Re-Emergence of Epizootic Epitheliotropic Disease Virus: Potential Effects and Development of Improved Diagnostics and Control Measures

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Executive Summary
Salmonid herpespesvirus 3, commonly known as Epizootic Epitheliotropic Disease Virus (EEDV) is a serious pathogen of lake trout (*Salvelinus namaycush*), a fish that is considered indigenous to the Great Lakes basin. The lack of solid data on the biological characteristics of this virus severely hampers the development of control strategies. With funds made available by GLFT, scientists joined forces to unravel some EEDV characteristics. We were able to document two EEDV-associated epidemics in hatchery lake trout. It was possible, for the first time, to sequence the full virus genome and decipher many of the genes it carries, which will aid in finding potential vaccines effective against EEDV. This study also provided evidence on the host range of EEDV and differences in susceptibility between lake trout strains. We were able to prepare virus-rich tissue homogenates from infected fish and use this material to standardize intraperitoneal and water-borne experimental infection protocols. These protocols were instrumental in studying the course of EEDV infection. Despite the lack of an *in vitro* propagation system for this virus, we were able to develop a number of molecular assays that are currently instrumental in EEDV diagnosis and made strides to develop a serological set up that would allow for both direct and indirect EEDV diagnosis. Fortunately, Virkon®, a commonly used disinfectant in aquaculture practices, proved effective in eradicating EEDV infectivity. In conclusion, this study has unquestionably increased our knowledge on EEDV and identified several targets of potential importance for the development of control strategies.

Background and Objectives:
In the 1980's, Great Lakes fishery managers were confronted with the emergence of a deadly disease of lake trout (*Salvelinus namaycush*) in 7 hatcheries in 3 states. These outbreaks resulted in the destruction of 15 million fish and were of particular concern due to the reliance of the basin-wide Lake Trout Rehabilitation Program on these hatchery fish. Despite initial difficulties identifying the etiological agent of these disease outbreaks, it was eventually determined that a herpesvirus was the cause; namely, the Epizootic Epitheliotropic Disease Virus (EEDV; **Bradley et al. 1988; 1989**) that was later identified as the Salmonid Herpesvirus 3 (SalHV3) and placed in the family Alloherpesviridae (**Waltzek et al. 2009**).

During the 1980s, fishery managers adopted stringent disease control measures that limited the movement of hatchery-reared lake trout within the Great Lakes basin. It was then believed that EEDV had been eradicated, however, in 2003, an episode of EEDV erupted in the Les Voigt State Fish Hatchery (LVSFH) with low mortalities. Tissues from affected lake trout allowed investigators to develop a diagnostic PCR assay (**Kurobe et al. 2009**). The authors used the developed assay in detecting EEDV in archived skin and ovarian fluid samples originating from multiple lake trout stocks in the USA. In 2012, another EEDV epizootic occurred at Marquette State Fish Hatchery (MSFH) in Michigan, killing 20% of their production lake trout. Although much remains unknown, the re-emergence of EEDV proves that the virus is still present in a highly pathogenic form and continues to appear and then disappear. Further details on the resurgence of EEDV described in **Appendix I** in the accepted manuscript entitled "Resurgence of Salmonid Herpesvirus-3 infection (Epizootic Epitheliotropic Disease) in hatchery propagated Lake Trout *Salvelinus namaycush* in Michigan."

The prevalence, pathogenicity mechanisms, and overall importance of interspecies transmission in the evolution of alloherpesviruses (e.g., EEDV/SalHV3) will remain unclear until a larger sampling of genomes can be scrutinized. Therefore, genome sequencing is essential for identifying genes and ultimately the evolutionary relationships that are at play between EEDV and lake trout in terms of transmission, pathogenicity, immunity, and latency. The ability to compare the genome of EEDV with other known pathogenic alloherpesviruses (e.g., the other

salmoniviruses, channel catfish virus, and koi herpesvirus) may cast light on potential virulence factors, vaccine development, antigenic sites, and EEDV ecology.

Prior to this study, most of what we knew on the biology of EEDV came from a few publications (**Bradley et al. 1988; 1989; McAllister & Herman 1989**). The authors were unable to grow the virus in commonly used fish cell lines such as FHM, RTG-2, CHSE-214, EPC, or RTH-149. Bradley et al. (1989) were able to reproduce mortality and gross pathology observed in spontaneous outbreaks. Moreover, Bradley et al. (1989) and McAllister and Herman (1989) challenged other salmonids such as rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Atlantic salmon (*S. salar*), and Chinook salmon (*O. tshawytscha*) by cohabitation with infected lake trout and were unable to elicit mortality.

To this end, a team of eight scientists, representing academia, state, and federal agencies gathered to unravel more important scientific facts about EEDV. The study spanned three years with the following objectives:

1. To fully elucidate the biological and pathological properties of EEDV using genomic characterization.
2. To determine EEDV host range, disease course, and modes of transmission.
3. To develop diagnostic assays aiming at elucidating humoral immune mechanisms involved in defense against EEDV.
4. To develop specific and sensitive diagnostic assays for EEDV detection in fish including carrier and previously exposed fish.
5. To test the efficacy of current biosecurity practices in hatcheries for the inactivation of EEDV including egg disinfection.

Achievements:

OBJECTIVE 1: To fully elucidate the biological and pathological properties of EEDV using genomic characterization.

Task 1: EEDV purification:

For purposes of whole genome sequencing, frozen skin samples from EEDV infected lake trout were used for virus purification. The EEDV was purified using a continuous sucrose density gradient.

For the tasks of experimental infections, a large volume of pure virus, without losing its infectivity, was required. Production of such a stock proved extremely difficult via traditional purification methods as handling of herpesvirus particles disrupts the tegument and consequently affects the virus' ability to infect fish. Initial attempts at purification utilized ultracentrifugation through a glycerol gradient infected fish tissue supernatant. While this method was successful in concentrating the viral particles and was used previously by Bradley et al (1989) for virus visualization via transmission electron microscopy, the purified virus material was unable to replicate and produce cytopathic effects (CPE) on *Epithelioma Papulosum Cyprini* (EPC) cells nor could it reproduce the disease in naïve lake trout. Subsequently, both an Optiprep™ solution (Iodixanol, Sigma-Aldrich), and a Ficoll® 400 gradient was used in place of the glycerol. Following ultracentrifugation, a small amount of PCR-positive product was recovered. The "purified" product was inoculated onto lake trout fry cells as well as into naïve yearling lake trout (intraperitoneal injection). Again, no clinical signs or

mortalities were observed in the naïve fish and no CPE were produced in cultured cells, indicating a lack of infectivity. Due to these continued challenges, it was decided to use clarified tissue homogenate rather than purified virus for experimental infections in order to complete the studies in a reasonable timeline.

Task 2: Determining the EEDV genomic sequence:

Purified EEDV was digested with a mixture of DNases to remove non-viral DNA. EEDV was extracted using commercially available kits. The resulting DNA was randomly amplified by PCR. Products of >70 base pairs (bp) were selected by column purification and ligated to specific linkers for next generation sequencing using the Illumina MiSeq platform V3 chemistry kits (2 x 300 read length). The first Miseq run generated 3,200,000 reads of an average read length of 256 bp and the second Miseq run produced 9,695,820 reads of an average read length of 250 bp. The host (salmon) reads were removed from the dataset using Kraken and the de novo assembly was performed in SPAdes. The resulting contigs were subjected to BLASTX searches against a proprietary alloherpesvirus database. The analysis identified a large alloherpesvirus-related contig, which was then joined to other contigs manually by PCR and Sanger sequencing. The reconstructed genome was 183,944 bp in size, consisting of a long unique region (UL; 145,135 bp) linked to a short unique region (US; 25,425 bp) which is flanked by inverted repeats (RS; 6,692 bp; Figure 1). A total of 721,529 (5.6%) reads matched this sequence at an average coverage of 1,081 reads per nucleotide. A total of 125 open reading frames (ORFs) were predicted using GenemarkS and CLC Genomics Workbench, with 96 ORFs in the long unique region, 21 in the short unique region, and 4 in each repeat. The gene functions were predicted based on BLASTP searches against the NCBI GenBank non-redundant protein sequence database (Table 1).

Task 3: Determining EEDV virulence factors:

EEDV (SalHV3) encodes a suite of genes potentially involved in evading host immune surveillance, latency, and viral dissemination. For example, EEDV ORFs 1 and 2 encode tumor necrosis factor receptor genes which are known to act as decoy receptors that compete with death receptors for ligand binding and prevent apoptosis. ORFs 59 and 60 encode genes with immunoglobulin domains that are thought to protect virus-infected cells against lymphocyte cytotoxicity by engaging lymphocyte inhibitory receptors. EEDV also contains BCL-2 genes (ORFs 77 and 109) with apoptosis inhibitor domains. These genes are known to: 1) prevent premature death of the host cell (apoptosis), which would impair virus production, 2) enable efficient emergence from latency, 3) facilitate persistent infection, and 4) contribute to the avoidance of immune surveillance by the host. Viral encoded BCL-2 genes have previously been discovered in herpesviruses infecting mammals; however, this is the first discovery in an alloherpesvirus (i.e., fish herpesvirus; Antonsson and Martinou 2000).

Task 4: Phylogenetics of EEDV:

The amino acid sequence of the complete DNA polymerase of all known alloherpesviruses available in GenBank (Table 2) were aligned with that of EEDV (SalHV3) using the MAAFT alignment tool. The final dataset contained 451 amino acid (AA) characters (excluding gaps) and the Maximum Likelihood phylogenetic analysis using the IQ-TREE web server produced a well-resolved and supported tree (Figure 2). The salmonid herpesviruses form a clade (i.e., genus Salmonivirus), with EEDV (SalHV3) forming the sister group to the clade formed by Salmonid herpesvirus 1 and 2 (Figure 2). The salmonid herpesviruses 4 and 5 were not included in this analysis since a full sequence of their DNA polymerase gene is not available.

Reflections on the achievements in OBJECTIVE 1:

It was unfortunate that we were unable to purify EEDV without losing its infectivity. However, the ability to sequence the entire EEDV genome is considered a monumental achievement, as it revealed many of the unknown biological properties of EEDV. For example, the identification of the BCL-2 gene and other immunomodulatory genes potentially involved in EEDV (SalHV3) virulence is the first logical approach in developing effective mitigation strategies (e.g., vaccines). This knowledge can have direct benefits in identifying best control strategies of this virus, a matter that will benefit all those agencies (state, provincial, federal) managing the restoration and health of lake trout in the Great Lakes Basin.

Table 1. Genome annotation of the EEDV (SalHV3).

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF1	507-1373	289	tumor necrosis factor receptor	unnamed protein product [Oncorhynchus mykiss]	2.38E-36	CDQ67597	Tumor necrosis factor receptor superfamily member 6B	1.08E-49	cd10575
ORF2	1419-2093	225	tumor necrosis factor receptor	PREDICTED: LOW QUALITY PROTEIN: tumor necrosis factor receptor superfamily member 6B, partial [Merops nubicus]	1.11E-10	XP_008941277	Tumor necrosis factor receptor superfamily member 6B	7.30E-15	cd10575
ORF3	2910-3539	210	hypothetical protein	---NA---					
ORF4	3410-4717	436	hypothetical protein	---NA---					
ORF5	4789-5055	89	hypothetical protein	---NA---					
ORF6	5543-6547	335	hypothetical protein	---NA---					
ORF7	6802-7392	197	hypothetical protein	---NA---					
ORF8	7596-8681	362	hypothetical protein	PREDICTED: probable serine/threonine-protein kinase kinX isoform X1 [Salmo salar]	3.72E-04	XP_013998550			
ORF9	8807-14980	2058	coiled-coil domain containing protein	---NA---			Coiled-coil domain-containing protein 158	3.79E-08	pfam15921
ORF10	15002-15955	318	hypothetical protein	---NA---					
ORF11	15954-17030	359	hypothetical protein	---NA---					
ORF12	16921-18030	370	serine/threonine kinase	---NA---			Serine/threonine protein kinase	1.74E-04	COG0515
ORF13	18021-19490	490	protein kinase	hypothetical protein, partial [Staphylococcus pasteuri]	3.11E-19	WP_048803262	Protein Kinases	7.93E-06	cd00180
ORF14	19385-21097	571	AAA ATPase	ORF25 [Acipenserid herpesvirus 2]	2.67E-92	AEF97684	AAA domain	3.19E-10	pfam13245
ORF15	21450-22406	319	capsid triplex subunit 2	ORF27 [Acipenserid herpesvirus 2]	1.48E-44	AEF97686	Capsid triplex subunit 2	5.12E-16	PHA03260

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF16	22479-24560	694	capsid maturational protease	ORF28 [Acipenserid herpesvirus 2]	2.94E-42	AEF97687	capsid maturational protease	2.86E-38	PHA03369
ORF17	24467-25174	236	hypothetical protein	ORF29 [Acipenserid herpesvirus 2]	4.14E-16	AEF97688			
ORF18	24945-25634	230	hypothetical protein	ORF30 [Acipenserid herpesvirus 2]	5.01E-21	AEF97689			
ORF19	25932-26924	331	hypothetical protein	---NA---					
ORF20	27872-28888	339	hypothetical protein	---NA---					
ORF21	28962-29561	200	hypothetical protein	ORF80 [Acipenserid herpesvirus 2]	1.87E-06	AEF97690			
ORF22	29585-30439	285	hypothetical protein	ORF33 [Acipenserid herpesvirus 2]	1.27E-21	AEF97691			
ORF23	30294-31700	469	hypothetical protein	ORF34 [Acipenserid herpesvirus 2]	3.14E-107	AEF97692	uncharacterized protein	3.12E-94	PHA03336
ORF24	31564-32223	220	hypothetical protein	ORF35 [Acipenserid herpesvirus 2]	6.42E-06	AEF97693			
ORF25	32186-32917	244	hypothetical protein	---NA---					
ORF26	32986-34983	666	hypothetical protein	ORF37 [Acipenserid herpesvirus 2]	1.31E-98	AEF97695			
ORF27	34799-35512	238	hypothetical protein	---NA---					
ORF28	35525-39076	1184	hypothetical protein	ORF39 [Ictalurid herpesvirus 1]	2.02E-178	NP_041130			
ORF29	39070-39765	232	hypothetical protein	---NA---					
ORF30	39817-40728	304	hypothetical protein	ORF41 [Ictalurid herpesvirus 1]	1.16E-29	NP_041132			
ORF31	40727-41107	127	hypothetical protein	---NA---					
ORF32	41196-43916	907	hypothetical protein	ORF43 [Acipenserid herpesvirus 2]	6.81E-82	AEF97701			
ORF33	43866-44900	345	hypothetical protein	ORF44 [Acipenserid herpesvirus 2]	1.83E-58	AEF97702			

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF34	45949-46311	121	hypothetical protein	---NA---					
ORF35	46712-47860	383	hypothetical protein	ORF60 [Siberian sturgeon herpesvirus]	9.10E-36	ADC79712			
ORF36	48018-49622	535	hypothetical protein	---NA---					
ORF37	49810-50286	159	hypothetical protein	---NA---					
ORF38	50265-50732	156	hypothetical protein	---NA---					
ORF39	50725-51519	265	hypothetical protein	---NA---					
ORF40	51455-52396	314	hypothetical protein	---NA---					
ORF41	52398-54239	614	hypothetical protein	ORF54 [Acipenserid herpesvirus 2]	1.10E-64	AEF97714			
ORF42	54343-55584	414	hypothetical protein	---NA---					
ORF43	56954-57475	174	hypothetical protein	---NA---					
ORF44	57521-58432	304	hypothetical protein	---NA---					
ORF45	58748-60781	678	hypothetical protein	hypothetical protein [Leifsonia aquatica]	2.04E-05	WP_025156087			
ORF46	60948-61700	251	hypothetical protein	---NA---					
ORF47	61871-62659	263	hypothetical protein	---NA---					
ORF48	62931-63656	242	hypothetical protein	---NA---					
ORF49	63778-63948	57	hypothetical protein	---NA---					
ORF50	63958-64854	299	hypothetical protein	---NA---					
ORF51	64880-65653	258	hypothetical protein	---NA---					
ORF52	65741-66526	262	hypothetical protein	---NA---					
ORF53	66635-67390	252	hypothetical protein	---NA---					

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF54	67451-68248	266	hypothetical protein	---NA---					
ORF55	68342-69304	321	hypothetical protein	---NA---					
ORF56	69607-70533	309	hypothetical protein	---NA---					
ORF57	70897-71688	264	hypothetical protein	---NA---					
ORF58	71929-72882	318	hypothetical protein	---NA---					
ORF59	72963-73730	256	Immunoglobulin domain containing protein	PREDICTED: leucine-rich repeats and immunoglobulin-like domains protein 1 isoform X2 [Salmo salar]	2.53E-87	XP_014050970	Immunoglobulin domain	2.30E-06	PF13895.3
ORF60	73986-74735	250	Immunoglobulin domain containing protein	PREDICTED: leucine-rich repeats and immunoglobulin-like domains protein 1 isoform X2 [Salmo salar]	1.08E-92	XP_014050970	immunoglobulin domain	1.29E-05	cl11960
ORF61	75300-76082	261	hypothetical protein	---NA---					
ORF62	76786-77559	258	hypothetical protein	---NA---					
ORF63	77926-81636	1237	hypothetical protein	ORF56 [Acipenserid herpesvirus 2]	0	AEF97716			
ORF64	join(81809..84715, 84816..86567)		DNA polymerase	ORF57+58 [Acipenserid herpesvirus 2]	0	ACZ55868	putative DNA polymerase catalytic subunit	0	PHA03334
ORF65	86665-87207	181	dUTPase	dUTPase [Salmonid herpesvirus 1]	2.16E-40	AAC59317	deoxyuridine 5'-triphosphate nucleotidohydrolase	3.24E-30	PRK00601
ORF66	87350-88393	348	hypothetical protein	similar to ictalurid herpesvirus 1 ORF 48 encoded by GenBank Accession Number M75136 [Salmonid herpesvirus 1]	2.48E-106	AAC59318			
ORF67	88428-89432	335	hypothetical protein	---NA---					
ORF68	89665-90921	419	protein convertase	ORF47 [Acipenserid herpesvirus 2]	1.59E-56	AEF97705	Protein convertase	3.04E-28	cd04059

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF69	91068-95027	1320	membrane glycoprotein	glycoprotein, partial [Salmonid herpesvirus 3]	0	AGB07607	membrane glycoprotein	5.87E-97	PHA03332
ORF70	95099-96565	489	hypothetical protein	---NA---					
ORF71	96750-97607	286	hypothetical protein	---NA---					
ORF72	97607-98626	340	hypothetical protein	---NA---					
ORF73	98747-99985	413	serine/threonine kinase	---NA---			Serine/Threonine Kinase	1.43E-04	cd06606
ORF74	100097-100675	193	hypothetical protein	---NA---					
ORF75	100908-101132	75	hypothetical protein	---NA---					
ORF76	101439-101945	169	hypothetical protein	---NA---					
ORF77	102129-102659	177	apoptosis regulator, Bcl-2 protein	---NA---			Apoptosis regulator proteins, Bcl-2 family	2.00E-05	pfam00452
ORF78	102890-103255	122	macro domain containing protein	PREDICTED: O-acetyl-ADP-ribose deacetylase 1 [Cyprinodon variegatus]	3.31E-18	XP_015228945	Macro domain, Poa1p_like family	3.25E-25	cd02901
ORF79	103324-103785	154	macro domain containing protein	PREDICTED: O-acetyl-ADP-ribose deacetylase 1 [Nomascus leucogenys]	5.87E-12	XP_003266362	Macro domain, Poa1p_like family	1.14E-16	cd02901
ORF80	103837-104931	365	hypothetical protein	orf61, partial [Salmonid herpesvirus 2]	1.48E-107	CAA56949			
ORF81	join(104882..105763, 122252..122821, 123565..124368)	751	DNA packaging terminase subunit 1	DNA packaging terminase subunit 1 [Silurid herpesvirus 1]	0.00E+00	AVP72266	putative ATPase subunit of terminase	0	PHA03333
ORF82	105843-107894	684	primase	ORF63 [Acipenserid herpesvirus 2]	1.54E-98	AEF97717	putative primase	4.29E-06	PHA03330
ORF83	107891-109372	494	hypothetical protein	ORF64 [Acipenserid herpesvirus 2]	3.08E-77	AEF97718			

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF84	109692-114767	1692	hypothetical protein	PREDICTED: apoptotic chromatin condensation inducer in the nucleus-like isoform X3 [Sinocyclocheilus anshuiensis]	2.69E-09	XP_016316513			
ORF85	114770-116167	466	hypothetical protein	ORF66 [Acipenserid herpesvirus 2]	1.72E-18	AEF97720			
ORF86	116166-121235	1690	hypothetical protein	ORF67 [Acipenserid herpesvirus 2]	6.52E-116	AEF97721			
ORF87	121232-122449	406	hypothetical protein	unnamed protein product, partial [Salmonid herpesvirus 2]	4.50E-137	CAA49845			
ORF88	122849-123520	224	hypothetical protein	ORF70 [Ictalurid herpesvirus 1]	1.35E-28	NP_041161			
ORF89	124534-129111	1526	hypothetical protein	ORF72 [Ictalurid herpesvirus 1]	1.66E-33	NP_041162			
ORF90	131627-135640	1338	serine/threonine Kinase	ORF73 [Ictalurid herpesvirus 1]	1.27E-46	NP_041163	Serine/Threonine protein kinase	5.01E-22	smart00220
ORF91	135545-136312	256	RING-finger-containing E3 ubiquitin ligase	---NA---			RING-finger-containing E3 ubiquitin ligase	6.20E-03	COG5574
ORF92	139379-140851	491	hypothetical protein	---NA---					
ORF93	140670-142121	484	hypothetical protein	ORF78 [Ictalurid herpesvirus 1]	5.35E-25	NP_041168			
ORF94	142076-142711	212	hypothetical protein	---NA---					
ORF95	142909-143694	262	hypothetical protein	---NA---					
ORF96	143831-145024	398	hypothetical protein	---NA---			ribonuclease E	7.99E-06	PRK10811
ORF97	145839-147023	395	hypothetical protein	PREDICTED: collagen alpha-3(VI) chain [Monodelphis domestica]	1.76E-16	XP_007486135	Epstein-Barr virus nuclear antigen 3	1.26E-05	cl27975

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF98	147183-149456	758	hypothetical protein	---NA---					
ORF99	150324-151373	350	large tegument protein	---NA---			large tegument protein UL36	8.48E-05	PHA03247
ORF100	151437-151676	80	hypothetical protein	---NA---					
ORF101	151791-152759	323	protein kinases	ORF73 [Acipenserid herpesvirus 2]	8.78E-13	AEF97683	Catalytic domain of Protein Kinases	2.63E-19	cd00180
ORF102	152829-154043	405	hypothetical protein	---NA---					
ORF103	154191-155420	410	hypothetical protein	---NA---					
ORF104	155589-156404	272	hypothetical protein	---NA---					
ORF105	156580-156762	61	hypothetical protein	---NA---					
ORF106	156869-157687	273	hypothetical protein	---NA---					
ORF107	158528-160912	795	large tegument protein	---NA---			large tegument protein UL36	5.73E-05	PHA03247
ORF108	161292-162821	510	hypothetical protein	---NA---			superantigen-like protein	2.66E-03	PRK13042
ORF98	147183-149456	758	hypothetical protein	---NA---					
ORF99	150324-151373	350	large tegument protein	---NA---			large tegument protein UL36	8.48E-05	PHA03247
ORF100	151437-151676	80	hypothetical protein	---NA---					
ORF109	162943-164616	558	inhibitor of apoptosis domain containing protein	---NA---			Inhibitor of Apoptosis domain	1.48E-04	pfam00653
ORF110	164934-165038	35	hypothetical protein	---NA---					
ORF111	165492-165929	146	hypothetical protein	---NA---					
ORF112	166020-166724	235	hypothetical protein	---NA---					
ORF113	166773-167402	210	hypothetical protein	---NA---					
ORF114	168112-168915	268	hypothetical protein	---NA---					
ORF115	169133-169951	273	hypothetical protein	---NA---					
ORF116	169973-171262	430	hypothetical protein	---NA---					

Table 1. Continued.

ORF	Position	Product size (AA)	Predicted function	Best BLAST hit[a]			Best conserved domains hit		
				Description	E-value	Accession no.	Description	E-value	Accession no.
ORF117	171521-172123	201	hypothetical protein	---NA---					
ORF118	172592-173236	215	hypothetical protein	---NA---					
ORF119	173262-174242	327	hypothetical protein	---NA---					
ORF120	174582-176015	478	hypothetical protein	---NA---					
ORF121	176431-177246	272	deoxynucleoside kinase	PREDICTED: deoxynucleoside kinase-like [Diuraphis noxia]	1.59E-13	XP_015370112	Deoxynucleoside kinase	2.36E-20	pfam01712
ORF122	177404-177643	80	hypothetical protein	---NA---					
ORF123	177707-178756	350	large tegument protein	---NA---			large tegument protein UL36	8.48E-05	PHA03247
ORF124	179624-181897	758	hypothetical protein	---NA---					
ORF125	182057-183241	395	hypothetical protein	PREDICTED: collagen alpha-3(VI) chain [Monodelphis domestica]	1.76E-16	XP_007486135	Epstein-Barr virus nuclear antigen 3	1.26E-05	cl27975

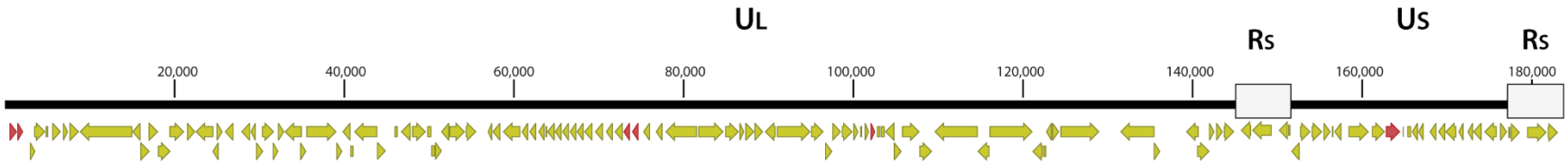

Figure 1. Schematic representation of the EDDV (SalHV3) genome.

Table 2. GenBank accession numbers for the alloherpesvirus sequences used in the phylogenetic analyses.

Species name (Virus abbreviation)	**DNA polymerase**
Ranid herpesvirus 2 (RaHV2)	ABG25576.1
Ranid herpesvirus 1 (RaHV1)	AAD12269.1
Cyprinid herpesvirus 3 (CyHV3)	AAX53082.1
Cyprinid herpesvirus 2 (CyHV2)	AKC02025.1
Cyprinid herpesvirus 1 (CyHV1)	AAX53084.1
Anguillid herpesvirus 1 (AngHV1)	ADA57818.1
Salmonid herpesvirus 2 (SalHV2)	ACD84537.1
Salmonid herpesvirus 1 (SalHV1)	ACD84535.1
Ictalurid herpesvirus 2 (IcHV2)	ACZ55873.1
Ictalurid herpesvirus 1 (IcHV1)	AAA88160.2
Acipenserid herpesvirus 2 (AcHV2)	ACZ55868.2
Esocid herpesvirus 1 (EsHV1)	KX198667
Silurid herpesvirus 1 (SiHV1)	KX198668
Salmonid herpesvirus 3 (SalHV3)	This study

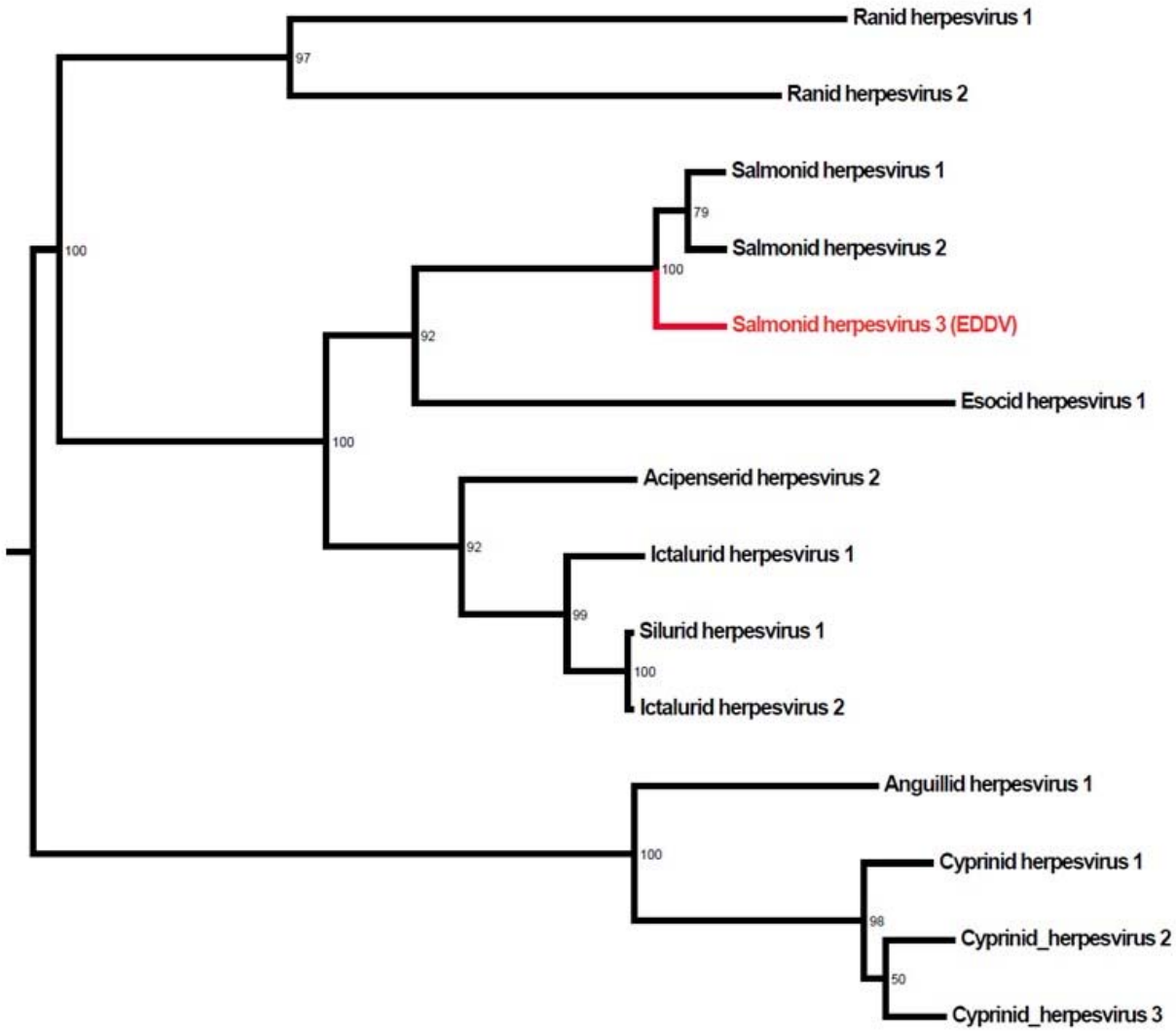

Figure 2. Phylogram depicting the relationship of EEDV (SalHV3) to other alloherpesviruses based on the amino acid (AA) sequences of the full length DNA polymerase gene.

<u>OBJECTIVE 2</u>: To determine EEDV host range, disease course, and modes of transmission.

The following species/strains were obtained from Michigan State Fish Hatcheries for use in host susceptibility challenges: lake trout [Lake Superior (LS) and Seneca Lake (SE) strains], brook trout, splake (Marquette State Fish Hatchery), brown trout, rainbow trout (Oden State Fish Hatchery), Atlantic salmon and coho salmon (Platte State Fish Hatchery), and muskellunge (Wolf Lake State Fish Hatchery). Lake herring were obtained from the Little Traverse Bay Band of Odawa Indians at their rearing facility in Levering, Michigan. Largemouth Bass were obtained from the La Crosse Fish Health Center. Mottled sculpin were collected from Brundage Creek during fall sampling efforts. Selected fish species are presented in Table 3. Fish were

acclimated at the Michigan State University Research Containment Facility (MSU-URCF) prior to use in experiments. Fish were fed commercial feed *ad lib.* and kept in flow-through PVC tanks (70L) at 10 ºC. Prior to use, a subsample of the fish were subjected to thorough clinical and laboratory investigation to exclude the presence of EEDV.

Table 3. Fish species selected.

Salvelinus namaycush	lake trout, Lake Superior strain
Salvelinus namaycush	lake trout, Seneca Lake strain
Salvelinus fontinalis	brook trout
Salvelinus namaycush X *Salvelinus fontinalis*	splake
Salmo salar	Atlantic salmon
Salmo trutta	brown trout
Oncorhynchus tshawytscha	Chinook salmon
Oncorhynchus mykiss	rainbow trout
Coregonus artedi	lake herring
Esox masquinongy	muskellunge
Micropterus salmoides	largemouth bass
Cottus bairdii	mottled sculpin

Preliminary injection trials were initiated using both “purified” product (obtained from experiments described in Task 1) or clarified tissue homogenate of fish that developed the disease following experimental infection with the virus and were showing the typical clinical signs of EEDV (Figures 3-5). In both cases, naïve yearling lake trout were injected IP. No mortalities were seen following injection with the Ficoll gradient product. Five separate infection groups of yearling lake trout were IP injected with EEDV positive tissue homogenate. All groups were infected with a clarified homogenate of tissues collected from a 2013 die-off of lake trout. Whole fish were stored at -80°C until ready for tissue collection and processing. The initial group of infected fish resulted in 80% mortality over 30 days, with tissues from 60% of the fish testing positive via PCR. The remaining four groups of fish showed no clinical signs and there was no mortality through the experimental period. Steroid injections using triamcinolone acetonide were used in an attempt to weaken the immune system during infection. Results showed that fish injected simultaneously with supernatants of infected fish tissue and corticosteroid succumbed to disease. Likewise, tissues from dead and dying fish contained high EEDV viral copies as determined by qPCR.

New infectious virus stock was obtained by serially infecting healthy lake trout (up to 7 fish passages). Following immersion exposure to 6th pass virus, 90% mortality was seen with initial mortalities occurring within 11 days post-infection. From each group of infected fish, skin samples were collected, processed and a tissue homogenate was stored at -80C for use in future infection trials. The 7th pass virus was titrated using quantitative PCR (9.5x10^{6}/µl) and then aliquoted in 15 ml tubes for use in all future trials.

Figure 3. Ocular hemorrhage following injection with EEDV infected tissue homogenate along with corticosteroids

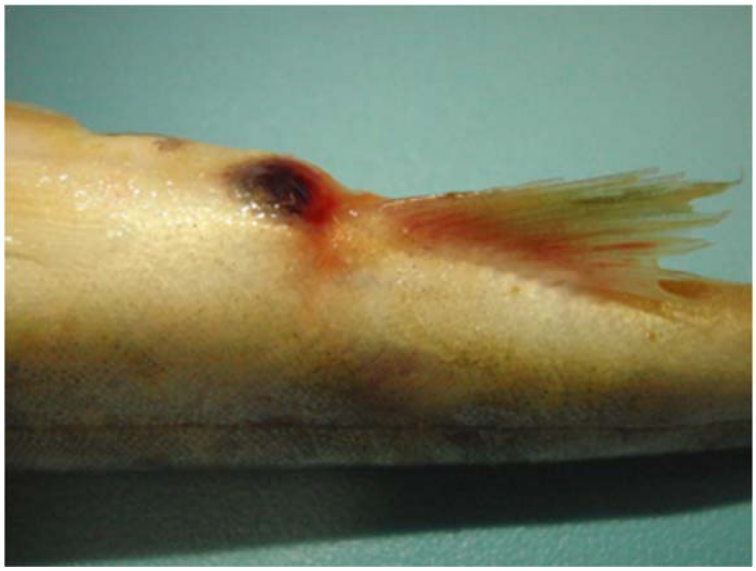

Figure 4. Fin and skin lesions along with protruded vents and signs of hemorrhagic enteritis following injection with EEDV infected tissue homogenate along with corticosteroids

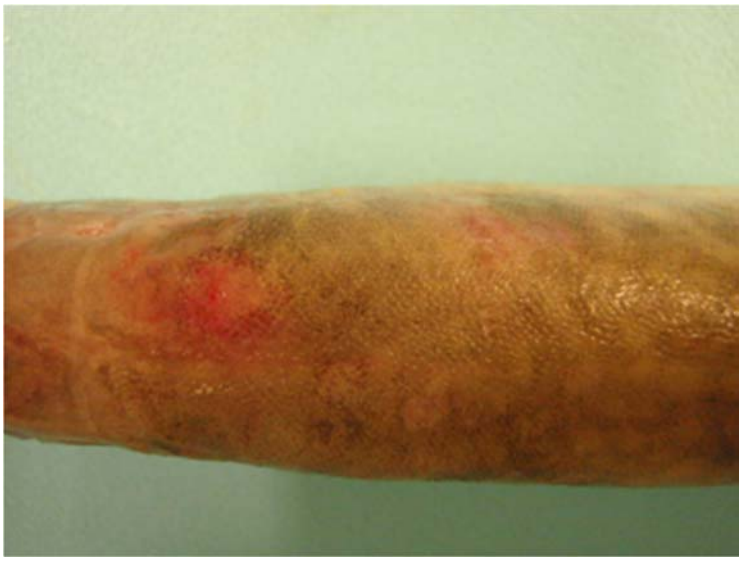

Figure 5. Skin erosion following injection with EEDV infected tissue homogenate along with corticosteroids

To elucidate the EEDV host range in multiple Great Lakes fish species, fish were intraperitoneally injected with EEDV at high and low doses (n=10 per fish species per challenge dose), monitored, and samples collected for viral detection using quantitative PCR. High dose fish received a total of $4.75x10^5$ copies per fish (50 µl of 7th pass virus stock mentioned previously). Low dose fish received 1:1000 dilution of this stock. Negative control fish received 50 µl of sterile tissue culture media. No lake herring, rainbow trout, largemouth bass, coho salmon, brown trout, brook trout or Atlantic salmon died in any of the challenge groups during this experiment (monitoring period was extended to ~33-62 days, based on the length of time for disease signs to develop in each species). Of note, 80% of the LS lake trout receiving the high virus dose died and showed characteristic EEDV disease signs, to include exophthalmia, ocular hemorrhages, corneal opacity, evidence of epithelial/mucus hyperplasia, and dermal hemorrhages. EEDV DNA was detected in 100% of high dose LS lake trout. In contrast, no high-dose SE lake trout died, despite the facts that EEDV was detected in a subset of the fish at the completion of the experiment, and that many showed significant disease signs (e.g., skin mottling, exophthalmia, fin hemorrhage, corneal opacity, lethargy, etc.). EEDV was also detected in one mottled sculpin (skin, low dose group); one splake (skin and K/S, high dose group); and four SE lake trout (skin, high dose group). Non-EEDV associated mortality unfortunately occurred in a subset of the experimental challenge groups (splake, muskellunge, mottled sculpin, LS lake trout). Further details regarding the host range experiments are presented in **Appendix II** in the manuscript entitled “Elucidating the host range of epizootic epitheliotropic disease virus (Salmonid herpesvirus-3)”.

Further experiments were performed on lake trout only. First and foremost, it was important to confirm the ability of EEDV to infect lake trout by immersion. This experiment was performed in triplicate. Three replicates of LS lake trout fingerlings (n=10 per challenge group) were infected with EEDV via immersion challenge in 600 ml of water: negative control (600 µl sterile tissue culture media), low dose (600 µl of 1:1000 dilution of 7th pass virus stock, or $9.5x10^3$ copies/ml water), and high dose (600 µl of 7th pass virus stock, or $9.5x10^6$ copies/ml water) groups.

Additionally, one group was intraperitoneally injected with 50 µl of 7th pass virus stock (4.75x10^{8} copies per fish) as a positive control group. Fish were monitored for 30 days and skin, gills and kidney/spleen pools were collected daily from all mortalities, and from all surviving fish at the completion of the 30 day observation period. Briefly, there were no mortalities in the low dose groups, while high mortalities were observed in the positive control group, as well as in two out of the three high dose groups. In Tank #2 of the control group, two fish died from non-EEDV related causes. From the two high dose groups that experienced mortalities, EEDV was detected in 90-100% of the skin tissues and in 100% of the gills and kidney/spleen pools tested. One fish from the third replicate high dose tank also tested positive for EEDV via PCR. More details are given in **Appendix III** in the manuscript entitled "Development and progression of gross and microscopic lesions in lake trout (*Salvelinus namaycush*) experimentally infected with Epizootic Epitheliotropic Disease Virus (salmonid herpesvirus-3)".

In order to estimate the median lethal dose (i.e., LD_{50}) using waterborne (i.e., immersion) challenge, LS lake trout fingerlings were immersed in water containing five viral dilutions (that ranged from 2.1x10^{1} to 1.01x10^{6}). For a negative control group, sterile tissue culture media was used in place of the virus dilutions. Mortalities were collected over a 30 day period, after which all surviving fish were euthanized. Skin tissues were tested for the presence of EEDV from all fish, and a subset of gills and kidney/spleen pools from fish that died were also assayed via qPCR. As per Reed & Muench (1938), the LD_{50} was calculated to be approximately 4.7x10^{4} viral copies per ml of immersion water. More details are given in **Appendix III**.

Determination of EEDV disease course:
80 fingerling lake trout were infected with EEDV via waterborne challenge, whereas 40 served as a negative control group. Fish were sampled on days 0, 1, 3, 6, 9, 12, 15, 18, 21, 28, 35, and 42 following infection. Gill, fin, skin, eye, brain, heart, spleen, liver, intestine and kidney tissues were collected from six infected fish and three control fish on each sample day through day 28. On days 35 and 42, 2 control fish were sampled. One additional whole fish from each group and a portion of each tissue was preserved in 10% buffered formalin and saved for histopathological analysis, and the remaining tissue was collected for EEDV detection via PCR. The first EEDV-positive tissue was found in eye tissue collected on day 9, followed by positive results in fins, skin, eyes, spleens, liver and kidneys collected on day 18. On day 21, EEDV was detected in every tissue type except for the heart. On day 28, all tissues from all six infected fish were EEDV-positive. On days 35 and 42, EEDV was detected in every tissue from at least two infected fish. More details are given in **Appendix III.**

Histopathologic examination was performed on a total of 754 tissues from 12 control fish and 67 infected fish. The major microscopic lesions were observed in the skin and fins, which included individual epithelial cell degeneration and inflammatory cell infiltration that eventually progressed to massive epithelial ulceration and epidermal loss. Hepatic lipidosis, which is a common finding in hatchery-reared fish that are fed artificial diets, was observed in nearly all control fish throughout the study and among infected fish until the last several sampling weeks, indicating a decreased appetite as the infection progressed. Also observed in the liver was individual hepatocellular necrosis and lymphohistiocytic perivascular inflammation. Additional lesions were observed in the spleen (lymphoid depletion and focal necrosis), omentum (lymphohistiocytic perivasculitis), heart (lymphohistiocytic perivasculitis and epicarditis), gills (proliferative branchitis), and kidney (hematopoietic cellular necrosis and depletion). More details are given in **Appendix III**.

One of the most important aspects that was investigated during this study (though not originally proposed) was to follow up on the kinetics of EEDV shedding into water. For this experiment, pit-tag microchipped Lake Superior strain lake trout that were intraperitoneally (IP) injected with EEDV were used. To assess virus shedding, each infected fish was housed individually in a static aerated aquarium for 8 hours, then water was assessed for the presence of EEDV DNA using quantitative PCR assay. Water sampling was conducted every seven days for up to 93 days post-infection (pi). Our results demonstrated that infected lake trout start to shed EEDV into the water as early as 9 days pi and shedding peaked ~three weeks pi and continued for up to ~9 weeks pi. Mortality began at day 40 pi, and viral shedding ceased ~ 70 days pi. Although mortality reached 73.9%, surviving fish ceased shedding and continued to grow. Findings of this study demonstrated that EEDV is shed in the water by infected hosts for extended periods of time, a matter that favors the dissemination of the virus. More details are given in **Appendix IV** in the manuscript entitled "Shedding of Salmonid herpesvirus-3 by infected lake trout (*Salvelinus namaycush*).

Task 7: Role of stressors:

Sub-task 7a: Determination of the role of stressors in the initiation of EEDV epizootics:

It was obvious in the 2012 and 2017 outbreaks that EEDV mortality occurs after rain events. We also noticed that increased stocking density encourages the eruption of outbreak in fish that survived EEDV (For details see **Appendix I**). In order to verify if stress hormones mediate the increased susceptibility to EEDV, four groups of EEDV-infected yearling lake trout were exposed to triamcinolone acetonide (4 mg per fish, intra-muscular injection), which is an immunosuppressive steroid. Mortality was observed within one week of infection. On the contrary our observation indicated that mortality take place three weeks post-infection without the exposure to stress hormones.

Reflections on the achievement in OBJECTIVE 2:

- Completion of molecular analysis for EEDV host species susceptibility confirmed the narrow host specificity of the virus. However, these experiments also show that susceptibility to EEDV varies substantially between two strains of lake trout, which may have important implications for future lake trout rehabilitation efforts.
- In regards to the immersion challenges, one concerning result was the lack of mortality in the third replicate of the high dose challenge. One possible explanation is the nature of herpesviruses, when they often do not undergo a full infection cycle until a later date. We have experienced a similar phenomenon when we kept surviving fish from the Marquette State Fish Hatchery and outbreaks erupted several months after the complete recovery of survivors.
- Concentrations of virus reported in the LD_{50} challenge vary slightly from expected 1:10 dilutions of the 7th pass EEDV stock. This is because the doses reported are exact calculations following quantitative PCR on the dilutions used for infection. Ten-fold dilutions were prepared from the 7th pass stock. These dilutions were then used for the LD_{50} challenge and a portion saved for DNA extraction and quantitative PCR.
- The initial plan was to sample seven fish per day from the infected tank during the disease course challenge. Based on previous infection trials and the relatively slow nature of this disease, it was decided to decrease the sample number to six in order to have one additional sampling day at the end of the trial.
- Following completion of the disease course challenge, it became clear the progression was much slower than initially anticipated, taking almost a month before mortality occurred and

tissues consistently tested positive for EEDV. In hindsight, challenges focusing on the period from 20-50 days post-infection and beyond would be beneficial in order to follow fish through the later stages of infection and recovery of disease.

- Analysis of microscopic lesions in tissues collected during the disease course challenge revealed primary lesions in the skin and fins with the addition of focal necrosis as well as lymphoid and hematopoietic cell depletion in multiple visceral organs. These changes corresponded with the identification of EEDV DNA using qPCR with the most severe lesions observed one month following viral exposure.
- Findings under this objective demonstrated that EEDV is shed in the water by infected hosts for extended periods of time, a matter that favors the dissemination of the virus.

OBJECTIVE 3: To elucidate humoral immune mechanisms involved in lake trout defense against EEDV.

Task 8: Production of hyperimmune antibodies against lake trout immunoglobulins:
We designed the IgT primers (Table 4) based on the available gene information for rainbow trout and other salmonids, and have cloned the IgT from lake trout and generated recombinant protein (r-IgT) in *E.coli*. The rIgT proteins were used to immunize rabbits for generation of polyclonal anti-IgT antibody. The specificity of the anti-sera was tested. The anti-IgT antibody can recognize IgT from both lake trout and rainbow trout serum samples. The polyclonal anti-IgT antibody can recognize sera samples from coho salmon, rainbow trout and pink salmon, as well as weak recognition of Atlantic salmon IgT indicating that IgT shares antigenic similarities among salmonids. Purified IgT was obtained in high enough amounts to generate poly- and monoclonal antibodies against it. Moreover, lake trout IgM from lake trout sera was purified and antibodies against the lake trout pure IgM was generated by immunizing rabbits and rats.

The IgT fractions can be detected by SDS-PAGE with polyclonal anti-IgT antibodies, but due to the quick digestion during the purification process, we could not get enough native IgT from lake trout sera for generation of anti-IgT antibodies.

Native IgM from lake trout sera was partially purified from lake trout serum by a single precipitation with 16% PEG and anion (Mono Q HR 5/5) and cation (Mono S HR 5/5) chromatography. The most concentrated fractions containing the lake trout IgM were pooled together and concentrated by Amicon filter through centrifugation. The concentrated fraction was then passed through gel filtration on Superdex 200 FPLC column (GE Healthcare). Elution profiles are shown in Figure 6. All the fractions were analyzed by loading fractions onto 4-15% SDS-PAGE at reducing and non-reducing conditions followed by Coomassie blue staining (Figure 7) and immunoblotting with anti-trout IgM/IgT polyclonal antibodies (Figure 6). Fractions (elution volume 8.5 & 9.0ml) that contained the pure Lake Trout IgM were collected, pooled, and outsourced for immunizing rabbit and rat to generate anti-lake trout IgM antibodies.

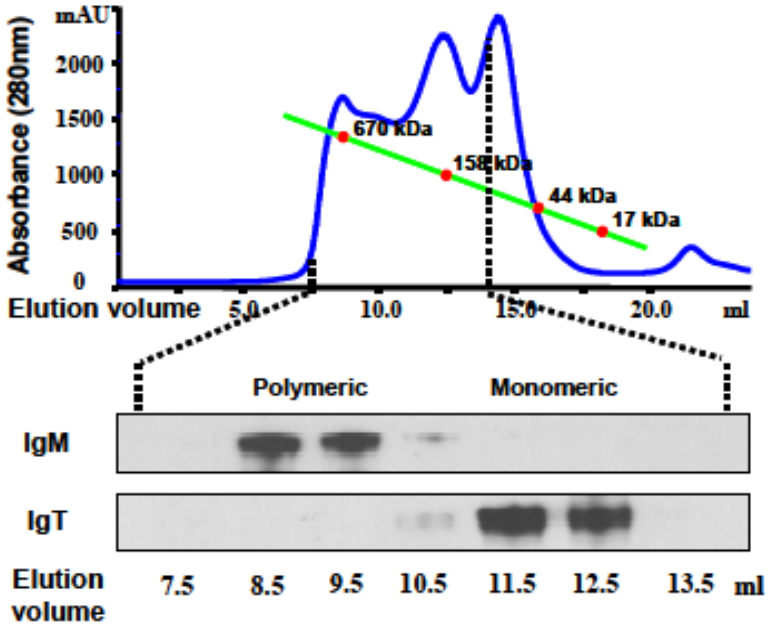

Figure 6. Elution profile of purified immunoglobulin with silver stained electrophoresis showing pure fractions of lake trout IgM and IgT

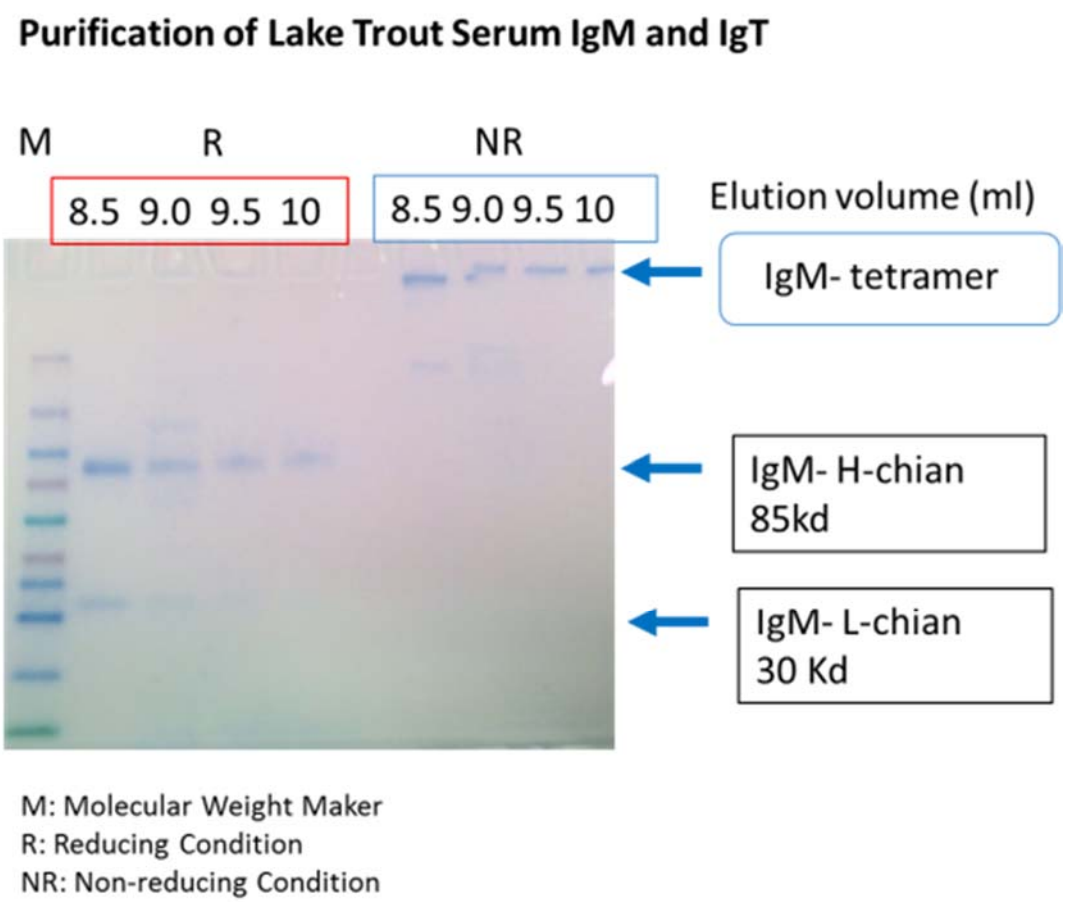

Figure 7. Commassie blue stained SDS-PAGE electrophoresis of fractions containing purified lake trout immunoglobulins (elution volumes 8.5 -10 ml, 0.5 ml/fraction) at reducing (R) and non-reducing (NR) conditions. Under the Non-reducing (NR) condition, the upper bands showed the tetramer of lake trout IgM (IgM-tetramer); Under the reducing (R) condition, the middle bands indicate the heavy chain of lake trout IgM (IgM-H chain) and the lower bands indicate the light chain of lake trout IgM (IgM-L-chain).

Table 4. IgT primers

IgT-F1：**R**GAGGAAGACAC**S**AAGAAGGA IgT-F2：GTGACTCTG**R**TGTGCCTGGT IgT-R1：ACTT**Y**GACAC**W**CTC**M**TCTCCT　　Tm: 64±2 ℃ Expect product: 250-290bp

Task 9: Assessment of immune responses against EEDV in lake trout:
After working out all reagents and optimization trials, all elements of the diagnostic assays are currently in place. The developed antisera are instrumental for the development of serological assays that will help us understand the immunosurveillance mechanisms against EEDV. That is something that did not exist before.

OBJECTIVE 4: To develop specific and sensitive diagnostic assays for EEDV detection in lake trout including carrier and previously exposed fish.

Task 10: Attempts to propagate EEDV in vitro:
Details are found in **Appendix V** in the manuscript entitled, "*In vitro* culture of lake trout cells (*Salvelinus namaycush*)".

Sub-task 10a: Attempt to develop cell lines from lake trout:
Primary cell cultures from adult lake trout (liver; Figure 8), yearling lake trout (fin and gonad) and lake trout sac fry (whole body; Figure 9) were created successfully. Culture flasks were monitored daily and sub-cultured when approaching 100% confluence. When exposed to samples containing Viral Hemorrhagic Septicemia Virus (VHSV) as well as Infectious Pancreatic Necrosis Virus (IPNV), the fry cells show significant CPE within 48 hours with majority of cell death occurring within 4-7 days (Figures 10&11).

Additionally, optimal growth criteria were established for these cells. When compared across multiple temperatures, serum concentrations and basal media types, it was determined that the lake trout cells grew ideally at 15°C with 15% fetal bovine serum in a Minimum Essential Medium (MEM)-based media (Figures 12-14). Further attempts were made on optimized cells to determine if they can support EEDV replication. As depicted in Figure 15a,b, there are some indications of cytopathic effects observed in the form of vacuolation and clustering. qPCR indicated that the virus DNA copies increased from the originally inoculated material, indicating the occurrence of some sort of virus replication.

Since many unsuccessful attempts to isolate EEDV have occurred since this disease first appeared in the 1980s, primary cell cultures of related species were also attempted. Skin and fin tissues were collected from lake herring (*Coregonus artedi*), treated with antibiotics, and then minced and trypsinized as detailed by Wolf and Quimby (1976). Tissue fragments were then planted into cell culture flasks. Trypsinized cell suspensions were neutralized with serum based cell culture media and placed into cell culture flasks. Several different cell culture media formulations were utilized in order to identify the most promising for future attempts. Only the L-15, supplemented with 20 % fetal bovine serum, was successful in producing enough primary lake herring cells to be successfully sub-cultured.

Fin and skin tissues were obtained and processed from more lake herring, and also bloater (*Coregonus hoyi*) cells are being grown in L-15. These cultures include: *Salvelinus namaycush* (Lake Trout Skin) to passage number 11; *Coregonus artedi* (Cisco Skin) to passage 46; *Coregonus artedi* (Lake Herring Skin I) to passage 7; *Coregonus artedi* (Lake Herring II) to passage 9; *Coregonus hoyi* (Bloater Fin) to passage 9, and *Coregonus hoyi* (Bloater Skin) to 10 passes. Additionally, we have successfully frozen, stored, and re-cultured the Cisco Skin cells.

Cell cultures of lake trout tissues have been produced and sub-cultured as follows: cultures of yearling gonads reached 29 sub-cultures, fry cells reached 44 sub-cultures. Characterization of the lake trout cells was performed using DNA barcoding to confirm species of origin was indeed lake trout (*Salvelinus namaycush*).

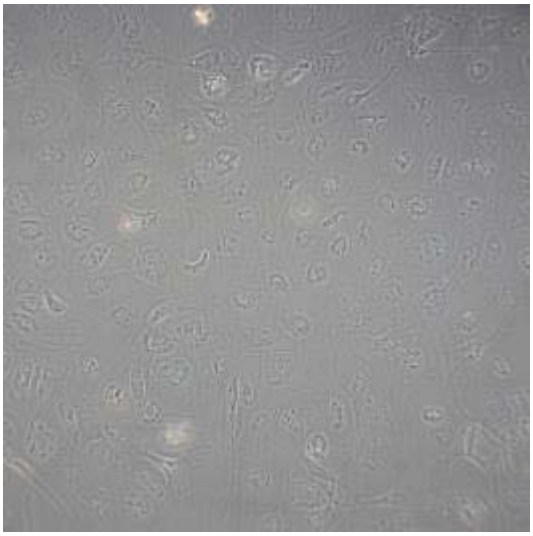

Figure 8. Adult lake trout liver cells in culture

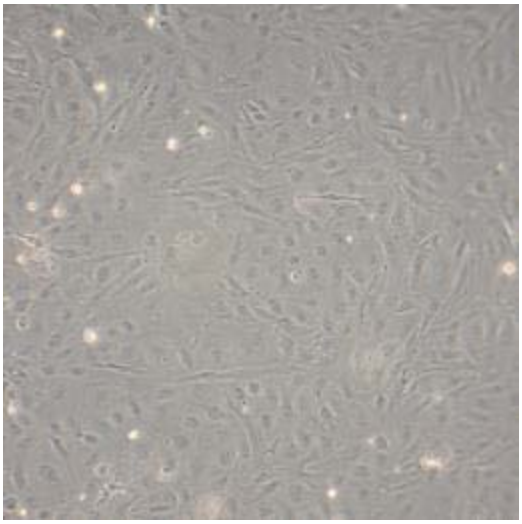

Figure 9. Lake trout fry cells in culture

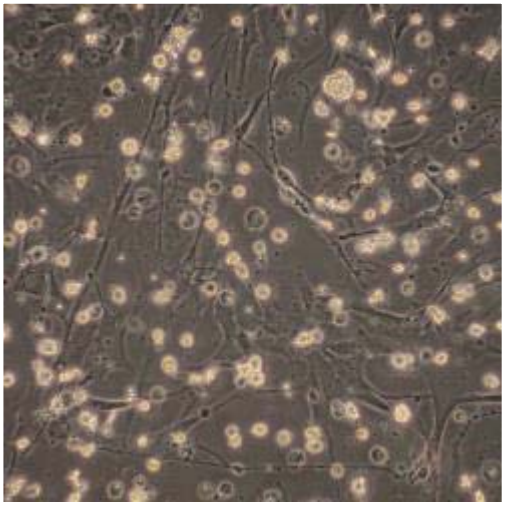

Figure 10. Suspicious CPE in lake trout (*Salvelinus namaycush*) fry cells following infection with VHSV

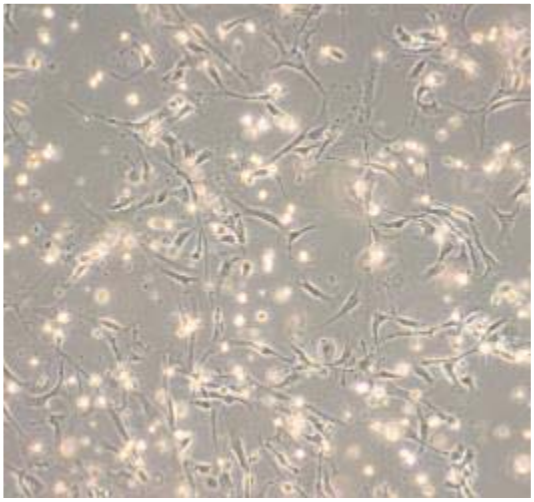

Figure 11. Suspicious CPE in lake trout (*Salvelinus namaycush*) fry cells following infection with IPNV

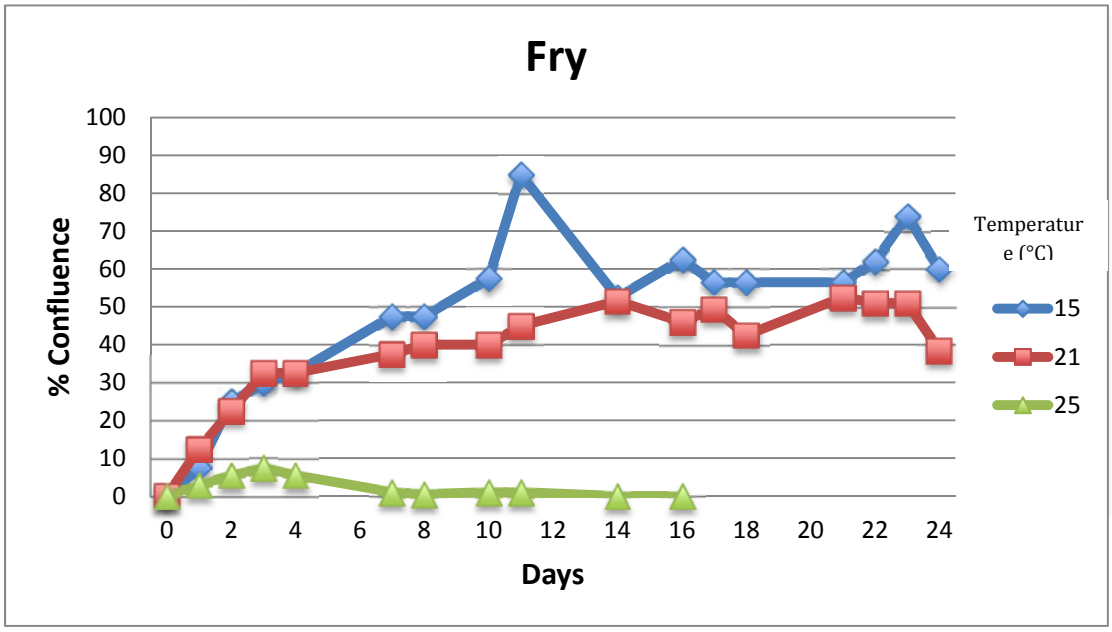

Figure 12. Growth curve of lake trout (*Salvelinus namaycush*) fry cells grown at three different temperatures (15°C, 21°C and 25°C)

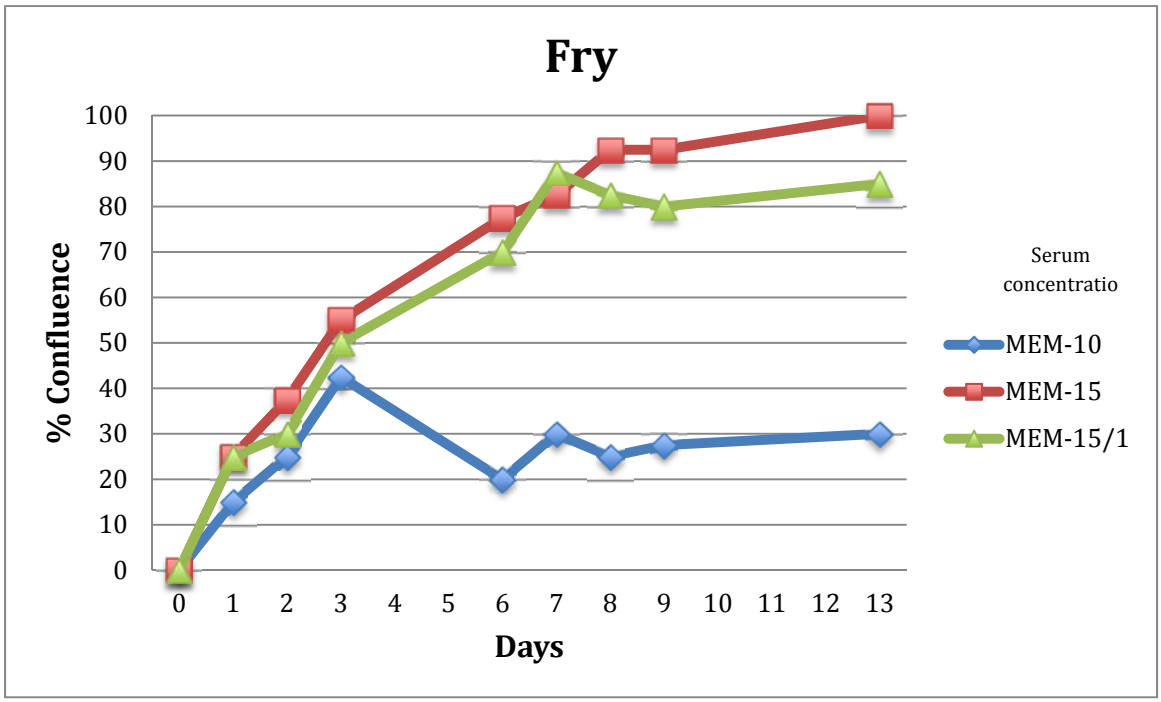

Figure 13. Growth curve of lake trout (*Salvelinus namaycush*) fry cells grown with three different serum concentrations: 10% FBS (MEM-10), 15% FBS (MEM-15) and 15% FBS + 1% lake trout serum (MEM-15/1)

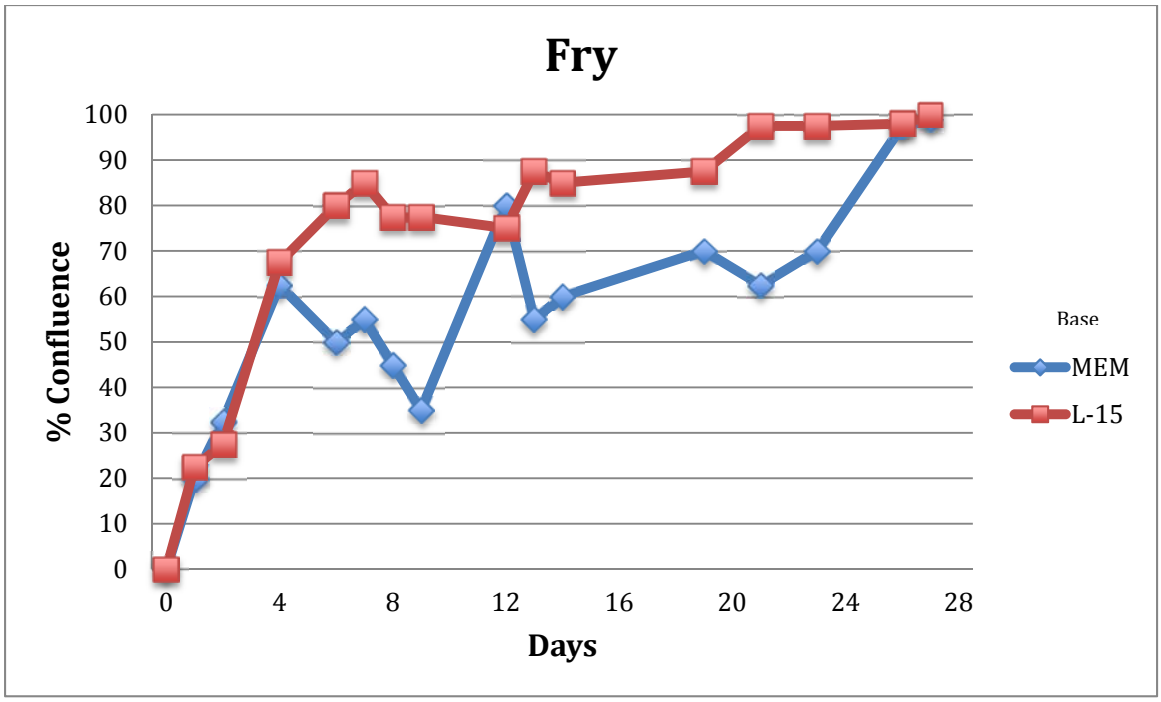

Figure 14. Growth curve of lake trout (*Salvelinus namaycush*) fry cells grown with two different base media: Minimum Essential Media (MEM) and Leibowitz (L-15)

Sub-task 10b: Attempt to propagate the virus on established fish cell lines:
Several attempts to propagate the virus on a number of established cell lines did not yield any virus propagation and therefore we focused on producing EEDV stocks from infected lake trout tissue.

Sub-task 10c: Inoculation of cell cultures with EEDV infected samples:
In order to test the temperature at which the virus will grow, infected cultures were placed at 4°C and at 15°C. Infected cells placed at both temperatures showed suspicious CPE in first passages yet unremarkable changes in subsequent passes. Quantitative PCR performed on samples prior to and after incubating on lake trout fry cells at 4°C for 5 days indicates an increase in viral DNA copy number (from 17 copies/ng DNA in the original material to 37 copies/ng of DNA in the inoculated cells). This indicated clearly that we have achieved some kind of EEDV replication, albeit very slow.

Lake trout fry and yearling gonad cells were seeded into 12.5cm^2 culture vessels and inoculated with EEDV-positive tissue homogenate and incubated at 9 degrees C. Following development of CPE or cell lysis, samples were collected and passed to fresh cells using either supernatant or cells that had been rinsed in maintenance media. Examples of lysis seen is shown in Figures 16&17.

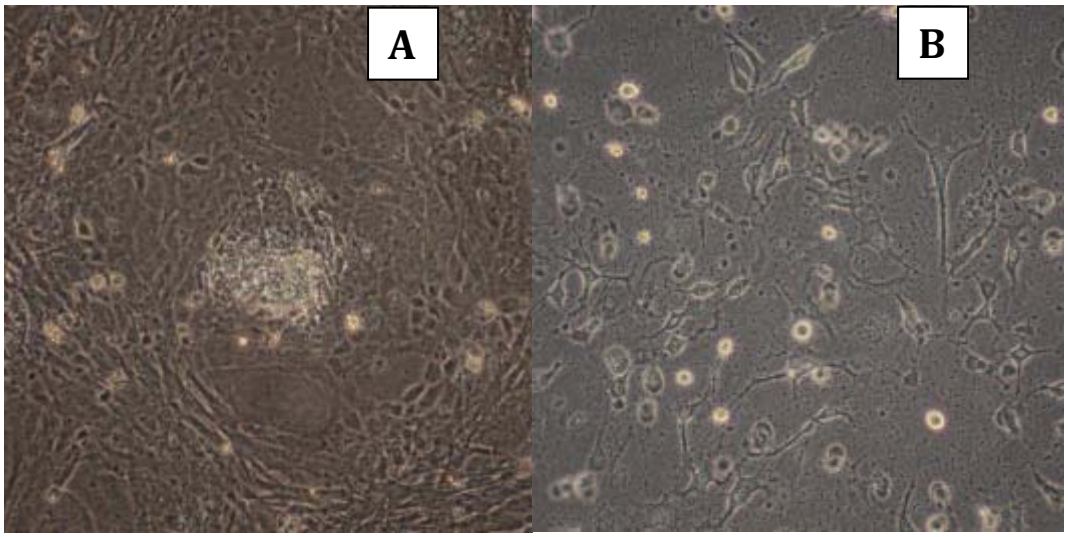

Figure 15. Suspicious cytopathic effect (CPE) in lake trout (*Salvelinus namaycush*) fry cells following inoculation with EEDV infected tissue suspension (MSU)

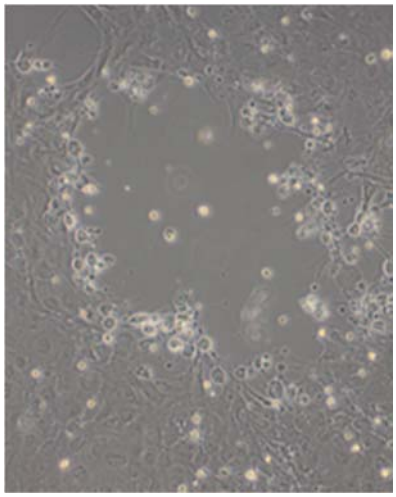

Figure 16. Fry cells infected with second pass EEDV+ cells

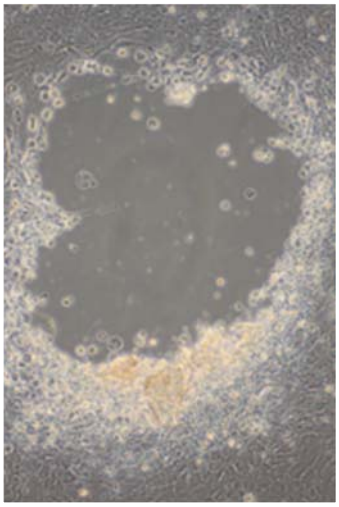

Figure 17. Gonad cells infected with second pass EEDV+ supernatant

<u>Task 11: Development of reliable EEDV molecular diagnostic assays:</u>

Sub-task 11a: Development of a sensitive real-time PCR assay specific for the detection of EEDV:

Based on the sequences of Alloherpesviridae members currently available in public databases and sequences performed at the Lamar Fish Health Center (USFWS), we developed a sensitive TaqMan® PCR assay to detect the presence of the terminase gene (GenBank EU349284) of EEDV in lake trout tissues. This assay was able to detect a linear standard curve over nine logs of plasmid dilution, and sensitive enough to detect single digit copies of EEDV. The mean PCR

efficiency of the assay was 99.4 ±0.06% (SD), with a 95% confidence limit of 0.0296 (R^2= 0.994). This real-time PCR assay was unexpectedly able to detect two additional herpesviruses, Atlantic salmon papillomatosis virus (ASPV/Salmonid herpesvirus 4), and Namaycush herpesvirus (NamHV/Salmonid herpesvirus 5), which both share high sequence identity with the EEDV terminase gene. With these unexpected findings, we subsequently designed three primer sets to confirm initial TaqMan® PCR assay positives and differentiate between EEDV, ASPV, and NamHV by detecting the glycoprotein genes via separate SYBR® Green real-time PCR assays (GenBank: EEDV- JX886027, ASPV- JX886028, and NamHV- KP686091).

We have successfully demonstrated the sensitivity of the TaqMan® PCR assay by detecting EEDV and NamHV in wild lake trout in a number of water bodies in the states of Pennsylvania, New York, and Vermont. Our preliminary findings indicate EEDV is more common in wild fish in the carrier or latent stage of infection than previously known. In combination with the TaqMan® PCR assay, we have shown the specificity of the three glycoprotein primer sets to differentiate these three Salmonivirus members via SYBR® Green real-time PCR technology. These assays have been confirmed by sequencing.

A qPCR was developed that is highly specific to salmonid Herpesvirus3 (EEDV) and can differentiate between it and salmonid herpesvirus 4 and 5. This newly developed assay has high sensitivity and specificity. The manuscript entitled “A Quantitative Polymerase Chain Reaction Assay for the Detection and Quantification of Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus 3)” has been published in the Journal of Aquatic Animal Health, Volume 28, Issue 1, pages 56-67, 2016. Details are given in **Appendix VI**, manuscript entitled “A quantitative polymerase chain reaction assay for the detection and quantification of epizootic epitheliotropic disease virus (EEDV; salmonid herpesvirus 3)”.

Sub-task 11b: Development of quantitative loop-mediated isothermal amplification (qLAMP):

We developed another molecular assay that is more time- and cost effective. Primers have been successfully designed and the test optimized. The test has also been validated and tested on a number of naturally and experimentally infected lake trout. This assay proved of equal sensitivity and specificity to the published qPCR assay by Glenney et al (2016) and was even able to detect the virus in mottled sculpin. Primers were specific enough that they did not recognize the closely related Salmonid Herpesvirus 4 or 5. Validation of the qLAMP assay was performed against the SYBR Green qPCR using previously known negative, low-, medium-, and high-positive tissue samples. Compared to the qPCR, the qLAMP has a diagnostic sensitivity of 84.3% and a diagnostic specificity of 93.3%. Details are given in **Appendix VII** in the manuscript entitled “Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3)”.

Sub-task 11c: Development of in situ hybridization (ISH) technique for the detection of EEDV:

A successful ISH technique has been developed and implemented by colleagues at the Michigan State University Veterinary Diagnostic Laboratory. The developed ISH technique shows a blue signal (NBT chromagen using an alkaline phosphatase labeled anti-digoxigenin detection system) and a nuclear fast red counter stain demonstrating presence of EEDV within cells of both the skin and gills associated with cellular necrosis.

This ISH technique has been instrumental in determining the distribution of EEDV throughout the course of disease and within particular lesions. Viral nucleic acid was observed within individual necrotic epithelial cells in the skin, within the nuclei of unremarkable gill lamellar

epithelial cells as well as in the nuclei of infiltrating mononuclear cells. Additionally, during late stages of disease, EEDV nucleic acid was detected in large numbers of mononuclear cells in the spleen as well as within blood vessels. Details are found in **Appendix VIII** in the manuscript entitled, “Progression of epizootic epitheliotropic disease virus (Salmonid herpesvirus 3) in target tissues and cells of its host, the lake trout (*Salvelinus namaycush*)”.

Task 12: To develop sensitive ELISA-based assays for detection of EEDV in clinical samples and quantitation of anti-EEDV antibodies in fish sera:

The ELISA assay to detect EEDV in clinical samples was developed and optimized using tissues of experimentally infected lake trout. To validate the assay, sera were collected from both Lake Superior and Seneca strain lake trout that were experimentally infected with either low or high EEDV dose along with negative control cohorts. The newly developed ELISA was used to assess the presence of EEDV in tissues. As expected, the ELISA assay did not find EEDV in the negative control fish. EEDV was detected by ELISA in 30% of the low dose group of LS lake trout but not in any of the SE lake trout that received the same dose. On the contrary, EEDV was detected in 50% of the high dose group of LS lake trout and 40% in SE lake trout that received the same dose. These finding again reflect the difference in susceptibility to EEDV by both strains of lake trout.

To quantitate anti-EEDV antibodies in fish sera, we developed rabbit anti- lake trout IgM and rat anti-lake trout IgM antibodies by immunizing rabbits and rats, respectively. The specificity to recognize lake trout IgM and the related antibody titer in the anti-sera were tested by ELISA. Figures 18 and Figure 19 showed the specific antibody titers. As shown in Figure 18 (immunized rabbit) and Figure 19 (immunized rat), after 1/10000 dilution of the anti-sera, the ELISA assay could specifically recognize the lake trout IgM from lake trout sera in comparison to the negative control (NC). The rabbit anti-lake trout IgM antisera showed higher antibodies titers than that in the rat antisera.

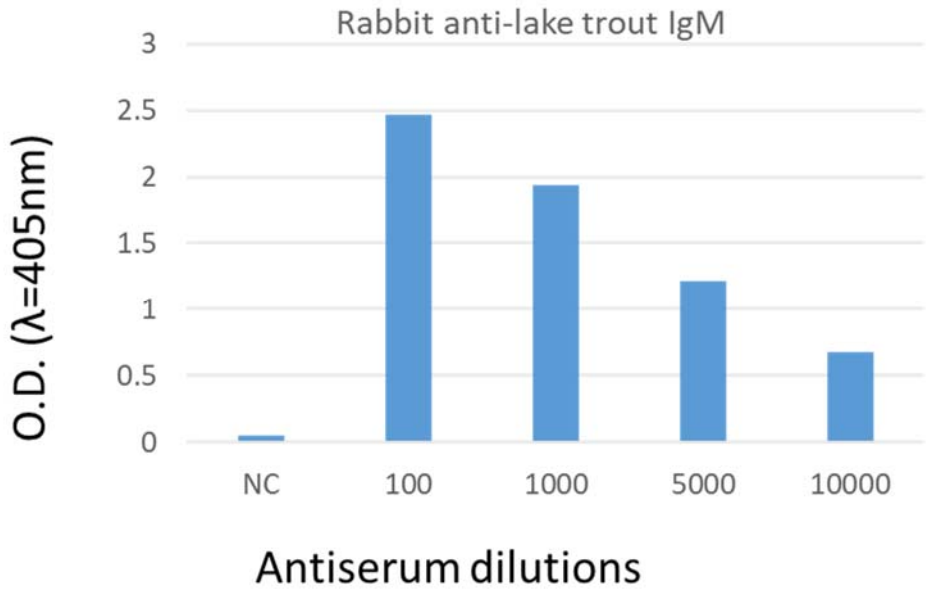

Figure 18: Generation of rabbit anti-lake trout IgM antibody. Rabbits were immunized by injection of purified lake trout IgM in Cocalico Biologicals, Inc., PA following their procedures. The prebleed (for negative control) and antiserum were harvested and stored in -80^{o}C until use.

The adult healthy lake trout sera were added into 96-well plate after 1:100 dilutions and incubated overnight at 4°C. After washing 3 times with PBS(1X), 200 microliter PBS, 1/100 diluted prebleed and serial diluted rabbit anti-lake trout IgM antiserum (1/100, 1/1000, 1/5000, 1/10000) were added into the plate and incubated for 2 hours at room temperature, and then following with the standard ELISA procedures for measurement of specificity and titers of antibody.

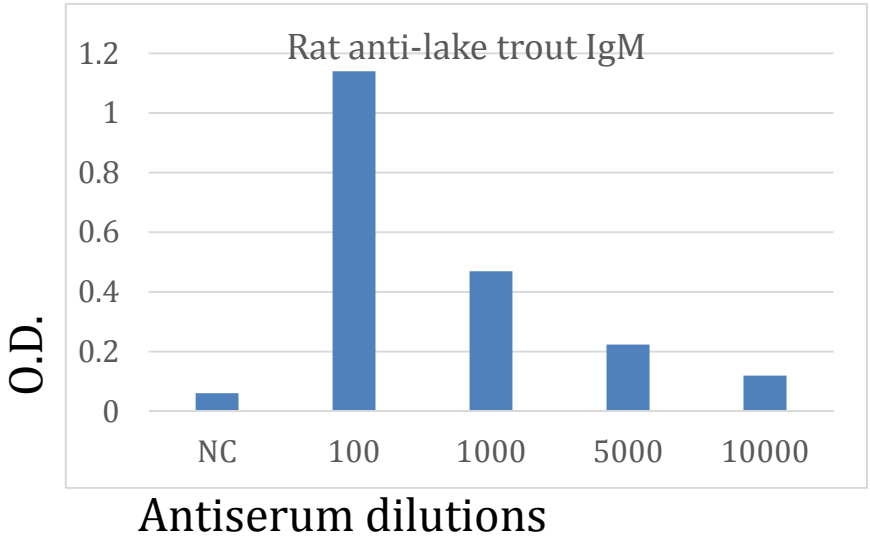

Figure 19: Generation of rat anti-lake trout IgM antibody. Rats were immunized by injection of purified lake trout IgM following their procedures (3 times immunization). The prebleed (for negative control) and antiserum were harvested and stored in -80°C until use. The adult healthy lake trout sera were added into 96-well plate after 1:100 dilutions and incubated overnight at 4°C. After washing 3 times with PBS (1X), 200 microliter PBS, 1/100 diluted prebleed and serial diluted rabbit anti-lake trout IgM antiserum (1/100, 1/1000, 1/5000, 1/10000) were added into the plate and incubated for 2 hours at room temperature, and then following with the standard ELISA procedures for measurement of specificity and titers of antibody.

By using the anti-lake trout IgM antibodies, we also investigated the immunological responses of IgM in the serum samples of lake trout experimentally infected at MSU by using ELISA. As shown in Figure 20, the total serum IgM were to be elevated in lake trout after experimental exposures to EEDV in a dose dependent manner.

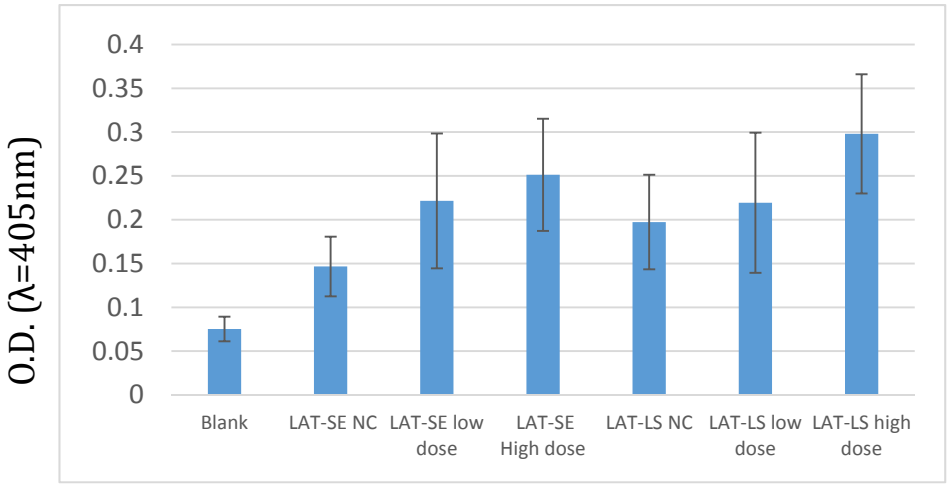

Figure 20: Immunological responses (serum IgM levels) of lake trout after EEDV exposures at low or high dosages. Lake trout serum samples (N=6-10 fish/group) were diluted 1/100 with PBS and then added into the 96-well plate for incubation at 4°C for overnight. The total IgM in the serum samples were then measured by using ELISA with the rat anti-lake trout IgM antibodies. (Mean±STD)

Armed with the newly developed reagents, we have developed an ELISA diagnostic method by coating the micro-well plates with EEDV positive homogenates, and incubated with the fish sera samples overnight, then we added the anti-lake trout IgM antibody and anti-Atlantic salmon IgM as well as anti-Rainbow trout IgM antibodies for specific fish species. The assay was a success and circulating antibodies against EEDV were detected in lake trout serum sera. This assay will be indispensable in determining if a lake trout population has been previously exposed to EEDV.

Highlights of Progress and Reflections in OBJECTIVE 4:

- One unanticipated benefit was the identification of another herpesvirus species in lake trout, *Namaycush* herpesvirus (NamHV), of which an important issue will be determining its pathogenicity – another project in itself.
- Another unanticipated benefit was the determination of the most promising cell culture media. In the first attempt, much work was done utilizing six different media formulations. Subsequent attempts allowed for six times more tissues being used with the best medium.
- The ISH technique has allowed for identification of viral targets both at a tissue and cellular level.
- A serological assays for EEDV detection and antibody quantitation were successfully developed for the first time.
- Two molecular assays have been developed that are specific and sensitive for EEDV detection.

<u>OBJECTIVE 5:</u> Test the efficacy of current biosecurity practices in hatcheries for the inactivation of EEDV, including egg disinfection.

Task 13: Efficacy of current disinfection techniques:

Sub-task 13a: Inactivation of EEDV on surfaces:

The inability to propagate EEDV *in vitro* delayed research under this objective. However, we devised an alternative method to assess the efficacy of various commercially available disinfectants on EEDV inactivation as follows. This experiment was done through *in vivo* challenges using live highly EEDV-susceptible fish as a means of detecting active virus. EEDV-laden nets were immersed in four commonly used disinfectants (i.e., iodophor, sodium hypochlorite, VirKon® and Nolvasan®) and then used to transfer naïve fish to experimental aquaria, thereby simulating virus transmission via fomites. Positive control groups were transferred in nets that are exposed to virus but not to disinfectants; a negative control group was transferred in nets that are not exposed to the virus. Exposed fish were maintained for >140 days and the presence of EEDV in experimentally exposed fish assessed via qPCR as described for previous tasks.

A clean and sterile fish net (first net) was soaked for 5 minutes in a bucket filled with water and containing a dose of EEDV corresponding to the LD_{100}. The first net was then pulled out and immediately soaked into another bucket containing diluted disinfectants, with the dilution and soaking period based on the recommended disinfectant administration and contact time that has been considered safe under field conditions. Using another clean and sterile fish net (second net), 20 live fish were moved out of the holding tank and directly transferred into the first net (virus-containing net). Fish were held in the first net for 20 seconds, then transferred into a clean and sterile glass aquarium filled with clean aerated water for 1 hour to allow virus infection to occur. Fish were then transferred into flow-though aquaria and observed for at least 60 days.

The three Virkon treatment replicates experienced 1/20, 1/20, and 0/20 mortalities, the three bleach replicates experienced 0/20, 0/20, and 2/20 mortalities, the three nolvasan replicates had 1/20, 1/20, and 1/20 mortalities, the Iodophor replicates had 0/20, 1/20, and 0/20 mortalities. In the case of Virkon positive control 80% mortality was achieved. Clinical signs among moribund or dead positive control fish were consistent with an EEDV infection including ocular hemorrhage, corneal opacity, exophthalmia, fin congestion and skin lesions overgrown by water mold. No EEDV-related clinical signs were observed in the fish that died in the treatment groups. Due to the slow progression of this disease, the experiment was extended beyond the originally planned 60 days (i.e., >140 days) in order to monitor disease development further.

In order to examine the Virkon treatment and control groups further, qPCR was performed on skin tissue collected from all fish. 18/20 positive control fish had detectible levels of EEDV DNA, while 0/60 Virkon treatment fish tested positive for the virus. For additional information, please see **Appendix IX** manuscript entitled, “The efficacy of a commonly used commercial hatchery disinfectant against epizootic epitheliotropic disease virus (EEDV). Thus, Virkon appeared efficacious in reducing the risk of EEDV transmission on contaminated fomites.

Sub-task 13b: Egg disinfection:

The inability to culture EEDV *in vitro* left us with limited amounts of infectious inoculum, a matter that precluded our ability to achieve an infectious dose that we felt would have reliably lead to EEDV infections in lake trout broodstock that are substantially larger than the fingerlings used in the experimental challenges described above. Nevertheless, we still attempted to test the efficacy of iodophore for preventing EEDV infections in eggs coming from naturally EEDV-infected lake trout broodstock in collaboration with Tom Jones (Fish Health Biologist, Vermont Fish and Wildlife Department, Montpelier, VT). Lake trout broodstock were obtained from Lake Champlain and were held in a quarantined area of the Ed Weed State Fish Hatchery (VT). The fins and ovarian fluid of 20 spawning female lake trout broodstock and the fins of 20 spawning

male lake trout were assayed for the presence of EEDV via qPCR. During this process, 1 female was EEDV positive (fin tissue only) and 2 males were EEDV positive (fin). Next, eggs and sperm from two EEDV negative pairs of fish were used to produce an EEDV negative group of fertilized eggs and a subset of those eggs were either iodophor disinfected or left as is (i.e., undisinfected). The creation of a second "EEDV positive" groups was attempted by using three combinations of eggs and sperm from the three EEDV positive broodstock and again, these were divided into iodophor disinfected and undisinfected groups. The eggs and fry from these different groups were then maintained separately and upon hatching, lake trout fry from two undisinfected eggs originating from the EEDV positive broodstock and lake trout fry originating from two iodophor-disinfected EEDV positive broodstock were euthanized and whole DNA extracted from the heads and/or whole fish (n=60 per group). Extracted DNA samples was then assayed for EEDV via qPCR in duplicate. All lots of lake trout fry were negative for EEDV. Because all lake trout fry originating from EEDV positive broodstock were EEDV negative, no fry from the EEDV negative broodstock were tested. Although the results from this experiment were inconclusive, it is nevertheless encouraging that EEDV was not detected in lake trout fry that originated from EEDV positive broodstock after iodophor egg surface disinfection.

Sub-task 13c: Ultraviolet (UV) Inactivation:
The inability to culture EEDV *in vitro* and the subsequent difficulties generating substantial infectious stocks via experimental challenges rendered this task impossible. However, we instead performed the EEDV shedding experiments that were described under Objective 2, which was possible with much smaller volumes of EEDV infectious inoculum.

Overall Conclusions

The work completed in the above listed studies has greatly added to our knowledge of the biologic and pathologic properties of EEDV and the interactions of the virus with its primary host species, the lake trout. We have expanded our understanding of the epidemiological factors that influence a natural epizootic, have developed a standardized experimental model, have uncovered the sequential pathology and distribution following viral infection and have also improved available options for research and diagnostic tools. All of these factors are vital to our ability to prevent and control future outbreaks of EEDV.

In order to begin the discussion as to how to prevent future outbreaks of EEDV, one must first understand what led to the initiation and spread of past epizootics. **Appendix I** describes the resurgence of EEDV in the State of Michigan as is highlighted by two mortality events in 2012 and 2017. The 2012 mortality event, the first of its kind since the early 1980s occurred in juvenile, fingerling lake trout of both the Lake Superior and Seneca Lake strains. Cumulative mortalities reached approximately 20% in all affected raceways, with the Lake Superior strain fish being affected sooner and to a more severe degree than the Seneca Lake strain. The disease appeared to be limited to only that age group and species, as none of the other fish on hatchery grounds at the time developed clinical disease or had detectible levels of EEDV genetic material. However, EEDV DNA was detected in mottled sculpin collected from upstream of the hatchery the following year, suggesting a potential virus reservoir in wild fish and hatchery source water. Following the mortality episode in the fall of 2012, EEDV was detected in two hatchery fish in 2013 and then not again until 2017 when mortalities once again occurred. What made the 2017 EEDV outbreak particularly interesting is the identification of the virus as well as development of clinical disease in older fish. All previous reports of EEDV-related mortalities have occurred in either fingerling or yearling aged fish whereas the fish experiencing mortalities in 2017 were two years old. In addition to highlighting the importance of strong biosecurity

practices in limiting the spread of disease, this publication serves to demonstrate the magnitude of work remaining in order to fully understand this devastating disease. Whether it be from exposure of naïve populations to carrier fish (e.g., hatchery water supply, fomite transfer), or recrudescence of disease in previously infected fish, it is clear that EEDV remains a threat to lake trout populations in the Great Lakes, and remaining chapters of this dissertation focus on some of the still unanswered questions. As the Lake Superior strain fish appeared, during the natural outbreak, to be more susceptible to EEDV than the Seneca Lake fish (e.g., earlier and more severe disease), the Lake Superior stain was used for the remainder of my studies.

Appendix III answered two important questions: 1) what is the necessary viral load or dose required to cause clinical EED? And 2) can morbidity and mortality be reproducibly initiated using an immersion bath method? In the first experiment, naïve, juvenile lake trout were exposed to a range of viral doses and monitored for development of clinical disease, which showed that exposure to a viral dose $\geq$ 4.7x104 viral copies per mL led to development of clinical disease consistent with that seen in natural EED outbreaks. A second study was performed exposing lake trout to high and low viral doses in triplicate in order to assess reproducibility of this immersion infection model. Results of this study showed that EED could be reproducibly initiated in experimentally challenged lake trout but that additional factors, such as individual fish variability and external stressors in a hatchery environment, also likely contribute to development of disease. Armed with a model of experimental disease challenge, we sought to address the temporal changes following an EEDV infection, whereby the sequential distribution of EEDV following viral exposure was followed to identify specific target tissues and cells. A newly developed quantitative PCR assay was used to compare viral loads among ten different tissues over twelve predetermined sampling days while a novel *in situ* hybridization assay was developed in order to visualize EEDV DNA within specific cells. These experiments widened our knowledge on the pathogenesis of EEDV and identified specific viral targets throughout the course of disease that are now highlighted for diagnostic purposes.

Additional experiments within **Appendix III** clarify the gross and histopathologic lesions throughout an EEDV infection. Primary lesions were observed in the skin and fins and correlated with qPCR data in that lesion severity increased over time, appearing to peak around Day 28-35 post infection. Interestingly, one of the first signs of disease was actually the lack of an abnormality, whereby healthy fish had moderate to severe hepatic lipidosis, once the fish began displaying clinical signs consistent with an active EEDV infection, the hepatic lipidosis went away, an indication that the fish ceased eating. Although a specific portal of EEDV entry was not identified, the severity of total epithelial damage of the skin by the end of the study likely resulted in hypo-osmotic shock and death.

In the studies of **Appendix V**, two novel cell lines of lake trout origin were established from multiple lake trout tissues and successfully expanded and sub-cultured out beyond 35 and 50 sub-cultures respectively. Optimal growth conditions for these cells were established, and DNA barcoding was used to prove lake trout as the species of origin. Moreover, these cell lines apparently supported the replication of multiple fish-pathogenic viruses, including Viral Hemorrhagic Septicemia Virus and Infectious Pancreatic Necrosis Virus. With a limited number of established cell lines originating from fish tissues, study of specific fish species and pathogens falls to the development of novel cell cultures. With no commercially available cell lines of lake trout origin, this work serves to provide an extremely useful research and diagnostic tool. Likewise, multiple manuscripts describing the development of new EEDV diagnostic assays resulted from this research (**Appendices VI, VII**).

Additional experiments described in **Appendix II** examined the potential for EEDV to cause infections and mortality in eleven fish species/strains that inhabit the Great Lakes basin was examined, a matter of importance given that previous EEDV host range studies were not able to assess the capacity for subclinical infections and/or virus loads. There was no evidence for EEDV replication and/or associated disease in 8/11 species (e.g., brook trout, *Salvelinus fontinalis*; brown trout, *Salmo trutta*; Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss;* coho salmon, *O. kisutch;* lake herring, *Coregonus artedii*; largemouth bass, *Micropterus salmoides*; and muskellunge, *Esox masquinongy*). Similarly, although EEDV was detected in one lone experimentally infected mottled sculpin, the low detected virus load and time of detection relative to experimental challenge (i.e., 8 days post infection (pi)) suggest that virus replication did not occur. On the contrary, EEDV was detected in one splake (lake trout x brook trout hybrid) relatively early in the experiment (i.e., 10 days pi), but virus loads exceeded the initial challenge dose ($3.84x10^7$ virus copies/mg skin compared to a challenge dose of $4.74x10^5$ virus copies/fish) and provided evidence that this hybrid may serve as a short term EEDV reservoir. Last, study results showed that two lake trout strains (e.g., Seneca and Lake Superior) not only varied in EED-associated mortality, but possibly also in the length of time that the EEDV infections persist.

Results from the EEDV shedding dynamics experiments (**Appendix IV**) proved that infected fish shed high quantities of EEDV into the water column and can do so for an extended period. In fact, the peak number of virus copies shed per fish per hour exceeded $9x10^8$ and exceeded the virus quantity that the fish were originally exposed to for eight weeks after challenge. Importantly, these quantities exceed the estimated median lethal dose of EEDV in lake trout as determined by Shavalier (2017) and demonstrate substantial transmission potential from host to host via the water column. Study results also showed that individual fish vary in their shedding rates and/or patterns and also suggest that host density is an important factor in EEDV shedding rates.

During the disinfectant experiments (**Appendix IX**), a potassium peroxymonosulfate-based hatchery disinfectant (Virkon® Aquatic) effectively prevented EEDV transmission on contaminated fomites (e.g., nets) to a highly susceptible lake trout strain (i.e., Lake Superior strain). In fact, EEDV was not detected in a single lake trout that was handled with an EEDV-contaminated but then Virkon® Aquatic disinfected net, compared to a 90% EEDV infection prevalence in lake trout handled with an undisinfected, EEDV-contaminated net.
In conclusion, despite the significant challenges posed by working with a virus that has yet to be cultured *in vitro*, the collective findings presented herein provide fishery managers, fishery biologists, and fish health professionals with valuable knowledge on the biological properties of EEDV so that improved strategies can be devised to prevent and control EEDV in Great Lakes hatcheries and beyond. Indeed, this research satisfied some of the unanswered questions surrounding EEDV. Much of this work focused on developing a standardized experimental model, uncovering the sequential pathology and distribution of EEDV in its host from infection to death, and improving research and diagnostic tools. Armed with additional knowledge of virus-host interactions and with improved diagnostic assays, fish health professionals and natural resource managers are better prepared to handle the next EEDV outbreak, to limit the spread of this virus, and to prevent additional mortalities. Armed with a model of experimental disease challenge, we were able to track gross and histopathologic lesions throughout the course of an EEDV infection. Diagnostic options for EEDV have been vastly improved through molecular and *in vitro* work. The culmination of this work leaves fish health and resource managers better armed to combat the spread of this deadly virus than every before.

Project deliverables produced in this project

This grant supported a PhD and a master's theses Funding support has been acknowledged in these **theses/dissertations**:

1. Shavalier, M. 2018. Investigations on the intricate interactions between Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) and its host, the lake trout (*Salvelinus namaycush*). 2018. Doctoral dissertation, available on ProQuest.
2. Purbayu, M.A. 2018. Elucidating the Properties of Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) Transmission to Facilitate Improved Disease Control. Master thesis, current status: in the process of being made available on ProQuest.

Funding support has been acknowledged in these **conference presentations**:

1. Shavalier M. Propagation of Lake Trout (*Salvelinus namaycush*) Cells in Vitro. Presented at the 40th Annual Eastern Fish Health Workshop, Mt. Pleasant, SC, March 2015.
2. Shavalier M. Propagation of Lake Trout (*Salvelinus namaycush*) Cells as a Tool for Pathogen Investigation. Presented at the Michigan State University, College of Veterinary Medicine, Phi Zeta Research Day, October 2015. Award for Best Oral Presentation by a Resident, Intern or Masters student.
3. Shavalier, M. and M. Faisal. Tissue Tropism of Epizootic Epitheliotropic Disease Virus (EEDV) in Naturally and Experimentally Infected Lake Trout (*Salvelinus namaycush*). Poster presented at the Michigan State University, College of Veterinary Medicine, Phi Zeta Research Day, October 2016.
4. Shavalier, M., T. Loch, and M. Faisal. Uncovering the Biological Properties of Epizootic Epitheliotropic Disease Virus. Oral presentation at the 42nd Annual Eastern Fish Health Workshop, East Lansing, MI, April 2017.
5. Shavalier, M. Re-emergence of Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus 3) in the Great Lakes Basin: Deciphering the Biologic and Pathologic Properties. Oral presentation for graduate research proposal seminar, East Lansing, MI, June 2017.
6. Li, J. Shavalier M., Faisal M. 2017. Re-emergence of EEDV in the Great Lakes basin. Developmental and Comparative Immunology Forum, Qingdao National Laboratory of Marine Sciences and Marine Technology. July 28-29. Qingdao, China.
7. Shavalier, M., M. Faisal, T. Thaiwong, and M. Kiupel. Progression of Salmonid Herpesvirus-3 in target tissues and cells of its host, the lake trout (*Salvelinus namaycush*). Oral presentation at the Michigan State University, College of Veterinary Medicine, Phi Zeta Research Day, East Lansing, MI, October 2017.
8. Shavalier, M. Investigations on the intricate interactions between Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) and its host, the lake trout (*Salvelinus namaycush*). Oral presentation for PhD dissertation defense seminar, East Lansing, MI, December 2017.
9. Purbayu, M.A. Elucidating the Properties of Epizootic Epitheliotropic Disease Virus (EEDV: Salmonid Herpesvirus-3) Transmission to Facilitate Improved Disease Control. Oral presentation for M.S. defense seminar, East Lansing, MI, August, 2018.

Appendices:

Funding provided by this grant supported multiple publications that are appended:

Appendix I: Faisal, M., Shavalier, M., Gunn Van Deuren, M., Standish, I., Winters, A., Loch, T.P., Glenney, G., Aho, J., Wolgamood, M., VanAmberg, J., Eisch, E. and Whelan, G. *Accepted pending revisions*. Resurgence of Salmonid Herpesvirus-3 infection (Epizootic Epitheliotropic Disease) in hatchery propagated Lake Trout *Salvelinus namaycush* in Michigan. Journal of Aquatic Animal Health.

Appendix II: Purbayu, M.A., Loch, T.P., Shavalier, M. and Faisal, M. *In preparation*. Elucidating the host range of epizootic epitheliotropic disease virus (Salmonid herpesvirus-3). To be submitted to Journal of Aquatic Animal Health.

Appendix III: Shavalier, M., Faisal, M., Fitzgerald, S. and Kiupel, M. *Accepted pending revisions*. Development and progression of gross and microscopic lesions in lake trout (*Salvelinus namaycush*) experimentally infected with Epizootic Epitheliotropic Disease Virus (salmonid herpesvirus-3). Journal of Veterinary Pathology.

Appendix IV: Faisal, M., Purbayu, M.A., Shavalier, M. and Loch, T.P. *In preparation*. Shedding of Salmonid herpesvirus-3 by infected lake trout (*Salvelinus namaycush*). To be submitted to Diseases of Aquatic Organisms.

Appendix V: Shavalier, M. and Faisal, M. *Under review*. *In vitro* culture of lake trout cells (*Salvelinus namaycush*). *In vitro* Cellular and Developmental Biology – Animal.

Appendix VI: Glenney, G.W., P.A. Barbash, and J.A. Coll. 2016. A quantitative polymerase chain reaction assay for the detection and quantification of epizootic epitheliotropic disease virus (EEDV; salmonid herpesvirus 3). Journal of Aquatic Animal Health 28:56–67.

Appendix VII: Zhang, Q., Shavalier, M., Standish, I., Glenney, G.W. and Faisal, M. *Accepted pending revisions*. Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3). Journal of Virological Methods.

Appendix VIII: Shavalier, M., Faisal, M. and Kiupel, M. *In preparation*. Progression of epizootic epitheliotropic disease virus (Salmonid herpesvirus 3) in target tissues and cells of its host, the lake trout (*Salvelinus namaycush*). To be submitted to Journal of General Virology.

Appendix IX: Purbayu, M.A., Loch, T.P., Shavalier, M. and Faisal, M. *In preparation*. The efficacy of a commonly used commercial hatchery disinfectant against epizootic epitheliotropic disease virus (EEDV). To be submitted to Journal of Fish Diseases.

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