DNA Vaccination Partially Protects Muskellunge *Esox masquinongy* Against Viral Hemorrhagic Septicemia Virus (VHSV-IVb)

Elena V. Millard[1], Scott E. LaPatra[2], Travis O. Brenden[3], Ashley M. Bourke[1], Scott D. Fitzgerald[1], Mohamed Faisal[1,3,*]

[1]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University
Mail Address: Food Safety and Toxicology Building 1129 Farm Lane, Room 174 Michigan State University, East Lansing, MI 48824

[2]Clear Springs Foods, Inc., Research Division, Buhl, Idaho 83316, USA
Mail Address: 1500 East 4424 North Clear Lakes Rd., Buhl, ID 83316

[3]Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University
Mail Address: 7 Natural Resources Building, Michigan State University, East Lansing, MI 48824

* Corresponding author: faisal@cvm.msu.edu, Tel.: +1 517 884 2019; fax: +1 517 432 2310.

Abstract.— A DNA vaccine (pVHSivb-G) containing the glycoprotein (G) gene of the North American viral hemorrhagic septicemia virus (VHSV) genotype IVb (VHSV-IVb) was developed to evaluate the immune response of Muskellunge *Esox masquinongy* following vaccination and its efficacy in protecting fish against VHSV-IVb challenge. Seven weeks (539 degree days) following vaccination with 10 µg of either pVHSivb-G or a control plasmid (mock vaccine), Muskellunge were challenged by immersion with 10^5 plaque-forming units (pfu/ml) of VHSV-IVb. Vaccinated fish had a relative percent survival (RPS) of 45% and significantly lower mean viral titers in tissues (4.2×10^2 pfu/g) compared to mock vaccinated fish (3.3×10^5 pfu/g). Neutralizing antibodies were detected 28 days (308 degree days) post-challenge (11 weeks post-vaccination [PV]) in 100% of pVHSivb-G vaccinated Muskellunge compared to only 11.8% of mock vaccinated Muskellunge, suggesting robust induction of a secondary, adaptive immune response. In addition, pVHSivb-G vaccinated Rainbow Trout *Oncorhynchus mykiss* challenged seven days (100 degree days) PV with the heterologous novirhabdovirus, infectious hematopoietic necrosis virus (IHNV), experienced a RPS of 76%, compared to a RPS of 38% for control fish, suggesting induction of an early and transient non-specific anti-viral immune response. This study provides an important starting point for VHSV-IVb vaccine development and useful information about the anti-viral immune response elicited by DNA vaccination in a non-domesticated fish species.

The Great Lakes viral hemorrhagic septicemia virus (genotype IV, sublineage b) (VHSV-IVb) is a reportable fish virus that has emerged in the Great Lakes region of North America within the past decade and caused large-scale mortality events of wild fish (reviewed in Kim and Faisal 2011a, 2011b). A number of controlled laboratory experiments confirmed the pathogenicity of this sublineage in a broad range of freshwater fish (Al-Hussinee 2010; Kim and Faisal 2010a; Goodwin and Merry 2011). The virus was especially pathogenic to Muskellunge *Esox masquinongy* (Kim and Faisal 2010b), a large, ecologically and recreationally important piscivore in freshwater systems. Great Lakes Muskellunge fisheries are generally managed through a combination of habitat protection and enhancement, fishing regulations, and stocking of hatchery-reared fingerlings. Concerns regarding effects of VHSV-IVb on wild fish populations as well as the potential introduction into federal and/or state/provincial fish hatcheries in the region prompted interest in the development of a vaccine for conferring protection to hatchery-reared fish prior to stocking.

VHSV is a member of the genus Novirhabdovirus (family Rhabdoviridae) along with several other fish pathogenic viruses including infectious hematopoietic necrosis virus (IHNV), Hirame rhabdovirus, and Snakehead rhabdovirus (Dietzgen et al. 2012). In novirhabdoviruses, the surface glycoprotein (G) is one of six proteins encoded by the single-stranded RNA genome (Dietzgen et al. 2012). Surface G proteins of VHSV and IHNV initiate host cell infection, induce production of neutralizing antibodies (Engelking and Leong 1989; Lorenzen et al. 1990; Bearzotti et al. 1995; Coll 1995) and cell-mediated immunity (Utke et al. 2008). DNA vaccines encoding the glycoprotein of VHSV (genotype I) and IHNV have been found to be efficacious in conferring protection to salmonids following virus challenge (Lorenzen et al. 1988; Anderson et al. 1996; Traxler et al. 1999; Corbeil et al. 2000; Lorenzen et al. 2000; Lorenzen et al. 2002; Lorenzen and LaPatra 2005; Kurath et al. 2007). The DNA vaccine construct, initially developed for IHNV (Anderson et al. 1996), and the European VHSV genotype I (Lorenzen et al. 1988; Heppell et al. 1998), consists of glycoprotein gene inserted downstream of the cytomegalovirus (CMV) promoter in the eukaryotic expression vector pCDNA3.1 (Invitrogen). Administered by intramuscular

(IM) injection, these DNA vaccines induce protection that is superior to killed and recombinant subunit vaccines (reviewed in Kurath et al. 2007; Purcell et al. 2012). Early anti-viral immunity affords cross-protection to other novirhabdoviruses within days of vaccination, and specific and long-lasting immunity develops later (Lorenzen et al. 2000; LaPatra et al. 2001; McLauchlan et al. 2003).

The specificity of the adaptive response prompted the development of a DNA vaccine containing the glycoprotein gene of VHSV-IVb. Studies with VHSV (Lorenzen et al. 1999; Lorenzen et al. 2000; Hart et al. 2012)) have shown sub-optimal protection when vaccination and challenge types differ. We assessed the new VHSV-IVb construct's protective efficacy against VHSV-IVb challenge and ability to elicit neutralizing antibody production. Additionally, the early non-specific anti-viral response elicited by vaccination was studied in Rainbow Trout challenged with IHNV.

<A> 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 for vaccine construction. The design of the construct was based on DNA vaccines against VHSV genotype I (Lorenzen et al. 1988; Heppell et al. 1998) and IHNV (Anderson et al. 1996). Plasmid construction, replication, and purification were outsourced as a custom project to Life Technologies (Carlsbad, CA). 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 in sterile phosphate buffered saline (PBS) and stored at -80°C until use.

Vaccination and viral challenge.— Juvenile Muskellunge obtained from Rathbun National Fish Hatchery (Iowa Department of Natural Resources, Moravia, IA) were reared to experimental size at the Michigan State University Research Containment Facility, East Lansing, MI. Fish were fed live Fathead Minnows *Pimephales promelas* that were certified free of VHSV and other reportable viruses. Tanks received single-pass water and water temperature was maintained at 11 ± 1°C for the duration of the study. Following a one-week acclimation period, 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. Each tank contained an approximately equal total fish biomass, though fish ranged in weight from 40 to 70 g (mean = 56 g, SD = 9). For vaccination, fish were first anesthetized by immersion in water containing 0.1 g/L tricaine methanesulfonate (MS-222) and 0.3 g/L sodium bicarbonate. Each fish was given a single IM 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. Vaccination was considered day 0 of the experiment.

Seven weeks (539 degree days) post vaccination (PV), fish were challenged by

immersion with 10^5 pfu/ml VHSV-IVb. The MI03 isolate from Muskellunge (Elsayed et al. 2006) was used for the challenge. 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 and Winton 1989). Fish from each tank were challenged for 90 minutes in separate glass aquaria containing 15 L of chilled (11°C), 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. Following challenge, fish were returned back to their respective tanks and monitored for 28 days (308 degree days). Severely moribund fish were euthanized and counted as mortalities. Relative percent survival (RPS) from vaccination was calculated by as the ratio of the mean cumulative percent morality (CPM) among the triplicate tanks of the vaccine treatment compared to that of the mock vaccine treatment RPS = [1-(mean CPM of pVHSivb-G treatment/mean CPM of mock vaccine treatment)] × 100 (Amend 1981).

Fish that survived to 28 days post-challenge (11 weeks PV) were euthanized with an overdose of MS-222 (0.25 g /L) buffered with sodium bicarbonate. Blood and tissue samples (kidney, spleen, heart, liver) were collected from all fish for antibody and virus testing respectively. Blood samples were kept at 4°C overnight prior to centrifugation (2500×g, 20 min, 4°C). Sera and tissues were stored at -80°C until testing.

Vaccination and bleeding of unchallenged fish.—In parallel with the aforementioned trial, a separate group of 24 Muskellunge (mean weight 75 g) were vaccinated as previously described, 12 with pVHSivb-G and 12 with empty plasmid. Fish were held in replicate tanks of 6 fish each and blood samples (0.5 – 0.75 ml/fish) were collected non-lethally from 10-12 fish per treatment group at time 0 (pre-vaccination) and at 7 and 11 weeks post-vaccination (PV) and tested for neutralizing antibodies.

Determination of neutralizing antibody titers.—Serum samples from vaccinated fish surviving the virus challenge, and from vaccinated, unchallenged fish were tested for

VHSV-IVb neutralizing antibodies by 50% plaque neutralization tests on EPC cells as previously described (Millard and Faisal 2012a, Millard and Faisal 2012b). Two-fold dilutions of 1:20 to 1:640 were tested. 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 were reported as the reciprocal of the highest serum dilution causing at least a 50% reduction in the average number of virus plaques compared to negative control sera. Neutralizing titers ≥160 were considered to be positive for antibody production (Millard and Faisal 2012b).

Quantification of VHSV-IVb load in tissues by plaque assay.—A pooled sample of kidney, spleen, heart and liver tissue collected from each fish (both mortalities and survivors [28 days post-challenge (PC)], was used for virus titration by plaque assay (Batts and Winton 1989). Tissues were diluted 1:10 (weight:volume) in minimum essential medium with Earle's salts (pH 7.5) supplemented with 10% tryptose phosphate broth (TPB), 100 IU /ml penicillin, 100 μg /ml streptomycin, 100 μg /ml gentamicin sulfate, and 2.5 μg /ml amphotericin. Following homogenization in a stomacher (high speed, 3 min), samples were centrifuged (2,500 × g, 30 min, 4°C), and tissue supernatant collected. For the plaque assay, each sample and a tenfold dilution series were inoculated 100 µL/well onto EPC monolayers grown in flat bottom 24-well plates and incubated for 30 minutes. EPC monolayers had been pretreated prior to inoculation for 10 min with 200 µL/well of 7% polyethylene glycol (PEG; 20,000 MW in in tissue culture media. Lastly, 1 mL/well methylcellulose overlay (0.75% in 2X concentrated tissue culture media) 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 were calculated as pfu/g of tissue. The lower and upper detection thresholds were 100 pfu/g and 3.0×10^7 pfu/g tissue, respectively. Virus titers greater than 3.0×10^7 were treated as 3.0×10^7 for calculations.

Cross-protection against IHNV.—To assess the efficacy of the pVHSivb-G DNA

vaccine in conferring an early anti-viral immune response, a challenge study involving Rainbow Trout was conducted. Rainbow trout approximately 4 g in weight were vaccinated with 1 µg of the pVHSivb-G vaccine, mock vaccine or the Apex ®-IHN vaccine (Novartis), a commercially available vaccine against IHNV. Another group of the same size fish were vaccinated with only PBS (negative control). Water temperature was maintained at approximately 14.5°C during the course of the experiment. 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) 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 analyses.— Survival, prevalence percentages, and virus titers were analyzed as a completely randomized design with subsampling where tanks were treated as experimental units (Hinkelmann and Kempthorne 1994). Data were analyzed using the GLIMMIX procedure in SAS Version 9.4 (SAS Institute, Inc. 2013). Survival rates and prevalence percentages were analyzed assuming a binomial distribution. Virus titer levels were analyzed assuming a normal distribution following log*e* + 1 transformation of the data. 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.

<A> Results

<*B*> Protection and viral titers after VHSV-IVb challenge

Cumulative percent survival (CPS) of pVHSivb-G vaccinated fish following a 7 week challenge of VHSV-IVb was 56% (SE = 6.2%) which was significantly greater than that of mock vaccine (19%, SE = 4.6%) ($F_{1,4}$ = 18.25, P = 0.0129) (Figure 1). The RPS of pVHSivb-G vaccinated Muskellunge was 45%. Mean days until death for pVHSivb-G vaccinated treatment and mock vaccine treatment were 12.4 (SE = 1.0) and 10.6 (SE =

0.5) days, respectively.

Of fish that survived virus challenge, fish that had been vaccinated against VHSV-IVb, had significantly lower infection prevalence (4%, SE = 3.0%) compared to the mock vaccine (82%, SE = 10.0%) ($F_{1,4} = 20.73$, $P = 0.0104$) (Table 1). Mean viral tissue titers for surviving, vaccinated fish was 4.9×10^2 pfu /g (SE = 4.6×10^2 pfu/g) versus 3.1×10^5 pfu /g (SE = 5.3×10^5 pfu/g) for mock vaccinated individuals. The differences in viral tissue titer was statistically significant after log+1 transformation ($F_{1,4} = 25.87$, $P = 0.0070$). Of the mortalities, VHSV-IVb was detected in most fish regardless of treatment group (pVHSivb-G vaccinated=95%; mock vaccinated=100%). Titers greater than 3.0×10^7 pfu /g, the maximum detection limit of the assay, were detected in 82% of dead fish (90 of 110 total combined mortalities).

<*B*>Primary and secondary adaptive immune response to vaccination

By 7 weeks PV, which corresponded to the timing of virus challenge in the parallel study, one of 12 pVHSivb-G vaccinated fish was seropositive (Table 2). By 11 weeks PV, however, neutralizing antibodies were produced by 60% (6 of 10) of pVHSivb-vaccinated fish. Neutralizing antibodies were detected in 100% of pVHSivb-G vaccinated survivors but only 12% of mock vaccinated survivors 28 days post virus challenge (Figure 2). Titers of mock vaccinated survivors ranged from 0 to 320, with the majority (59%: 10 of 17 fish) having titers less than 20. Titers of pVHSivb-G vaccinated surviving fish were greater; over half (55.6%) of seropositive fish had neutralizing titers greater than 640.

<*B*>Early protection provided against IHNV

Compared to mock vaccinated fish, pVHSivb-G and Apex-IHN vaccinated Rainbow Trout had significantly greater survival when challenged 7 days PV (pVHSivb-G: t = 3.72, df = 3, $P = 0.0337$; Apex-IHN: t = 4.74, df = 3, $P = 0.0178$) (Table 3). Differences in survival between pVHSivb-G and Apex-IHN vaccinated fish were not significant (t=1.54, df=3, P=0.2222). The relative percent survival of pVHSVivb-G vaccinated fish was 61.3% compared to 80.6% for Apex-IHN vaccine fish. Following challenge at 28

days PV, survival of fish vaccinated against the homologous virus was 92% compared to 47% and 42% for pVHSVivb-G and mock vaccinated fish, respectively. Survival of fish vaccinated against the homologous virus was significantly greater than both pVHSVivb-G ($t = 4.65$, $df = 3$, $P = 0.0188$) and mock vaccinated fish ($t = 4.32$, $df = 3$, $P = 0.0228$). Differences in survival between pVHSVivb-G and mock vaccinated fish were not statistically significant ($t = 0.51$, $df = 3$, $P = 0.6445$).The RPS of pVHSVivb-G vaccinated fish was <0%, compared to fish vaccinated against the homologous virus (85%). Only a few mortalities (< 4% CPM) occurred in unchallenged, PBS-injected fish during either trial (results not shown).

<A> Discussion

DNA vaccines against novirhabdoviruses in salmonids are among the most efficacious developed to date (Lorenzen and LaPatra 2005; Kurath et al. 2007). In this study, we developed a similar DNA vaccine construct, but inserted the G gene of the North American genotype IV (Great Lakes sublineage IVb) of VHSV. Results of challenge trials in both Muskellunge and Rainbow Trout suggest successful uptake and/or transfection of host cells with the pVHSivb-G DNA plasmid and subsequent expression of the VHSV-IVb G protein. The level of protection (RPS 45%) achieved in Muskellunge against VHSV-IVb immersion challenge at 7 weeks was significant, albeit moderate, compared to VHSV (Lorenzen et al. 1998; Heppell et al. 1998; Lorenzen et al. 2000) and IHNV (Corbeil et al. 2000) DNA vaccination studies in salmonids. A similar level of protection was achieved in Pacific Herring *Clupea pallasii* following VHSV-Ia vaccination and challenge with the North American VHSV IVa (Hart et al. 2012). The less than optimal protection could be due to innate differences in immune responses against DNA vaccination in non-salmonid fish species. Stress might also have a more immunosuppressive effect on species such as Muskellunge that are less adapted to culture compared to salmonids. By 7 weeks PV, corresponding to the timing of viral challenge, neutralizing antibodies were only beginning to be developed to VHSV-IVb in vaccinated, unchallenged fish. It is possible that a greater level of protection may have been observed had Muskellunge had a longer period of time to mount a protective response prior to exposure.

Partial protection despite a low seroprevalence of neutralizing antibodies in vaccinated, unchallenged fish has been reported previously for VHSV DNA vaccination (Lorenzen et al. 1998; Kim et al. 2000; McLauchlan et al. 2003,). These findings suggest that other immune mechanisms (i.e. cell-mediated immunity, non-neutralizing antibodies) play a role in the adaptive VHSV-IVb response, although it is also possible that low levels of neutralizing antibodies (<160, or even below detection limit of assay) confer protection to infected individuals. Indeed, sera with low neutralizing titers (20-40), and even non-neutralizing sera, from IHNV-challenged and DNA- vaccinated fish were still found to be protective in vivo following passive transfer (LaPatra et al. 1994; Traxler et al. 1999).

Vaccination primed the adaptive immune respond for a robust secondary response following 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 high titers that ranged from 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 and 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 a secondary immune response to challenge and not just a primary adaptive response to vaccination. These findings suggest that a booster dose of vaccine administration may have resulted in better protection.

Vaccination with pVHSivb-G significantly reduced viral prevalence and titers in fish surviving viral challenge. This 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 and Faisal 2012). 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 after challenge has been reported in Rainbow Trout (Lorenzen et al. 2000; Lorenzen et al. 2009) and Pacific Herring (Hart et al. 2012) that had been vaccinated against VHSV genotype I.

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. Early phase protection after VHSV DNA vaccination in salmonids is mediated by non-specific anti-viral immune mechanisms (e.g. interferon system, Mx protein) that can provide cross-protection against other fish rhabdoviruses within days PV (Lorenzen et al. 1998; Boudinot et al. 1998; LaPatra et al. 2001; McLauchlan et al. 2003; Purcell et al. 2012). This early cross-protection does not seem to occur in all species though based on a recent study in Pacific Herring (Hart et al. 2012). Cross-protection was lost at the later time-point following the induction of specific immunity as found in previous studies (Lorenzen et al. 1988; LaPatra et al. 2001).

Rhabdoviral pathogens continue to represent a significant threat to the aquaculture industry worldwide. DNA vaccines have proven to be a useful tool and are currently being used on a commercial scale to prevent IHNV outbreaks in cultured Atlantic Salmon *Salmo salar* in Canada (Salonius et al. 2007). 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.

<A> Acknowledgments

The authors thank Niels Lorenzen and Katja Einer-Jensen for critical review of the VHSV-IVb DNA vaccine construct and sequence, and the Great Lakes Fishery Trust for funding this work (#2012.1257).

<A> References

Al-Hussinee L., P. Huber, S. Russell, V. LePage, A. Reid, K.M. Young, E. Nagy, R.M. W. Stevenson, and J.S. Lumsden. 2010. Viral haemorrhagic septicaemia virus IVb experimental infection of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), and Fathead Minnow, *Pimphales promelas* (Rafinesque). Journal of Fish Diseases 33:347-360.

Alonso M., M. Johnson, B. Simon, and J.A. Leong. 2003. A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. Vaccine 21:1591-600.

Alonso M., P.P. Chiou, and J.A. Leong. 2011. Development of a suicidal DNA vaccine for infectious hematopoietic necrosis virus (IHNV). Fish and Shellfish Immunology 30:815-823.

Amend D.F. 1981. Potency testing of fish vaccines. Developments in Biological Standardization 49:447-454.

Anderson E.D., D.V. Mourich, S.C. Fahrenkrug, S. LaPatra, J. Shepherd, and J.A. Leong. 1996. Genetic immunization of Rainbow Trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 5:114-122.

Batts W.N., and J.R. Winton. 1989. Enhanced detection of infectious hematopoietic necrosis virus and other fish viruses by pretreatment of cell monolayers with polyethylene glycol. Journal of Aquatic Animal Health 1:284-290.

Bearzotti M., A.F. Monnier, P. Vende, J. Grosclaude, P. de Kinkelin, and A. Benmansour. 1995. The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. Veterinary Research 26:413-422.

Boudinot P., M. Blanco, P. de Kinkelin, and A. Benmansour. 1998. Combined DNA immunization with the glycoprotein gene of viral haemorrhagic septicaemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in Rainbow Trout. Virology 249:297-306.

Coll J.M. 1995. The glycoprotein G of rhabdoviruses. Archives of Virology 140:827-51.

Corbeil S., G. Kurath, and S.E. Lapatra. 2000. Fish DNA vaccine against infectious

hematopoietic necrosis virus: Efficacy of various routes of immunisation. Fish and Shellfish Immunology 10:711-723.

Dietzgen R.G., C.H. Calisher, G. Kurath, I.V. Kuzmin L.L. Rodriguez, D.M. Stone, R.B. Tesh, N. Tordo, P.J. Walker, T. Wetzel, and A.E. Whitfield. 2012. Family - Rhabdoviridae. Pages 686-713 *in* A.M.Q. King, M.J. Adams, E.B. Carstens, and E.J. Lefkowitz, editors. Virus taxonomy. Elsevier, San Diego.

Elsayed E., M. Faisal, M. Thomas, G. Whelan, W. Batts, and J. Winton. 2006. Isolation of viral haemorrhagic septicaemia virus from Muskellunge, *Esox masquinongy* (Mitchill), in Lake St Clair, Michigan, USA reveals a new sublineage of the North American genotype. Journal of Fish Diseases 29:611-619.

Engelking H.M., and J.C. Leong. 1989. The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. Virus Research 13:213-230.

Goodwin A.E., and G.E. Merry. 2011. Mortality and carrier status of Bluegills exposed to viral hemorrhagic septicemia virus genotype IVb at different temperatures. Journal of Aquatic Animal Health 23:85-91.

Hart L.M., N. Lorenzen, S.E. LaPatra, C.A. Grady, S.E. Roon, J. O'Reilly, J.L. Gregg, and P.K. Hershberger. 2012. Efficacy of a glycoprotein DNA vaccine against viral haemorrhagic septicaemia (VHS) in Pacific Herring, *Clupea pallasii* Valenciennes. Journal of Fish Diseases 35:775-779.

Heppell J., N. Lorenzen, N.K. Armstrong, T. Wu, E. Lorenzen, K. Einer-Jensen, J. Schorr, and H.L. Davis. 1998. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology 8:271-286.

Hinkelmann, K., and O. Kempthorne. 1994. Design and analysis of experiments, volume I. Wiley, New York.

Kim R., and M. Faisal. 2010a. Experimental studies confirm the wide host range of the Great Lakes viral haemorrhagic septicaemia virus genotype IVb. Journal of Fish Diseases 33:83-88.

Kim R., and M. Faisal. 2010b. The Laurentian Great Lakes strain (MI03) of the viral haemorrhagic septicaemia virus is highly pathogenic for juvenile Muskellunge,

Esox masquinongy (Mitchill). Journal of Fish Diseases 33:513-527.

Kim R., and M. Faisal. 2011a. Emergence and resurgence of the viral hemorrhagic septicemia virus (Novirhabdovirus, Rhabdoviridae, Mononegavirales). Journal of Advanced Research 2:9-23.

Kim R., and M. Faisal. 2011b. Emergence of viral hemorrhagic septicemia virus in the Laurentian Great Lakes. Pages 113-122 *in* R.C. Cipriano, A.W. Bruckner, and I.S. Shchelkunov, editors. Bridging America and Russia with shared perspectives on aquatic animal health. Khlaed bin Sultan Living Oceans Foundation, Landover, Maryland.

Kim R., and M. Faisal. 2012. Shedding of viral hemorrhagic septicemia virus (genotype IVb) by experimentally infected Muskellunge (*Esox masquinongy*). Journal of Microbiology 50:278-284.

LaPatra S.E., K.A. Lauda, and G.R. Jones. 1994. Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. Diseases of Aquatic Organisms 20:119-126.

LaPatra S.E., S. Corbeil, G.R. Jones, W.D. Shewmaker, N. Lorenzen, E.D. Anderson, and G. Kurath. 2001. Protection of Rainbow Trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 19:4011-4019.

Kurath G., M.K. Purcell, and K.A. Garver. 2007. Fish rhabdovirus models for understanding host response to DNA vaccines. CAB Reviews 2:1-12.

Lorenzen E., K. Einer-Jensen, T. Martinussen, S.E. LaPatra, and N. Lorenzen. 2000. DNA vaccination of Rainbow Trout against viral hemorrhagic septicemia virus: a dose response and time course study. Journal of Aquatic Animal Health 12:167-180.

Lorenzen E., K. Einer-Jensen, J.S. Rasmussen, T.B. Kjær, B. Collet, C.J. Secombes, and N. Lorenzen. 2009. The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. Vaccine 27:3870-3880.

Lorenzen N., and S.E. LaPatra. 2005. DNA vaccines for aquacultured fish. Revue Scientifique et Technique de l'OIE 24:201-213.

Lorenzen N., E. Lorenzen, K. Einer-Jensen, J. Heppell, and H.L. Davis. 1999. Genetic

vaccination of Rainbow Trout against viral haemorrhagic septicaemia virus: small amounts of plasmid DNA protect against a heterologous serotype. Virus Research 63:19-25.

Lorenzen N., E. Lorenzen, K. Einer-Jensen, J. Heppell, T. Wu, and H. Davis. 1998. Protective immunity to VHS in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish and Shellfish Immunology 8:261-270.

Lorenzen N., E. Lorenzen, K. Einer-Jensen, and S.E. LaPatra. 2002. DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in Rainbow Trout. Fish and Shellfish Immunology 12:439-453.

Lorenzen N., N.J. Olesen, and P.E. Vestergaard-Jargensen. 1990. Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. Journal of General Virology 71:561-567.

McLauchlan P.E., B. Collet, E. Ingerslev, C.J. Secombes, N. Lorenzen, and A.E. Ellis. 2003. DNA vaccination against viral haemorrhagic septicaemia (VHS) in Rainbow Trout: size, dose, route of injection and duration of protection--early protection correlates with mx expression. Fish and Shellfish Immunology 15:39-50.

Millard E.V., and M. Faisal. 2012a. Development of neutralizing antibody responses in Muskellunge, *Esox masquinongy* (Mitchill), experimentally exposed to viral haemorrhagic septicaemia virus (genotype IVb). Journal of Fish Diseases 35:11-18.

Millard E.V., and M. Faisal. 2012b. Heterogeneity in levels of serum neutralizing antibodies against viral hemorrhagic septicemia virus genotype IVb among fish species in Lake St. Clair, Michigan, USA. Journal of Wildlife Diseases 48:405-415.

Olesen N.J., and P.E.V. Jørgensen. 1986. Detection of neutralizing antibody to Egtved virus in Rainbow Trout (*Salmo gairdneri*) by plaque neutralization test with complement addition. Journal of Applied Ichthyology 2:33-41.

Purcell M.K., K.J. Laing, and J.R. Winton. 2012. Immunity to fish rhabdoviruses. Viruses 4:140-166.

Salonius K., N. Simard, R. Harland, and J.B. Ulmer. 2007. The road to licensure of a

DNA vaccine. Current Opinion in Investigational Drugs 8:635-641.

Traxler G.S., E. Anderson, S.E. LaPatra, J. Richard, B. Shewmaker, and G. Kurath. 1999. Naked DNA vaccination of Atlantic Salmon *Salmo salar* against IHNV. Diseases of Aquatic Organisms 38:183-190.

Utke K., H. Kock, H. Schuetze, S.M. Bergmann, N. Lorenzen, K. Einer-Jensen, B. Köllner, R.A. Dalmo, T. Vesely, M. Ototake, and U. Fischer. 2008. Cell-mediated immune responses in Rainbow Trout after DNA immunization against the viral hemorrhagic septicemia virus. Developmental and Comparative Immunology 3:239-252.

Table 1. Virus prevalence and tissue titers in Muskellunge following vaccination and challenge with VHSV-IVb. Fish surviving through 28 days after challenge were considered survivors. Different letters indicate statistical significance compared to plasmid controls ($P < 0.05$).

	Prevalence (SE)	Mean titer (pfu/g)	SE (pfu/g)	Range (pfu/g)
Mortalities				
pVHSivb-G	95% (3.6%)	$>2.1 \times 10^7$	$>2.1 \times 10^6$	$0 – >3.0 \times 10^7$
plasmid controls	100%	$>2.7 \times 10^7$	$>9.2 \times 10^5$	$4.1 \times 10^5 – >3.0 \times 10^7$
Survivors				
pVHSivb-G	4% (2.8%)	$4.2 \times 10^{2\ b}$	2.9×10^2	$0 – 1.1 \times 10^4$
plasmid controls	82% (9.5%) [a]	3.3×10^5	1.9×10^5	$0 - 3.0 \times 10^6$

491 Table 2. Infection prevalence and titers of neutralizing antibodies following DNA
492 vaccination against VHSV-IVb. Blood was drawn non-lethally from 10-12 fish from each
493 treatment group prior to vaccination and at 7 and 11 weeks post-vaccination.
494

Time	Seropositive prevalence	Neutralizing antibody titer
		pVHSivb-G treatment
Pre-vaccination	8%	<20 (10/12), 40 (1/12), 160 (1/12)
7 weeks	8%	<20 (9/12), 40 (2/12), 320 (1/12)
11 weeks	60%	20 (2/10), 40 (1/10), 80 (1/10), 320 (2/10), ≥640 (4/10)
		Mock vaccinated treatment
Pre-vaccination	0%	<20 (10/11), 40 (1/11)
7 weeks	8%	<20 (9/12), 40 (1/12), 80 (1/12), 160 (1/12)
11 weeks	10%	<20 (8/10), 20 (1/10), 320 (1/10)

495

496 Table 3. Mean cumulative percent survival (CPS) of Rainbow Trout (*Oncorhynchus*
497 *mykiss*) challenged by immersion 7 days (100 degree days) and 28 days (400 degree
498 days) post-vaccination (PV) with 10^5 pfu/ml IHN virus. Different letters indicate statistical
499 significance within each challenge compared to plasmid controls ($P < 0.05$).
500

	7 days PV			28 days PV		
Treatment	CPS	SE	RPS	CPS	SE	RPS
plasmid control	38	6.9	0.0	47	7.1	0.0
Apex®-IHN	88[a]	4.6	80.6	92[b]	3.9	84.9
pVHSivb-G	76[a]	6.1	61.3	42	7.1	0.0

501

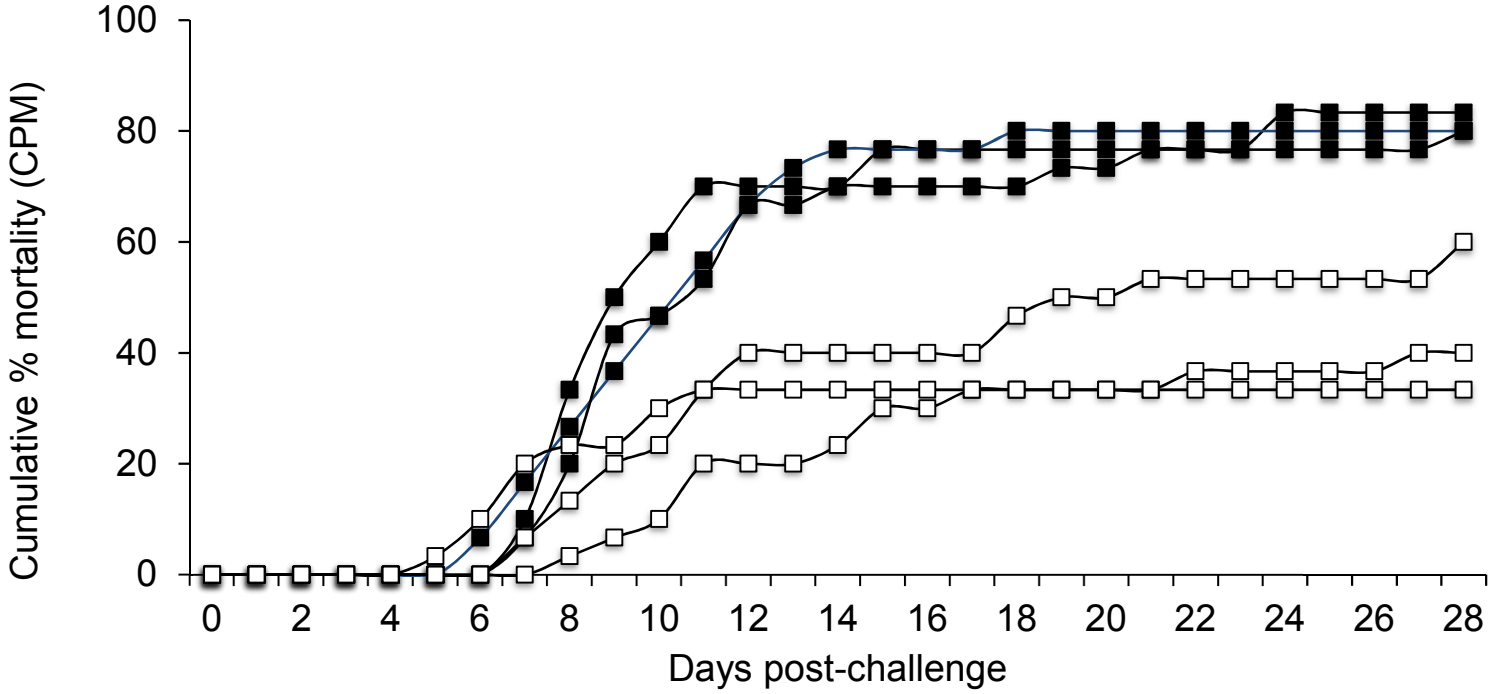
Figure Captions

Figure 1. Development of cumulative percent mortality (CPM) in triplicate tanks of Muskellunge (*Esox masquinongy*) vaccinated with pVHSivb-G DNA vaccine (open squares) or the empty plasmid (i.e., mock vaccine) (closed squares) and challenged with VHSV-IVb 7 weeks (539 degree days) post-vaccination. Mean cumulative percent mortality for pVHSivb-G and mock vaccinated fish was 81.1% and 44.4%, respectively.

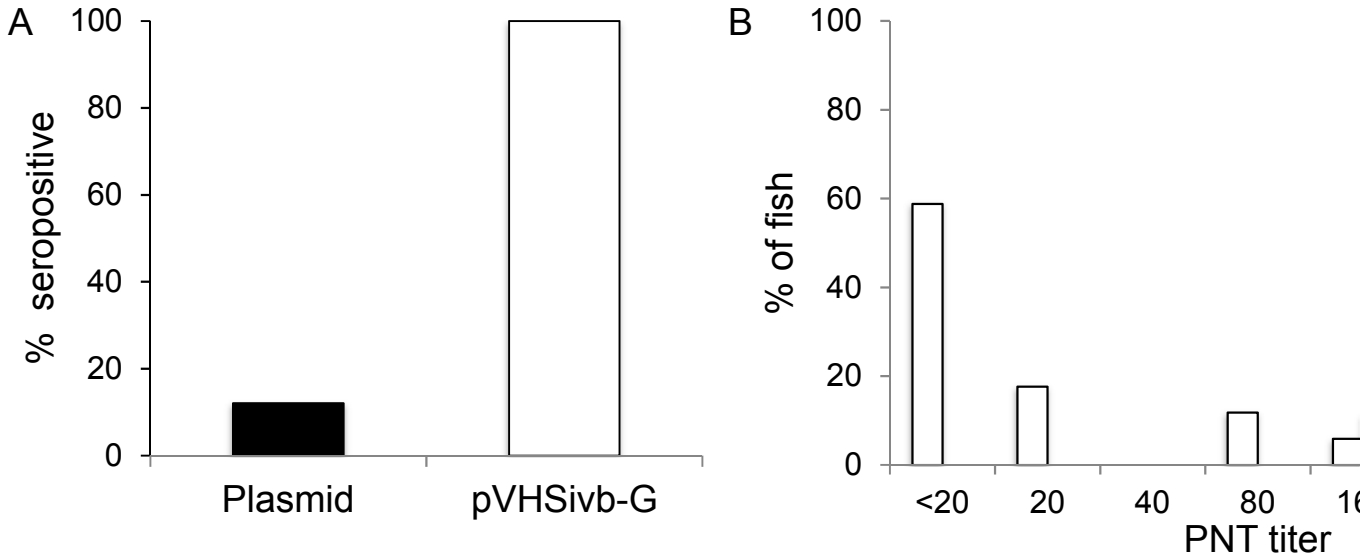
Figure 2. A) Percent of fish with neutralizing titers and B) distribution of titers in vaccinated Muskellunge surviving challenge with VHSV-IVb. Fish were challenged at week 7 and survivors sampled 28 days post-challenge (11 weeks post-vaccination).

515 Figure 1. Faisal

516



520 Figure 2 Faisal



521