1}$ VHSV-IVb (0.1 $\mu g \cdot well^{-1}$) and the optimal dilution of rabbit hyperimmune serum was 1:512,000. Using these conditions, rabbit serum control wells resulted in an OD of approximately 1.0 and the difference between percent inhibitions of positive and negative fish sera controls was maximized. Positive fish sera controls effectively inhibited rabbit serum from binding and resulted in inhibition of color development by 86% and 84% compared to rabbit control wells. The negative control serum resulted in only 3-6% inhibition. Using these concentrations, results indicated that the cELISA was capable of making a clear distinction between muskellunge sera with and without antibodies to VHSV-IVb. These reagent concentrations were used for all subsequent assays.

Several treatments of the fish control sera were tested during preliminary trials. Heat-treatment of the serum resulted in lower non-specific binding of negative fish sera. Incubating overnight in diluent containing 1%NFDM also reduced non-specific binding. Using the final cELISA protocol and reagent concentrations, a small trial was done to determine whether fish sera should be screened at a dilution higher than 1:10. A panel of fish sera with and without VHSV-IVb antibodies was tested at dilutions of 1:10, 1:20, 1:40 and 1:80 (data not shown). At a serum dilution of 1:10, optimal positive/negative ratios for fish sera were achieved, so all test sera for this study were screened at the

single, fixed dilution of 1:10. This method of testing was selected over an end-point dilution technique so that large numbers of sera could be screened at once.

Establishment of a positive-negative threshold for muskellunge. The distribution of inhibition values of sera from naïve fish used to establish the positive-negative threshold is shown in Figure 5.2. Of the 60 naïve muskellunge, sera from 50 fish (83.3%) exhibited less than 5% inhibition of the rabbit serum, and 58 fish (96.7%) showed less than 10% inhibition. The observed range of the inhibition values in naïve muskellunge was 0% to 18.8% (mean = 3.0%; SD = 3.5%). A positive-negative threshold of 14.6% inhibition was obtained using the 95^{th} quantile. For the purpose of this study, inhibition values ≥14.6% were considered positive for VHSV-IVb antibodies. Using the traditional "mean + 3 SD" approach would have resulted in a very similar threshold (13.5%).

Use of sera from fish surviving experimental VHSV-IVb infection to test assay. Inhibition values from fish that survived the single VHSV-IVb challenge are shown in Figure 5.3. Inhibition was first detected 7 weeks PI (33.3% of fish). The highest proportions of cELISA positive fish were detected 11 weeks (66.7%) and 15 weeks (100%) PI. Of the 18 fish sampled after the onset of antibody production (between 7 and 15 weeks PI), eight fish (44.4%) blocked the rabbit hyperimmune serum from binding to VHSV-IVb during the assay. Inhibition values of seropositive fish ranged from 31.6% to 69.7% (mean = 51.0%; SD = 11.9%). The range in inhibition for the 16 mock-challenged fish in contrast was 0.0% to 3.1% (mean = 1.3%; SD = 1.4%).

Of the 22 muskellunge that survived two VHSV-IVb exposures, inhibition levels above the threshold were detected in sera of 7 fish (31.8%) sampled 67 weeks PI (45 weeks post-second exposure) and in 10 fish sampled 79 weeks PI (57 weeks post-second exposure) (Table 5.2). Inhibition values of seropositive fish ranged from 14.7% to 46.1% (mean = 25.2%; SD = 8.2%).

Application of the assay to field-collected sera of muskellunge. Inhibition values of all wild fish are shown in Figure 5.4 by location and year and data are summarized in Table 5.1. Seroprevalence is given as apparent (versus true) seroprevalence by definition since sensitivity and specificity of this assay has not been determined. Of the 38 muskellunge collected from Lake St. Clair, 33 fish (86.8%) had antibodies to VHSV-IVb based on the threshold of 14.6 % inhibition. Seroprevalence was 83.3% in 2010 and 90.0% in 2011. Inhibition values for all Lake St. Clair muskellunge ranged from 0.0% to 86.8% (mean = 55.6%; SD = 26.9%). Inhibition values of seropositive fish from Lake St. Clair ranged from 22.2% to 86.8 % (mean = 63.1%; SD = 19.7%). Mean positive antibody titer was slightly greater in May 2010 (mean = 68.2; SD = 18.3) compared to May 2011 (mean = 58.9; SD = 20.3).

Of the 24 muskellunge from Lower Fox River tested by cELISA, nine fish (37.5%) had antibodies to VHSV-IVb. Antibodies were detected in 60.0% of fish in April 2010 and 85.7% of fish in May 2012. No antibodies were detected in fish sampled in May 2011.

Inhibition values of all seropositive fish ranged from 17.1% to 53.7 % (mean = 32.8%; SD = 12.0%). The mean antibody titer was higher in fish sampled in April 2010 (mean = 41.5; SD = 13.2) compared to May 2012 (mean = 28.5; SD = 9.7).

All 78 muskellunge collected from Butternut Lake (n = 18) and Lake Hudson (n = 60) were negative for VHSV-IVb antibodies. For Butternut Lake fish, inhibition values ranged from 0.1% to 8.1% (mean = 2.5%; SD = 2.0%). For Lake Hudson fish, inhibition values ranged from 0.0% to 11.3% (mean = 2.9%; SD = 2.9%).

Thornapple Lake, like Butternut Lake and Lake Hudson, was considered to be VHSV-free based on past viral testing. Muskellunge in Thornapple Lake, however, showed evidence of an immune response against VHSV-IVb. Of the 60 fish collected in 2008 and 2010, 25 fish (41.7%) had inhibition levels greater than the threshold. Seroprevalence and mean positive antibody titers were greater for fish sampled in April 2008 compared to 2010. In 2008, 16 of 30 muskellunge (53.3%) were seropositive. Inhibition values for cELISA-positive fish in 2008 ranged from 18.3% to 80.5% (mean = 51.8%; SD = 16.7%). In 2010, 9 of 30 muskellunge (30.0%) had antibodies to VHSV-IVb. Inhibition values of seropositive fish in 2010 ranged from 19.2% to 60.8 % (mean = 39.2%; SD = 13.6%). Inhibition values for all Thornapple muskellunge ranged from 0.0% to 80.5% (mean = 21.2%; SD = 24.7).

Comparison of cELISA with PNT results. Results by cELISA and PNT agreed in classifying fish as positive or negative for antibodies to VHSV-IVb for the majority of sera tested (Figure 5.5). Overall, results were in agreement for 69 of 88 fish tested by both assays (78.4%, Cohen's kappa coefficient = 0.55). For the experimentally exposed fish alone, 29.6% were positive by cELISA and 33.3% were positive by PNT. Agreement between the tests was 88.9% (24 of 27 fish) (Cohen's kappa coefficient = 0.74). Of the observations that were in agreement, 7 of 24 fish (29.2%) were positive by both assays and 17 of 24 fish (70.8%) were negative by both assays. For the wild muskellunge from Lake St. Clair and Lower Fox River, 41 fish (67.2%) were positive by cELISA and 53 fish (86.9%) were positive by PNT. Agreement between the tests for wild muskellunge was 73.8% (45 of 61)(Cohen's kappa coefficient = 0.3). For the samples that agreed, 39 of 45 fish (86.7%) were positive by both assays and 6 of 45 fish (13.3%) were negative by both assays. There were several sera from experimentally challenged and wild muskellunge that were positive by only one assay. Of results that disagreed, 16 fish were PNT positive/cELISA negative and 3 fish were PNT negative/cELISA positive. Over half (56.3%) of the sera that were PNT positive/cELISA negative were from muskellunge sampled from the Lower Fox River in May 2011. There was some correlation between neutralizing titers and cELISA percent inhibition values. The Spearman correlation coefficients were 0.80 ($P < 0.0001$) and 0.6 ($P < 0.0001$) for experimentally exposed and wild fish, respectively.

DISCUSSION

In the current study, we developed a cELISA for the detection of antibodies in sera of fish previously exposed to VHSV-IVb under experimental and natural (field) conditions. Competition and inhibition ELISAs are used to quantify a substance (in our case, serum antibodies to VHSV-IVb) by measuring its ability to interfere with an established pre-titrated direct or indirect ELISA system (Crowther 2000). In our assay, microplates coated with purified VHSV-IVb were used as the solid phase. Fish test sera and rabbit serum (as a source of polyclonal antibody to VHSV-IVb) were added sequentially and bound rabbit immunoglobulin was then detected with a commercially available enzyme-labeled conjugate. When optimizing reagent concentrations, we found that inhibition of the rabbit antiserum was most apparent when the purified virus was used at a non-saturating concentration (1.0 $\mu g \cdot mL^{-1}$) and when the maximum absorbance in the absence of inhibition was approximately 1.0 OD. In wells that previously received fish serum containing antibodies to VHSV-IVb, the polyclonal antibody was effectively outcompeted (or blocked) from binding, thus inhibiting enzymatic color change in those wells. This variation of the competitive assay is sometimes referred to as a blocking ELISA (Crowther 2000, Jordan 2005). Expressed on a scale of 0 to 100 percent inhibition of the competing antibody, results are proportional to antibody activity in the test sample (Wright et al. 1993). The presence of immunoglobulins in several sera that produced inhibition was confirmed in preliminary trials using an indirect ELISA system with a monoclonal antibody produced against muskellunge immunoglobulin M (Kaattari et al., unpublished). The main reason for developing a competitive ELISA was however to provide a test that could be used on multiple Great Lakes fish species, even on the

same plate, without the need for developing species-specific immunoglobulin reagents as would be required for indirect ELISAs. Application of the assay for testing antibodies in other species was beyond the scope of this initial study.

Fish sera were heat-treated (Dixon et al. 1994, Kibenge et al. 2002) and pre-incubated with nonfat milk, as suggested by Kim et al. (2007), and purified virus was used to coat plates to minimize the possibility for false positive reactions. These measures were undertaken since some untreated sera from normal fish can exhibit high levels of non-specific binding (Olesen et al. 1991, Kibenge et al. 2002, Kim et al. 2007). Using the aforementioned conditions, sera from 60 juvenile muskellunge reared in a hatchery setting and considered to be naïve to VHSV-IVb (e.g. never exposed and never infected) were tested by the cELISA. Most sera produced inhibition values less then 5% indicating only a low level of non-specific binding. With these data, we established a threshold (14.6% inhibition) above which, inhibition would be unlikely to be due to non-specific binding. For the purposes of this study, we considered sera with inhibition ≥14.6% to be positive for antibodies to VHSV-IVb. While the threshold provided a means for us to interpret our results, we recognize that serological thresholds are often dynamic and can be biased based on extrinsic (e.g. diet, presence of other pathogens) and intrinsic factors (e.g. species, age, sex) of the host, as well as by laboratory testing methods. A large number of sera of known antibody-negative and antibody-positive status would be needed in order to refine the threshold in the future; however, this is

difficult in the absence of a true gold standard reference test for determining VHSV-IVb seropositivity.

Using sera from experimental trials, we determined that the cELISA could detect antibodies to VHSV-IVb in some fish that had been previously exposed to the virus. The threshold provided a clear distinction between seropositive and seronegative fish. Antibodies were first detected in fish sampled 7 weeks post-immersion exposure and the highest percentage of seropositive fish were detected later, between 11 and 15 weeks PI. Low levels of inhibition could still be distinguished in sera from another group of muskellunge that was experimentally challenged approximately one year prior to sampling. It is important to consider that not all fish exposed to the virus by immersion necessarily became infected, and furthermore, not all infected fish necessarily seroconvert.

When the cELISA was applied to wild populations of muskellunge, VHSV-IVb antibodies were detected in three of five populations tested. Muskellunge from two populations (Butternut Lake, WI and Lake Hudson, MI) were negative for antibodies based on the threshold. Muskellunge from both locations were expected to be seronegative as VHSV has never been detected from fish from these locations. Antibodies were detected in 60 to 90% of muskellunge from Lower Fox River sampled in 2010 and 2012 and in 80 to 90% of fish from Lake St. Clair sampled in 2010 and 2011. These two locations were considered enzootic for VHSV-IVb. The virus has been present in Lake St. Clair since at

least 2003 and in Green Bay and its connected waters since 2007 (reviewed in Faisal et al. 2012). Prior to sampling, VHSV-IVb was last confirmed from fish in these systems in the spring and early summer 2009. Seroconversion may be due to recent exposure(s), indicating the persistence of the virus in these populations. Alternatively, given the longevity the muskellunge, it is possible that fish were survivors from the last known occurrence of VHSV in these areas (2009). At the time of sampling, fish may have been fully recovered or may have been asymptomatic carriers of the virus, perhaps in tissues not sampled or at levels below detection limits of cell culture isolation. The high seroprevalence detected in fish from Lake St. Clair and Lower Fox River likely indicates that some immunity has been established to VHSV-IVb in these populations. While the association between virus neutralizing antibodies and protective immunity against fish rhabdoviruses is well established (reviewed in Lorenzen & LaPatra 1999, Purcell et al. 2012), less is known about the role of binding antibodies. Binding antibodies in general function to opsonize pathogens for removal by phagocytes.

Antibodies were also detected in wild muskellunge from Thornapple Lake. Approximately 50% of muskellunge were seropositive in 2008 and 30% of fish were seropositive in 2010. Thornapple Lake is an inland lake in Michigan that was not considered to be positive for VHSV-IVb based on previous testing by virus isolation of reproductive fluids and/or sera from 60 muskellunge from this population collected yearly since 2006. The finding that fish in Thornapple Lake had prior exposure to VHSV-IVb was not surprising given this lake's high angler use and proximity to VHSV-IVb

positive regions of the Lake Michigan watershed. Although the presence of cross-reactive antibodies could be an alternative explanation for this finding, this is unlikely given that no other fish rhabdoviruses are known to occur in Michigan. Our results suggest that the use of serological tests for detecting VHSV-IVb antibodies in fish sera can increase the likelihood of detecting, indirectly, the presence of the virus in a population (or water system) in the absence of clinical disease outbreaks. Given the longevity of antibodies compared to the time in which the virus can be isolated, the likelihood of detecting VHSV exposure based on antibodies could be expected to be higher than the likelihood of detecting the virus itself on most sampling occasions, the exception being when fish are sampled during outbreaks of clinical disease. This was shown recently for cultured rainbow trout naturally infected with VHSV-I in Denmark (Schyth et al. 2012). This has especially important implications when clinically healthy fish are being tested, as is done for surveillance purposes. In fish that have high levels of circulating antibodies, virus may be cleared even more rapidly from tissues following infection, further decreasing the time-frame in which VHSV could be isolated.

In the absence of providing preliminary estimates of diagnostic sensitivity and specificity, due to the fact that we do not have a gold standard reference test for determining true antibody status, Cohen's kappa was used to compare agreement with the PNT. An overall kappa value of 0.55 indicated there was a moderate level of agreement beyond chance in the assays' ability to differentiate seropositive and seronegative muskellunge (Landis & Koch 1977). Agreement was not necessarily

expected due to differences in the types of antibodies each assay measures and this has been discussed in the context of several fish rhabdoviruses (Olesen et al. 1991, Ristow et al. 1993, Smail & Snow 2011). While the PNT measures antibodies directed against neutralizing epitopes of viral glycoproteins, immunoassays measure binding antibodies that could react with any exposed virus proteins (e.g. nucleoprotein, matrix protein). Given the greater diversity of antibodies theoretically detectable by the cELISA, it is interesting that the majority of discrepant results in this study came from sera that were actually PNT positive/ cELISA negative and this requires further study. Binding antibodies have been found to persist longer than neutralizing antibodies in sera of rainbow trout following experimental infection with VHSV-I (Olesen et al. 1991, Encinas et al. 2011). This was not evident in the current study for muskellunge following experimental infection with VHSV-IVb for the time period tested (1-15 weeks PI) but it is possible that an extended sampling period or sample size would have revealed less agreement and correlation. Less agreement and correlation was detected for wild fish and this could reflect differences in sampling time in relation to exposure time and number of exposures. Further studies are needed to characterize the types of antibodies detected by the cELISA and the kinetics of production and duration of neutralizing and binding antibodies against VHSV-IVb in Great Lakes fish.

The cELISA has the potential to be a useful, non-lethal research and/or surveillance tool to detect fish that have survived infection with VHSV-IVb. Compared to the PNT, the cELISA assay has a faster turn-around time for results and is easier to perform and less

costly in terms of both technician time and materials. We suspect the further use of the cELISA as a supplement to VHSV-IVb surveillance efforts in the Great Lakes will reveal a wider distribution of the virus than is currently known.

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APPENDIX

Figure 5.1. Map of Michigan and Wisconsin, USA showing sites where sera were collected from muskellunge (*Esox masquinongy*) during health surveys of wild fish, 2005 to 2012. Fish sampled from the Anchor Bay and Detroit River sites are collectively referred to as Lake St. Clair, MI muskellunge in this study. Muskellunge sampled from the Lower Fox River, WI are primarily residents of Green Bay waters of Lake Michigan except during spawning.

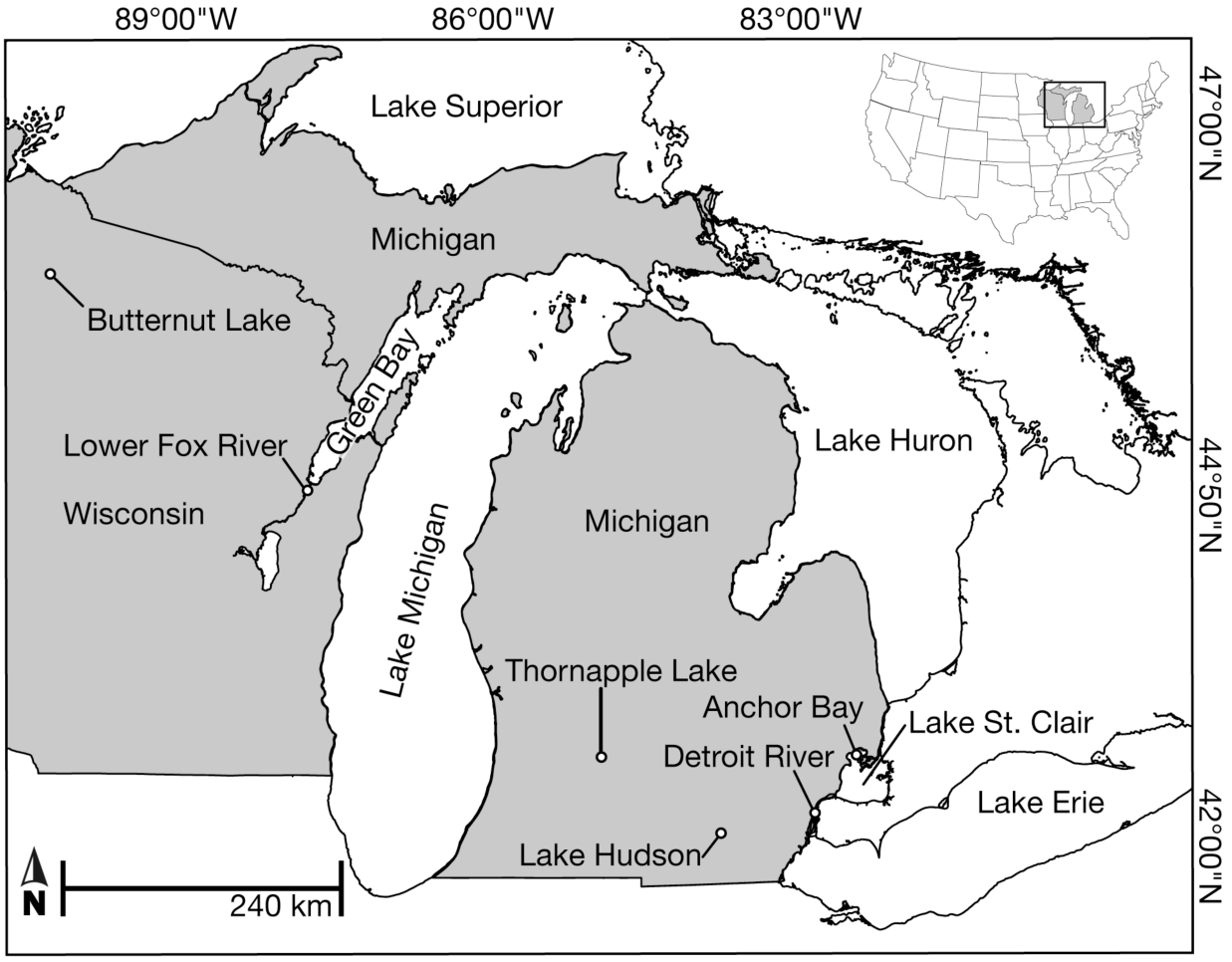

Figure 5.2. Distribution of cELISA percent inhibition values from sera of unexposed muskellunge (n = 60) that were used to establish a positive-negative threshold of 14.6 % inhibition (dashed line).

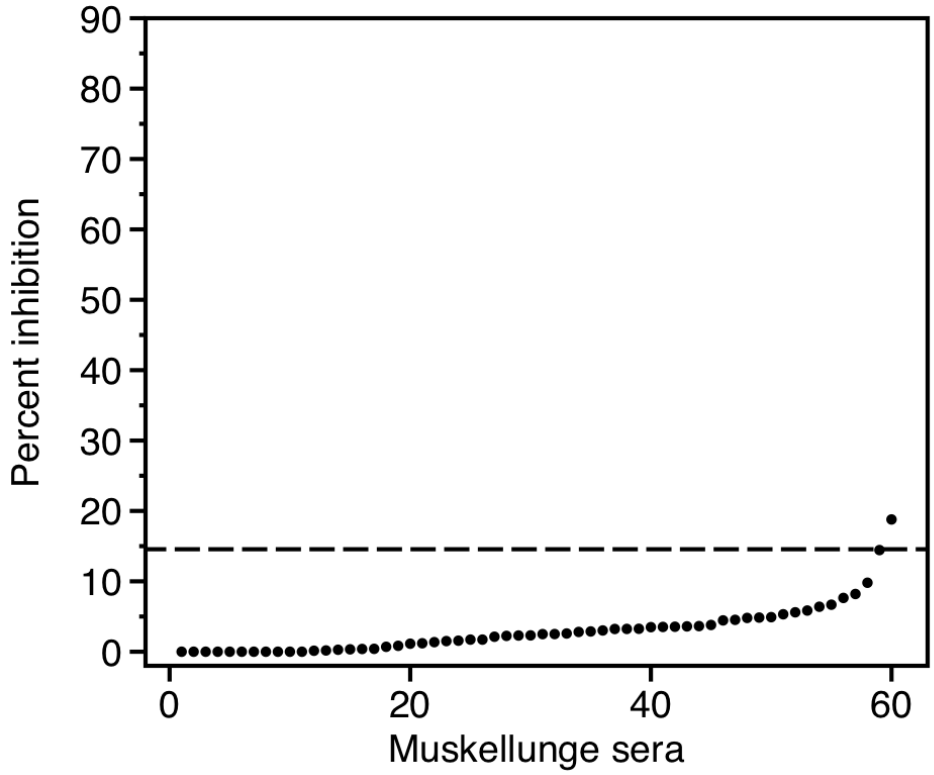

Figure 5.3. Percent inhibition values by cELISA of muskellunge (n = 27) sampled between 1 and 15 weeks after immersion challenge with VHSV-IVb. Samples with percent inhibition values ≥14.6 (dashed line) are positive for antibodies to VHSV-IVb. Horizontal lines represent the mean percent inhibition.

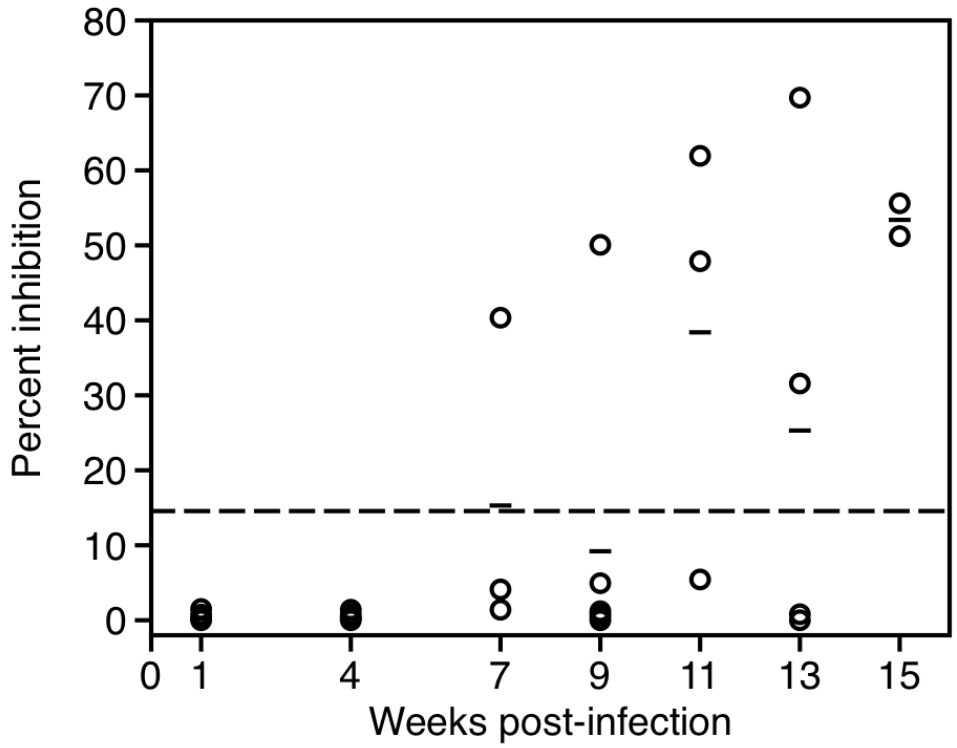

Figure 5.4. Distribution of percent inhibition values by cELISA of sera from 200 wild muskellunge by sampling location and year. Samples with percent inhibition values ≥14.6 (dashed line) are positive for antibodies to VHSV-IVb.

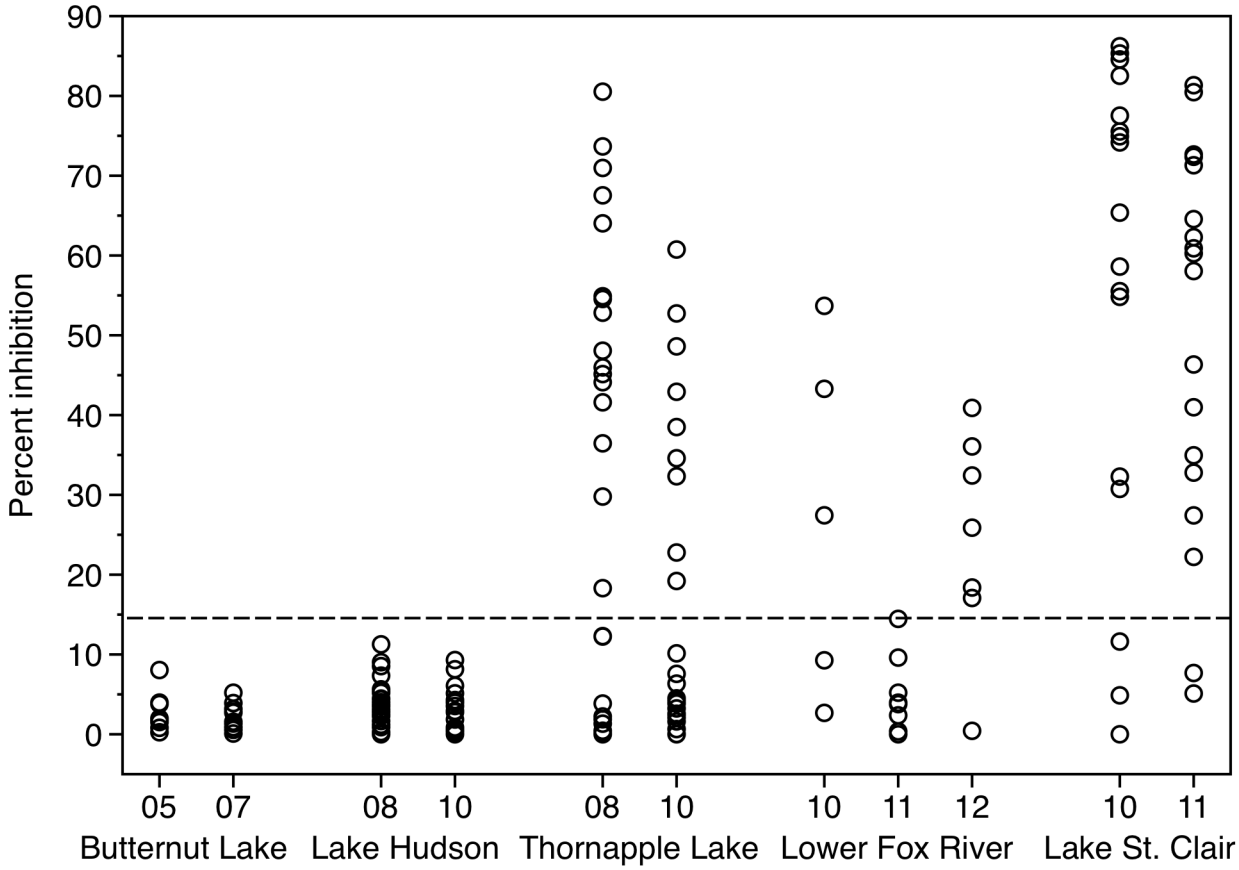

Figure 5.5. Comparison between cELISA and 50% plaque neutralization test (PNT) using sera from 38 wild muskellunge from a Lake St. Clair, Michigan (circles), 23 muskellunge from Lower Fox River, Wisconsin (diamond) and 27 experimentally exposed muskellunge sampled 1-15 weeks post-exposure (triangles). Titers ≥14.6 percent inhibition (cELISA) and ≥160 (PNT) are considered positive for VHSV-IVb antibodies. In order to display all titers, PNT titers were arbitrarily transformed by adding two and resulting values plotted on a logarithmic scale.

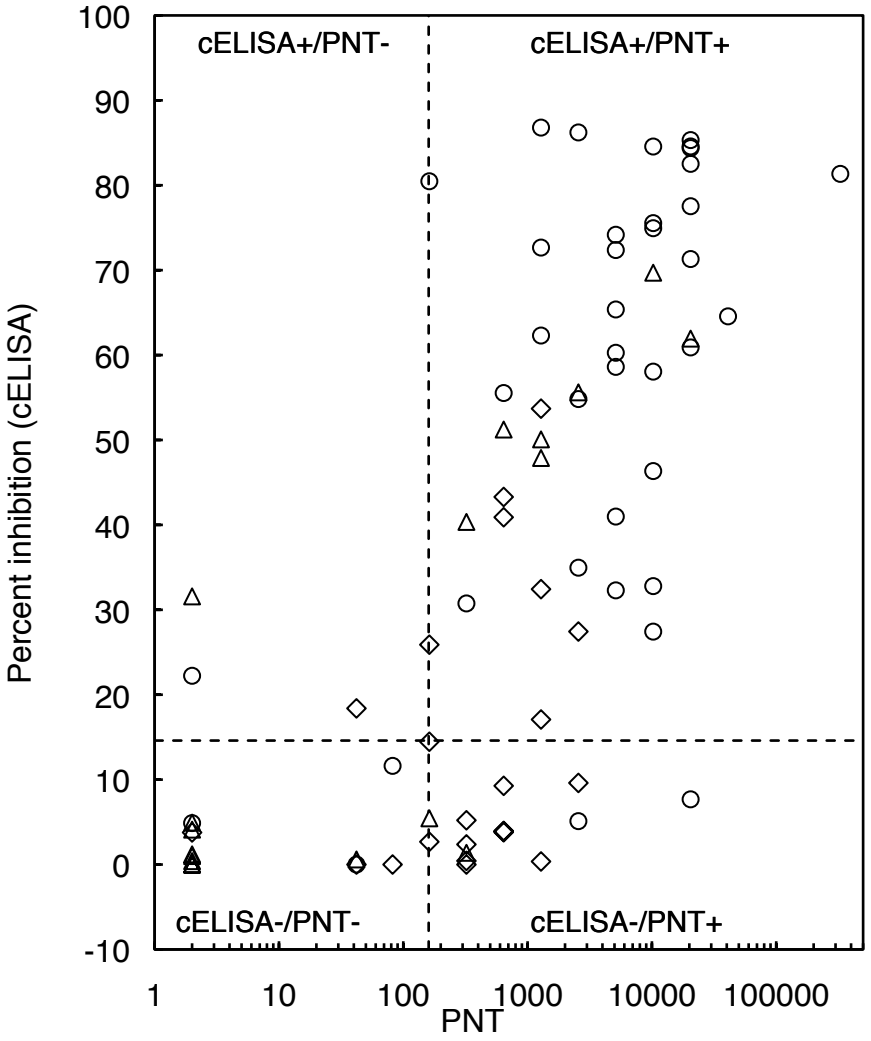

Table 5.1. Competitive ELISA results for 200 free-ranging muskellunge shown by location and sampling event. Fish were sampled between 2005-2012 from water bodies in Michigan (MI) and Wisconsin (WI). Sera with percent inhibition values ≥14.6 by cELISA are positive for antibodies to VHSV-IVb. Tissues and/or non-lethal samples (ovarian fluid, sera) from all fish were negative for VHS virus by cell culture isolation. A positive VHSV-IVb status indicates that the virus had been isolated from fish from that water system prior to the first sampling event. Confidence intervals (CI) were calculated using R (R Core Team 2012). SD = standard deviation

					Percent inhibition		
Location	VHSV-IVb status	Sampling event	No. of fish	% cELISA positive (95% CI)	Mean positive (SD)	Overall mean (SD)	Range
Butternut Lake, WI	Neg.	2005, Apr 2	8	0	-	2.8 (2.5)	0.3 - 8.1
		2007, Apr 24	10	0	-	2.2 (1.6)	0.1 - 5.2
Lake Hudson, MI	Neg.	2008, Apr 11	30	0	-	3.3 (2.9)	0.0 - 11.3
		2010, Apr 7	30	0	-	2.4 (2.9)	0.0 - 9.8
Thornapple Lake, MI	Neg.	2008, Apr 11	30	53.3 (35.8 - 70.1)	51.8 (16.7)	28.8 (27.9)	0.0 - 80.5
		2010, Mar 31	30	30.0 (16.4 - 48.3)	39.2 (13.6)	13.6 (18.6)	0.0 - 60.8
Lower Fox River, WI	Pos.	2010, Apr 28	5	60.0 (20.4 - 90.0)	41.5 (13.2)	27.3 (21.7)	2.7 - 53.7
		2011, May 10	12	0	-	4.0 (4.3)	0.0 - 14.5
		2012, May 1	7	85.7 (41.9 - 98.0)	28.5 (9.7)	24.5 (13.8)	0.4 - 40.9
Lake St. Clair, MI	Pos.	2010, May 5-19	18	83.3 (59.1 - 94.5)	68.2 (18.3)	57.7 (29.3)	0.0 - 86.2
		2011, May 26-Jun 3	20	90.0 (67.6 - 97.5)	58.9 (20.3)	53.6 (25.1)	5.1 - 86.8

Table 5.2. Percent inhibitions of muskellunge testing positive (≥14.6% inhibition) for VHSV-IVb antibodies by cELISA on at least one time point sampled. Fish were challenged at 0 and 22 weeks with VHSV-IVb by immersion and blood was sampled from 22 survivors 67 and 79 weeks post-infection (45 and 57 weeks post-second exposure). SD = standard deviation

	Percent inhibition	
Fish number (no.)	67 weeks (45 weeks)	79 weeks (57 weeks)
1	14.7	17.9
2	16.3	18.2
3	23.4	29.2
4	33.2	33.4
5	20.6	23.6
6	31.5	29.8
7	9.7	21.5
8	28.4	46.1
9	11.2	25.1
10	11.4	16.3
No. seropositive (%)	7 (31.8 %)	10 (50.0 %)
No. fish total	22	20[a]
Mean positive (SD)	24.0 (7.3)	26.1 (9.0)

[a] No blood could be collected from 2 fish

CHAPTER 6

VACCINATION WITH A PLASMID CONTAINING THE VHSV-IVB GLYCOPROTEIN GENE PROTECTS MUSKELLUNGE (ESOX MASQUINONGY) AGAINST CHALLENGE

INTRODUCTION

The objective of this study was to evaluate the immune response of fish following vaccination with a new DNA vaccine against the Great Lakes strain of viral hemorrhagic septicemia virus (VHSV). The Great Lakes VHSV (genotype IV, sublineage b) is a reportable fish virus that has emerged in the Great Lakes region of North America within the past decade (reviewed in Kim and Faisal 2011a,b). The virus has been responsible for large-scale mortality events of wild fish. A number of subsequent laboratory trials have confirmed the pathogenicity of this isolate in a broad range of freshwater fish hosts, many of which are important from both an ecological and economical standpoint to Great Lakes fisheries (Al-Hussinee et al. 2010, Kim & Faisal 2010b, Goodwin & Merry 2011). Strict biosecurity measures and a limited distribution of VHSV in North America have restricted the virus to wild populations. Concerns regarding effects of VHSV-IVb on wild fish populations as well as the potential introduction of VHSV-IVb into Great Lakes hatcheries have prompted this investigation. Muskellunge have a high susceptibility to VHSV-IVb and have an important role as a top predator in Great Lakes ecosystems (Kim & Faisal 2010c). Vaccination could provide a level of immunity in stocked fish.

VHSV is a member of the genus *Novirhabdovirus* (family *Rhabdoviridae*) along with several other fish pathogenic viruses such as infectious hematopoietic necrosis virus (IHNV), Hirame rhabdovirus, and Snakehead rhabdovirus (Dietzgen et al. 2012). DNA vaccines encoding the immunogenic glycoprotein (G) of VHSV (genotype I) and IHNV

are highly efficacious in conferring protection to salmonids following severe virus challenge (Anderson et al. 1996, Lorenzen et al. 1998, Corbeil et al. 2000, Lorenzen et al. 2000, Lorenzen et al. 2002, Lorenzen & LaPatra 2005, Kurath et al. 2007). The surface G protein is one of six proteins encoded by the single-stranded RNA genome of fish novirhabdoviruses (Dietzgen et al. 2012). The surface G proteins of VHSV and IHNV initiate host cell infection and induce production of protective and neutralizing antibodies (Engelking & Leong 1989, Lorenzen et al. 1990, Bearzotti et al. 1995, reviewed by Coll 1995). The basic VHSV and IHNV DNA vaccine construct consists of the G gene inserted downstream of the CMV promoter in the eukaryotic expression vector pCDNA3.1 (Invitrogen).

DNA vaccines in salmonids induce cellular and humoral immunity (reviewed in Kurath et al. 2007, Purcell et al. 2012). A robust innate immune response is evident within days after vaccination that is cross-protective against other fish viruses. Specific immunity develops within several weeks and is characterized by high efficacy, loss of cross-protection, and development of neutralizing antibodies. Protection 1-2 years after vaccination after neutralizing antibodies have declined suggests establishment of long-term immunological memory. In the current study, the efficacy of a VHSV-IVb DNA vaccine was tested in muskellunge. We also measured neutralizing and binding antibodies to assess the adaptive, humoral response to vaccination. In a follow up study, the ability of the construct to induce an innate, antiviral response was tested in rainbow trout (*Oncorhynchus mykiss*) following an early challenge with IHN virus.

MATERIALS AND METHODS

Ethics statement. Michigan State University (MSU) Institutional Biosafety Committee approved protocols involving recombinant nucleic acid molecules according to guidelines required by the National Institute of Health (registration #3280). Live fish experiments at MSU were designed and carried out with oversight by MSU's Institutional Animal Care and Use Committee (AUF # 09/10-140-00 and 02/10-013-00).

Construction of eukaryotic expression plasmid pVHSivb-G. A plasmid encoding the glycoprotein (G) gene (GenBank # GQ385941) of the VHSV-IVb isolate MI03 (Elsayed et al. 2006) was used. The design of the construct was based on DNA vaccines against VHSV genotype I and IHNV (Anderson et al. 1996, Heppell et al. 1998, Lorenzen et al. 1998). Plasmid construction, replication, and purification were outsourced as a custom project to Life Technologies. Briefly, the full-length open reading frame of the VHSV-IVb (G) gene (1524 bp) was assembled from synthetic oligonucleotides and/or PCR products. An EcoRI restriction site sequence followed by a kozak consensus sequence ending in the initial Met (start) codon of the G gene was added to the 5' end. An XbaI restriction site sequence was added following the termination codon of VHSV-G. The fragment was cloned into the eukaryotic expression vector pcDNA_3.1+ (Invitrogen) using EcoRI and XbaI restriction sites. The resulting plasmid is hereafter referred to as pVHSivb-G following the naming scheme of other fish

rhabdoviral DNA vaccines. The plasmid was replicated in *Escherichia coli* K12 cells and subsequently purified out for use as the vaccine. The pcDNA_3.1+ vector without the G gene insert was replicated in *E. coli* cells for use as a plasmid control (or mock) vaccine. The pDNA purity and concentration for both plasmid preparations were determined by UV spectroscopy. DNA sequencing of the plasmid confirmed the correct sequence and orientation of the insert. The plasmid DNA was diluted to a concentration of 1 mg mL^{-1} in sterile phosphate buffered saline (PBS) and stored at -80°C until use.

Muskellunge vaccination and challenge. Juvenile muskellunge were obtained outside the Great Lakes basin from the Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, IA) and reared to experimental size at the University Research Containment Facility, Michigan State University, East Lansing, MI. Fish were fed live fathead minnows (*Pimephales promelas*) obtained outside the basin and tested to be free of VHSV and other reportable viruses. This same cohort of muskellunge was used for the pilot virus challenge and vaccination trials to assess protection and development of antibodies (Figure 6.1). Tanks received single-pass water and water temperature was maintained at 11 ± 1°C.

To analyze the effect of vaccination on survival following lethal VHSV-IVb challenge, a protection trial was carried out. Two treatments (vaccination with pVHSivb-G or the empty plasmid) were assigned to 3 tanks each. Muskellunge (n = 180) were randomly assigned to tanks so that each tank had 30 fish. Fish ranged in weight from 40 to 70 g

(mean = 56 g, SD = 9) but each tank had an approximately equal total biomass. Fish were given several days to acclimate to the experimental tanks prior to vaccination. On day 0, fish were anesthetized by immersion in water containing 0.1 g L^{-1} tricaine methanesulfonate (MS-222) and 0.3 g L^{-1} sodium bicarbonate. Each fish was given a single intramuscular injection of 10 μg of the pVHSivb-G vaccine or 10 μg of the empty plasmid in a volume of 50 μl sterile PBS. The vaccine was administered in the right dorsal epaxial muscle, anterior to the dorsal fin.

Seven weeks later (539 degree days), fish were challenged by immersion with 10^5 pfu mL^{-1} VHSV-IVb. The MI03 isolate from muskellunge (Elsayed et al. 2006) was used. The virus was grown in the *epithelioma papulosum cyprini* (EPC) cell line from the fathead minnow (ATCC® CRL-2872) and titer determined by plaque assay (Batts & Winton 1989). Fish from each tank were challenged in separate glass aquaria containing 15 L of chilled, aerated water to which the virus had been added. The density of fish in each challenge aquaria was approximately 112 g or 2 fish L^{-1}. The immersion challenge lasted 90 min and fish were returned back to their respective tank. Fish were monitored for a period of 28 days during which morbidity was selected as an end-point whenever possible. Mean cumulative percent mortality (CPM) of triplicate tanks of each vaccine treatment were used to calculate relative percent survival (RPS) according to

the following formula: RPS = [1-(average CPM of pVHSivb-G tanks/average CPM of plasmid control tanks)] × 100 (Amend 1981).

After 28 days post-challenge (PC; 11 weeks PV) surviving fish were euthanized using an overdose of MS-222 (0.25 g L^{-1}) buffered with sodium bicarbonate. Tissues were collected from all fish for virus titration. A blood sample was also collected from all fish and a subset, representing 85% of survivors, was tested for antibodies to VHSV-IVb. The subset tested included all plasmid control fish (n = 17) and an approximately equal number of pVHSivb-G survivors (n = 18; 6 fish selected at random from each triplicate tank).

The dose that was used for challenge of vaccinated fish (10^5 pfu mL^{-1}) was determined in a pilot challenge (Figure 6.1) to account for the possibility that older and/or larger muskellunge might have a lower VHSV-IVb susceptibility. Groups of 5 fish each were challenged by immersion with doses ranging from 0 to 10^7 pfu mL^{-1} and mortality recorded for 28 days (Table 6.1).

Quantification of VHSV-IVb in tissues by plaque assay. The mixed sample of kidney, spleen, heart and liver tissue collected from mortalities and survivors [28 days post-challenge (PC)] was used for virus titration by plaque assay. Tissues were stored at -80°C until testing. Tissues were diluted 1:10 (weight: volume) in minimum essential

medium (MEM) with Earle's salts supplemented with 10% tryptose phosphate broth (TPB), 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 100 μg mL^{-1} gentamicin sulfate, and 2.5 μg mL^{-1}amphotericin and adjusted to pH 7.5 with 1 M tris buffer. Tissues were homogenized in a stomacher on high speed for 3 min. Homogenate was transferred into a tube and centrifuged (2,500 × g, 30 min, 4°C). Tissue supernatant was transferred to a 1.5 mL microcentrifuge tube and a 10-fold serial dilution series was performed in tissue culture media. Tissue culture media, hereafter referred to as MEM-5-T, was Earle's salt-based MEM with 10% TPB, 5% fetal bovine serum, 2 mM L-glutamine, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 100 μg mL^{-1} gentamicin sulfate, 2.5 μg mL^{-1}amphotericin and 12 mM tris buffer. Tissue supernatant and three dilutions of each sample were plaque-assayed on confluent EPC cells grown in flat bottom 24-well plates. Briefly, media was removed from cell monolayers and cells were pre-treated for 10 min with 200 μL well $^{-1}$ of 7% polyethylene glycol (PEG; 20,000 MW; Batts and Winton 1989). The PEG was a 7% solution in tissue culture media (MEM-5-T). Samples were inoculated in a volume of 100 μL well $^{-1}$ and incubated for 30 min at room temperature. Finally, 1 mL $well^{-1}$ methylcellulose overlay (0.75% in 2X concentrated MEM-5-T) was added to restrict the spread of the virus. Plaque assays were incubated at 15°C for 6 days and then fixed and stained with 1% crystal violet in 50% formalin. Virus titers are calculated as pfu $gram^{-1}$ of tissue. The lower and upper detection thresholds were 100 pfu $gram^{-1}$ and 3.0×10^{7} pfu $gram^{-1}$ tissue, respectively.

Detection of an adaptive humoral immune response. A serological analysis of vaccinated, unchallenged muskellunge was conducted in parallel in order to determine if protection observed in the challenge study might be due to induction of an adaptive, humoral immune response (Figure 6.1, antibody response trial). Ten muskellunge per treatment (pVHSivb-G or plasmid control) were vaccinated on the same day and same manner as described above and fish were held in replicate tanks of 5 fish each. Blood samples were collected non-lethally from each fish on week 0 (prior to vaccination) and at 7 and 11 weeks post-vaccination (PV). We selected larger fish (75 g) for this group intentionally to increase the likelihood we could collect blood non-lethally. Another small group of vaccinated muskellunge was held in a divided tank to serve as supplementary or replacement blood donors in the case that we were unable to obtain sufficient quantity of blood from an individual. On average, blood was drawn from 2 of these additional fish per treatment at each time-point. Blood samples were kept at 4°C overnight prior to centrifugation (2500 × g, 20 min, 4°C). Sera were stored at 80°C until testing.

Antibody testing. Samples were tested for VHSV-IVb neutralizing G antibodies by 50% PNT on EPC cells as previously described (Millard & Faisal 2012b). Pooled serum from naïve lake trout (*Salvelinus namaycush*) was used as a source of complement in the assay. Positive and negative fish sera controls were included each day the assay was performed. Neutralizing antibody titers are reported as the reciprocal of the highest

serum dilution causing at least a 50% reduction of virus plaques compared to negative control sera. Sera were tested in 2-fold serial dilutions from 1:20 to 1:640. Neutralizing titers ≥160 are considered to be positive for neutralizing antibodies (Millard & Faisal 2012b).

Sera were also tested for binding antibodies using a VHSV-IVb competitive enzyme-linked immunosorbent assay (cELISA) as described in Millard et al. (in press). The assay uses a polyclonal rabbit antiserum to compete with fish sera for purified VHSV-IVb binding sites. Antibody results are reported as percent inhibition according to the formula: [1- (Average $OD_{405/490}$ fish serum / Average $OD_{405/490}$ rabbit serum control)] × 100. Percent inhibition ≥ 14.6 are positive for antibodies to VHSV-IVb.

Study 2: Rainbow trout vaccination and cross-protection challenge. To assess the efficacy of the pVHSivb-G DNA vaccine to confer early antiviral immunity, a challenge study was carried out in S. LaPatra's laboratory. Rainbow trout (4 g) were vaccinated with 1 μg of the pVHSivb-G vaccine, empty plasmid (plasmid control), or the Apex-IHN® vaccine (Aqua Health Ltd). Another group of the same size fish were vaccinated with only PBS (negative control). Water temperature was maintained at approximately 14.5°C. On 7 days PV (100 degree days), duplicate groups of 25 fish each from each treatment were challenged by immersion with a lethal dose (10^5 pfu mL^{-1}) of IHNV (strain 220-90; LaPatra et al. 1994). The PBS-injected control group was not challenged

at this time. Mortalities were recorded for a period of 28 days at which point survivors were euthanized. For comparison, the same challenge trial and 28 day observation period was repeated with different fish at a later time-point (28 days, 400 degree days PV).

Data analysis. Survival, prevalence percentages, and virus titers were analyzed as a completely randomized design with subsampling. Even though we administered the vaccine to individual fish, by virtue of fish being pooled into tanks by treatment, we could not treat fish as independent units. Instead, for the purpose of analyses, tanks were considered experimental units and fish were observational units. Tank survival rates and prevalence percentages were arcsine square root transformed and virus titer levels were $\log_e + 1$ transformed prior to analysis. Data were analyzed using the MIXED procedure in SAS Version 9.2 (SAS Institute, Inc. 2010). For the cross-protection challenges, differences in survival among the different treatments were tested using pair-wise comparisons of least-squares means. For all tests, the type-I error rate was set at 0.05.

RESULTS

Efficacy of pVHSivb-G in conferring protection to muskellunge. Muskellunge were vaccinated with 10 μg of pVHSivb-G or the plasmid without VHSV-G gene insert (plasmid control) and challenged 7 weeks PV with a lethal dose (10^5 pfu mL^{-1}) of

VHSV-IVb by immersion. Kinetics of mortality development are shown in Figure 6.2. The virus dose used was the lowest dose that resulted in ≥ 60% mortality in a pilot VHSV-IVb challenge study conducted several months prior with muskellunge of the same age and size (Table 6.1). There was a significant effect of vaccination on survival, $F_{1,4}$ = 22.71, p = 0.0089. Plasmid control tanks experienced percent mortality of 83.3%, 80.0% and 80.0% (mean = 81.1%, SE = 4.2%). Fish died between day 6 and 28 PC with a steep incline in mortality between 7 and 11 days PI that accounted for 80% of the overall mortality. The mean days until death was 10.6 days (SE = 0.5). Fish vaccinated with pVHSivb-G experienced mortality of 60.0%, 33.3% and 40.0% (mean = 44.4%, SE = 5.3%). The mean days until death was 12.4 days (SE = 1.0). The RPS of pVHSivb-G vaccinated muskellunge was 45.2% compared to plasmid-vaccinated fish.

External gross pathology of fish that died from either treatment included severely pale (white) gills, areas of diffuse and petechial hemorrhage near and inside the mouth and fins, and diffuse and ecchymotic hemorrhage on cranium and along dorsal surface. Gross pathology of internal organs included swollen and pale spleens, pale heart, hyperemia of renal, gastrointestinal and liver vessels, congested and swollen kidney tissue, and focal areas of hemorrhage of swim bladder, liver, and visceral adipose tissue.

Quantification of VHSV-IVb in tissues of mortalities and survivors. A mixed tissue sample from all mortalities and all surviving vaccinated fish (28 days PC) were titrated

for VHSV-IVb by virus plaque assay (Table 6.2). The majority of fish that died had high virus titers regardless of vaccination treatment as expected. VHSV-IVb was detected by virus plaque assay in tissues from 100% of plasmid control fish and 94.7% (SE = 3.6%) of pVHSivb-G vaccinated fish. Titers of most fish from both groups (81.8%, 90 of 110 fish) were greater than 3.0×10^7 pfu gram^{-1} which was the maximum detection limit of the assay. Of the plasmid vaccinated group, titers ranged from 4.1×10^5 to $>3.0 \times 10^7$ pfu gram^{-1}. Titers of pVHSivb-G vaccinated fish ranged from 0 to $>3.0 \times 10^7$ pfu gram^{-1}.

Surviving fish from the pVHSivb-G vaccinated group had a significantly lower infection prevalence than plasmid control fish, $F_{1,4} = 21.99$, $p = 0.0094$. Tissues of 82.4% (SE = 9.5%) of the plasmid-control vaccinated fish were virus positive. In contrast, only 4.0% (SE = 2.8%) of pVHSivb-G vaccinated fish were positive. There was also a significant difference in mean virus titer between vaccinated groups, $F_{1,4} = 28.22$, $p = 0.0060$ (Figure 6.3). The overall mean titer for pVHSivb-G vaccinated survivors was 4.2×10^2 pfu gram^{-1} (SE = 2.9×10^2 pfu gram^{-1}). Virus titers in tissues of surviving plasmid control fish ranged from 0 to 3.0×10^6 pfu gram^{-1} (mean = 3.3×10^5 pfu gram^{-1}, SE = 1.9×10^5 pfu gram^{-1}). The mean virus titer was 100 fold higher in one of the plasmid control tanks compared to the others (Figure 6.3).

Analysis of the adaptive humoral immune response to pVHSivb-G. Sera from vaccinated, unchallenged fish and challenge survivors were tested by 50% PNT and cELISA to determine if VHSV-IVb vaccination induced an adaptive humoral immune response (Table 6.3). By 7 weeks PV, that corresponded to the timing of virus challenge in the parallel study, only one of 12 pVHSivb-G vaccinated fish were seropositive (neutralizing antibody titer of 320). By 11 weeks PV, neutralizing antibodies were produced by 60.0% (6 of 10) of pVHSivb-vaccinated fish. Titers of seropositive fish were 320 (2 of 6 fish) and ≥640 (4 of 6 fish). Two fish (one per treatment) had neutralizing titers prior to vaccination, and two additional sera from the plasmid control group were neutralizing at later time points. Unfortunately there was not enough serum left to retest these four samples.

Sera from a subset of challenge survivors collected 28 days PC (11 weeks PV) was also tested for neutralizing antibodies (Table 6.4). Neutralizing antibodies were detected in 100.0% of pVHSivb-G vaccinated fish but only 11.8% of plasmid controls. Titers of pVHSivb-G vaccinated fish ranged from 160 to ≥640. Ten of the 18 seropositive fish (55.6%) had neutralizing titers ≥640. Titers of plasmid control fish ranged from 0 to 320, and the majority of fish had titers of <20 (58.8%, 10 of 17 fish).

Binding antibodies against VHSV-G protein were not detected by cELISA in any of the vaccinated, unchallenged fish at week 0, 7 or 11 PV based on the threshold of 14.6% inhibition (Table 6.3). To follow up on this result, sera were collected again from these

fish at 15 and 20 weeks PV. All sera were still negative for antibodies by cELISA (data not shown). Binding antibodies were produced by a few of the challenge survivors. Antibodies were detected in two VHSivb-G vaccinated fish and one plasmid control fish. For VHSivb-G vaccinated fish, titers ranged from 0.0 to 23.0% inhibition (mean = 4.7%, SD = 6.2%). For the plasmid vaccinated survivors, titers ranged from 0 to 21.4% inhibition (mean = 5.3%, SD = 5.4%).

***Saprolegnia* infection post-challenge.** An outbreak of *Saprolegnia* sp. occurred following virus challenge of muskellunge. The fungus was found on some moribund and dead fish between 8 and 28 days PC from 5 of 6 tanks. The number of dead fish with the observed fungus was 8 plasmid-vaccinated fish and 14 pVHSivb-G vaccinated fish. Virus titers in internal organs of most fish with external fungus were high (between 2.9×10^4 and $>3.0 \times 10^7$) with the exception of two fish that were negative. The pVHSivb-G tank with the lowest CPM (33.3%) had the highest amount of mortalities with *Saprolegnia* (9 of 10 fish). For these reasons, the fungus was not likely to be the cause of most mortality observed in this study though its involvement cannot be ruled out. Fish were treated with formalin several times in an attempt to control the infection. The fungus was not found on any vaccinated, unchallenged fish.

Study 2: Rainbow trout vaccination and cross-protection challenge. In a follow up study, juvenile rainbow trout were immunized with 1 μg of the pVHSivb-G vaccine, Apex-IHN® vaccine, or an empty plasmid and protection was evaluated following

challenge with IHN virus at several time points PV. Pair-wise comparisons of least-squares means between treatments indicated that pVHSivb-G ($t = 4.88$, $df = 3$, $p = 0.0165$) and Apex-IHN® vaccination ($t = 6.86$, $df = 3$, $p = 0.0063$) resulted in significantly lower mortality after the 7 day challenge compared to the plasmid control. Differences in survival of pVHSivb-G and IHN vaccinated groups were not statistically significant ($t = 1.98$, df = *3*, $p = 0.1416$). Percent mortality of replicate tanks with each group is shown in Table 6.5. The mean CPM of plasmid control tanks was 62.0% (SE = 6.9%). The mean CPM of the pVHSivb-G vaccinated fish was 24.0% (SE = 6.1%) and the mean CPM of Apex-IHN® vaccinated fish was 12% (SE = 4.6%). The RPS of pVHSivb-G and Apex-IHN® treatment groups was 61.3% and 80.6%, respectively.

Following the later challenge (28 days PV) vaccination protected fish only against challenge with the homologous virus as expected**.** The mean CPM of pVHSivb-G vaccinated fish was 58.0% (SE = 7.1%), which was similar to the mean CPM of the plasmid control fish (52.9%, SE = 7.1%). As such, the RPS of pVHSivb-G fish was <0. As expected, pair-wise comparisons of least-squares means indicated rainbow trout vaccinated with Apex-IHN® were significantly protected against IHN challenge compared to plasmid-vaccinated ($t = 3.47$, $df = 3$, $p = 0.0402$) and pVHSivb-G vaccinated ($t = 3.76$, df = 3, $p = 0.0328$) fish. Differences in survival between plasmid—vaccinated and pVHSivb-G treatments were not statistically significant ($t = 0.29$, $df = 3$, $p = 0.7909$). The mean CPM of Apex-IHN® vaccinated fish was 8.0% (SE = 3.9%). The

RPS of Apex-IHN® vaccinated fish was 84.9%. Only a few mortalities (< 4% CPM) occurred in unchallenged, PBS-injected fish during either trial.

DISCUSSION

DNA vaccines against fish novirhabdoviruses for salmonids are among the most efficacious DNA vaccines developed to date (Lorenzen & LaPatra 2005, Kurath et al. 2007). In typical laboratory trials of the IHNV and VHSV DNA vaccines, duplicate or triplicate groups of fish are given a single IM injection of 0.1 to 1 μg of vaccine and challenged by immersion 4-10 weeks later (reviewed in Kurath et al. 2007). A virus dose producing ≥ 60% CPM is used for challenge as this increases the validity of the RPS calculation (Johnson et al. 1982). In salmonids, DNA vaccines are highly efficacious under these circumstances, providing nearly 100% protection. In this study, we developed a similar DNA vaccine construct but containing the G gene of the emerging VHSV-IVb Great Lakes genotype. Results of challenge trials in both muskellunge and rainbow trout indicate successful uptake and/or transfection of host cells with the pVHSivb-G DNA plasmid and subsequent expression of the VHSV-IVb G protein. In muskellunge, immune responses mounted against the expressed G protein protected fish from a severe VHSV-IVb immersion challenge 7 weeks (539 degree days) later. Fish vaccinated with a 10 μg dose of pVHSivb-G had a significant, albeit moderate, level of protection (45% RPS) compared to fish vaccinated with the empty plasmid. The challenge dose used in this study resulted in 81% mortality in plasmid control fish and

this level of mortality is consistent with typical post-vaccination challenge trials of IHNV and VHSV salmonids (Kurath et al. 2007). Nevertheless it is important to note that this is a much higher dose than fish would likely encounter in the wild and as such, we expect that muskellunge would be protected to an even greater degree under natural circumstances.

The VHSV-IVb DNA vaccine primed the adaptive immune response of muskellunge for virus challenge as evident from the analysis of sera from survivors sampled 4 weeks PC (or 11 weeks PV). Neutralizing antibodies were produced by 100% of the pVHSivb-G vaccinated survivors tested and most sera had titers of 320 to ≥640. In contrast, low levels of neutralizing antibodies were detected in sera from only 12% of the plasmid control survivors, and this is consistent with an unprimed response expected by 4 weeks after VHSV-IVb challenge in this species (Millard & Faisal 2012a). The seroprevalence of pVHSivb-G vaccinated survivors was higher also compared to pVHSivb-G vaccinated, *unchallenged* fish at this same time point (11 weeks PV), indicating an anamnestic immune response to challenge and not just a primary adaptive response to vaccination.

Sera collected from vaccinated, unchallenged fish showed that neutralizing antibodies against the G protein were only beginning to develop by 7 weeks PV, corresponding to the timepoint in which fish from the protection trial were challenged. The fact that only a single fish had seroconverted based on neutralizing titers ≥ 160 may be the reason for

the moderate level of protection. However, numerous studies have found that detectable neutralizing antibodies is not necessary for protection of DNA-vaccinated salmonids following rhabdovirus challenge (reviewed in Kurath et al. 2007). Sera with low neutralizing titers (20-40), even non-neutralizing sera, from IHNV-challenged and DNA-vaccinated fish can still provide protection *in vivo* following passive transfer (Traxler et al. 1999, LaPatra et al. 1994). It is possible then that neutralizing titers <160, and even below the PNT detection limit (<20), are protective against VHSV-IVb in muskellunge as well. We consider PNT titers 160 and above to be positive for neutralizing antibodies in muskellunge sera and this is conservative compared to some other research groups. However, non-specific reactions of normal sera and/or fluctuations in titers from normal day-to-day variability can occur with this assay (Olesen & Jørgensen 1986, Millard & Faisal 2012b) making low levels of neutralization difficult to interpret. We suspect this was the cause for the neutralizing titers detected in a few of the pre-vaccination and plasmid-control sera in this study.

Although the neutralizing antibody response is often used as an assessment of protective immunity induced by DNA vaccination in salmonids, non-neutralizing (binding) antibodies and specific cell-mediated cytotoxicity are expected to be involved in protection as well (Lorenzen et al. 1998, Lorenzen et al. 2000, Kurath et al. 2007). In this study, binding antibodies were not induced by vaccination and as such were unlikely to have contributed to survival of muskellunge against challenge. Low levels of antibodies were detectable by cELISA by 4 weeks PC in sera of a few challenge

survivors from both treatment groups. We can conclude that it was VHSV-IVb infection, and not vaccination, that induced binding antibodies in these survivors. Muskellunge surviving the pilot virus challenge also produced cELISA-binding antibodies when sera were tested between 6 and 12 weeks PC (data not shown) and binding antibodies after VHSV-IVb immersion challenge has been reported previously for this species as well (Millard et al. in press). The specificity of these antibodies detectable after VHSV-IVb infection to the G protein though is unknown since whole, purified VHSV is used as the coating phase in cELISA. It is possible that there are differences in the native VHSV-IVb G protein conformation, the G protein expressed by muskellunge cells, and the G proteins on the plate after virus purification and this requires further investigation. High-titer neutralizing sera from both survivors and vaccinated, unchallenged fish were negative by cELISA confirming that at least some types of neutralizing G antibodies are not detectable by VHSV-IVb cELISA, which was unclear based on results in a previous study (Millard et al. in press).

The fact that pVHSivb-G significantly reduced viral prevalence and titers after challenge is important to consider from a fisheries management aspect. Infected muskellunge surviving VHS shed high titers of virus for several months and may reassume shedding following a stressful circumstance (Kim & Faisal 2012). The role of virus shedders in amplifying infection was clear in this study. All tanks were challenged with the same virus load, yet 4 weeks later viral tissue loads were considerably higher in fish kept together in one of three plasmid control tanks. Vaccinating fish prior to stocking in the

Great Lakes could be done in an effort to establish herd immunity among wild populations. Furthermore, it could help reduce virus transmission by decreasing viral load, and presumably the amount of shed virus, in fish that do become infected. Neutralizing antibodies likely played a role in clearing infection in pVHSivb-G vaccinated survivors. A reduced persistence of virus in tissues of VHSV DNA vaccinated fish after challenge has been reported in rainbow trout (Lorenzen et al. 2000, 2009) and Pacific herring (*Clupea pallasii*) (Hart et al. 2012).

In a follow-up study, a low dose of the pVHSivb-G vaccine protected rainbow trout against virulent IHN virus challenge at an early time point (7 days PV) suggesting induction of a robust innate immune response by this species. This result also suggests that the pVHSivb-G plasmid is functional, and mediates appropriate expression of the protein upon injection. Early phase protection after VHSV DNA vaccination in salmonids is mediated by non-specific antiviral immune mechanisms (*e.g.* interferon system) that can provide cross-protection against other fish rhabdoviruses within days PV (reviewed in Kurath et al. 2007, Purcell et al. 2012, Lorenzen et al. 1998, LaPatra et al. 2001). This cross-protection does not seem to occur in all species though based on a recent study in Pacific herring (Hart et al. 2012). It will be interesting to follow up on this aspect of the immune response in muskellunge and other species. Following the later challenge at 28 days PV, only fish vaccinated with Apex®-IHN were protected against challenge with the homologous virus. This was expected based on the transition to specific immunity

that occurs within several weeks after rhabdoviral DNA vaccination in rainbow trout (Lorenzen et al. 2002a, LaPatra et al. 2001).

The protection conferred by pVHSivb-G DNA vaccination in muskellunge was less then that conferred to salmonids by VHSV-I DNA vaccination despite a similar construct and study design. Muskellunge are evolutionarily different compared to salmonids and immune responses probably differ in some ways. Furthermore, stress may have a stronger immunosuppressive effect on species such as muskellunge that are less domesticated to aquaculture conditions compared to rainbow trout. This idea is supported by results from a recent study by Hart et al. (2012) with Pacific herring vaccinated with VHSV-genotype I DNA vaccine and challenged with VHSV-IVa, to which they are highly susceptible. Although a higher level of protection may have been observed in herring with a homologous DNA vaccine, combined with our study, it seems as if the typical response of salmonids to DNA vaccines does not necessarily apply to other fish species. It is also possible that the vaccine dose used overwhelmed the immune system leading to a suppressive effect, though other studies using similar doses on a per weight basis still observed a high level of protection (Lorenzen et al. 2000).

Live minnows fed to muskellunge were likely the source of the external *Saprolegnia* infection that occurred PC. With the exception of two fish, viral titers of fish that died with fungus were sufficient to cause mortality, suggesting viral infection was the cause of

death. We cannot rule out an effect on our results, however, since compromise of the skin barrier may have lead to re-infection from virus shed into the water. The fact that the fungal outbreak occurred post-challenge is suggestive of reduced immune function that could be due to destruction of hematopoietic and immune tissues by the virus.

Rhabdoviral pathogens represent a significant threat to the aquaculture industry in North America including the Great Lakes region. This study provides an important starting point for VHSV-IVb vaccine development and provides useful information about the antiviral immune response to DNA vaccination in a non-domesticated fish species. While no DNA vaccines have been approved to date in the U.S. for aquacultured species, the Apex®-IHN DNA vaccine was approved for commercial use as a preventative strategy against IHNV infection for cultured Atlantic salmon (*Salmo salar*) in Canada in 2005 (reviewed in Salonius et al. 2007). DNA vaccines with inducible promoters of fish origin and mechanisms capable of limiting the duration of the vaccine in tissues have been developed (Alonso et al. 2011, Alonso et al. 2003). Should the development of DNA vaccination against VHSV-IVb continue in the future, incorporation of some of these safety mechanisms would seem appropriate.

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APPENDIX

Figure 6.1. Schematic. A pilot virus challenge was first carried out with a group of 28 unvaccinated muskellunge (*Esox masquinongy*) in order to determine the dose to be used for the protection challenge (Table 6.1). On day 0, two trials were started. For the protection trial, 90 fish (3 tanks of 30 fish each) were vaccinated with the pVHSivb-G DNA vaccine and 90 fish (3 tanks of 30 fish each) were vaccinated with the empty plasmid vaccine. Fish were then challenged with 10^5 plaque-forming units mL^{-1} VHSV-IVb by immersion 7 weeks post-vaccination (PV). After 28 days, blood and tissues were collected from surviving fish for virus titration (Table 6.2) and serology (Table 6.4). For the antibody response trial, another group of fish was used. Blood was drawn non-lethally from 10-12 fish from each vaccine treatment group prior to vaccination, and on week 7 and 11 PV for serological testing (Table 6.3). Fish were not challenged at any point.

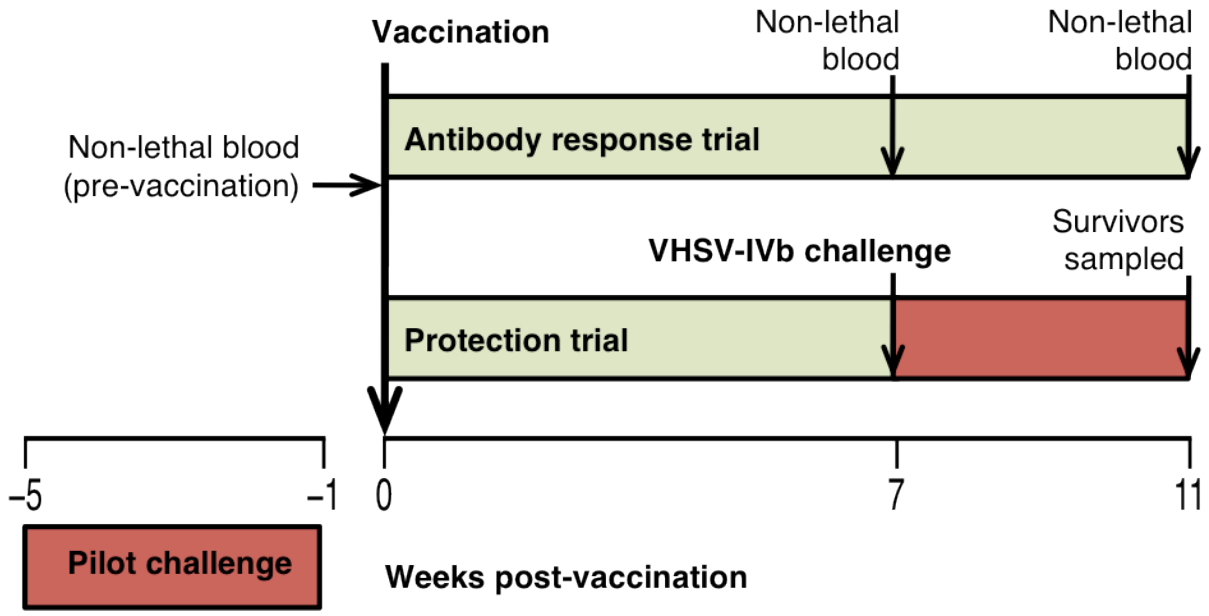
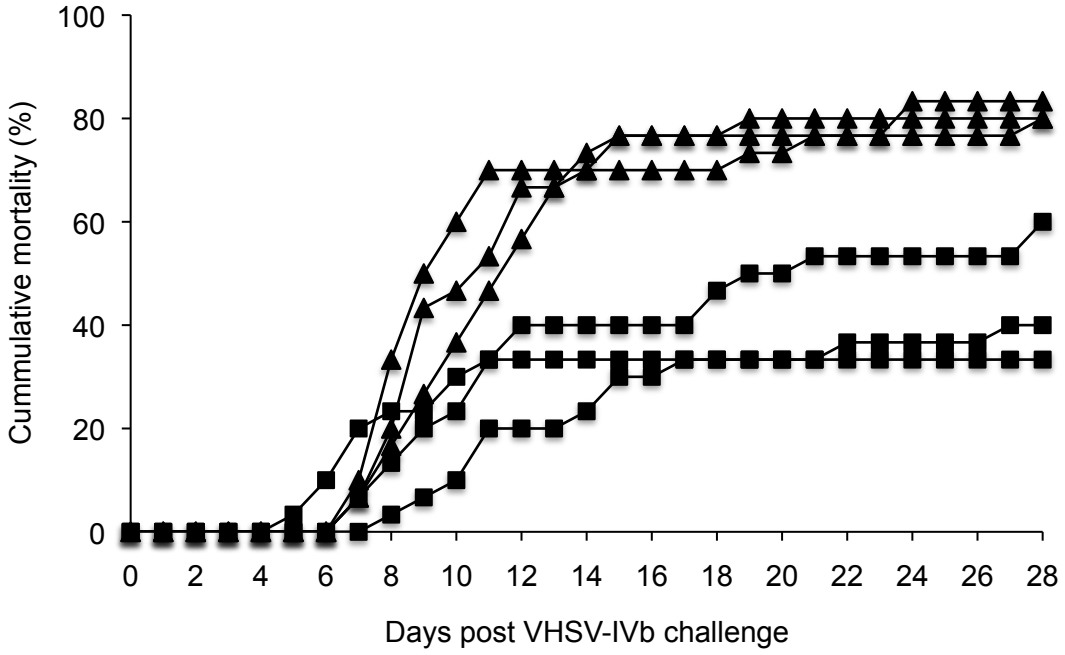


Figure 6.2. Development of cumulative percent mortality (CPM) in triplicate tanks of muskellunge (*Esox masquinongy*) vaccinated with pVHSivb-G DNA vaccine (squares) or the empty plasmid (triangles) and challenged with VHSV-IVb 7 weeks (539 degree days) post-vaccination. The day of virus challenge is considered day 0. The pVHSivb-G significantly reduced mortality compared to the control ($p = 0.0089$). The relative percent survival (RPS)[a] of pVHSivb-G vaccinated muskellunge was 45.2%.



[a] RPS = [1-(average CPM of pVHSivb-G groups/average CPM of plasmid control groups)] × 100

Figure 6.3. Virus titers in tissues of vaccinated muskellunge (*Esox masquinongy*) 28 days after virus challenge with VHSV-IVb. A-C designations represent triplicate tanks within each treatment. Titers were transformed by adding one in order to depict titers of 0 plaque forming units gram^{-1}.

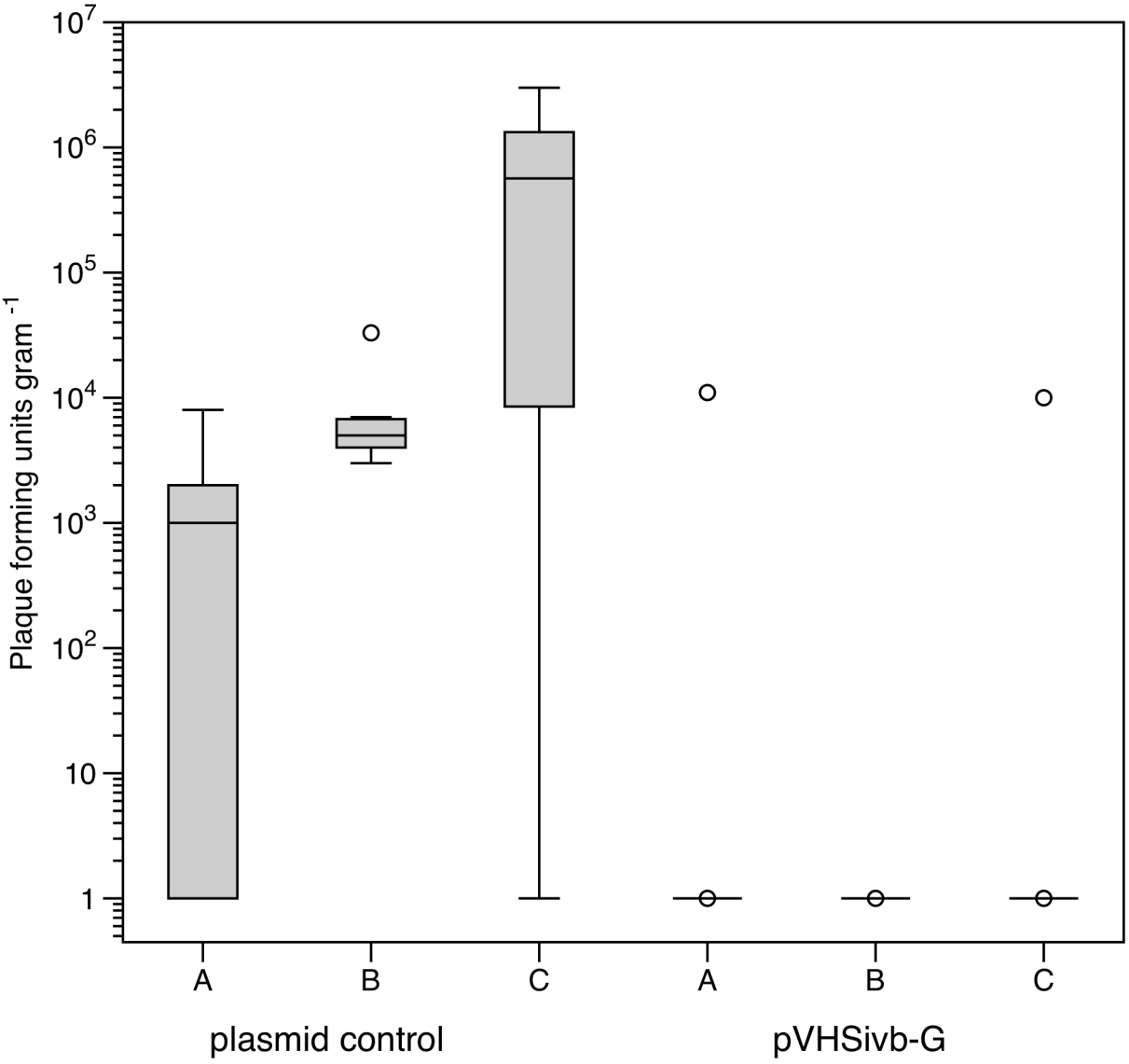


Table 6.1. A pilot study was conducted in order to determine the minimum VHSV-IVb dose that would be lethal to ≥60% of muskellunge (*Esox masquinongy*) by immersion. Five fish were infected at each dose, except for control fish (n = 3). A dose of 10^5 plaque-forming units (pfu) mL^{-1} was chosen for challenge of the vaccinated fish.

Challenge dose (pfu mL^{-1})	Percent mortality
0	0.0
10^3	20.0
10^4	40.0
10^5	100.0
10^6	80.0
10^7	100.0

Table 6.2. VHSV-IVb infection prevalence and tissue titers of mortalities and survivors. Fish were vaccinated with an empty plasmid or the pVHSVivb-G DNA vaccine and challenged with VHSV-IVb 7 weeks (539 degree days) post-vaccination. Data from survivors is from fish sampled 28 days post virus-challenge. Virus titers are in plaque-forming units $gram^{-1}$ of tissue.

	Mortalities [a, b]		**Survivors**	
	plasmid control	pVHSivb-G	plasmid control	pVHSivb-G
Infection prevalence	72/72 (100.0%)	36/38 (94.7%)	14/17 (82.4%)	2/50 (4.0%) [c]
Standard error (%)	0	3.6	9.5	2.8
Mean virus titer	$>2.7 \times 10^7$	$>2.1 \times 10^7$	3.3×10^5	4.2×10^2 [c]
Standard error	$>9.2 \times 10^5$	$>2.1 \times 10^6$	1.9×10^5	2.9×10^2
Virus titer (range)	4.1×10^5 - $>3.0 \times 10^7$	0 - $>3.0 \times 10^7$	0 - 3.0×10^6	0 - 1.1×10^4

[a] Excludes data for 3 mortalities due to improper storage.

[b] Virus titers greater than 3.0×10^7 were treated as 3.0×10^7 for calculations.

[c] pVHSivb-G vaccinated survivors had a lower infection prevalence ($p = 0.0094$) and mean tissue viral titer ($p = 0.0060$) compared to plasmid control survivors.

Table 6.3. Antibody titers of muskellunge (*Esox masquinongy*) vaccinated with pVHSivb-G or the plasmid control. Blood was drawn non-lethally from 10-12 fish from each treatment prior to vaccination (0d) and 7 and 11 weeks later. Fish were not challenged at any point with VHSV-IVb. Sera were tested for neutralizing G antibodies by 50% plaque neutralization test (PNT) and for binding G antibodies by VHSV-IVb cELISA. Parentheses indicate number of sera with the specified titer.

Treatment	No. PNT positive/no. tested [a]	Neutralizing antibody titers	No. cELISA positive/ no. tested	cELISA percent inhibition range	Mean cELISA percent inhibition (SD)
pVHSivb-G					
0d	1/12	<20 (10), 40 (1), 160 (1)	0/10	0.2 - 6.7	3.2 (2.1)
7 weeks	1/12	<20 (9), 40 (2), 320 (1)	0/10	0 - 8.4	2.7 (2.7)
11 weeks	6/10	20 (2), 40 (1), 80 (1), 320 (2), ≥640 (4)	0/10	1.2 - 6.2	3.3 (1.6)
plasmid control					
0d	1/12	<20 (10), 40 (1), ≥640 (1)	0/10	0 - 6.4	3.3 (2.1)
7 weeks	1/12	<20 (9), 40 (1), 80 (1), 160 (1)	0/10	0 - 8.4	2.0 (1.4)
11 weeks	1/10	<20 (8), 20 (1), 320 (1)	0/10	1.2 - 6.2	1.3 (1.3)

[a] 50% PNT titers of ≥160 are positive for VHSV-IVb neutralizing antibodies

Table 6.4. Neutralizing antibody titers in vaccinated muskellunge (*Esox masquinongy*) surviving challenge with VHSV-IVb. Fish were sampled 28 days post-challenge.

	pVHSivb-G	**plasmid control**
PNT Titer	Number sera (%)	Number sera (%)
<20	0	10 (58.8%)
20	0	3 (17.6%)
40	0	0
80	0	2 (11.8%)
160	2 (11.1%)	1 (5.9%)
320	6 (33.3%)	1 (5.9%)
≥640	10 (55.6%)	0
Number of fish tested	18	17
% positive (≥160)	100.0%	11.8%

Table 6.5. Cumulative percent mortality (CPM) of rainbow trout (*Oncorhynchus mykiss*) challenged by immersion 7 days (100 degree days) and 28 days (400 degree days) post-vaccination with 10^5 pfu mL^{-1} IHN virus. Number designations 1 and 2 indicate replicate tanks. Relative percent survival (RPS) = [1-(average CPM of vaccinated fish/average CPM of plasmid control fish)] × 100

	No. fish		No. dead		CPM		Mean CPM	RPS
	1	2	1	2	1	2		
7d IHNV challenge								
plasmid control	25	25	14	17	56.0	68.0	62.0	0
Apex®-IHN	25	25	4	2	16.0	8.0	12.0 [a]	80.6
pVHSivb-G	25	25	7	5	28.0	20.0	24.0 [a]	61.3
28d IHNV challenge								
plasmid control	27	24	14	13	51.9	54.2	52.9	0
Apex®-IHN	25	25	0	4	0.0	16.0	8.0 [b]	84.9
pVHSivb-G	25	25	13	16	52.0	64.0	58.0	<0

[a] Significantly less than plasmid control: Apex®-IHN ($p = 0.0063$) and pVHSivb-G ($p = 0.0165$).

[b] Significantly less than plasmid control ($p = 0.0402$) and pVHSivb-G ($p = 0.0328$).

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH

The studies performed in fulfillment of this dissertation have improved our understanding of the immune response of fish against a rhabdoviral pathogen, viral hemorrhagic septicemia virus (VHSV). Specifically, studies elucidated some kinetics of the adaptive, humoral immune response of a susceptible host, the muskellunge, to VHSV-type IVb. The combined experimental and field studies performed herein shed light on virus trafficking and how wild populations have adapted to the presence of VHSV-IVb over time. Two serological assays have been applied for the first time to the study of Great Lakes fish and VHSV-IVb. These assays, the 50% plaque neutralization test (PNT) and competitive ELISA (cELISA), will open a new horizon for future serological studies in the Great Lakes and elsewhere in North America, where the VHSV genotype IV is endemic.

CONCLUSIONS

From the experimental study detailed in Chapter 2, we concluded that muskellunge mount a pronounced neutralizing antibody (NAb) response as part of their adaptive immune defense against VHSV-IVb, and that this response can be detected by an *in vitro* method, the PNT. The immersion dose of VHSV-IVb influenced the NAb response, as well as the onset and duration of viremia. Medium and high immersion doses of virus [4×10^3 and 10^5 plaque forming units (pfu) mL^{-1}] produced a state of viremia, indicating a systemic infection, while the lowest dose of virus (10^2 pfu mL^{-1}) did not. An earlier

onset [2 days post-infection (PI) vs. 6 days] and longer duration (11 weeks vs. 6 weeks) of viremia was apparent in fish infected with high vs. medium doses. Muskellunge produced NAbs (≥160) by 5-7 weeks PI, levels peaked between 9-16 weeks and were detectable at least until 17 weeks PI. The onset of NAb production corresponded to a rapid clearance of the virus from systemic circulation in fish infected with a medium dose of the virus indicating that antibodies were critical in limiting systemic infection. The lowest immersion dose resulted in a reduced proportion of fish producing antibodies suggesting that a certain threshold of infection is required for induction of the NAb response in this species. In fish infected with a highest dose of the virus, less NAb were detected compared to the medium dose group and this could be indicative of a) extensive damage of kidney and spleen that led to a reduced ability to mount an immune response or b) NAb were not detectable because they were saturated with virus and subsequently cleared from circulation (this is a possibility especially since high dose fish remained viremic for a longer period of time). The findings of this study were indispensible for the better understanding of the host immune response against this emerging pathogen.

One objective of this study initially was to compare the relationship of isolatable virus from tissues and serum antibody levels throughout the disease course. By combining serological results of this study for the medium dose group, with virus tissue data from these same fish from a parallel study (Kim 2010), it is clear that a trend exists (Figure 7.1). During acute to chronic disease (~1-6 weeks PI), virus isolation remains superior in

detecting exposed fish. However, from the onset of antibody production through the end of the study period, there is clear trend in the proportion of fish being virus positive (in tissues) going down and the proportion of antibody-positive fish going up. Furthermore, antibodies were more consistently detected across multiple sampling periods later in the disease course compared to virus isolation. Combined with results from wild fish studies, we can conclude that combined virus isolation and serological testing would increase the diagnostic ability to detect VHSV-IVb in a given population—by being able to not only detect infected fish, but also those that are recovering from past infection, and those with established immunity.

A DNA vaccine encoding the glycoprotein (G) of VHSV-IVb was developed and proved to be a useful tool for studying adaptive immunity in muskellunge (Chapter 6). Exposure to the VHS DNA vaccine (pVHSivb-G) followed by VHSV-IVb challenge increased seroprevalence and titers of survivors compared to challenge or vaccination alone. By only four weeks after challenge, neutralizing antibodies were produced in elevated levels (320 to ≥640) in 100% of pVHSivb-G vaccinated survivors. This suggests that the G protein was expressed by host cells and presented to lymphocytes during the primary phase of the adaptive immune response. Vaccination conferred significant, albeit moderate, protection against a lethal VHSV-IVb challenge 7 weeks (539 degree days) post-vaccination. Considering that the dose used in challenge was far higher than what could be environmentally realistic during disease outbreaks, fish vaccinated with the pVHSivb-G will, most probably, be able to combat the disease under natural conditions.

Protection was mediated, at least in part by, neutralizing antibodies. The fact that vaccinated, unchallenged controls did not have a robust nAB response by 7 weeks suggests NAb levels below the PNT detection threshold (<20) and/or cell-mediated immunity also played a role in protection. Some studies have suggested the role of non-neutralizing (binding) antibodies against the G protein as a protective mechanism particularly in vaccinated survivors that do not have NAb. For muskellunge, non-neutralizing antibodies are not likely to play a role in protection following vaccination, as the pVHSivb-G vaccine did not induce production of any cELISA detectable antibodies. Finally, pVHSivb-G vaccination, and its induced immune mechanisms, drastically limited viral replication and/or increased viral clearance as evidenced by significantly reduced viral loads in vaccinated survivors compared to plasmid control survivors. Like VHSV genotype I DNA vaccines, the pVHSivb-G vaccine also protected rainbow trout against an early challenge with a different rhabdovirus, proving that the expressed VHSV-IVb G protein also induced innate antiviral mechanisms.

As concluded in Chapters 3- 5, fish residing in Lake St. Clair, a VHSV-IVb enzootic lake, show evidence of virus exposure based on the presence of circulating antibodies. Prior to this investigation, one hypothesis for the absence of die-offs in this ecosystem after initial fish kills of 2005-2006 was that some fish had established immunity to the virus. We detected a high seroprevalence in muskellunge over multiple years after the 2006 die-off events. Given the protective nature of NAbs against rhabdoviruses, we can be

confident that a significant percentage of sub-adult to adult fish of this species have established immunity and are not at immediate risk of a VHSV-IVb outbreak.

Parallel testing by virus isolation and serological assays over a multiple year period allowed a better understanding of disease dynamics. In general, the presence of VHSV-IVb in the Lake St. Clair ecosystem was more likely to be indicated based on serological vs. virus isolation evidence. The sampling period following 2006 mortality events revealed a number of fish positive for both antibodies and virus, indicating an early convalescent stage of disease. In contrast, recovered populations sampled a year later in 2007 and in 2010 (a year after a 2009 mortality event) were antibody positive and virus isolation negative.

Heterogeneity in antibody prevalence and titers existed between resident Lake St. Clair fish species when tested by PNT (chapter 3) and cELISA (chapter 4). Muskellunge, smallmouth bass, freshwater drum, and northern pike were the only 4 of 15 species that had NAb. In chapter 4, binding antibodies by cELISA were detected in these species (except northern pike) and also channel catfish. Muskellunge had the greatest seroprevalence and titers of both neutralizing (and binding) antibodies compared to other species. Based on this, muskellunge are a good serological indicator species for VHSV-IVb presence in an ecosystem. This is likely related to aspects of this species life history (e.g. longevity, top predator, spawning congregation behaviors) and VHSV-IVb susceptibility, all of which favor increased virus exposures. Our results support previous

studies that suggest that neutralization and ELISA-based binding assays are detecting different types of antibodies. Samples with high titers of antibodies by cELISA had high, low or negative PNT results, and vice versa.

The cELISA was also used to screen other populations of muskellunge for virus exposure (Chapter 5). Antibodies were detected in muskellunge from Lower Fox River/Green Bay, Wisconsin and Thornapple Lake, MI. Results suggest that the cELISA can be used to monitor the persistence of the virus in enzootic areas and detect virus exposure in areas previously considered negative for the virus. Experimental results showed that the antibody response by cELISA in some muskellunge lasts over a year after exposure. Thus, the detection of antibodies in a population indicates exposure could have occurred at least up to one year prior to the sampling event. The cELISA is a valuable and efficient test for screening fish sera for antibodies against VHSV-IVb. Results from Chapter 4 suggest that the developed assay can be applied to many different species without the need for species-specific reagents.

In summary, the detection of a robust neutralizing response in muskellunge in the wild and after experimental infection and vaccination proves their susceptibility to VHSV-IVb is not due to an inability to mount an antibody response to this virus. This piece of knowledge is critical to understanding the long-term effects that VHSV-IVb will have on populations of this important predator and game species in the Great Lakes, whose

sport and commercial fisheries are estimated in billions of dollars of revenue to the state of Michigan.

FUTURE RESEARCH DIRECTIONS AND APPLICATIONS

Like with every scientific study, one ends up with more questions than answers. Future studies could build on the novel findings of studies detailed in this dissertation, including, but not limited to the following:

Variation in presence, type, and titers of antibodies that we observed in these studies are likely due in part to differences in host immune response mounted against VHSV-IVb. It appears there are definitely differences both between individuals of the same species as well in between species. As such, additional studies on innate and acquired, humoral and cell-mediated defenses against VHSV-IVb are warranted. Fish are estimated to consist of ~27,000 species, most of which have not been studied and their basic physiological mechanisms unknown, not to mention their host defense mechanisms. For example, it is currently unknown whether binding antibodies are preferentially induced over NAb by some fish species in the defense against VHSV. Do some species lack any adaptive humoral mechanisms and rely instead on innate and/or adaptive cell-mediated immunity? These questions are important from both basic and applied sciences perspectives. Answers to these questions would help characterize immune responses of different fish species, help determine what types of tests could

best be used to detect immune responses in a population, and what type of vaccines (and adjuvants) may be best suited for different species.

However, it is unclear at this time whether vaccination against VHSV-IVb in hatcheries within the Great Lakes is desired or feasible. If DNA vaccines are not likely to be approved in the near future in the U.S. for aquacultured species, it is reasonable to reconsider at this time whether DNA vaccine development for VHSV-IVb is a priority. If so, the reduced protection in muskellunge following vaccination compared to what is reported for salmonids would be interesting to investigate further. Future studies could investigate the use of multiple vaccine and virus challenge doses and timeframes of challenge post-vaccination. Tissues collected during the DNA vaccine trial could be used to assess induction of innate or adaptive immune genes.

In terms of VHSV-IVb ecology and population health, several future studies are needed. Detection of high seroprevalence of NAb in a wild population likely suggests the risk of VHSV-IVb outbreak in that species is unlikely. However, what level of herd immunity is needed to prevent an outbreak and how does the level of herd immunity in one fish species influence the likelihood of virus outbreaks in other fish species? It would be interesting to further study the roles of carrier fish in VHSV-IVb transmission and disease dynamics. For example, can muskellunge, or other species with high levels of antibodies, still shed the virus or does shedding only resume if antibodies decline past a certain threshold? On that note, what is the role of salmonids and other fish species that

become infected but do not develop clinical disease? Perhaps these fish act as carriers and have role in viral amplification and transmission by shedding the virus.

The unique relationship between the MSU-Aquatic Animal Health Laboratory and the Michigan DNR allows for research findings to almost immediately become useful from a management standpoint. The following are reasonable suggestions for how information from this dissertation can be applied to future VHSV-IVb surveillance and regulatory testing in Michigan. Modification and implementation of the suggestions below require conversations and planning with Michigan regulatory agencies.

- **For VHSV-positive management (enzootic) areas**: the persistence of the virus could be monitored from now on using a non-lethal serological assay alone. This would reduce the number of fish sacrificed for purposes of screening for virus presence. Long-lived, susceptible species such as muskellunge (or northern pike, smallmouth bass) would be good species to use for this based on our studies. Acute disease outbreaks in VHS positive areas would still be submitted as diagnostic cases and presence of the virus confirmed by traditional tests.

- **For VHSV-surveillance and VHSV- free zones:** we recommend the addition of serological testing to traditional virus isolation testing. Choice of fish for each assay should definitely differ; for example a short-lived, VHSV-IVb susceptible species that is quick to reproduce like bluegill, or better yet round gobies, an invasive species in the Great Lakes, would be ideal to target for virus isolation. A long-lived, susceptible,

larger species that can be sampled non-lethally would be ideal for serological analysis. Finances and staff time will be a limiting factor; it seems reasonable that this could be implemented on a limited number of sites for several summers and then results assessed to determine future testing priorities. It is important to determine immediately what the finding of antibodies in the absence of virus means from a management standpoint. For example, we are confident that the detection of antibodies means that Thornapple Lake muskellunge population has been exposed. However, this has not led to reclassification of this lake as a VHS-positive lake to date.

- Using the most sensitive techniques for understanding the distribution of VHSV-IVb is critical from a management standpoint. It is therefore important to consider that sensitive quantitative RT-PCR assays combined with serological testing could be considered as an alternative to the conventional virus isolation tests.

Parallel results of VHS isolation and serology may reveal a variety of testing patterns that would allow for a better understanding of disease dynamics on a population level. For example, virus isolation *and* antibody positive status could indicate a recent VHSV-IVb exposure. Virus negative status with concurrent high seroprevalence would indicate a recovered population that has likely established immunity. Absence of virus and antibodies may suggest a naïve population that is at immediate risk if the virus enters. It would be interesting to study further what factors influence the relationship between

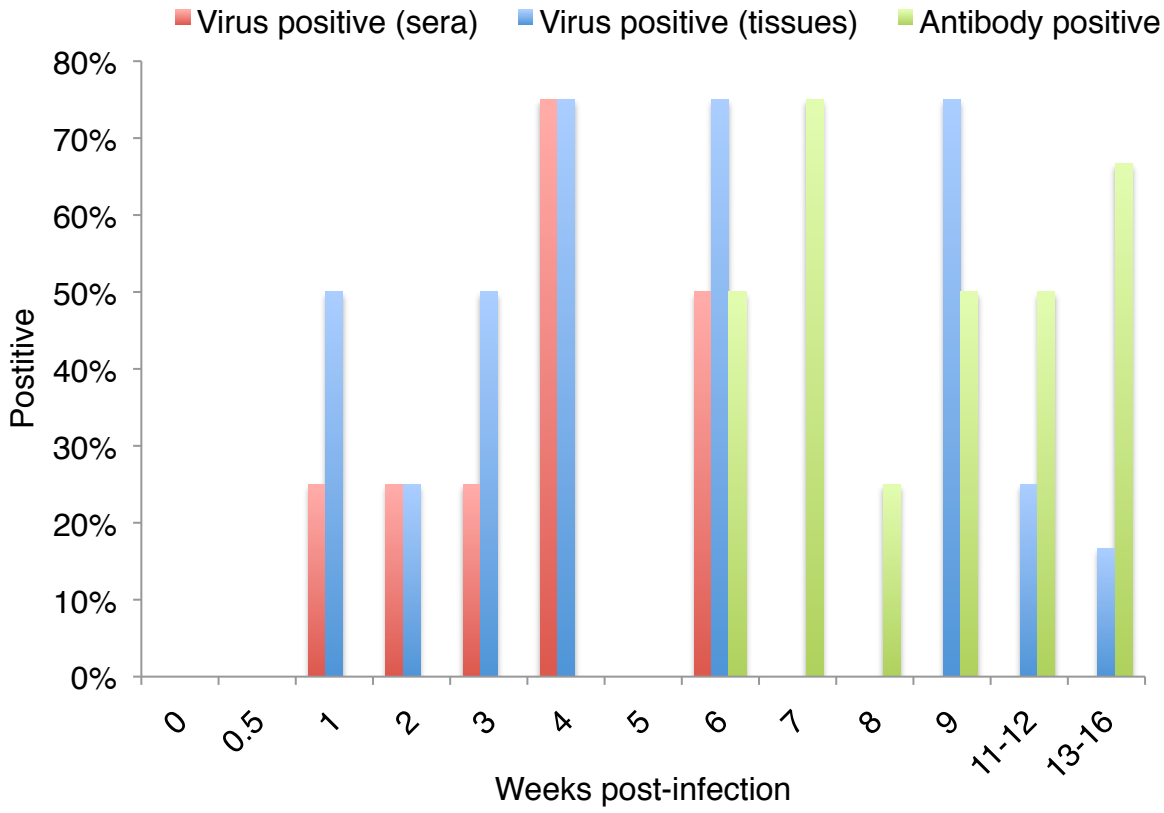
presence/absence of neutralizing and binding antibodies. Some studies have suggested that neutralizing antibodies decline sooner than do binding antibodies. It has been found for infectious hematopoietic necrosis virus, for example, that increased frequency of exposures lead to a more diverse antibody repertoire (Ristow et al. 1993). Therefore it would be interesting to sample a population throughout the course of a year including before, during, and after outbreak(s) of VHSV-IVb. These will allow a better estimation of when fish were exposed based on the presence of antibodies and a better understanding of how immunity plays a role in protecting wild fish from virus outbreaks.

Finally, while not the sole intent of the present investigation, it appears that the cELISA is a valuable and efficient test for screening fish sera of many species for antibodies against VHSV-IVb. As this is the main reason for developing the cELISA, further research should aim to examine sera from naïve/unexposed individuals of numerous fish species in order to estimate negative/positive thresholds. This is necessary as we expect that innate or intrinsic levels of background inhibition exist in some or all fish species, and that levels likely vary based on species. Alternatively, one threshold could be used; this would simplify the assay but likely result in a reduced sensitivity for some species and a reduced specificity for other species. The likelihood of false negatives and false positives, respectively, then would need to be taken into consideration in light of the results. In that respect, the same is true regarding thresholds for the PNT. In terms of further assay testing, it would be useful to measure intra- and inter-assay variability on the PNT and cELISA and compare sensitivity and specificity for detecting

antibodies throughout the disease course in several species. While NAb are known to be protective, more studies are needed to determine the types and protective nature of the antibodies detectable by cELISA.

APPENDIX

Figure 7.1. Percent of sampled fish with virus in sera, tissues (kidney, spleen and/or heart), and neutralizing antibodies over the course of VHSV-IVb infection and recovery. Muskellunge were infected by immersion in a medium dose of the virus (4×10^3 pfu mL^{-1}). Four to five fish are represented at each time-point, except early in the disease course (0 week = 3 fish, 0.5 week = 20 fish, 1 week = 8 fish). Mortality rate of fish dying with clinical signs consistent with VHSV-IVb infection peaked between day 6 and 16 post-infection. This graph was added to emphasize that the combination of both conventional virus isolation assays along with antibody testing allowed more consistent detection of exposed fish throughout the disease course.



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