INVESTIGATIONS ON THE INTRICATE INTERACTIONS BETWEEN EPIZOOTIC EPITHELIOTROPIC DISEASE VIRUS (SALMONID HERPESVIRUS-3) AND ITS HOST, THE LAKE TROUT (*SALVELINUS NAMAYCUSH*)

By

Megan Ann Shavalier

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ABSTRACT

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Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) is an Alloherpesvirus (Order *Herpesvirales*) capable of causing severe morbidity and mortality in lake trout (*Salvelinus namaycush*). After several decades of absence, EEDV re-emerged in the Great Lakes basin and was the causative agent of substantial mortality events in fingerling and two year old lake trout in 2012 and 2017 respectively. This resurgence highlighted the challenges associated with managing an infectious disease when many questions remain regarding its biological and pathological properties. As a result, several studies were designed in order to advance our knowledge of viral targets, disease progression, and the availability of research models and diagnostic assays. As EEDV cannot be propagated *in vitro*, I first aimed to determine the *in vivo* viral exposure dose required to cause clinical disease consistent with that seen in natural outbreaks of EEDV. Results revealed that 10^3 viral copies per mL of immersion bath water is not a sufficient dose to produce clinical disease, while 10^6 viral copies per mL of water can produce up to 100% mortality. Utilizing this predetermined dose range, I then assessed the temporal course of an EEDV infection by determining the sequential distribution of virus and identification of specific viral target tissues and cells using quantitative PCR and *in situ* hybridization assays. Following exposure of naïve juvenile lake trout to EEDV, the virus first targeted the epidermis of the skin and fins followed by the epithelial lining of primary and secondary gill lamellae and eventually infection of endothelial cells and monocytes resulting in

viremia and disseminated infection of multiple visceral organs. However, viral titers remained significantly higher among external tissues compared to visceral organs throughout the study. Subsequently, in order to elucidate a more comprehensive understanding of the pathologic changes associated with EEDV infection, I next examined sequential gross and pathological alterations in lake trout tissues. After an extended incubation period, severe pathology was first observed grossly and microscopically in the cutaneous epithelium followed by the hematopoietic organs and vessels during the later viremic stage of disease. Following these advancements in our understanding of EEDV-lake trout interactions and associated pathology, my focus shifted to expanding future experimental and diagnostic capabilities. First, I produced two novel cell lines of lake trout origin. With the limited number of commercially available aquatic cell lines, and none originating from lake trout, diagnosis of, and research into lake trout specific immunology or disease pathology, has been hampered. The successful production of primary cultures from adult liver, yearling fin, yearling gonad and fry body cells of lake trout origin, contributes to what was previously a void in salmonid tissue culture options. Without an established *in vitro* model of EEDV replication to date, there was a need for a diagnostic assay that was not only sensitive and specific, but also time and cost effective, leading to the development of a loop mediated isothermal amplification (LAMP) assay for the quantitative diagnosis of EEDV in fish tissues. This assay is highly specific for the EEDV glycoprotein gene, is cost effective, and has the potential for commercialization and use in field conditions. The end result of this dissertation is the uncovering of new insights on EEDV ecology, and a significant advancement in our understanding of EEDV-lake trout interactions.

This dissertation is dedicated to Ann Fee Elliott, the woman who taught me to treasure the natural world around us and to do so with grace and compassion and love.

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KEY TO ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium sulfate
AciHV	Acipenserid herpesvirus
AFS-FHS	American Fisheries Society Fish Health Section
AngHV	Anguillid herpesvirus
ASP(V)	Atlantic Salmon Papillomatosis (Virus)
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp(s)	Base pair(s)
CA	Cytophaga agar
CCV	Channel Catfish Virus
CHSE	Chinook salmon embryo
CO1	Cytochrome *c* oxidase 1
CPE	Cytopathic effect
Ct	Threshold cycle
CyHV	Cyprinid Herpesvirus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Department of Natural Resources
dNTP	Deoxynucleotide triphosphate
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity

EEDV	Epizootic Epitheliotropic Disease Virus
ELISA	Enzyme-linked immunosorbent assay
EPC	*Epithelioma Papulosum Cyprini*
FBS	Fetal bovine serum
FHM	Fathead minnow
GLB	Great Lakes Basin
GLFHC	Great Lakes Fish Health Committee
GSRV	Golden Shiner Reovirus
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
HSM	Hsu Shotts medium
IcHV	Ictalurid Herpesvirus
IDT	Integrated DNA Technologies
IHNV	Infectious Hematopoietic Necrosis Virus
IP	Intraperitoneal
IPNV	Infectious Pancreatic Necrosis Virus
ISH	*In situ* hybridization
kbp	Kilobase pairs
KCl	Potassium chloride
KF	Koi fin
KHV	Koi herpesvirus
LAMP	Loop mediated isothermal amplification
LS	Lake Superior strain

MALT	Mucosa-associated lymphoid tissue
MEM	Minimal essential medium
MgCl2	Magnesium chloride
MgSO4	Magnesium sulfate
MS-222	Tricaine methanesulfonate
MSFH	Marquette State Fish Hatchery
NCBI	National Center for Biotechnology Information
OIE	World Organization for Animal Health
OMV	*Oncorhynchus masou* Virus
p.i.	Post-infection
P1	First pass
P2	Second pass
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RTG	Rainbow trout gonad
RTH	Rainbow trout hepatoma
SalHV	Salmonid Herpesvirus
SALT	Skin-associated lymphoid tissue
SE	Seneca Lake strain
spp.	Species
SVCV	Spring Viremia of Carp Virus

TSA	Trypticase soy agar
USA	United States of America
UV	Ultraviolet
VHSV	Viral Hemorrhagic Septicemia Virus
WSS	White sturgeon spleen
WSSK	White sturgeon skin

Introduction

Epizootic Epitheliotropic Disease Virus (EEDV) is a serious pathogen of lake trout (*Salvelinus namaycush*), one which the aquaculture and scientific communities yet know little about. Officially named Salmonid Herpesvirus-3, an Alloherpesvirus (Order *Herpesvirales*) (*1*), this virus was initially described in the 1980s following mass mortality events in Midwestern state and federal hatcheries (*2–4*). Epizootics were notably preceded by stressor events associated with either standard hatchery operations or environmental changes and were characterized by rapid mortalities (up to 90% cumulative) and behavioral changes such as spiral swimming patterns, ataxia, and lethargy with intermittent hyperexcitability (*2*, *4*). Electron microscopy revealed the presence of viral particles with characteristics (e.g., size, capsomere number) consistent with a herpesvirus, however as the affected species and clinical signs were inconsistent with the previously described salmonid herpesivirus-1 or -2 outbreaks, it was determined this was a novel virus and was designated Salmonid Herpesvirus-3, Epizootic Epitheliotropic Disease Virus (*3*).

Early research focused on disease etiology and pathogen identification with multiple researchers demonstrating the ability to produce clinical disease in experimentally challenged juvenile lake trout (e.g., intraperitoneal injection, immersion bath, cohabitation) consistent with natural epizootics (*3*, *4*). Following the 1980s mortality events, hatchery staff and managers instigated stringent disease control measures which included depopulation of lake trout within the hatchery system and cessation of all lake trout movement within the Great Lakes basin (*5*). EEDV then remained undetected in the Great Lakes for several decades, and it was believed to have been eradicated, until mortality events once again occurred in Wisconsin and Michigan hatcheries (*5*) (*Chapter 2*).

This re-emergence of EEDV in Great Lakes hatchery lake trout populations is particularly concerning due to the potential for mass casualties in such a valuable species. The lake trout is currently under intensive population management by state and federal regulatory agencies in an attempt to mitigate past declines caused by invasive species, habitat destruction and overfishing (*6*–*12*). These fish, found throughout much of northern North America, are particularly valuable within the Great Lakes as they are a native, apex predator, have a stabilizing effect on many ecosystems, and are prized by both sports and commercial fishermen (*6*, *13*). These fish are slow growing, and don't reach sexual maturity until 7-10 years of age (*6*), meaning that were an infectious agent, such as EEDV, to decimate a hatchery population of lake trout, it would be particularly devastating due to the extended length of time required to recover and bolster wild populations once again. Throughout this study, lake trout will serve as our model species as they are historically the species most affected by EEDV infections.

While it has been established that EEDV remains a threat to the ongoing recovery efforts with Great Lakes lake trout populations, our knowledge of the pathogenesis of this deadly virus, and our ability to detect and study it, have gone largely unchanged in the past 30 years. While molecular diagnostic techniques have advanced to the point where genome sequencing and primer design for PCR-based assay detection are possible (*1*, *14*), there as of yet are no available cell cultures capable of supporting viral replication. While attempts were made following the initial EEDV epizootics to produce a susceptible cell line, minimal cytopathic effect was appreciable, any such cultures are no longer preserved (*15*), and past efforts demonstrated that established cell lines (e.g., CHSE-214, EPC, RTG-2) do not support the growth of EEDV (*3*, *4*). This lack of a powerful research and diagnostic tool is particularly detrimental to efforts aimed at uncovering the complex biological and pathological properties of EEDV. Consequently, there is

a dire need to better characterize the interactions between EEDV and its host, the lake trout through improved diagnostic assays and research models.

To that end, Chapter 1 of this dissertation presents a review of literature encompassing the extent of current knowledge concerning EEDV, the history of associated disease outbreaks in lake trout, diagnostic options, and past research, focusing on other closely related Alloherpesviruses where EEDV-specific answers do not yet exist. The culmination of this summary was used as a basis for formulation of the study objectives.

Chapter 2 of this dissertation focuses on detailing the two most recent epizootic mortality events contributable to EEDV, which occurred in Michigan's Upper Peninsula in the fall of 2012 and 2017. I highlight certain epidemiological aspects of the outbreak such as the temporal and spatial spread between and among lake trout strains, and discuss diagnostic confirmation of viral infection and ongoing screening within and without the hatchery. I also demonstrate our capability of reproducing clinical disease in a controlled laboratory environment through the experimental infection of juvenile lake trout with infectious tissue homogenate collected from naturally diseased fish. Chapter 2 serves to highlight the need for improved screening protocols, biosecurity and readiness of reactionary plans should there be additional EEDV epizootics in the future and also brings to light the scope of questions left unanswered regarding the biology and pathogenesis of EEDV.

In order to address these crucial questions, we first require an understanding of the dose-dependent effects of experimental exposure to EEDV and knowledge of whether clinical disease can be reproduced in a controlled laboratory environment. Based on the pilot study from Chapter 2, in Chapter 3, we design two studies; the first aimed at determining the viral load necessary to cause clinical disease and the second with a goal of developing an immersion model that

appropriately mimics a natural route of infection. Following development, this model could subsequently be used for future experimental studies focused on improving our understanding of EEDV disease ecology.

In Chapter 4, I utilized the predetermined dose range to infect a group of naïve juvenile lake trout with a moderate dose of EEDV that allowed the tracking and temporal localization of viral DNA throughout the course of infection. Identification of EEDV DNA within certain tissues collected on specific days was reinforced through the development and use of an *in situ* hybridization assay, which allowed for the visualization of EEDV genetic material within target cells. Identification of EEDV cellular and tissue targets throughout a course of disease provides key information concerning the pathogenesis of the virus, its interactions with the host fish, and potential ideal diagnostic targets.

Following up on these results, Chapter 5 focuses on identifying and characterizing sequential pathology in a multitude of organs through gross and histopathologic study. While comprehensive histopathologic studies have been performed on several of the other Alloherpesviruses (*16*, *17*) previous studies of EEDV have been limited to analysis during and after a mortality event (*2*, *4*), often resulting in subtle lesions becoming obscured by the advanced severity of disease.

Armed with the novel information uncovered in the first four chapters, I then turned to improving diagnostic capabilities in the next two sections. Chapter 6 discusses the development of novel cell cultures of lake trout origin. All previous attempts at EEDV propagation in cell culture have been unsuccessful (*3*, *4*) and despite the one time production of lake trout cells (*15*), there are currently no established cell lines of lake trout origin. As the species specificity of many herpesviruses translates into host-specific cell lines, we created primary cultures of lake

trout cells and assessed their viability for use in EEDV diagnosis and research. As novel cell lines continue to evolve and improve, additional molecular diagnostic assays were also being developed. Chapter 7 outlines the design of a novel loop mediated isothermal amplification (LAMP) assay for the detection and quantification of EEDV. LAMP assays have become popular due to their reasonable cost, comparable specificity and sensitivity to PCR assays and the high potential for commercialization into a kit that can be performed in a field setting, a fact that is particularly appealing for fish health professionals (*18*, *19*).

Lastly, Chapter 8 summarizes my overall conclusions and provides suggestions for future research. The culmination of the methodology and analyses of this dissertation is an improved understanding of the pathogenesis of EEDV as well as improved diagnostic options, all of which can be used to prevent or limit future spread of this virus.

Chapter 1

Literature Review

1. Fish Pathogens

1.1. Overview

In comparison to other vertebrate classes, fish have an extremely high level of species diversity and make up a vast proportion of the earth's biomass (*20*). Due to this, changes such as over-exploitation, habitat modification and the introduction of invasive species, which result in population level alterations, have the potential for dramatic ecological consequences (*21–23*). Aquatic species have been heavily stocked for decades in an attempt to balance the effects of such changes (*24*, *25*). While stocking can be an effective tool for population management, it can also lead to decreased evolutionary potential and genetic integrity (*26–28*), and it has been suggested that captive reared fish display a decreased level of fitness compared with those born in the wild (*26*). Certainly there are inherent risks and stress associated with the density of captive fish as well as day-to-day hatchery management practices.

Plumb & Hanson (2011) (*29*) state that infectious diseases of fish (e.g., caused by viral, bacterial and parasitic agents), are constraining the expansion of the aquaculture industry. Often, mass mortality events are caused by the eruption of endemic diseases, after fish are exposed to stressful conditions such as those experienced with intensive captive rearing (*29*). In general, fish react more quickly to environmental changes than terrestrial animals due to their poikilothermic nature, resulting in substantial morbidity and mortality due to handling stress, abrupt water temperature changes or water chemistry alterations (*29*). A compromised immune system is another disadvantageous side effect of increased stress, leaving the fish more susceptible to infectious diseases (*29*). Many aquatic diseases occur on a seasonal trend based on cyclical water temperatures and the presence of susceptibly aged fish in the environment (*30*, *31*). Good

biosecurity practices are vital to preventing disease outbreaks as well as limiting their spread within a hatchery (*32*).

Disease manifestation can take on many forms, however one common early clinical sign, regardless of pathogen, is a change in behavior such as cessation of feeding activity or inability to remain upright (i.e., loss of equilibrium). Lethargy, listlessness, or crowding around water inlets are additional clues to the presence of diseased fish (*29*). Unfortunately, most frequently, external signs of infectious disease are nonspecific and there are very few aquatic diseases with overt pathognomonic clinical signs (*29*), leading researchers, veterinarians and fishery managers to pursue additional diagnostics in order to effectively manage captive populations of fish.

1.2. Immunology

Every pathogen has an optimal or primary point of entry into its host. In fish, these sites are most commonly the intestines, gills or skin (*29*). Fish possess a mucous layer over their epithelium which can provide protection against certain pathogens, however if this layer is damaged, by human handling either at a hatchery, or via catch-and-release for example, then that fish is at an increased risk for water-borne infections both pathogenic and opportunistic (*29*). Nutritional status and environmental conditions also play a large role in the ability of a fish to fight off disease. For example, adult fish are at a higher risk of acquiring infectious diseases during spawning season when many have compromised immune systems due to a cessation of feeding entirely (*29*).

The skin of all vertebrates is the first line of defense against disease and consists of an epidermis and dermis (*33*). Fish skin functions to provide mechanical, chemical and immune barriers to injury and pathologic invasion (*34*). Unlike mammals, fish epidermis consists of

living cells capable of mitotic division in all layers (*34*). Primary epithelial cells include the filamentous Malpighian cells and glandular, mucus-secreting goblet cells (*34*). Teleost fish skin has a cuticle (i.e., mucous) layer covering the epidermis (*34*). This mucous layer is continuously moving downstream, which reduces pathogen access to epithelial cells similarly to the muco-ciliary escalator in pulmonate animals (*34*). The mucous also contains immunoglobulins and enzymes capable of neutralizing many microorganisms. Stressors that alter or disturb this mucous layer can increase a fish's susceptibility to infectious pathogens (*34*).

As with other vertebrates, in addition to the physical barriers, fish have several natural mechanisms of disease resistance including natural killer cells, the phagocytic activity of neutrophils and macrophages, and serum components such as interferons and complement (*29*). In teleost fish, IgT likely plays a major role in the neutralization of pathogens both on the skin and within the gut (*33*). Trout skin-associated lymphoid tissue (SALT) functions very similar to mammalian mucosal-associated lymphoid tissue (MALT) and contains populations of B and T cells capable of responding to immune stimuli (*33*, *35*).

1.3. Treatment

Treatment of aquatic diseases can, at times, be challenging and disheartening, as there is but a short list of approved therapies and medications available, mainly due to limitations concerning environmental levels of certain chemicals and tissue withdrawal times prior to human consumption. Fortunately, the use of vaccines is beginning to become generally more acceptable and feasible in the aquaculture industry. The earliest aquatic vaccines date back to the 1940s when a *Aeromonas salmonicida* preparation was fed to trout in an attempt to prevent furunculosis (*29*, *36*). Leong and Fryer (1993) (*37*) explained that an aquaculture vaccine needs

to demonstrate specific features: 1) adequate immunoprotection under intensive conditions, 2) effectiveness when the fish is most susceptible, 3) long term protection, 4) protection against all serotypes, 5) be easily administered, 6) be safe for the fish, and 7) be cost effective. There is a concern however that vaccinated fish could become reservoirs or carriers for diseases (*29*) if, for example, a modified live vaccine reverted to a pathogenic strain or if the vaccine limited clinical signs but not pathogen infection. With the relatively short list of approved medications and vaccinations available for use in fish, often times aquatic animal health programs focus efforts on prevention of outbreaks and limiting affected individuals after a pathogen is detected. These goals can be achieved through the rigorous screening of any novel fish populations entering a hatchery, public education regarding movement between bodies of water, strong biosecurity practices within hatcheries and frequent disease surveillance within hatchery fish. The failure of these goals is potentially disastrous as fish in their aquatic habitats are vulnerable to infection with a myriad of pathogens, the most serious of which are fish-pathogenic viruses.

2. Fish Viruses

2.1. Overview

Viruses represent a potential for catastrophic losses of fish kept under intensive rearing conditions (*29*). Many of the recent viral outbreaks have occurred following exposure of infected fish populations to environmental stressors (*29*). The Great Lakes support a more than $7 billion fishery industry and encompasses commercial, recreational, and tribal fisheries (*38*). Fish stocking has occurred in the Great Lakes for more than 50 years with goals of bolstering the sports and commercial fishing industries as well as restoring and rehabilitating specific fish populations. The Great Lakes Fish Health Committee (GLFHC) developed a “Great Lakes Fish

Disease Control Policy and Model Program" with a goal of coordinating disease management between fishery agencies throughout the region (*39*). The model program serves as a guideline for fishery managers, fish health professionals and policy makers in regards to hatchery management, fish health testing and transportation within the Great Lakes Basin, and applies to all fish species that have the potential to harbor transmissible pathogens within the Great Lakes Basin (*39*). Specific goals include prevention of fish pathogen introduction and spread within the basin as well as providing classification of hatchery disease status (*39*). Agency responsibilities include developing regulations to eradicate or minimize fish pathogens, limiting the rearing and release of infected fish, preventing the transportation and importation of infected fish, and developing response plans in case of disease outbreaks (*39*).

Detection of viral infection in fish involves virus isolation in tissue culture (gold standard), electron microscopic examination, and serologic assays (*29*). Following isolation from cell culture, virus identification can be confirmed using such techniques as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), or loop mediated isothermal amplification (LAMP) (*19*, *40*).

2.2. OIE reportable diseases

There are currently twelve fish-specific diseases listed as reportable to the World Organization for Animal Health (OIE) (*41*). Of the 12, one is a fungal infection (*Aphanomyces invadans*), one is an ectoparasite (*Gyrodactylus salaris*) and the remaining ten are viruses (*41*). All of these pathogens are reportable due to their potential to cause significant mortalities in a variety of fish species or populations and have very limited treatment options. While vaccinations are a popular area of study, there are few commercially available vaccines for the

above-mentioned viruses. As a group, these viruses have the potential to cause mass mortalities and have dramatic economic and ecologic effects on a wide range of both marine and aquatic fish species.

3. Order *Herpesvirales*

Two of the OIE reportable fish viruses are members of the order *Herpesvirales*. Koi Herpesvirus (KHV) or Cyprinid Herpesvirus-3, causes an acute viremia in all forms of carp including common carp (*Cyprinus carpio*) and koi (domesticated *Cyprinus carpio*) (*16*, *42*), while *Oncorhynchus masou* virus (OMV; Salmonid Herpesvirus-2) causes tumors, ulcerative skin lesions, and mass mortalities in multiple salmonid species in Japan (*42*, *43*).

Herpesviruses have been isolated from every class of vertebrates as well as from a single invertebrate (*1*, *44*). In general, each herpesvirus is closely associated with a single host species, however individual hosts may be affected by multiple species of herpesviruses (*44*), indicating a co-evolution of virus and host; as hosts evolve and become extinct, so too do their herpesviruses (*44*). Herpesviruses have large genomes with a highly conserved yet complex viral structure (*44*, *45*). The virus particles are typically 200-250 nm in diameter with a double stranded DNA genome ranging from 125 to 245 kbp long, packed densely inside an icosahedral capsid (*44*, *45*). The capsid contains 162 capsomeres and is embedded in a tegument matrix layer. The outermost layer is a lipid bilayer envelope derived from the host cell (*46*).

Prior to the 1980s, designation as a herpesvirus was made based on virion morphology plus antigenic and biologic properties (*44*, *46*), however recently, complete genome sequencing has allowed for the construction of phylogenetic trees (*44*). The current viral taxonomy has herpesviruses within the order *Herpesvirales*, which consists of three families: Herpesviridae,

Alloherpesviridae and Malacoherpesviridae (*44*) (Figure 1.1). The family Herpesviridae contains viruses that infect mammals, birds and reptiles and is further divided into three subfamilies: Alphaherpesvirinae (primary hosts mammals, birds, reptiles), Betaherpesvirinae (primary hosts mammals) and Gammaherpesvirinae (primary hosts mammals) (*1*, *46–51*). The family Alloherpesviridae contains those viruses infecting fish and amphibians while Malacoherpesviridae contains the single virus isolated from an invertebrate host (*1*, *47*, *48*). While gene order is highly conserved among closely related herpesviruses, there is a high degree of divergence between the three main "boughs", making it difficult to prove a single common viral ancestor using amino acid sequences (*44*). None of the genes common to all three herpesvirus families are unique to herpesviruses alone (*44*), and it has been suggested that herpesviruses may share a common fundamental ancestor with the T4 bacteriophages (*44*). Division between the mammalian and fish viruses occurred approximately 400 million years ago and extant lineages have lost most remnants of common inheritance unlike the divisions among the mammalian viruses, which occurred only 200 million years ago and retain many related genes (*44*). There are several examples where multiple herpesviruses affect a single species such as the nine human herpesviruses and the five equine herpesviruses. These groups likely survived due to the occupation of separate biological niches. There are also examples of closely related viruses that affect different species such as Varicella zoster virus in humans (chickenpox) and Simian Varicella Virus (SVV) in old-world monkeys, indicating viral divergence may have occurred following host speciation (*44*).

4. Family *Alloherpesviridae*

4.1. Classification

The family Alloherpesviridae encompasses over 14 known herpesviruses associated with clinical disease in fish and consists of four genera: Batrachovirus (primary hosts frogs), Cyprinivirus (primary hosts carp and eel), Ictalurivirus (primary hosts catfish and sturgeon), and Salmonivirus (primary hosts salmon and trout) (*52*) (Figure 1.2). While many of these herpesviruses cause only mild or unapparent disease under natural conditions, they can often be fatal in either immune compromised or naïve fish such as in captive bred populations or aquaculture facilities (*45*). To date, all characterized alloherpesviruses have the ability to cause disease in just a single species, which is a characteristic also displayed in the difficulty of cell culture isolation (*45*).

Within the Alloherpesviridae family there are at least 12 conserved genes (*45*). Regions within these conserved genes have been used to establish primers for PCR amplification including regions of the polymerase, terminase and glycoprotein genes. Coevolution of the Alloherpesviruses with their hosts is only supported at the most distal phylogenetic branches (*1*). There is not a close relationship between the herpesviruses that infect cyprinid and ictalurid fishes despite both fish families being members of the Ostariophysi superorder (*1*). The two herpesviruses that infect sturgeon are not sister taxa even though sturgeons are an ancient fish lineage (*1*), however the herpesviruses that infect salmonids do form a monophyletic clade.

4.2. Clinical presentation and latency

Most of the alloherpesviruses are epitheliotrophic, replicate in epithelial cells, and cause pathology such as epidermal and branchial necrosis, hypertrophy, or hyperplasia, and formation

of papillomas or adenocarcinomas (*45*). Clinical signs are often mild in mature fish and severe in immature fish resulting in high mortalities in fry and fingerlings (*53*). Latency has been demonstrated in five of the Alloherpesviruses (Cyprinid Herpesvirus-1 and -3, Salmonid Herpesvirus-2, Anguillid Herpesvirus-1 and Ictalurid Herpesvirus-1) via detection of viral DNA in fish without infectious virus (*54–61*). While the Alloherpesviruses have diverged from members of the Herpesviridae family, they maintain many similar biological and pathological properties including high host specificity and ability to develop latency (*1*, *45*).

4.3. Specific Alloherpesviruses

4.3.1. Genus Cyprinivirus

Cyprinid Herpesvirus-1 (CyHV-1) is also known as Carp Herpesvirus, Carp Pox Virus and Herpesvirus Cyprinid (*47*). Carp pox is one of the oldest fish diseases on record, dating back to the Middle Ages, and the causative agent, CyHV-1, currently has a world-wide distribution (*47*, *62*). The most common clinical sign associated with CyHV-1 is the presence of epidermal growths on either common carp or koi carp (*47*). CyHV-1 leads to a biphasic infection with an acute, lethal systemic disease in young carp and a recurring, nonlethal, proliferative skin disease in adult fish (*63*, *64*).

Cyprinid Herpesvirus-2 (CyHV-2) is called Goldfish Hematopoietic Necrosis Virus and also has a global distribution (*47*, *65*). Epizootics result in high mortalities in all ages of goldfish (*Carassius auratus*) with minimal gross external lesions, however the liver and kidney may be pale and enlarged on necropsy with white, granular nodules in the spleen (*66*). Upon histopathologic examination, mild to severe, multifocal to diffuse, coagulative necrosis has been

seen in the kidney and spleen (*65*). Goodwin et al. (2006) (*67*) reported that CyHV-2 has been detected in apparently healthy goldfish, which suggests that a latent infection can be established.

The third Cyprinivirus, CyHV-3, is called Koi Herpes Virus, Carp Nephritis Virus or Gill Necrosis Virus (*16*, *68*) and causes disease in both common and koi carp (*47*). CyHV-3 has a global distribution and infection results in mass mortalities in all ages of carp, common or koi (*16*, *69*, *70*). Secondary bacterial and fungal infections are common with CyHV-3, and upon necropsy, typical findings include enlarged kidney and spleen, flaccid and mottled heart, hyperplasia and/or hypertrophy of the branchial epithelium and fusion of the secondary lamella (*16*). The primary mode of entry is through the skin and fins leading to secondary disease in the gills (*34*, *71*). Infections with KHV are reportable to the World Organization for Animal Health (OIE) as this virus is of specific concern to the global ornamental fish trade.

According to current classification by the International Committee on Taxonomy of Virusses, Anguillid Herpesvirus 1 (AngHV-1) is also a member of the Cyprinivirus genus. AngHV-1 is known as Eel Herpesvirus or Herpesvirus Anguillae, was first isolated from Japanese eel (*Anguilla japonica*) in the 1980s (*72*), and has since spread throughout eel farms in Europe (*73*). While it is suspected that this virus is ubiquitous in wild eel populations, when the virus is present in dense captive populations, fatal disease outbreaks can occur (*73*). Clinical signs associated with AngHV-1 outbreaks include skin and gill erythema and necrosis, liver necrosis, systemic hemorrhaging and mortality rates ranging from 1-7% (*72*, *73*).

4.3.2. Genus Ictalurivirus

Ictalurid Herpesvirus-1 or Channel Catfish Virus (IcHV-1, CCV) was first characterized following isolation from juvenile channel catfish (*Ictalurus punctatus*) during a mass mortality

event in the southern United States in the late 1960s (*17*, *74*). Natural outbreaks of CCV most frequently occur in fingerling catfish, and when water temperatures are above 27°C, mortalities can reach 90% within the span of a couple weeks (*45*). Common clinical signs include erratic swimming, exophthalmia, a distended abdomen and fin hemorrhages (*45*), with gross pathology including yellow ascites and a swollen kidney and spleen. CCV appears to have high host specificity with only channel catfish, blue catfish (*Ictalurus furcatus*) and their hybrid showing any level of susceptibility (*45*). In order to limit commercial losses, many producers attempt to decrease stress and crowding of juvenile catfish, particularly during the times of year when water temperatures are ideal for CCV replication (*45*). Additionally, several experimental vaccines have been developed including a DNA vaccine (*75*), an attenuated live vaccine (*76*) and multiple recombinant vaccines (*77*, *78*) with varying degrees of protection.

Ictalurid Herpesvirus-2 (IcHV-2) was first isolated in 1994 from two farms in northern Italy experiencing mass mortalities in black bullhead catfish (*Ictalurus melas*) (*79*). Throughout these mortality events, fish exhibited abnormal behavior including spiral swimming patterns and developed multifocal hemorrhages along the abdomen, fin base and in many internal organs (*79*, *80*). Under experimental conditions IcHV-2 was infectious to both black bullhead as well as channel catfish suggesting a broader host range than CCV (*80*). While at this time IcHV-2 has been limited to European waters, were it to spread to areas with heavier channel catfish production such as the southern United States, catastrophic losses could occur (*80*).

The third member of the genus Ictalurivirus is Acipenserid Herpesvirus-2 (AciHV-2) or White Sturgeon Herpesvirus-2. This virus was initially detected in ovarian fluid of apparently healthy captive white sturgeon (*Acipenser transmontanus*) broodstock in the early 1990s in California, USA (*81*). The novel virus was isolated from sturgeon spleen (WSS-2) and skin

(WSSK-1) cells following development of cytopathic effect (CPE) characterized by focal rounding, enhanced refractility, enlargement and detachment of spleen cells as well as syncytia formation and discrete, grape-like clusters in skin cells (*81*). Experimentally challenged fish developed lethargy and erratic swimming behavior within 10 days of exposure, followed by the development of discrete, raised hypochromatic lesions on the head and pectoral fins with cumulative mortalities reaching 80% (*81*). One particular concern with this virus is its potential to contribute to increased mortalities in juvenile captive bred sturgeon due to chronic skin diseases (*81*).

4.3.3. Genus Salmonivirus

The salmonid herpesviruses are particularly concerning in the Great Lakes basin due to the importance both economically and ecologically of the four trout species, three salmon species and one trout hybrid in the region that are all potentially at risk of viral exposure or infection. There are currently three confirmed members of the Salmonivirus genus: Salmonid Herpesvirus-1, Salmonid Herpesvirus-2, and Salmonid Herpesvirus-3; and two proposed viruses: Salmonid Herpesvirus-4 and Salmonid Herpesvirus-5.

Salmonid Herpesvirus-1 (SalHV-1), also known as Herpesvirus Salmonis or Steelhead Herpesvirus, was first isolated from healthy rainbow trout in the state of Washington (*82*). Distinguishing itself from previously described aquatic herpesviruses, SalHV-1 required water temperatures below 10°C for replication (*82*). Electron microscopy demonstrated morphological characteristics consistent with other herpesviruses, and syncytia plus nuclear inclusions were seen in cell culture (*82*). Clinical findings associated with SalHV-1 infection include increased mortalities in rainbow trout fry, with erratic swimming and loss of motor control, generalized

darkening, exophthalmia, ocular hemorrhage, abdominal distention, pale gills, ascites and visceral pallor and/or edema (*83*).

Salmonid Herpesvirus-2 (SalHV-2, *Oncorhynchus masou* Virus, OMV) was first isolated in RTG-2 and CHSE-214 cell lines following inoculation of tissues collected from masou salmon, (*Oncorhynchus masou*) coho salmon (*Oncorhynchus kisutch*), sockeye salmon (*Oncorhynchus nerka*) and rainbow trout (*84–87*). Beginning in 1970, Sockeye salmon fry in Japan began having increased mortalities (up to 80%) following generalized darkening and behavioral changes (*84*). A syncytia-forming virus was isolated from RTG-2 cells and a similar virus was isolated from ovarian fluid of adult masu salmon a few years later (*84*, *88*). Designated OMV, this virus was shown to be significantly pathogenic and oncogenic to juvenile salmonids (*43*, *88*) with epithelial tumors developing in 12-100% of fish surviving initial infection (*89*). Following the implementation of iodine disinfection of eyed eggs, this virus is now only rarely detected in Japanese hatcheries (*88*).

Doszpoly et al. (2013) (*90*) proposed a new virus as SalHV-4 to be called Atlantic salmon papillomatosis virus (ASPV). This disease was first reported in the 1950s in both wild and farmed fish in Scandanavia, Scotland and Russia (*90*). ASP is a benign skin disease mainly affecting young Atlantic salmon (*Salmo salar*), although occasionally adult fish returning to spawn can become infected (*91*). Common clinical signs include slowly forming areas of focal epithelial hyperplasia and petechial hemorrhage followed by end-stage large pale papilloma-like lesions. Secondary opportunistic infections are also common (*90*). Wolf (1988) (*92*) and Shchelkunov et al. (1992) (*93*) both described a viral agent found inside the proliferative epithelial cells of the papilloma lesions with morphology consistent with a herpesvirus. They were however, unable to isolate the virus in cell culture. Based on the sequencing of three partial

gene fragments, Doszpoly classified ASP as an Alloherpesvirus and sister-species to SalHV-3 with the proposition of designating ASP as SalHV-4.

Salmonid Herpesvirus-5 was initially described following isolation from apparently healthy lake trout (*Salvelinus namaycush*) in Keuka Lake, New York in 2011 (*94*). While fish exhibited no clinical signs of disease and kidney samples inoculated onto EPC and CHSE-214 cells demonstrated no cytopathic effect, tissue samples were PCR-positive for a novel herpesvirus, most closely related to salmonid herpesviruses -3 and -4 (*94*).

SalHV-3, known as Epizootic Epitheliotropic Disease Virus (EEDV), is the salmonid herpesvirus of most concern within the Great Lakes basin today (*2–4*) and will be discussed in great detail below.

5. Epizootic Epitheliotrophic Disease Virus (EEDV)

5.1. Initial epidemics

Salmonid Herpesvirus-3 (Epizootic Epitheliotropic Disease Virus; EEDV) is an Alloherpesvirus and a serious pathogen of lake trout (*Salvelinus namaycush*), one that the aquaculture and scientific communities yet know little about. This virus was initially described in the 1980s following mass mortality events in Midwestern state and federal hatcheries, which were preceded by potential stressor events associated with either environmental changes or standard hatchery operations. These epizootics were characterized by rapid mortalities, spiral swimming patterns, ataxia, gasping at the surface and lethargy with intermittent hyperexcitability, specific to juvenile lake trout (*3*). Reported mortalities ranged from 15% to greater than 95% and over a million fish either died or were euthanized following onset of clinical disease (*2*).

Over the next several years, many similar natural epizootics were associated with environmental or handling stress and occurred following the subjection of apparently healthy lake trout to stress caused by standard hatchery operations (*3*, *5*). Each new epizootic lasted for two to three months and younger fish (fry versus fingerlings versus broodstock) appeared to be more susceptible (*3*). Following routine tagging operations in 1986, yearling lake trout at the Iron River National Fish Hatchery experienced severe mortalities, followed shortly by mortalities in fingerling lake trout as well (*4*). Mortalities at the Iron River NFH reached almost 100% with this outbreak (*4*). Attempted treatment options included the addition of sodium chloride, formalin, benzalkonium chloride, teramycin, erythromycin and malachite green but all were ineffective at preventing the onset of disease or improving clinical signs (*3*). Additionally, the use of oxytetracycline had no therapeutic effect on diseased fish and did not prevent disease transmission to uninfected fish (*3*). Secondary infections with *Pseudomonas* spp. and *Aeromonas* spp. were found in several epizootics but were not considered the inciting event (*3*).

5.2. Pathogen identification

Histopathologic examination performed during the original epidemics revealed branchial inclusions (9-15 μm in diameter) with densely packed coccoidal particles interspersed along the secondary lamellae (*2*). Some fish also had marked epithelial hyperplasia and hypertrophy of the primary and secondary lamellae as well as a generalized thickening of the secondary lamellae and the interlamellar regions with increased mucus accumulation (*2*). Transmission electron microscopy revealed an organism in the inclusion bodies present in samples from multiple epidemic sites that was 9-25 um in diameter, observed along the secondary lamellae (*2*). Systemic lesions consistent in the outbreaks were renal glomerulitis, renal tubule degeneration

and epithelial hyperplasia (*2*). Electron microscopy revealed icosahedral virus particles that were both enveloped and unenveloped. The unenveloped particles measured 100-105 nm in diameter, had 5 capsomeres per capsid side and a hollow center. The enveloped particles had a diameter of 220-235 nm (*3*). The total number of capsomeres (i.e., 162) is consistent with a member of the Herpesviridae family and as all herpesviruses are enveloped, the finding of unenveloped particles may be due to the fragility of the viral envelope (*3*). Immunofluorescent data was not supportive of a Chlamydial agent and eventually, it was concluded that a herpes virus was responsible for these epizootics in juvenile lake trout (*3*) and was designated Epizootic Epitheliotrophic Disease Virus.

5.3. Disease management

Brood stock strains were developed from wild populations in the Great Lakes in the 1980s in order to begin a lake trout restoration program (*5*), however, outbreaks of EEDV occurred in the first year progeny in both state and federal hatcheries (*5*). EEDV outbreaks also occurred in fish raised on well water from eggs obtained from the Apostle Islands and Michigan hatcheries (*5*). The original source of infection was difficult to establish in both instances, based on complexity of hatchery water use and fish movement between facilities (*5*). Depopulation was recommended and a total of approximately 15 million fish were destroyed due to EEDV (*5*). Severe restrictions were put in place regarding the movement of lake trout within the Great Lakes Basin (*5*). In hopes of obtaining a "disease-free" strain of lake trout, eggs were collected and shipped in from a federal hatchery in the state of Wyoming (*5*). Egg collections from wild lake trout in the Great Lakes for use in stocking resumed in the year 2000 (*5*).

5.4. Early pathogenesis studies

Virus propagation was attempted on FHM, RTG, CHSE-214, EPC or RTH cell lines, however all were unsuccessful (*3*). *In vivo* experimental infections were performed using an infectious filtrate of the unknown virus (*3*) and within 32 days resulted in clinical signs such as spiral swimming, ataxia, and lethargy with intermittent hyperexcitability, consistent with the primary epizootic. Mortalities began at 33 days post-exposure and 100% mortality occurred within the following week. No mortalities were observed when the filtrate was passed through a 220 nm filter prior to infection (*3*), which may have been due to clumping of viral particles. Fish exposed to purified virus began dying at 35 days post-exposure with 100% mortality occurring by 43 days post-exposure.

Gross lesions included secondary saprolegnia infections, hemorrhaging in the eyes and at fin bases, fin degeneration, and renal swelling. Histopathologic examination revealed epidermal hyperplasia, hypertrophy and necrosis with intranuclear inclusion bodies as well as lamellar edema, renal tubular degeneration and dilation of renal glomeruli (*3*).

In order to further characterize the etiologic agent behind these mortality events, McAllister & Herman (1989) (*4*) performed additional experimental infections. Naïve lake trout fingerling died following exposure to either moribund lake trout or to filtered skin homogenates and scrapings (*4*). Clinical signs developed beginning 7-9 days following viral exposure via cohabitation (*4*). From skin homogenates and scrapes, the virus was able to pass through 0.45 and 0.22 μm average pore diameter membrane filters (*4*) and clinical signs occurred 5-7 days following exposure. Multiple fish species of the family Salmonidae were exposed to EEDV in order to examine virus host range. No brown trout (*Salmo trutta*), rainbow trout or Atlantic salmon died following exposure via either cohabitation or a waterborne route. One brook trout

(*Salvelinus fontinalis*) died, however EEDV was ruled out as the cause of death (*4*). Histopathologic findings in tissues from experimentally infected lake trout were similar for both methods of exposure and included epithelial hyperplasia with lymphocytic infiltrates, hydrophic cells and necrosis as well as macrophages with cellular debris in the kidneys and vacuolated hepatocytes (indicative of reduced glycogen reserves) (*4*). No cytopathic effect was noted on cell culture (primary or secondary passages) (*4*), however viral particles were detected in the epidermis (ellipsoidal to spherical particles) and were 150-200 nm in diameter (*4*).

6. Lake trout (*Salvelinus namaycush*)

EEDV is of particular concern within the Great Lakes Basin due to the vital importance of the lake trout. The lake trout is one example of a species currently under intensive population management by regulatory agencies including the United States Fish and Wildlife Service and Michigan Department of Natural Resources (DNR). Lake trout are found naturally throughout much of northern North America, and are the largest of the chars, a genus within the Salmonidae family, which also includes the brook trout and their hybrid, the splake (*6*). Lake trout are extremely valuable to the commercial fishing trade in the Great Lakes, second only to whitefish in both pounds produced and economic value (Michigan DNR commercial fishing report 2001-2013), as well as to sports fishermen who prize these fish for their size and their fight when on a line (*6*). There is also an intrinsic value placed on the species, as they are one of the only native apex predators in the Great Lakes, and posses a life cycle well adapted to these waters, utilizing a wide range of habitats and resources, which results in a stabilizing effect on local fish communities across the basin (*13*).

Prior to the 1940's, Lake Michigan contained one of the world's largest populations of lake trout (*13*). Unfortunately, this period of history saw the inadvertent introduction of two invasive species to the Great Lakes Basin: the sea lamprey (*Petromyzon marinus*) which prey upon the fish which had previously been apex predators in the Great Lakes, and also the alewife (*Alosa pseudoharengus*) which outcompeted the lake herring (*Coregonus artedi*), the primary food source of lake trout (*10*, *13*). Along with overfishing and habitat degradation, the introduction of these invasive species led to the depletion of native lake trout populations in Lakes Ontario, Erie and Michigan (*6–9*, *11*, *12*, *95*). Both sport and commercial fishing industries suffered collapses following the decline of this dominant predator (*13*). In response, state and federal management agencies developed programs to regulate harvest and create self-sustaining lake trout populations across the nation. This included the introduction of these fish into new habitats (e.g., Lake Tahoe, California and Harding Lake, Alaska) and bolstering native wild stock populations (e.g., Lake Michigan) (*6*). Unfortunately, these lake trout rehabilitation efforts have encountered many hurdles including emerging infectious diseases, continued predation by sea lamprey, and inadvertent overfishing following the introduction of Pacific salmon into the Great Lakes (*13*).

7. Diagnostic Tools

7.1. Cell culture

The use of cell culture has become a staple in the study of virology, toxicology, carcinogenesis, immunology, endocrinology, aquaculture, and more (*96*, *97*). Tissue culture has been successfully used in research laboratories as an alternative to whole animal models for many years now (*96*) and is often the first step in the diagnosis of and surveillance for aquatic

viruses in populations of interest or concern. While American Type Culture Collection (ATCC) carries over 3,400 commercially available distinct cell lines, there are less than 20 that are derived from fish tissues. Several members of Alloherpesviridae have been isolated in cell culture including Cyprinid Herpesvirus-1 (Carp Pox) and -2 (Koi Herpesvirus) in KF-1 cells (*16*, *63*), Acipenserid Herpesvirus-1 (White Sturgeon Herpesvirus-1) in WSS-1 cells (*98*), and Salmonid Herpesvirus-1 (Herpesvirus Salmonis) and -2 (Oncorhynchus Masou Virus) in both RTG and CHSE-214 cells (*82*, *99*, *100*). As evidenced by the cell lines used to isolate these viruses, herpesviruses are not only host-species-specific *in vivo* but also *in vitro*, with the carp herpesviruses being isolated using cell lines of carp (i.e., cyprinid) origin and the salmonid herpesvirus being isolated in cell lines originating from *Oncorhynchus* species (i.e., Chinook salmon and rainbow trout). Unfortunately there are currently no established cell lines originating from *Salvelinus* fish (i.e., lake trout or brook trout).

In vitro propagation of EEDV was attempted by both Bradley et al. (1989) (*3*) and McAllister and Herman (1989) (*4*), yet both were unsuccessful. Established cell lines including CHSE-214, EPC, FHM, McCoy, RTG-2, and RTH-149 were inoculated with tissue homogenates from EEDV-infected fish and showed no evidence of cytopathic effect (*3*, *4*). Without the ability to isolate the EED virus in cell culture, diagnosis initially depended on observation of viral particles by transmission electron microscopy (*5*) and more recently, PCR (*5*, *14*).

One solution to the lack of available aquatic cell lines is the development of novel primary cell cultures. Normal somatic cells are not immortal and will trigger senescence either via cell cycle arrest or via shortening of telomeres (*96*). In order to take primary cultures and immortalize them, either or both of these mechanisms must be overcome. Frequently, this is done by inducing expression of a telomerase reverse transcriptase protein, which can be

accomplished with the use of a eukaryotic plasmid (*96*). One of the concerns with maintaining an immortal cell line is the alteration of original characteristics over time (*96*). As the number of passages increases, cell lines often begin to behave differently as they are placed under selective pressures (*96*). Cheng, Spitsbergen and Bowser (1990) (*15*) aimed to produce EEDV-susceptible cell lines of lake trout origin and while they were able to produce a number of cell cultures and observed plaque formation in a few infected wells, they were unable to produce consistent results in subsequent passes (*15*). Unfortunately, these cell cultures no longer exist and there are no commercially available cells of lake trout origin.

7.2. Imaging

In order to address and elucidate the pathology and host-pathogen interactions of these herpesviruses, many researchers turn to the use of advanced imaging assays such as histopathology, electron microscopy and *in situ* hybridization. Such tools can be used not only in the diagnosis of known pathogens, but also in advancing our knowledge of the complexities behind viral disease progression, tissue and cellular targets and pathogenesis.

Furihata et al. (2005) (*87*) performed experimental challenges with *Oncorhynchus masou* Virus (OMV; Salmonid Herpesvirus-2) and rainbow trout and demonstrated the development of hemorrhagic and necrotizing alterations in the spleen, kidney, liver and intestines (*87*). Wolf and Smith (1981) (*101*) produced clinical disease in juvenile rainbow trout experimentally challenged with Herpesvirus Salmonis (Salmonid Herpesvirus-1) (*101*). Histopathologic examination of these fish revealed a systemic disease with extensive and degenerative changes in many tissues including renal tubular necrosis, necrosis and sloughing of gut mucosa and the presence of syncytia in pancreatic acinar tissue.

Comprehensive histopathologic studies have been completed on several of the non-salmonid Alloherpesviruses as well, including Koi Herpesvirus (KHV; Cyprinid Herpesvirus-3) and Channel Catfish Virus (CCV; Ictalurid Herpesvirus-1). Histopathologic examination of koi fish experimentally challenged with KHV revealed prominent lesions in multiple organs including nuclear degeneration of myocardial cells, necrotic hematopoietic cells in the kidney, and capillary and venous congestion within the valvula cerebelli, with the principal lesions being seen in the gill filaments (e.g., swollen, vacuolated respiratory epithelial cells with nuclear degeneration) (*16*). Wolf et al. (1971) (*17*) experimentally challenged juvenile channel catfish with CCV and reported development of a hemorrhagic disease with systemic edema, renal, hepatic and enteric necrosis as well as necrosis of the renal hematopoietic tissues (*17*).

During the original EEDV outbreak in the 1980s, some preliminary histopathology was performed on an undisclosed number of affected lake trout where branchial tissues were collected and examined using light and electron microscopy. Changes included epithelial hyperplasia and hypertrophy of the primary and secondary gill lamellae as well as branchial inclusions along the secondary lamellae (*2*). Additional organs collected from a limited number of fish revealed renal glomerulitis, renal tubule degeneration and epithelial hyperplasia of the skin, nares, upper palate and alimentary tract.

7.3. Molecular techniques

7.3.1. PCR

Without the ability to isolate EEDV in cell culture, recent diagnosis has involved the use of molecular techniques including PCR. In 2009 a diagnostic quantitative PCR (qPCR) assay for detection of EEDV was developed based on the terminase gene sequences for the three salmonid

herpesviruses described at that time (*1*, *5*). This assay uses a *Taq* polymerase and has a reported detection limit of 10 viral copies (*5*). Following the identification and molecular description of salmonid herpesviruses -4 and -5, it was determined that this initial qPCR assay was unable to distinguish between the later three viruses (*14*). At this time, a novel assay was designed using SYBR Green and primer sets that recognized sequences on the glycoprotein gene, which was capable of quantifying and differentiating between all five salmonid herpesviruses (*14*).

7.3.2. LAMP

Loop-mediated isothermal amplification (LAMP) assays have become a promising method of virus detection due to their rapid speed, cost effectiveness (compared to the relatively expensive qPCR), specificity and sensitivity (*19*), as well as their potential for commercialization (*18*). LAMP assays have several advantages when compared to PCR, first of which is the performance of amplification under isothermal conditions negating the need for expensive laboratory thermal cyclers (*19*). Additionally, LAMP makes use of three primer sets rather than just one, resulting in increased specificity and decreased background noise and non-specific binding (*19*). Finally, LAMP has the potential to be commercialized and used in peripheral laboratories with minimal staff training and no specialized equipment (*102*). In an aquaculture setting, such a tool would be invaluable in that pathogen identification could occur on site at a hatchery in a short period of time, allowing for more rapid instigation of treatment protocols. In fact, LAMP assays have been designed and implemented for the identification of the aquatic pathogens such as fathead minnow nidovirus (*103*) and *Edwardsiella tarda* (*104*).

8. Knowledge Gaps and Study Objectives

Our current knowledge of EEDV is based mainly on case reports and studies following the initial disease outbreaks in the 1980s (*2–4*) with the addition of improved molecular diagnostic assays over the past few years (*5*, *14*). Despite the catastrophic losses of a valuable native fish species caused by this virus, with its apparent disappearance for up to a decade at a time, coupled with its challenging diagnosis, our knowledge base regarding the biologic and pathologic properties of EEDV remains mostly unchanged over the past several decades; a fact that recent events have thrown into sharp focus. While initially believed to have been eradicated by depopulation of infected fish, increasing intra- and inter-hatchery biosecurity and decreasing transportation of fish between at-risk bodies of water (*5*), the continued identification of EEDV genomic material coupled with recent disease outbreaks (*5*, *14*) demonstrates the virus' continued presence in the Great Lakes basin and underscores the need for further research focused at improving our understanding of this deadly pathogen.

It is currently believed that EEDV is host species specific and has no significant deleterious effects on exposed salmonid species other than lake trout (*3*) and potentially its hybrid (*105*), however with the recent re-emergence of the virus after an extended quiescent period, it is unknown whether or not this remains true for current active virus strain(s). While previous studies have shown that EEDV is specific for lake trout (*4*) it is still possible that other species may develop unapparent infections and/or act as disease reservoirs or carriers. The identification of viral targets and at risk populations is vital to preventing additional losses from EEDV infections.

Current disease prevalence in hatchery and wild lake trout is unknown due to lack of accurate and practical diagnostic tests (*5*). Vertical transmission potential is unknown, however

EEDV DNA was detected in ovarian fluid of spawning adult lake trout, indicating a potential for vertical or egg-associated transmission (*5*). Detection of viral DNA was also found in the skin of non-clinical juvenile lake trout (*5*) suggesting the potential for latency. While this data highlights the continued presence of EEDV in the Great Lakes basin, many questions remain regarding origin of and pathogenesis behind these positive tissue samples.

The most likely targets for viral entry into the body are via the skin or fins, based on the results of histologic and electronmicroscopic examination of tissues from infected fish (*3*). Unfortunately, while pathogenesis studies have been performed and analyzed for many of the aquatic herpesviruses, there is a lack of extensive, controlled histopathologic studies on EEDV as much of the previous work with this virus has focused primarily on improving diagnostics through genome sequencing, assay development and tissue culture trials. The result is that while there are reports of pathologic changes observed in severely diseased fish collected during mortality events, the early stages of infection remain a mystery (*3*, *4*).

The continued presence of EEDV in the Great Lakes basin presents a major threat to established rehabilitation efforts, yet unfortunately there remains a large knowledge gap surrounding EEDV, which is preventing the effective management and prevention of this disease. The potential for outbreaks in either hatcheries or wild lake trout populations is particularly sobering without the knowledge of how to effectively manage such an epizootic. To that end, this dissertation was designed with the following study objectives:

Objective 1. Shed light on the details of two recent EEDV epizootics. Within the past six years, there have been two mortality episodes among captive lake trout at a state fish hatchery in northern Michigan. With the identification of EEDV as the causative agent in these epizootics, a virus which had not caused such mortalities in almost 30 years, it is clear that this virus still

constitutes a major threat to lake trout rehabilitation efforts in the Great Lakes basin. A complete and thorough understanding of when and how and why these mortality events occurred is vital to not only the furthering of knowledge of EEDV ecology, but also in preventing future disease outbreaks.

Objective 2. Establish a repeatable model of infection capable of mimicking natural EEDV disease in controlled laboratory environments. As an *in vitro* model of viral replication has not yet been established for EEDV, in order to proceed with experimental challenges aimed at improving the understanding of EEDV disease ecology, two things must first occur: 1) a stock of infectious virus must be produced from the tissues of naturally infected fish, and 2) it must be shown that this stock is repeatedly capable of producing clinical EED in controlled laboratory conditions. Previously in our laboratory, similar studies have been performed using Viral Hemorrhagic Septicemia Virus (*106*), and Fathead Minnow Nidovirus (*107*) which served as a guide for development of an *in vivo* model of EEDV replication and disease development. Initially, a stock of infectious EEDV tissue homogenate was produced via *in vivo* serial passages through naïve juvenile lake trout. Next, in order to mimic a natural route of infection, a new group of lake trout were exposed to EEDV via immersion bath at a wide range of doses so to determine a range at which clinical disease is produced. Based on the results of that trial, additional challenges were performed, in replicate, in order to assess the reproducibility of experimentally induced morbidity and mortality.

Objective 3. Determine the sequential distribution of EEDV among various tissues and cell types using qPCR and in situ hybridization. Quantitative PCR and *in situ* hybridization have been utilized as tools to identify tissue targeted entry points of Cyprinid Herpesvirus-3 in koi and common carp (*71*) as well as to determine that gill, kidney and spleen are the primary tissue

targets of Cyprinid Herpesvirus-2 in Prussian carp (*Carassius auratus gibelio*) (*108*). Based on these study designs, in order to evaluate the tissue and cellular targets of EEDV in lake trout hosts, juvenile fish were exposed to a moderate dose of EEDV via immersion bath, after which ten separate tissues were collected over the course of 42 days. Viral load amongst tissues and visualization of viral genetic material within specific cell types over the course of the study was evaluated.

Objective 4. Determine the extent to which EEDV infection sequentially alters individual organs and tissue layers both grossly and microscopically in its natural host, the lake trout. Many comprehensive histopathologic studies have been performed with members of the Alloherpesviridae family (*87*, *90*, *101*, *109*), however EEDV is not one of them. Current understanding of EEDV-caused pathology is limited to analysis of lesions collected during mortality events, which naturally lack details regarding the early stages of disease. In order to assess the gross and microscopic pathology associated throughout the early stages of an EEDV infection through development of severe morbidity or mortality, a complete set of tissues was collected from multiple fish on pre-determined sampling days. Microscopic lesions were scored in order to help evaluate the progression of disease severity over time.

Objective 5. Develop and characterize a cell culture system of lake trout origin. Cell culture has become a vital tool in aquatic animal medicine and research. Unfortunately there are often instances where a species-specific cell line is required, however the number of commercially available cell lines originating from fish tissue is severely lacking, and none exist from lake trout. In order to rectify this, tissues were collected from three different groups of lake trout (i.e., broodstock, yearling and fry), digested and seeded into cell culture flasks to produce primary cultures. Upon becoming confluent, flasks were subcultured, and eventually viral

susceptibility to three different aquatic viruses was assessed as well as determining optimal growing conditions for newly established cell cultures (e.g., incubation temperature, growth medium, and serum concentration).

Objective 6. Develop and evaluate the sensitivity and specificity of a quantitative loop mediated isothermal amplification (LAMP) assay for the detection of the EEDV virus by comparison to the SYBR green quantitative PCR assay already in use. LAMP assays have become favored in molecular diagnostics as they are equally as sensitive and specific as PCR, more cost effective to develop and run, and have the potential for commercialization and use in field environments (*19*). In the absence of a cell line capable of supporting EEDV replication, molecular assays become the primary mode of diagnosis. A novel quantitative LAMP assay was optimized in the presence of calcein to $MgCl_2$ and dNTP concentration as well as incubation temperature. Diagnostic sensitivity and specificity were established and compared to real-time qPCR as was the capability of the assay for viral load quantification.

APPENDIX

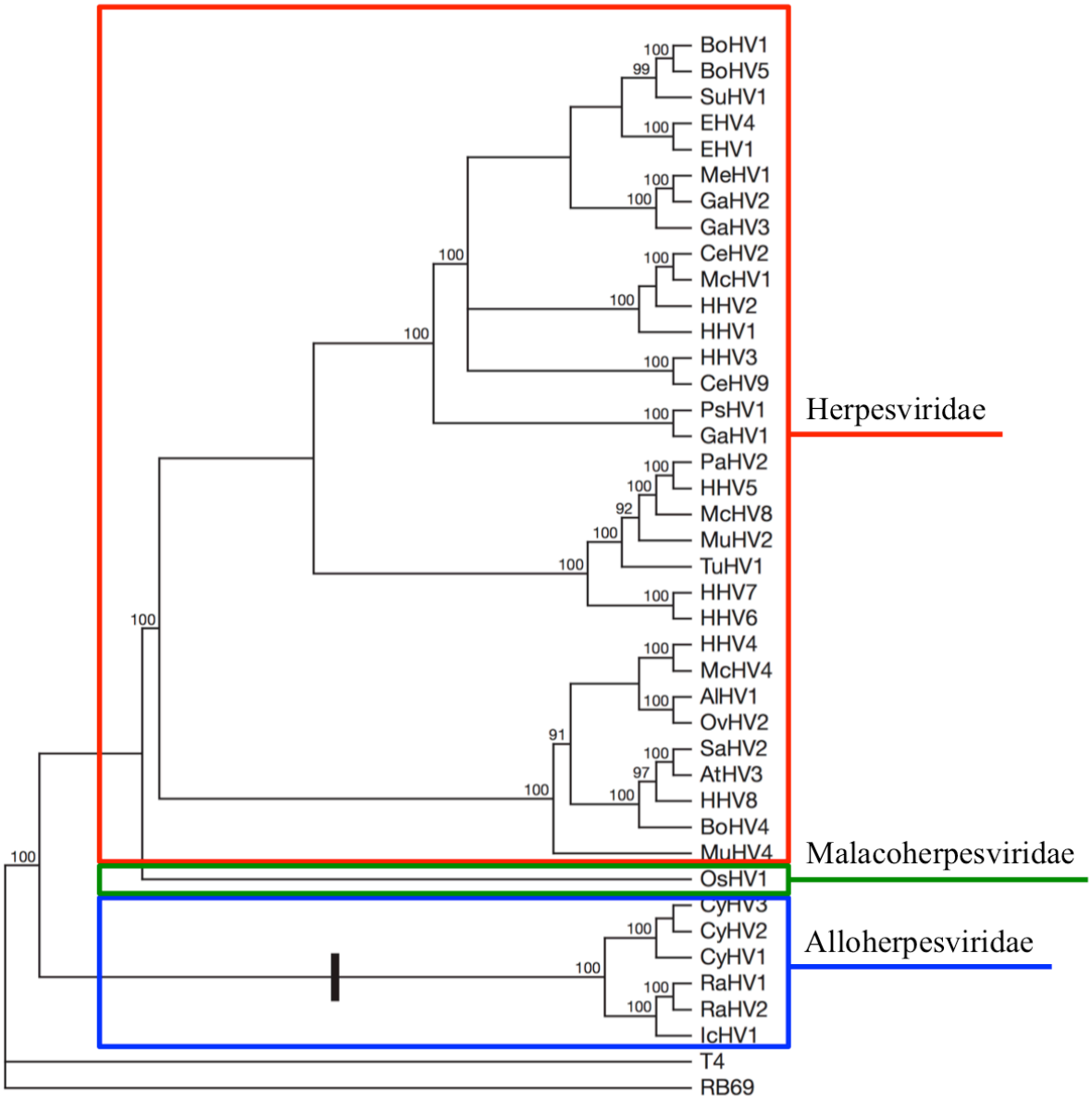

Figure 1.1 Cladogram depicting relationships of select viruses within the order *Herpesvirales* and between the families Herpesviridae, Malacoherpesviridae and Alloherpesviridae (modified from Waltzek et al. (2009) (*1*)).

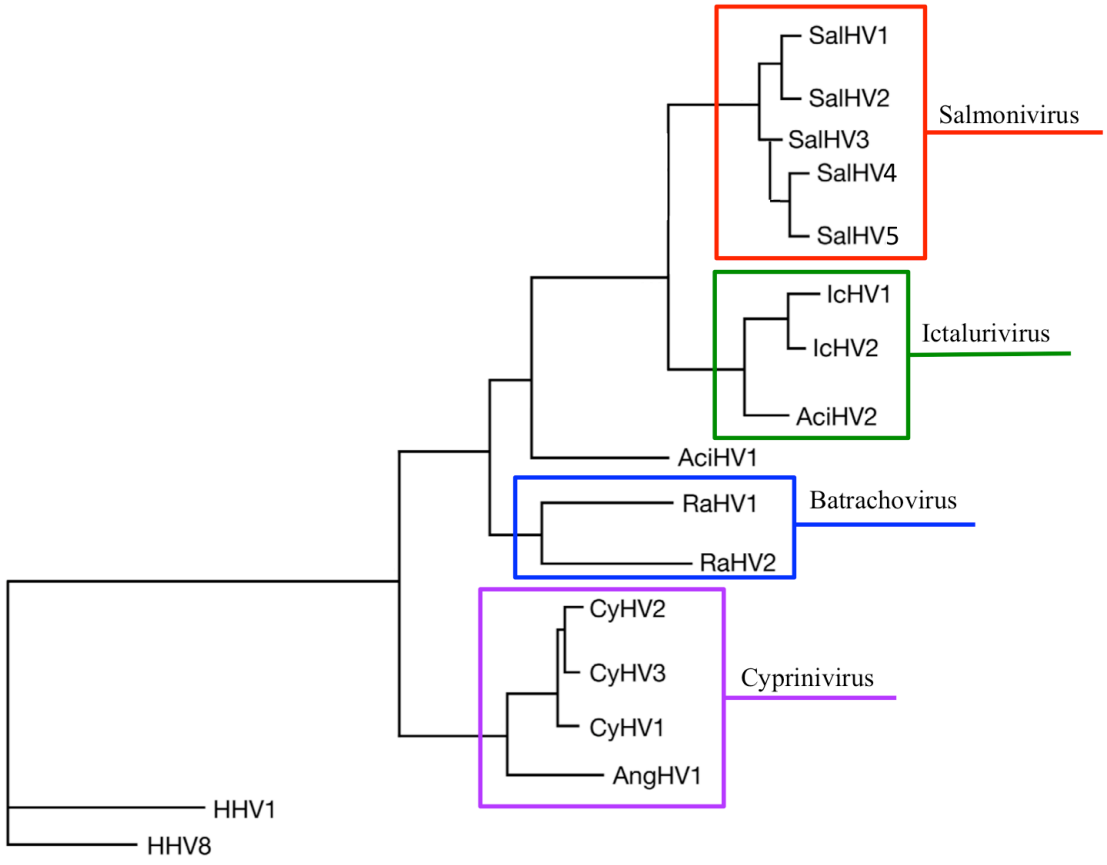

Figure 1.2 Phylogram depicting relationships of the four genera within the Alloherpesviridae family (modified from Waltzek et al. (2009) (*1*) and Glenney et al. (2016) (*14*)).

Chapter 2

Resurgence of Salmonid Herpesvirus-3 infection (Epizootic Epitheliotropic Disease) in hatchery propagated lake trout (*Salvelinus namaycush*) in Michigan

1. Abstract

Over the past century, populations of the economically and ecologically important North American lake trout (*Salvelinus namaycush*) have been threatened throughout the Great Lakes basin (GLB) due to over-fishing, habitat destruction, introduction of invasive species, and emerging infectious disease. To combat these declines, state and federal fishery management agencies undertook substantial fishery conservation efforts, including more stringent regulation of sport and commercial catch limits and increasing hatchery propagation of lake trout that are stocked into GLB waterways. One state fish hatchery involved in these rehabilitation efforts experienced mass mortality events in the fall of 2012 and of 2017. In 2012, following a period of abnormally heavy rain, hatchery staff observed abnormal behavior followed by increased mortalities in two strains of lake trout fingerlings that reached upwards of 20% and totaled a loss of approximately 100,000 fish. In 2017, following another heavy rain season, 6-8% of two-year old lake trout experienced morbidity and mortality similar to that observed in 2012. During both episodes, brook trout (*Salvelinus fontinalis*) and splake (lake trout x brook trout hybrid) reared in flow-through systems receiving water from diseased lake trout remained clinically unaffected. Molecular analyses revealed that all lots of affected lake trout were infected with Salmonid Herpesvirus-3 (Epizootic Epitheliotropic Disease Virus, EEDV). Further sampling detected EEDV in apparently healthy 5-year old lake trout, and in wild mottled sculpin (*Cottus bairdii*). Rivers' postulates were fulfilled by exposing naïve lake trout to infectious material, resulting in similar disease signs. Despite going undetected for many years, these two EEDV episodes clearly demonstrate the continued presence of this deadly virus in the Great Lakes basin.

2. Introduction

The lake trout (*Salvelinus namaycush*) is an invaluable constituent of the fish fauna residing within the Laurentian Great Lakes Basin of North America (*13*). In addition to their intrinsic ecological value as a native species, lake trout are also a valuable commercial and sports fishery commodity (*6*). Unfortunately, Great Lakes lake trout fisheries have steadily declined due to overfishing, habitat degradation, predation by the sea lamprey (*Petromyzon marinus*), and alewife (*Alosa pseudoharengus*) invasion (*6–9*, *11*, *12*, *95*). As a result, state and federal fishery management agencies developed programs to regulate lake trout harvest and create self-sustaining wild populations (*6*), which included bolstering depleted wild populations by the stocking of hatchery-raised lake trout as well as supplementing wild populations in water bodies with low natural egg survival rates (*6*).

During their tenure in hatcheries, lake trout are susceptible to a number of infectious diseases (*110*, *111*), including one particularly devastating disease caused by Epizootic Epitheliotropic Disease Virus (EEDV; *Herpesvirales*, *Alloherpesviridae*). This herpesvirus, also known as Salmonid Herpesvirus-3, led to the loss of approximately 15 million juvenile, hatchery-reared lake trout in the 1980s in seven state and federal hatcheries across three states in the Great Lakes region (*2–4*). In an attempt to control this virus and limit its spread, fishery managers opted for depopulation and disinfection of affected hatcheries, along with the implementation of movement restrictions for Great Lakes basin lake trout (*5*). It appeared these control efforts were largely successful because reports of EEDV outbreaks, characterized by a rapid onset of mortality in young (< 2 years of age) lake trout, hyperplastic lesions of the skin and gill epithelia, ocular hemorrhage (*2*), and secondary infections (*5*) ceased. However, the virus was detected in the reproductive fluids of wild, spawning adult lake trout in Lake Superior,

Wisconsin as well as in the skin of both apparently healthy hatchery-raised juvenile lake trout and those experiencing mortalities (severity of mortalities unreported) (*5*).

Herein, we report two mortality events associated with EEDV that occurred in lake trout at a state fish hatchery in Michigan's Upper Peninsula during the fall of 2012 and 2017. This apparent resurgence of EEDV following decades of covertness highlights the need to better understand the biological properties of this virus, along with the intricacies of the host-virus interactions.

3. Materials and Methods

3.1. Lake trout mortality events

In September of 2012, Michigan's Upper Peninsula experienced several days of heavy rain, resulting in the flooding of many smaller streams and creeks including Cherry Creek, which is a surface water source for Marquette State Fish Hatchery (MSFH; Marquette County, Lake Superior watershed). At the time, MSFH was raising two strains of lake trout (i.e., Lake Superior (LS) and Seneca Lake (SE) strains), brook trout (*Salvelinus fontinalis*), and splake (lake trout x brook trout hybrid), all of which were housed in covered, outdoor raceways (12,786-14,793 gallons) with an average of 90,000 fish per raceway. As a whole, the hatchery receives both well water and spring water from nearby Cherry Creek; production aged fish receive Cherry Creek water at an approximate rate of 1,200 gallons per minute. As an additional precaution, all water supplying broodstock fish is passed through an ultraviolet filter before entering the raceways. Among the production fish, the brook trout, LS lake trout, and a portion of the SE lake trout were receiving first pass water, whereas the splake and the remainder of the SE lake trout were receiving second pass water and were housed immediately downstream of the first pass raceways

(Figure 2.1). Shortly after the period of abnormally heavy rains, mortality began to climb in both strains of juvenile (approximately 8 months post-hatch) lake trout (Figure 2.2). Between October 2nd and November 8th 2012, moribund lake trout from two lots were collected live for clinical examination. Over the five-week period, diagnostic examinations were performed on a total of 60 LS and SE lake trout (30/strain).

In September of 2017, elevated mortality was once again reported at MSFH, also following a heavy rain event, this time in one lot of 2-year old LS lake trout. Moribund fish (n = 10) were collected live and sent for clinical examination.

3.2. Clinical examination

Upon receipt at the laboratory, fish were euthanized with an overdose of tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.25 mg/mL) and immediately subjected to gross external pathological examination. Wet mounts of gill tissues and skin lesions were prepared and examined for presence of parasites, fungi and bacteria via light microscopy. Next, fish were surface disinfected with 70% ethanol and gross internal pathological examination and aseptic tissue collections performed following guidelines presented in the American Fisheries Society Fish Health Section (AFS-FHS) Blue Book (2016) (*40*).

3.3. Bacteriology

For primary bacterial isolation, 10 µL sterile disposable loops were used to streak kidney tissues directly onto trypticase soy agar (TSA; Remel Inc., San Diego, California, USA), Hsu Shotts medium (HSM) (*112*), and cytophaga agar (CA) (*113*), which were incubated aerobically at 22°C (TSA and HSM) or 15°C (CA) for up to seven days. Additionally, representative brain

and gill tissues, as well as tissues from skin/muscle lesions, were streaked onto HSM and/or CA due to suspicion of flavobacterial involvement. Resultant bacterial growth was recorded, sub-cultured, and identified as recommended in the AFS-FHS Blue Book (*40*). Specifically for *F. psychrophilum,* molecular confirmation was performed as previously described (*114*).

3.4. Virus isolation

Kidney, spleen and heart tissue samples were aseptically collected, diluted 1:4 (w/v) with Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen). Tissues and diluent were then homogenized and centrifuged at 4,700 x *g* for 30 minutes, and the supernatant clarified by a second centrifugation at 2,700 x *g* for 20 minutes. The final supernatant was used to inoculate cell cultures of *Epithelioma papulosum cyprini* (EPC) (*115*) and Chinook salmon embryo (CHSE-214) (*116*) cell lines and examined for cytopathic effects as per the guidelines of the AFS-FHS Blue Book (2016) (*40*). Skin, fin, gill (2012 and 2017), and eye (2017 only) tissues were collected and stored at -20°C for further molecular diagnostics (see below).

3.5. Histopathology

Skin, muscle, fin, gill, eye, kidney, spleen, heart, and liver tissues, as well as transverse and sagittal whole body sections were collected from representative fish and preserved in phosphate-buffered 10% formalin for histopathological assessment. After embedding within

paraffin, tissues were sectioned at 5 μm, stained with hematoxylin and eosin (H&E) (*117*) and examined under a light microscope.

3.6. Molecular analysis

In the years following the 2012 mortality episode and as EEDV molecular assays were being designed and improved, two novel salmonid herpesviruses (i.e., SalHV-4; Atlantic salmon papillomatosis virus (*90*), and SalHV-5; Namaycush Herpesvirus (*94*)) were identified and found to possess some terminase gene sequence similarity with EEDV (*14*). However, glycoprotein gene sequence analysis allowed for development of highly sensitive qPCR assays specific to each of salmonid herpesviruses -3, -4, and -5 (*14*). Therefore, in the present study, three separate PCR assays were employed (*14*):

a) End-point PCR (terminase gene; amplifies DNA from SalHV-3, -4, and -5) (*14*)
b) TaqMan qPCR (terminase gene; amplifies DNA from SalHV-3, -4, and -5) (*14*)
c) SYBR Green qPCR (glycoprotein gene; amplifies DNA from SalHV-3 only) (*14*)

End-point PCR was used in the 2012 and 2017 EEDV outbreaks for gene sequencing and phylogenetic analyses (see below) whereas the TaqMan qPCR was used for screening purposes from 2012 through the summer of 2016 until the SYBR Green qPCR assay was developed and optimized to definitively identify EEDV infected fish (*14*).

As the available molecular assays changed between 2012 and 2017, so too did the knowledge of EEDV tissue tropism and optimal diagnostic samples (data not shown). As a result, early EEDV testing following the 2012 mortality episode was performed on pools of

kidney, spleen and gill tissues lethally collected from juvenile fish, while mucous was non-lethally collected from adult broodstock in order to allow for screening of a larger number of fish without significantly influencing broodstock availability for future fish production. Historical samples from 2007-2012 consisted of kidney, spleen and heart pools previously collected for virological screening and stored at -20°C.

For viral DNA extraction, one of two extraction methods was used. For the TaqMan qPCR, the MagMax^{TM} 96 Viral RNA isolation kit (Life Technologies, Grand Island, New York, USA) was used manually, following manufacturer's instructions. Samples were lysed using Proteinase K and Lysis buffer (Qiagen, Germantown, MD, USA), and incubated in a water bath at 55°C for 1 hour. Following lysis, samples were centrifuged at 21,000 x *g* for 10 minutes and the supernatant used in the extraction process. Following the development of the SYBR Green qPCR assay, viral DNA extractions were performed manually using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) after tissue digestion (*14*). Following all nucleic acid extractions, DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA) and diluted to a standard concentration using nuclease free water.

All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA). Both the TaqMan and SYBR Green assays were performed as described previously (*14*) with the exception that the total reaction volume of the SYBR Green assay was 20 μL; 30-60 ng total DNA was added to each qPCR reaction. Using the Mastercycler ep *realplex*^{2} S accompanying software at the manufacturer's default settings,

samples were considered positive based on a threshold setting of the computer default noiseband for the TaqMan assay and 10% maximum florescence for the SYBR Green assay with a limit of 35 cycles for all samples. Positive extraction controls consisted of EEDV-positive tissue samples from diseased lake trout collected during a natural EEDV outbreak. Positive amplification controls and standards were produced for both the TaqMan and SYBR Green assays as previously described (*14*). Negative controls consisted of water as well as negative tissue extraction controls from disease-free lake trout.

3.7. Gene sequencing and phylogenetic analysis

Representative samples (n = 4 per episode) that were positive via the TaqMan qPCR (in 2012) or SYBR Green qPCR (in 2017) were selected for endpoint PCR and subsequent gene sequencing and phylogenetic analysis. Amplicons for sequence analysis were produced using primers 194F (5' - TAG TCT GAT CCC CCT CAT GC - 3') and 249R (5' - GTC GAG TCC GAC ACC AGA TT - 3'), which amplify a 324 bp fragment of the terminase gene (*14*). Each 50 µL reaction mixture was comprised of 25 µL 2x Go-Taq Green Master Mix (Promega, Madison, Wisconsin, USA), 250 mM of each primer, 50 ng of DNA template, and DNase-free water. Cycling parameters consisted of an initial denaturation step at 95°C for 15 minutes followed by 35 cycles of 94°C for 30 seconds, 59.5°C for 30 seconds and 72°C for 1 minute with a final step of 72°C for 10 minutes and were carried out in a Mastercycler Pro Thermal Cycler (Eppendorf, Hamburg, Germany). Amplicons and a 1 kb molecular ladder (Roche Applied Science, Penzberg, Germany) were combined with SYBR Green (Cambrex Bio-Science, Lonza Group, Basel, Switzerland), electrophoresed through a 1.5% agarose gel at 50V for 45 minutes, and visualized under ultraviolet light. Amplicons were then purified using a QIAquick PCR

Purification Kit (Qiagen, Hilden, Germany) and Sanger sequenced at the Michigan State University Research Technology Support Facility using both the forward and reverse primers.

For phylogenetic analyses, contigs were assembled using the contig assembly program in Bioedit Sequence Alignment Editor (*118*). Multiple sequence alignment was done using ClustalW in the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0) (*119*), whereby reference terminase gene sequences for Salmonid Herpesvirus-1 through -5 were downloaded from GenBank (NCBI) and included in the alignment (a total of 303 bases were included in the final data alignment set). The optimal model for phylogenetic reconstruction was assessed in MEGA 6.0 and the model with the lowest Bayesian Information Criterion (Kimura Two Parameter model with gamma distribution, K2+G) was selected. Neighbor-joining analysis was carried out in MEGA 6.0 with 1,000 resamplings. Bayesian analysis was conducted in MRBAYES version 3.1.2 (K2+G model) (*120*). The Markov chains ($n = 4$) were run for up to one million generations, with a stopping rule in place once the analysis reached an average standard deviation of split frequencies of <0.01. Two independent analyses were conducted, with the initial 25% of Markov chain Monte Carlo samples being discarded as burnin and sampling occurring every 500 generations. Results from Bayesian analyses were visualized in FigTree v1.3.1 (*121*).

3.8. Pilot experimental challenges

To confirm the virulence of the EEDV strain associated with the 2012 hatchery disease outbreak and fulfill Rivers' Postulates, pilot experimental challenges were performed. Juvenile LS strain lake trout (approximately 6 months post hatch) were collected from MSFH while maintained on a closed (i.e., well) water system and transported live to the Michigan State

University - University Research and Containment Facility (East Lansing, Michigan). The originating lot of fish used for experimental challenges was not present in the hatchery system during either mortality episode. Upon receipt, fish were held in a 680 L fiberglass tank supplied with continuous flow-through oxygenated well water (12.0 ± 1.0°C). Fish were fed 1.0 mm sinking feed (BioOregon, Westbrook, Maine, USA) daily and allowed to acclimate to laboratory conditions for at least one month prior to use in experimental challenges. Sixty fish were randomly collected, clinically examined, and determined to be free from fish pathogens as per the guidelines of the AFS-FHS Blue Book (2016) (*40*). Likewise, EEDV qPCR confirmed an absence of EEDV. Experimental challenges were performed in accordance with the Institutional Animal Care and Use Committee.

All experimental challenges were performed in 42 L continuous, flow-through tanks receiving oxygenated, chilled, well water (9.0 ± 0.5°C), and fish were allowed to acclimate for a minimum of 48 hours to experimental conditions prior to start of challenges. A stock of infectious EEDV was produced from the skin of MSFH-naturally infected lake trout following mortality. Skin was homogenized in a sterile phosphate buffered saline solution (PBS; pH 7.5 ± 0.5; Sigma-Aldrich, St Louis, Missouri, USA) at a 1:3 (w/v) ratio, and clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C.

Next, LS strain lake trout (n = 5 challenge group and n = 5 control group) were anesthetized using tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.1 mg/mL) then IP injected with either EEDV stock ($1.22x10^7$ viral copies per fish) or sterile PBS. Following recovery from anesthesia, fish were transferred back to experimental tanks for the duration of the studies.

All fish were fed and monitored daily for development of clinical signs of disease, morbidity or mortality for 2 months following injection. Any moribund fish displaying severe clinical signs such as altered behavior, inability to maintain balance, gasping for air or significantly pale gills was euthanized with MS-222 (0.25 mg/mL). At the end of the two-month period, surviving fish were euthanized. Skin tissues were collected from all fish immediately following death and tested for the presence of EEDV using the TaqMan qPCR protocol described above.

3.9. Intra- and extra-hatchery EEDV surveillance

After the 2012 mortality event, all lots of fish at the hatchery were screened for the presence of EEDV. Between the fall of 2012 and the spring of 2013, pools of kidney, spleen and gill were collected from a total of 120 juvenile LS lake trout, 240 juvenile SE lake trout, 480 juvenile brook trout, and 240 juvenile splake while mucous was collected non-lethally from 60 adult brook trout and 270 adult LS lake trout. Routine EEDV surveillance screening continued among MSFH lake trout through 2017 for both production fish (n = 60 per lot) and broodstock fish (n = 10 per lot) with the testing of kidney, spleen, gill, fin, skin and eye tissues (Table 2.1).

Additionally, wild fish were collected by standard electrofishing from Cherry Creek, upstream of MSFH, and tested for the presence of EEDV (Table 2.2). In 2012, 70 each of brook trout, brown trout (*Salmo trutta*), and mottled sculpin (*Cottus bairdii*) were collected from Cherry Creek with 60 of each of these three species collected and tested in 2013. From 2014-2017, 60 mottled sculpin per year were collected from Cherry Creek for EEDV screening.

4. Results

4.1. Description of MSFH mortality events

In 2012, approximately 10 days after the heavy rains, juvenile LS lake trout began exhibiting decreased appetites, evidenced by a lack of interest in food. Within three days, the LS lake trout in raceways 5a and 5b (Figure 2.1) began displaying flashing and were lethargic. Similar changes were noted 10 days later in the SE lake trout in raceway 6, followed one month later by the SE lake trout in raceways 4a and 4b. Affected fish developed multifocal skin pallor that in some instances became overgrown with white. The mortality episode spanned over 200 days, with the cumulative mortality by raceway ranging from >15% to >25% (Figure 2.2) and total losses exceeding 100,000 lake trout. Based upon initial clinical examinations, disease signs, and the detection of *Flavobacterium psychrophilum* in external lesions (see below), affected lake trout initially received an immersion treatment with Chloramine-T (Halamid®, Syndel USA, Ferndale, Washington, USA; 10 mg/mL for 1 hour for 3 consecutive days, repeated a second time after two days of no treatment). After no discernible improvements, this was followed by two treatment courses with Florfenicol medicated feed (Aquaflor; Merck Animal Health, Madison, New Jersey, USA; 10 mg/kg body weight per day for 10 days), which resulted in only a slight and brief decrease in mortality.

Likewise, in late September 2017, 2-year old LS lake trout (20-27 cm in length) at MSFH began developing disease signs similar to those seen in 2012. Initial mortalities were approximately 5 fish per day with an additional 12-15 showing signs of morbidity by day. These levels of mortalities continued through the following month.

4.2. Clinical examination

During the 2012 mortality event, both strains of moribund lake trout showed a number of external disease signs, including ocular hemorrhage with or without corneal opacity (Figure 2.3a, 2.3b), gill pallor, multifocal to diffuse skin " blotchiness" (Figure 2.3c) that was sometimes accompanied by erythema (Figure 2.3d), ulcerations that penetrated through the epidermis and dermis of the skin into the underlying muscle (Figures 2.3e, 2.3f), as well as ulcers that were overgrown by water mold hyphae (Figure 2.3g). In some instances, ulceration progressed to the point where the caudal fin had eroded completely (Figure 2.3h) and was also accompanied by a yellowish discoloration (Figure 2.3i), which is commonly associated with flavobacteria (see below). Other notable disease signs included erythema of the fins (Figure 2.3d), oral cavity, isthmus and ventrum, and excess mucus production of the skin. Internally, occasional hemorrhage within the adipose tissue, hemorrhagic enteritis, splenomegaly, and renal congestion were observed. Clinical findings were similar between both strains of lake trout; however, those seen in the LS strain tended to be more severe than those in the SE strain.

During the 2017 outbreak, disease signs similar to the 2012 outbreak were observed and were once again predominated by ocular hemorrhage (Figure 2.4a), varying degrees of skin ulceration (Figure 2.4b, 2.4c), and overgrowth by water mold (Figure 2.4d). In both the 2012 and 2017 outbreaks, microscopic examination of skin and gill preparations revealed the presence of occasional monogeneans (*Gyrodactylus* spp.), gliding filamentous bacterial rods consistent with flavobacteria, and aseptated hyphae consistent with the oomycete *Saprolegnia*.

4.3. Bacterial and viral isolation

Flavobacterium psychrophilum was recovered from a portion of the external lesions, and motile *Aeromonas* spp. were occasionally recovered in kidney cultures, albeit in relatively low quantities. No signs of viral replication were observed following cell culture inoculation.

4.4. Histopathology

Histopathological findings in EEDV-infected lake trout collected during the 2012 outbreak included corneal epithelial necrosis and/or ulceration (Figure 2.5a), epithelial necrosis and ulceration of the skin (Figure 2.5b), lamellar edema (Figure 2.5c), gill epithelial swelling/hypertrophy (Figure 2.5d), proteinaceous exudate within both Bowman's space and the renal tubules (Figure 2.5e), renal tubular epithelial necrosis (Figure 2.5f), and multifocal necrosis of the renal interstitium (Figure 2.5f). Similar microscopic changes were observed in EEDV-infected 2 year old LS lake trout in 2017; however, individual necrosis of the gill lamellar epithelium and a moderate dermatitis in the skin were also observed, as was hemosiderosis within the spleen.

4.5. Molecular identification

PCR-based molecular assays clearly demonstrated the presence of a Salmonid Herpesvirus in affected MSFH lake trout tissues from 2012. Skin, gill and kidney/spleen tissues from 16 fish (8 LS and 8 SE strain lake trout) tested positive via endpoint and TaqMan qPCR, although sequencing was required for confirmation of EEDV identity (see below). EEDV was detected in the LS lake trout from 7/8 gill samples, 7/8 kidney/spleen samples and 8/8 skin samples and in the SE lake trout from all 24 tissues tested. Viral gene copy number per reaction

in the LS lake trout ranged from 30-$2.3x10^3$ (median = 201) in the gills, to 14-656 (median = 134) in the kidney/spleen, and $1.98x10^4$-$9.80x10^5$ (median = $9.22x10^4$) in the skin. Viral gene copy number per reaction in the SE lake trout ranged from $1.92x10^3$-$9.63x10^4$ (median = $1.26x10^4$) in the gills, to 10-$5.45x10^4$ (median = 40.6) in the kidney/spleen, and 270-$1.44x10^4$ (median = $2.56x10^3$) in the skin.

The presence of EEDV in the 2017 mortality event was confirmed using the SYBR green qPCR assay, as well as with endpoint PCR and gene sequencing (see below). All tissues collected from moribund fish were positive for EEDV: 10/10 skin lesions ($2.57x10^4$-$1.42x10^6$; median = $2.04x10^5$ copies) and 10/10 eye tissues ($8.68x10^3$-$2.38x10^7$; median = $1.38x10^6$ copies).

4.6. Sequencing and phylogenetics

Amplification and sequencing of a portion of the EEDV terminase gene from four naturally infected lake trout from each of the 2012 and 2017 EEDV outbreaks led to the generation of gene fragments totaling 311-322 bps in length. Percent similarity analysis revealed that 3 out of 4 MSFH EEDV isolates from 2012 and 4 out of 4 2017 isolates were 100% similar to the Salmonid Herpesvirus-3 isolate from Wisconsin (accession # EU349284) at this locus, whereas 1 2012 isolate was 99.7% similar (310/311 bp) to the Wisconsin reference isolate. Phylogenetic analyses placed the eight MSFH EEDV isolates into a robustly supported clade (i.e., posterior probability and bootstrap values >70) that also contained the Wisconsin reference isolate, which shared a most recent common ancestor with Salmonid Herpesvirus-4 and -5 (Figure 2.6).

4.7. Pilot experimental challenges

In the EEDV experimentally challenged fish, 80% mortality was reached at 29 days post-infection with previous mortalities occurring at days 6, 13, and 20 post-infection. EEDV was detected in multiple tissues from infected fish that died on days 13, 20, and 29 post-infection. The virus was found in the skin/fin (3/5 fish; $9.40x10^4$-$2.50x10^6$; median = $5.85x10^5$ copies per reaction), gill (2/5 fish; $4.61x10^3$-$5.26x10^3$; median = $4.93x10^3$ copies per reaction), and kidney/spleen (3/5 fish; 360-$2.72x10^3$; median = 868 copies per reaction) of experimentally challenged fish. Clinical signs were consistent with those seen in the natural epizootics and included ocular hemorrhage (Figure 2.7a), skin pallor, erosions and ulcerations (Figure 2.7b, 2.7c), with congestion and erosion of the fins. The control group experienced only a single mortality, and no evidence of EEDV infection was detected in any control fish.

4.8. Intra- and extra-hatchery EEDV surveillance

Using qPCR as detailed above, EEDV was not detected in any of the adult brook trout or lake trout broodstock tested in 2012. Of the 120 juvenile LS lake trout and 240 juvenile SE lake trout tested in 2012 following the mortality episode, EEDV was detected in 24 fish (21-113 virus copies per reaction; median = 36) and 21 fish (20-1,828 virus copies per reaction; median = 69) respectively. It is interesting to note that while beyond the established cut off of 35 cycles, amplification was observed from a total of 132 additional juvenile lake trout tested in 2012. Likewise, while no juvenile splake or brook trout had detectible levels of EEDV, amplification was observed after 35 cycles in two brook trout samples and four splake samples (kidney, spleen, and gill tissues). Following the 2012 mortality event, EEDV screening at MSFH continued

through 2017 as detailed in Table 2.1, including the testing of stored historical samples from 2007 and 2011.

During routine surveillance in 2017, EEDV was detected in 8 adult (5 years post-hatch) and 5 juvenile (2 years post-hatch) LS strain lake trout in fin and eye tissues ranging from 127 to $1.1x10^7$ viral copies in the fin (median = $3.89x10^3$) and $6.9x10^3$ to $2.5x10^5$ viral copies in the eye (median = $1.28x10^5$). Of note, these samples were collected just prior to the appearance of disease signs in the affected lot.

EEDV screening in fish collected from Cherry Creek (i.e., the surface water system feeding MSFH) is detailed in Table 2.2. All brook trout and brown trout collected were EEDV-negative (n = 150 and n = 185 respectively). All mottled sculpin were EEDV-negative except for five pools (n = 25 fish) in 2013.

5. Discussion

The lake trout-lethal herpesvirus, EEDV, has re-emerged in at least one hatchery within the Great Lakes basin, where it was once again associated with substantial mortality, severe disease signs, and high viral loads in multiple strains of fingerling lake trout, with the addition of disease signs in 2 year old lake trout and virus detection in 5 year old lake trout as well. Moreover, laboratory experiments aimed at fulfilling Rivers' postulates confirmed the ability of the causative virus strain to produce clinical signs and mortality consistent with those seen in natural EEDV outbreaks, despite the absence of secondary invaders (e.g., oomyetes, *F. psychrophilum*, etc.) that were present in the natural outbreaks. The resurgence of EEDV after decades of an apparent absence is both surprising and perplexing. On one hand, it is known that some human and animal herpesviruses can run a covert, low-level infection in which the host

survives and becomes a viral reservoir within a system (*122*). On the other hand, sub-lethal infections are uncommon among the other fish-pathogenic alloherpesviruses, although shedding of infectious virus has been detected following survival from Cyprinid Herpesvirus-3 infection (an OIE reportable pathogen) (*45*, *123*, *124*). In the case of EEDV, the 1980s reports demonstrated its high pathogenicity to lake trout and suggested that survival of infected fish was unlikely. This concept prevailed until Kurobe et al. (2009) (*5*) developed a novel PCR assay based on the terminase gene sequence and reported the presence of EEDV in apparently healthy lake trout collected from Wisconsin waters, signifying that EEDV may be capable of causing sub-lethal infections within lake trout. Indeed, such infections have subsequently been reported for EEDV (i.e., Salmonid Herpesvirus-3) and Salmonid Herpesvirus-5 in wild, clinically normal, adult lake trout throughout the northeastern United States (*14*, *94*). In this context, it is possible that the MSFH lake trout harbored a sub-clinical EEDV infection prior to the fall of 2012 and that stressors, such as the heavy rain events that preceded each of the outbreaks and resulted in an influx of sediment-laden water into hatchery rearing units, led to clinical outbreaks of EEDV.

Alternatively, it is possible the virus found its way into the hatchery via the source water, as, in 2013, EEDV DNA was detected in mottled sculpin residing upstream of MSFH in Cherry Creek, the tributary supplying water to the affected fish. Indeed, it was a surprise to detect EEDV DNA in non-salmonids, as alloherpesviruses are known to be highly species-specific. Detection of viral DNA on external tissues alone would be questionable as to whether these fish were truly infected, or if EEDV genetic material was present in the water only. However, the detection of EEDV DNA within pools of kidney, spleen and heart tissues raises the possibility that these fish were truly infected, although conclusive determination of whether the virus was active or not

was unfortunately not possible. Nevertheless, experiments examining the susceptibility of sculpin to EEDV are currently underway.

Finally, it is also possible that the lake trout broodstock harbored a sub-clinical EEDV infection and acted as a source of infection for the progeny in 2012. Although there are no reports of EEDV vertical transmission, Kurobe et al. (2009) (*5*) detected EEDV DNA in the ovarian fluids of spawning lake trout from Lake Superior, and both Salmonid Herpesvirus-3 and -5 have been detected in the ovarian fluid of clinically normal lake trout from Lake Champlain, Vermont (*14*). Likewise, it was suggested that broodstock may be a source of infectious virus after multiple EEDV outbreaks in the 1980s occurred in juvenile lake trout reared on well water (*4*, *5*), indicating a source of infection other than the water supply. Additionally, EEDV outbreaks occurred in first year progeny from wild source broodstock in the 1980s (*5*), and while infection source tracking wasn't possible at the time, the more recent detection of EEDV genomic material in apparently clinically normal fish (*5*, *14*) allows for the supposition that these broodstock were harboring an undetected EEDV infection.

The EEDV outbreak in 2017 was equally surprising given that: a) the virus had not been detected in MSFH since 2012 despite regular surveillance of broodstock and production fish with the highly sensitive TaqMan and SYBR green qPCR assays; b) it led to mortality in 2-year old lake trout as opposed to fingerlings or yearlings, which to our knowledge has not been reported previously; and c) it was associated with severe signs of disease and high virus loads in older fish. Importantly, during each mortality event, detection of EEDV was limited to a single cluster of raceways (Figure 2.1), further supporting the importance of biosecurity within the hatchery. As was the case in the 2012 outbreak, heavy rains preceded the 2017 mortality episode, during which raceway water temperatures were also within what is believed to be the optimal

temperature range (i.e., 9.0 ± 1.0°C) for EEDV outbreaks. Thus, this study suggests that EEDV is not only capable of causing mortality and frank disease in fingerlings and yearlings, but older fish as well, further demonstrating the effect this virus can have on lake trout rehabilitation efforts. Typically, only two year classes of production lake trout are housed on hatchery grounds at any given time, the youngest of which are held in indoor "nursery" raceways on well water until the older fish (in the Production Building) are stocked into the wild. Because of this process, a mortality event in production aged fish, while problematic to that year's stocking goals, can potentially be compensated for in following years with alterations in the number of spawning family pairs and stocking management plans. Significant mortalities in captive broodstock however have the potential to be catastrophic to lake trout rehabilitation and population management as these fish are frequently used to produce many consecutive years worth of production fish. Additionally, lake trout are slow maturing fish, taking typically 6-7 years to reach sexual maturity, meaning were a hatchery to lose a younger lot of broodstock to EEDV, it could take close to a decade to rebuild a new line of reproductive stock.

The 2012 disease outbreak also revealed some interesting epidemiological aspects of EEDV within a hatchery environment. First, it is noteworthy that in 2012, the LS lake trout were the first to show clinical signs of disease, followed by the SE lake trout receiving 2nd pass water that included water from the affected LS lake trout rearing units, followed lastly by the SE lake trout that were housed next to the affected LS lake trout (Figure 2.1). As noted previously, the source of EEDV (e.g., source water, covert infections, and/or broodstock) for this outbreak is unknown, but the pattern of EED initiation in 2012, coupled to the fish strain, suggests that susceptibility to EEDV may vary by lake trout strain. Interestingly however, comparison of viral loads between strains during the 2012 mortality event revealed comparable levels of EEDV

between the two strains in the kidney, spleen and skin while the SE lake trout had higher viral loads in the gills. However, in future screening the virus was detectible either at comparable levels between strains (2012), or only in the LS lake trout (2017). Knowledge of strain variation in disease resistance and research into specific genetic markers provides resource managers with the option to focus or tailor management strategies toward producing more resistant strains of fish or protecting more susceptible ones. Such a strain variation in susceptibility of rainbow trout (*Oncorhynchus mykiss*) to the Infectious Pancreatic Necrosis Virus (IPNV) has been well documented (*125*). Armed with the ability to experimentally induce clinical EED, future experiments can focus on dissecting these potential strain variations as well as more closely examining the non-lake trout species susceptibility to EEDV.

In addition, the observed temporal pattern of disease also suggests that a water borne route of transmission (i.e., from LS strain fish in raceways 5a and 5b to SE strain fish in raceway 6), without the need for direct fish to fish contact, may be important in EEDV contagion. Similarly, the initiation of disease signs and mortality in SE lake trout maintained next to, not up- or down-stream from, the LS lake trout that first showed disease signs, highlights the importance of biosecurity and the potential for virus spread without rearing unit interconnectivity. Lastly, brook trout, which were reared next to the affected SE lake trout, and splake, which were receiving 2nd pass water from the EEDV-infected SE and LS lake trout rearing units, never developed EED. These findings are in line with the reports of Bradley et al. (*2*) and McAllister & Herman (*4*) as no other salmonids on hatchery grounds during the 1980s EEDV outbreaks experienced mortalities, and experimental challenge of brook trout did not result in clinical disease or mortalities (*3*, *4*). Interestingly, although there is no peer-reviewed data, it has been reported that lake trout hybrids can be experimentally infected with EEDV (*105*).

Gross and histopathologic findings in these cases were consistent with those seen in the initial description of EEDV in the 1980s. As the name of this virus implies, the most significant microscopic lesions noted in this study were in the skin and gill epithelia, which may contribute to death of the host through osmoregulatory impairment and/or respiratory dysfunction. Additionally, the outer layers of the skin and gills serve as an important line of defense against fish pathogens, and as a result, any insult to this layer can predispose the affected host to a suite of opportunistic microbial pathogens, as was observed in this study in the form of *F. psychrophilum, Aeromonas* spp., and water mold infections in the more progressed EEDV-associated skin lesions.

Recent advancements in phylogenetics have allowed for improvements in our knowledge of the relationship between fish herpesviruses (*1*). Gene sequencing and phylogenetic analyses performed in this study confirmed the identity of the etiological agent as EEDV and also showed that seven of the eight isolates from the 2012 and 2017 outbreaks were identical to the salmonid herpesvirus 3 reference isolate (*1*) over the sequenced portion of the terminase gene. One isolate displayed a single nucleotide polymorphism (SNP) when compared to the other seven MSFH isolates and the reference isolate (Figure 2.6). Of note, this SNP led to an amino acid shift from a glutamine to a leucine (data not shown), but its effects on the functionality of the terminase gene product, which involve packaging viral DNA into the virus capsid (*126*), are currently unknown. Nevertheless, this study confirms the continued presence of highly similar EEDV strains in multiple Great Lakes states, a matter of grave concern in the context of lake trout rehabilitation and conservation efforts in the Laurentian Great Lakes.

These recent outbreaks of EEDV within the Great Lakes basin have highlighted the magnitude of work remaining to be completed in order to fully understand this devastating

disease. As molecular diagnostic assays continue to improve, other testing strategies must improve to match. This includes identifying and screening all at risk populations, particularly gametes and live fish slated to enter a hatchery system as well as focusing diagnostic efforts on sample collections most likely to highlight an EEDV infection by identifying viral target tissues. Increasing our working knowledge of lake trout immunology will allow for the identification of previously exposed fish and potential susceptibility differentiation between strains of lake trout, leading to alterations in management strategies to produce larger numbers of more resistant fish.

Efforts must be made to culture this virus, as the current lack of an EEDV-susceptible cell line hampers both diagnostic options as well as research opportunities. Without the ability to culture and produce an infectious stock of EEDV, frozen tissues and epizootic-surviving fish, both potentially containing active EED virus are of vital importance. Possession of these unique materials will allow this laboratory to perform this much-needed research. Moreover, further research into the pathogenesis and biological properties of this deadly virus will provide fishery management agencies with the tools and information necessary to not only prevent future outbreaks of EEDV, but also continue the successful rehabilitation of lake trout populations across North America.

APPENDIX

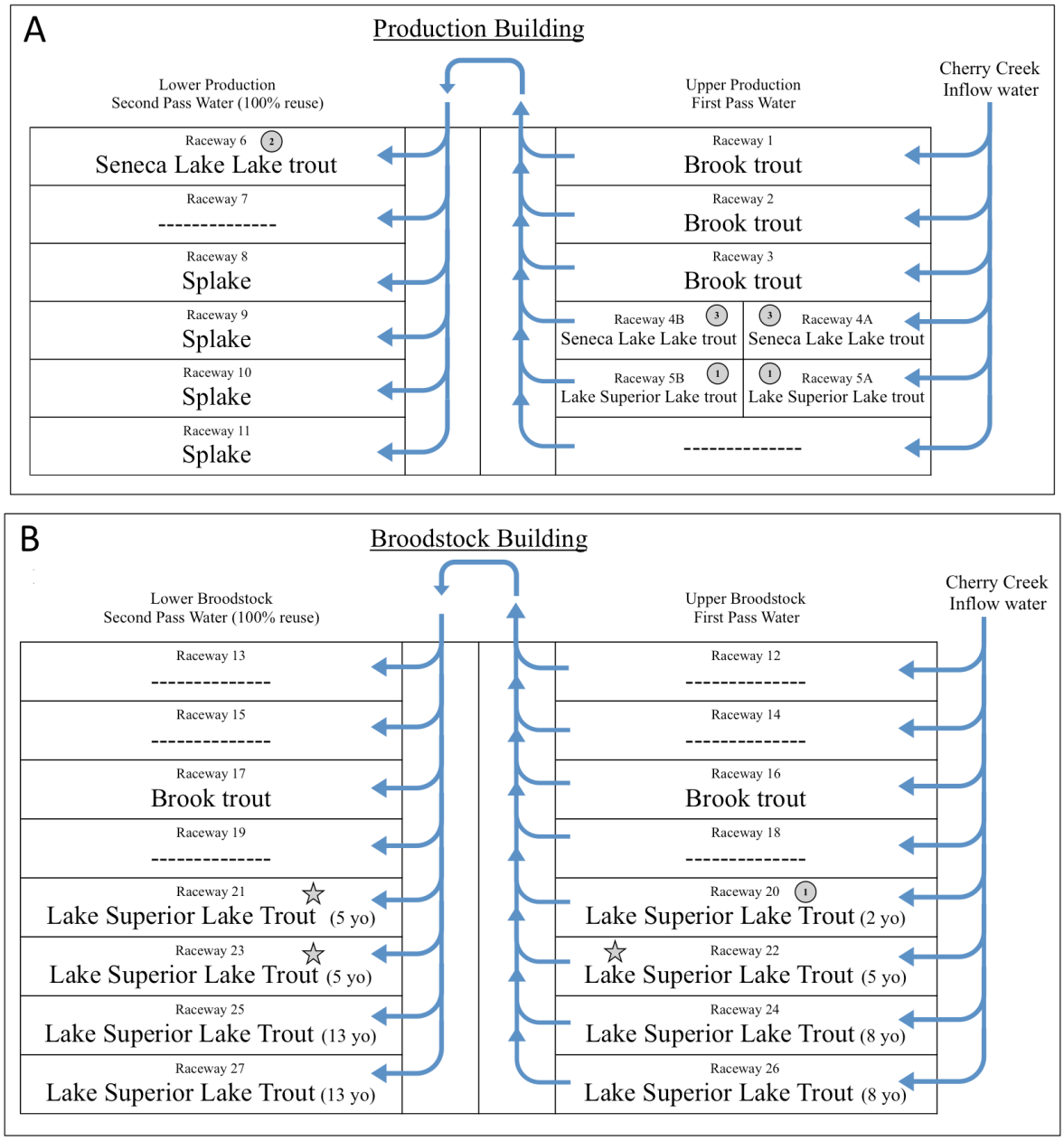

Figure 2.1 Layout depicting water flow within spatially separated broodstock and production raceway buildings at Marquette State Fish Hatchery; (A) production raceways in 2012; (B) broodstock raceways in 2017. Numbered circles represent order of disease progression through production lake trout in 2012 and broodstock in 2017. Stars indicate raceways from which EEDV genomic material was detected with no associated mortalities.

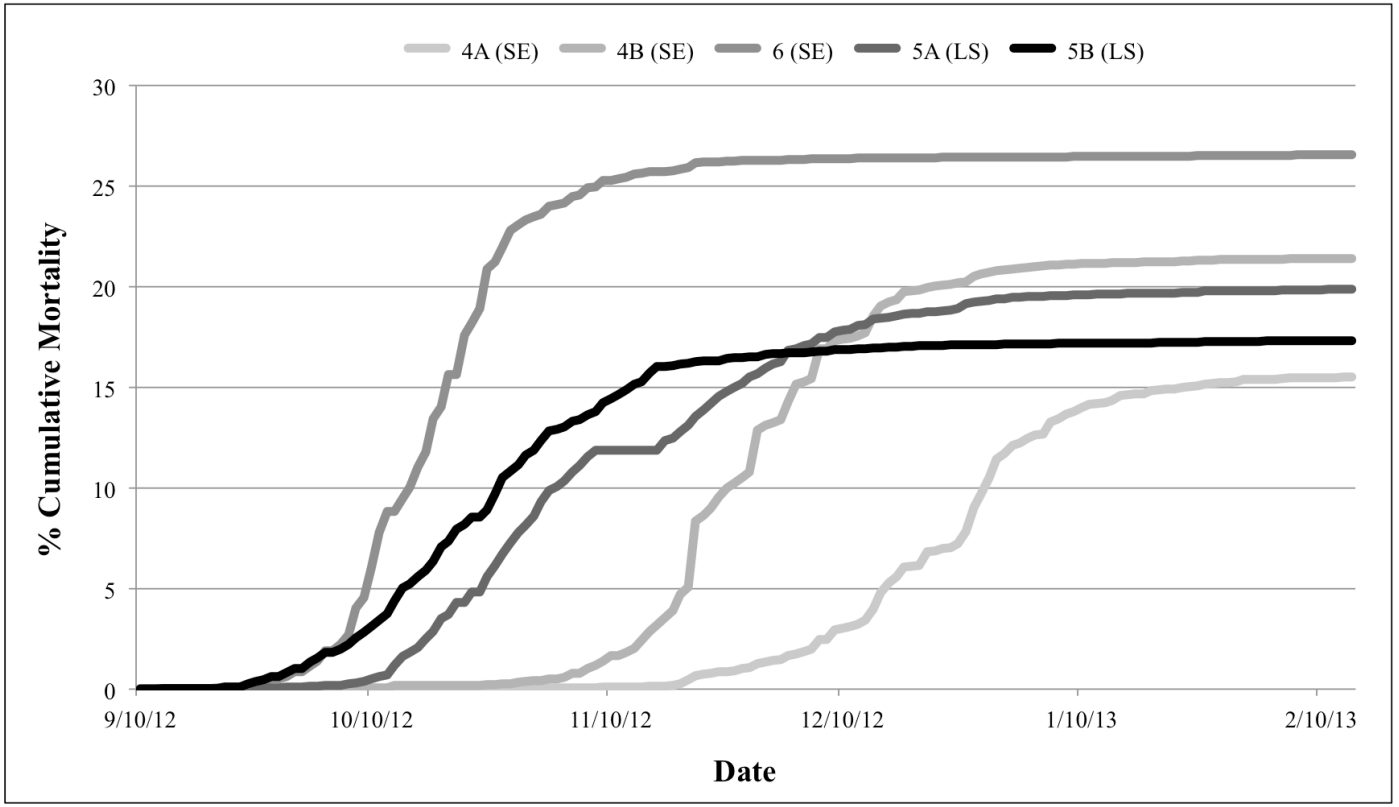

Figure 2.2 Cumulative percent mortalities during the 2012 mortality event at MSFH among juvenile production lake trout, by rearing unit raceway. Prior to mortality event, production fish numbers were approximately as follows: (4A) 125,105 fish; (4B) 124,782 fish; (5A) 75,031 fish; (5B) 76,085 fish; and (6) 56,167 fish.

Year Tested	Species (Strain)	Age	Tissue Tested	EEDV-positive
2007	Lake trout (LS)	Adult	KSp	0/11
2011	Lake trout (LS)	Juvenile	KSpH	0/6
	Lake trout (SE)	Juvenile	KSpH	0/20
	Brook trout	Adult	M	0/60
	Brook trout	Juvenile	KSpG	0/480
2012	Lake trout (LS)	Adult	KSpH, M	0/270
	Lake trout (LS)*	Juvenile	KSp, G, Sk	**8/8**
	Lake trout (LS)	Juvenile	KSpH, G	**24/120**
	Lake trout (SE)*	Juvenile	KSp, G, Sk	**8/8**
	Lake trout (SE)	Juvenile	KSpH, G	**21/240**
	Splake	Juvenile	KSpG	0/240
	Lake trout (LS)	Adult	KSpG	0/30
2013	Lake trout (LS)	Juvenile	G, F, KSpG	**2/300**
	Lake trout (SE)	Juvenile	G, F, KSpG	0/240
	Splake	Juvenile	G	0/60
2014	Lake trout (LS)	Juvenile	G	0/60
	Lake trout (SE)	Juvenile	G	0/80
	Lake trout (LS)	Adult	SkG	0/20❖
2016	Lake trout (LS)	Juvenile	G, SkG	0/70§
	Lake trout (SE)	Juvenile	G	0/60
	Lake trout (LS)	Adult	F, E	**8/80**❖
2017	Lake trout (LS)	Juvenile	F	**5/120**❖
	Lake trout (LS)*	Juvenile	Sk, E	**10/10**❖
	Lake trout (SE)	Juvenile	F	0/120❖

Table 2.1 Number of positive fish detected by EEDV screening at Marquette State Fish Hatchery, 2007-2017 (number positive / number tested). Sampling during mortality episodes denoted by a (*). Tissues tested included kidney (K), spleen (Sp), heart (H), mucous (M), gill (G), skin (Sk), fin (F), and eye (E) and were either pooled (i.e., KSpH) or individual (i.e., K, Sp, H). All samples tested using EEDV TaqMan qPCR as described, except where indicated: (❖) tested with EEDV SYBR green qPCR, (§) tested with both qPCR assays.

Year	Species	Tissue	EEDV-positive
2011	Brook trout	Kidney/Spleen/Heart	0/4
	Brown trout	Kidney/Spleen/Heart	0/11
	Mottled sculpin	Kidney/Spleen/Heart	0/12
2012	Brook trout	Kidney/Spleen/Heart	0/14
	Brown trout	Kidney/Spleen/Heart	0/14
	Mottled sculpin	Kidney/Spleen/Heart	0/14
2013	Brook trout	Kidney/Spleen/Heart	0/12
		Gills	0/12
	Brown trout	Kidney/Spleen/Heart	0/12
		Gills	0/12
	Mottled sculpin	Kidney/Spleen/Heart	**2/12**
		Gills	**3/12**
2015	Mottled sculpin	Gills	0/12
2016	Mottled sculpin	Gills	0/12
2017	Mottled sculpin	Fin	0/12❖

Table 2.2 EEDV screening in Cherry Creek, 2011-2017. No sampling was associated with a mortality episode. All testing performed in pools of five fish per pool. All samples tested using EEDV TaqMan qPCR as described, except where indicated: (❖) tested with EEDV SYBR green qPCR.

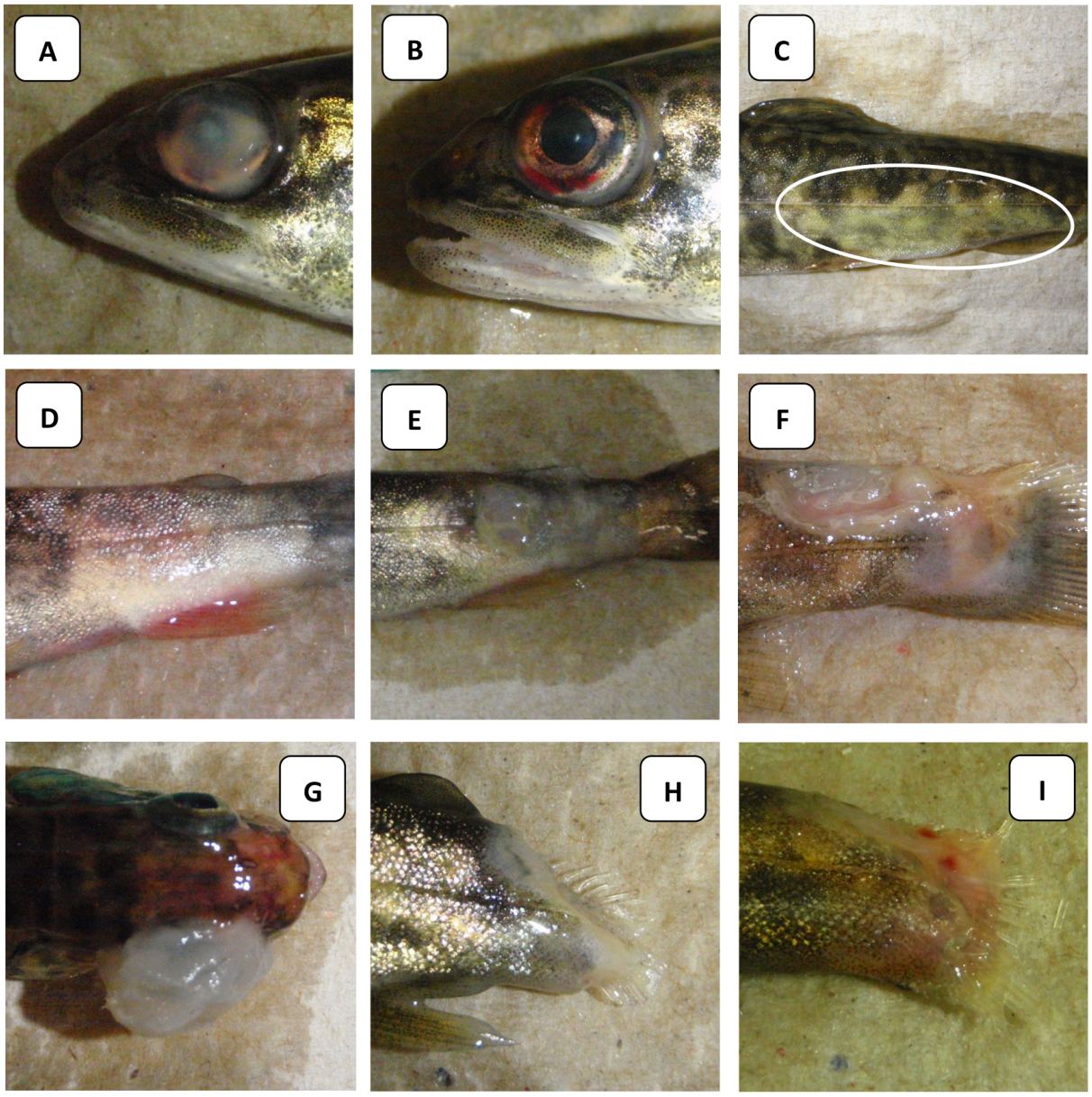

Figure 2.3 Gross clinical signs exhibited by lake trout naturally infected with EEDV in 2012; Lake Superior lake trout (A, D, E, G) and Seneca Lake lake trout (B, C, F, H). (A) advanced stage ocular degeneration with hemorrhage and corneal opacity; (B) ocular hemorrhage; (C) diffuse skin "blotchiness", dermal erosion and excess mucous production; (D) dermal erosion, "blotchiness" and erythema, anal fin congestion; (E) dermal erosion, ulceration of trunk and caudal peduncle; (F) caudal peduncle ulceration, necrosis and dermal erosion; (G) ocular degeneration with substantial water mold overgrowth; (H) caudal fin ulceration with exposed vertebrae; (I) caudal fin ulceration, necrosis, petechial hemorrhage and yellow discoloration, exposed fin rays.

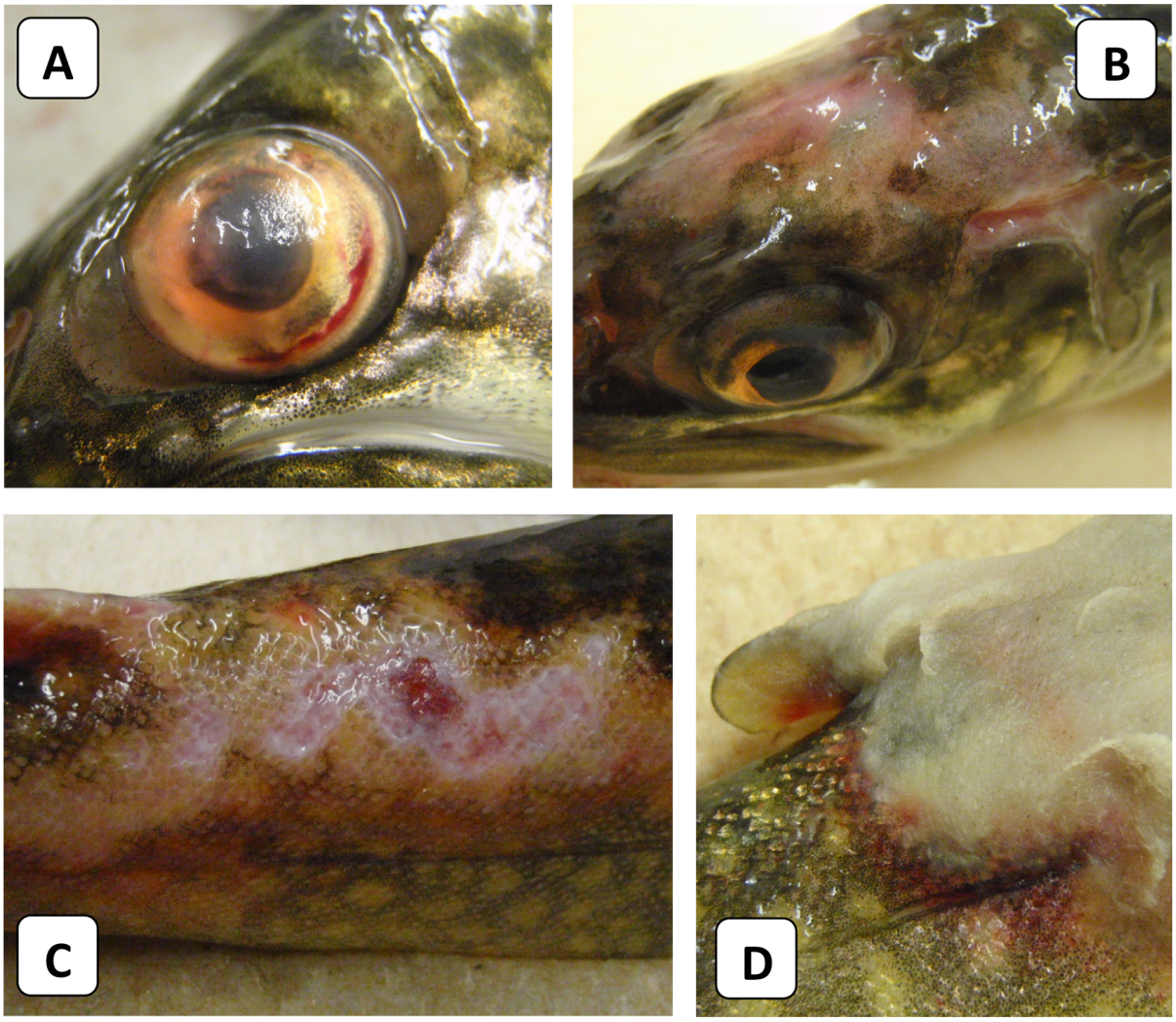

Figure 2.4 Gross clinical signs exhibited by Lake Superior strain lake trout naturally infected with EEDV in 2017. (A) ocular hemorrhage and ulceration; (B) cranial epithelial erosion, ulceration and hemorrhage; (C) skin erosion, ulceration and hemorrhage of trunk and dorsum; (D) skin ulceration with secondary overgrowth and hemorrhagic margins.

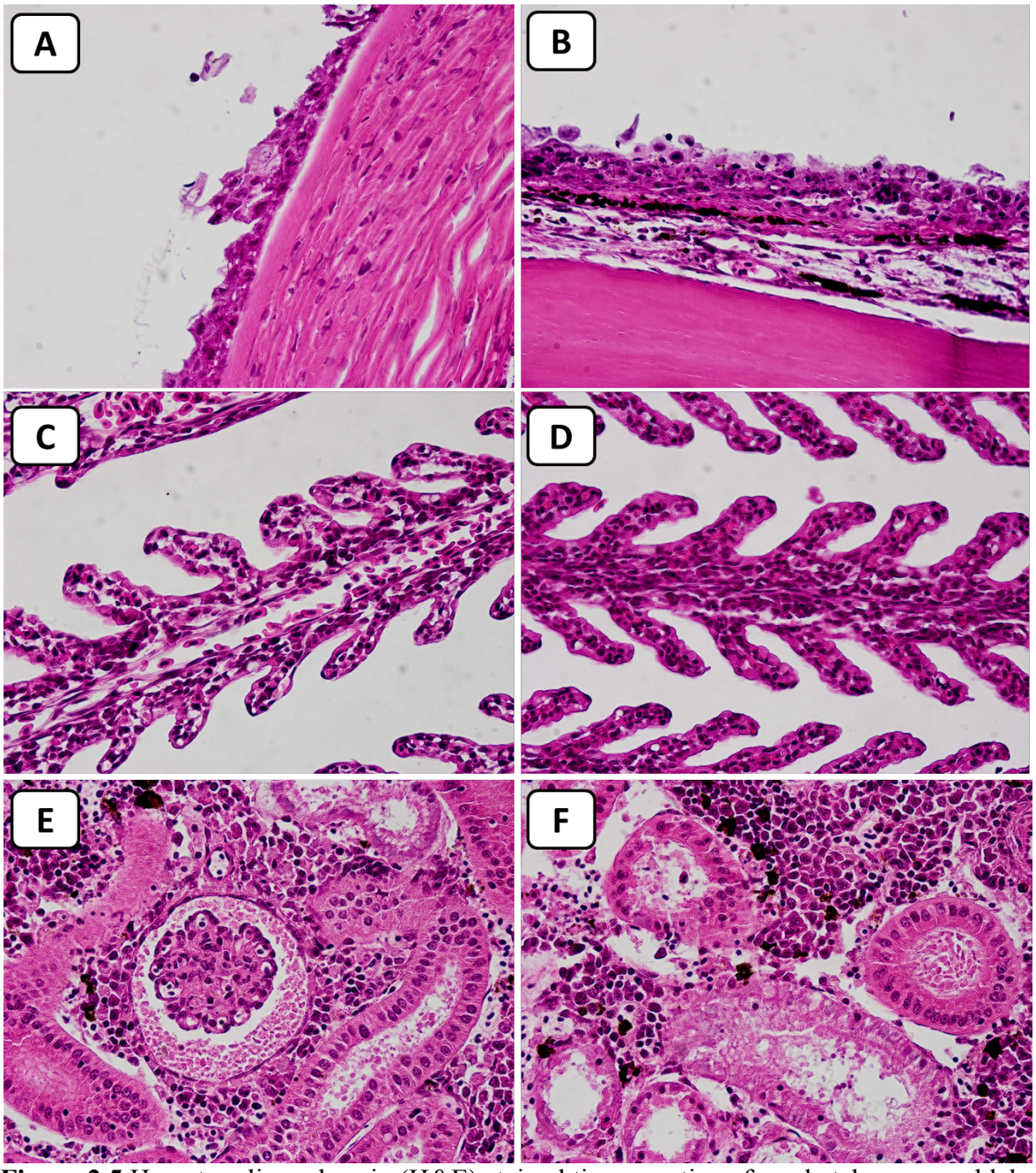

Figure 2.5 Hematoxylin and eosin (H&E) stained tissue sections from hatchery-reared lake trout that were naturally infected with Epizootic Epitheliotropic Disease Virus. A) corneal epithelial ulceration and necrosis (400x magnification); B) epithelial ulceration and necrosis of the skin (400x magnification); C) gill lamellar edema (400x magnification); D) gill epithelial swelling/hypertrophy (400x magnification); E) proteinaceous exudate within both Bowman's space and the renal tubular epithelium (400x magnification); and F) renal tubular epithelial necrosis and multifocal necrosis of the renal interstitium (400x magnification).

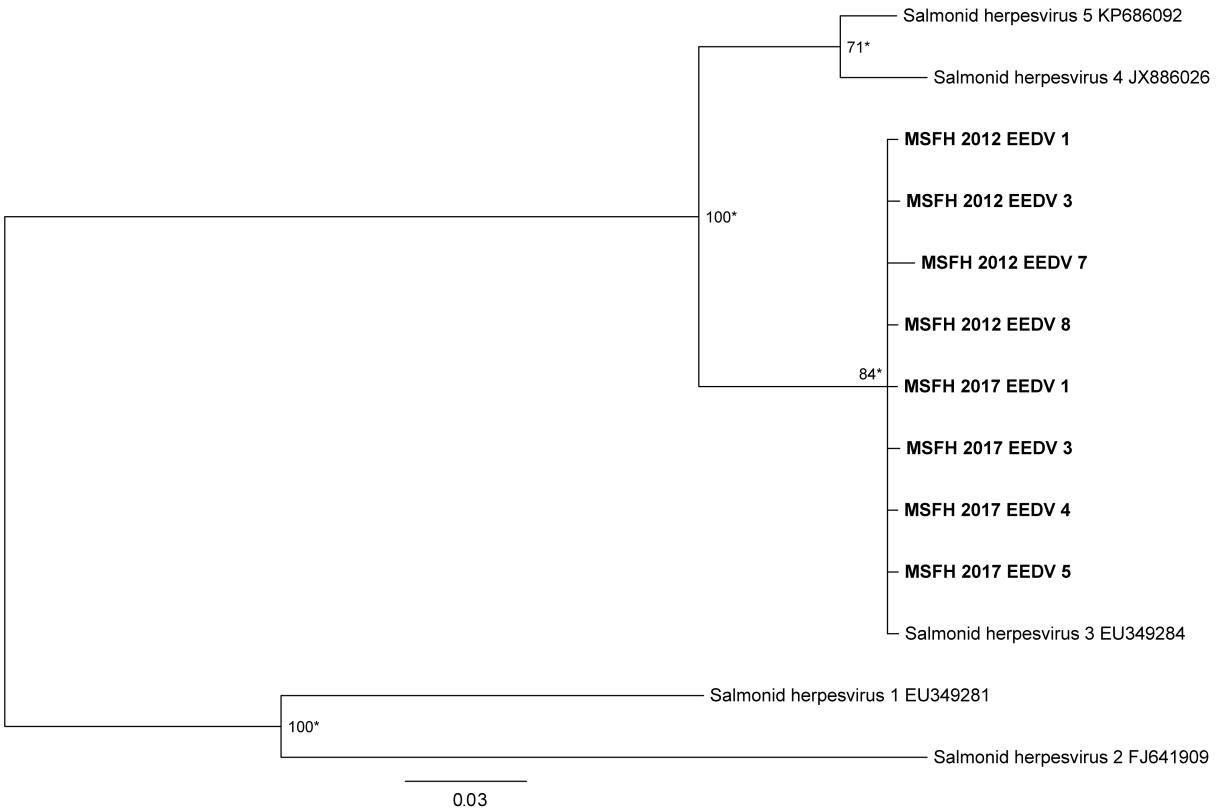

Figure 2.6 Dendrogram depicting the relationships of eight Epizootic Epitheliotropic Disease Virus (EEDV) isolates (denoted in bold) from the 2012 and 2017 hatchery outbreaks with isolates representing the five currently described salmonid herpesviruses. The dendrogram was generated in MRBAYES 3.1.2 (*120*) using the Kimura Two Parameter model with gamma distribution based upon the lowest Bayesian Information Criterion. The Markov chains (n = 4) were run until an average standard deviation of split frequencies of < 0.01 was attained. Two independent analyses were conducted, with the initial 25% of Markov chain Monte Carlo samples being discarded as burnin. Posterior probabilities ≥ 70 are displayed at the nodes, where an * denotes that the same node was supported in Neighbor-Joining analysis (i.e., boot strap value ≥ 70). The final data set contained 303 bp of the terminase gene.

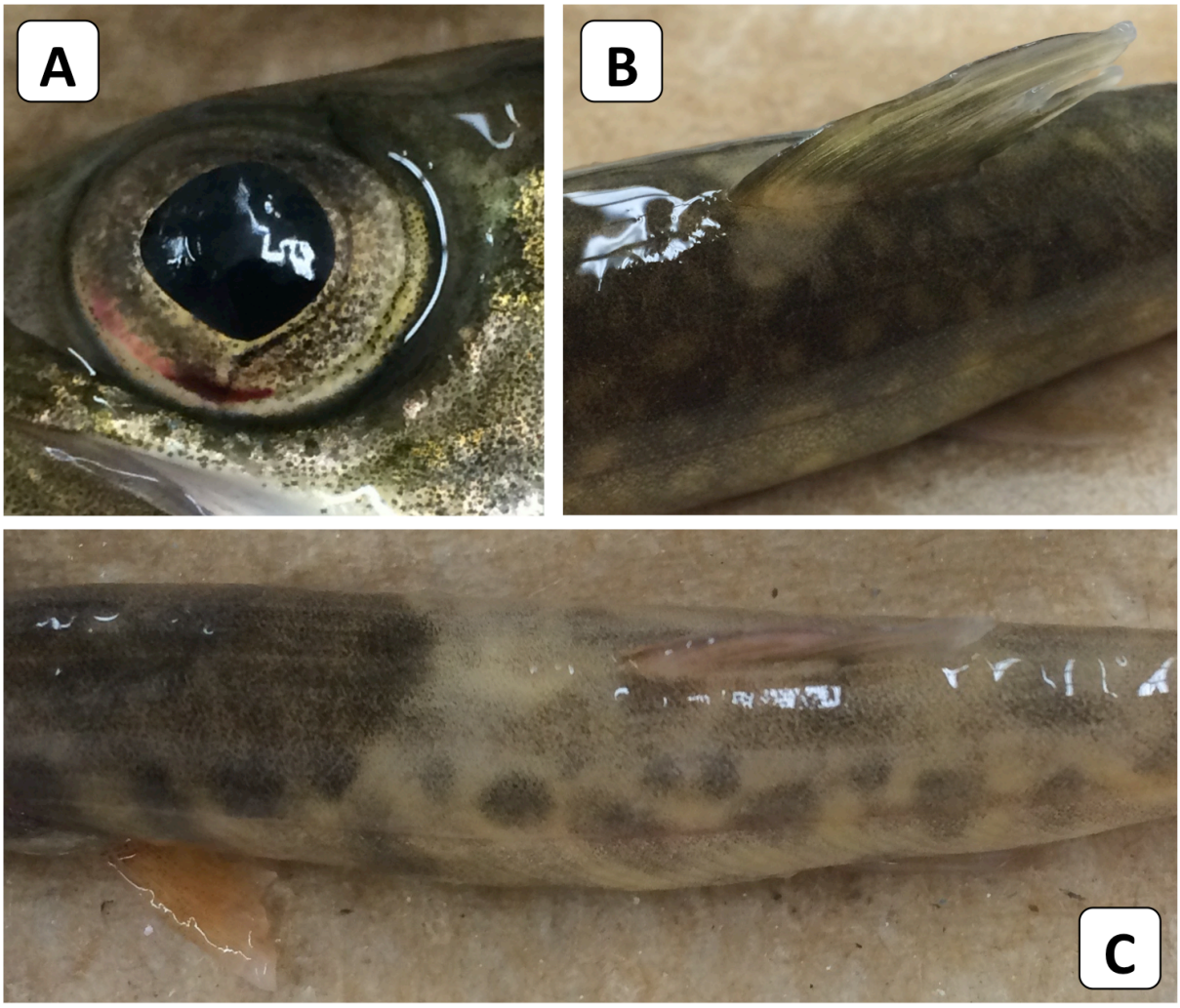

Figure 2.7 Gross pathology associated with experimentally challenged lake trout. (A) ocular hemorrhage; (B) multifocal pallor, skin and fin erosion; (C) generalized pallor with skin and fin erosion.

Chapter 3

Evaluation of the dose-dependent effects following *in vivo* infection with Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3)

1. Abstract

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) is the Alloherpesvirus (Order *Herpesvirales*) of most concern in the Great Lakes basin today, due to its potentially catastrophic effect on lake trout (*Salvelinus namaycush*) rehabilitation efforts. Although it was initially described in the early 1980s, there are many questions remaining unanswered regarding the virus' disease ecology, due in part do a lack of an *in vitro* model to support its replication. As an alternative, we explored the feasibility of using an *in vivo* model with naïve, juvenile lake trout to propagate the virus and study the dose-dependent effects of an EEDV infection. Primordial rounds of experimental challenges utilized both intraperitoneal injections and immersion bath challenges in order to establish consistent viral stocks of clarified skin homogenates as well as to maintain and increase virulence in available viral stocks by avoiding long-term freezing. Mortalities were seen as early as 6 days post viral challenge with cumulative percent mortalities ranging from 66-100% and viral titers in stock batches of EEDV increased approximately 1,000-fold between 1st and 6th passages. After production of an adequate volume of 7th passage EEDV stock, a study was performed in order to examine morbidity and mortality following dose-dependent immersion challenge with EEDV, allowing us to calculate the relative expected doses of EEDV required to produce a range of mortality percentages (e.g., 50% at $4.70x10^4$ versus 90% at $8.83x10^5$). Following identification of a viral dose range capable of inducing morbidity and mortality, immersion bath challenges were performed in triplicate in order to address repeatability of the immersion model. These immersion challenges served to highlight that 10^3 viral copies per mL of water is not a sufficient dose to produce clinical disease, while 10^6 viral copies per mL of water can produce up to 100% mortality. This information will allow researchers to proceed with additional studies aimed at

uncovering more specific aspects of EEDV biology and pathology such as tissue tropism and the effect of stressors on development of clinical disease, furthering our knowledge of this highly destructive virus.

2. Introduction

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3), Family Alloherpesviridae, Order *Herpesvirales*, is of special concern in the Great Lakes basin (GLB) due to its catastrophic potential on the rehabilitation efforts of lake trout (*Salvelinus namaycush*) populations in North America. First reported in the early 1980s, EEDV outbreaks resulted in the loss of greater than 15 million lake trout before seemingly disappearing for almost three decades (*2–4*). While EEDV genetic material has been detected by molecular assay in apparently healthy lake trout (*5*, *14*), it wasn't until the fall of 2012 that infection with this virus once again led to a mass mortality event in a Michigan hatchery (*Chapter 2*). The re-emergence of this virus in Michigan's Upper Peninsula has highlighted the dire need to expand our understanding of the detailed interactions between this pathogenic herpesvirus and its host. Due to the inability to replicate this virus in a cell culture system, investigators have been unable to design and implement research projects to understand the virus' pathogenic mechanisms or to develop effective control strategies. As a result, a need arose to explore the feasibility of using standardized and reproducible *in vivo* models to propagate the virus and determine its effects in a dose dependent manner. Herein, we hypothesized that exposing naïve, juvenile lake trout to clarified EEDV-positive tissue homogenate would result in clinical disease consistent with that seen in the natural epizootics. The development of such a model would be vital to the success of future experimental studies and improving our understanding EEDV disease ecology.

3. Materials and Methods

3.1. Fish and maintenance

Juvenile, Lake Superior strain lake trout (6 months post hatch) were obtained from Marquette State Fish Hatchery (Marquette, Michigan) for use in experimental studies. Due to a history of EEDV at the hatchery, prior to their use in experimental challenges, the originating rearing lot for these fish was determined to be free of EEDV using the real time, quantitative PCR (qPCR) assay as detailed below. Additionally, these fish were determined to be free of reportable diseases outlined by the OIE Aquatic Code (*41*) and the American Fisheries Society – Fish Health Section Blue Book (2016) (*40*) via external and internal clinical examination as well as bacterial and viral screening (n = 60 fish randomly collected).

Upon receipt, experimental fish were held in a 680-liter fiberglass tank with continuous, flow-through, oxygenated well water (12.0 ± 1.0°C) at the Michigan State University – University Research and Containment Facility (East Lansing, Michigan) in accordance with the Institutional Animal Care and Use Committee. Fish were fed *ad lib* with 1.0 mm sinking feed (BioOregon, Westbrook, Maine, USA) and allowed to acclimate to standard laboratory conditions for at least one month prior to use in experimental challenges.

All experimental challenges were performed in 42-liter flow-through tanks receiving chilled, oxygenated well water. Studies were performed at water temperatures ranging from 8-12°C; for experiments performed at lower temperatures, fish were allowed to acclimate to the colder water temperatures for a minimum of 48 hours prior to the start of experimental challenges.

3.2. EEDV in vivo serial passages and stock production

A stock of infectious EEDV was produced from the skin of naturally infected lake trout collected during a natural outbreak. Skin was homogenized in a sterile phosphate buffered saline solution, (pH 7.5 ± 0.5; Sigma-Aldrich, St Louis, Missouri, USA) at a 1:3 (w/v) ratio, and clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C. The initial EEDV stock was then passed through multiple groups of naïve juvenile lake trout via either an intraperitoneal injection or an immersion bath challenge. For the intraperitoneal challenges, fish were anesthetized using tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.1 mg/mL) then injected with 300-400 µL of virus stock solution and allowed to recover from sedation prior to return to flow-through aquaria for the duration of the study. For the immersion bath challenges, fish were transferred to glass aquaria containing pre-determined and combined volumes of virus stock and water. Fish were held in these static aquaria at a constant water temperature with continuous aeration for 1 hour, after which time they were transferred back to their flow-through aquaria for the duration of the study. Following virus exposure, fish were monitored daily for development of morbidity or mortality, where upon death or development of severe clinical disease (e.g., loss of equilibrium, difficulties respiring), the fish were euthanized with MS-222 (0.25 mg/mL), and their skin collected and processed as described above with the substitution of Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen) rather than PBS. Skin homogenates created from multiple individual fish within each passage were pooled together to create new batches of EEDV stock from which a new group of naïve lake trout were

infected in turn. This process continued until an adequate volume of 7th passage stock was produced for use in all future studies. Viral exposure route and dose are presented in Table 3.1.

3.3. Virus detection and quantification

Without the use of a susceptible cell line, identification and quantification of EEDV in challenge fish, passage stocks and experimental tissues was achieved using qPCR as described by Glenney et al. (2016) (*14*). Briefly, DNA extractions were performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA). Approximately 10 mg of tissue was used for each extraction and eluted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All PCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with a total reaction volume of 20 µL. Each reaction contained 10 µL SYBR Select Master Mix (2x; Life Technologies, Grand Island, New York, USA), 1.0 µM of forward and reverse primers and 50 nmol total DNA template. Positive control standards for quantification were produced using known positive skin samples following the method outlined by Glenney et al. (2016) (*14*). Positive extraction controls consisted of known positive skin samples; MEM was used for the negative extraction control and nuclease free water was used for a PCR negative control.

3.4. Experimental challenges

3.4.1. Dose range determination

Following the challenges outlined in Table 3.1, our first experiment extrapolated on preliminary results in order to examine the dose range or viral load required in order to lead to infection and clinical EED. This study included a total of 60 fish and six treatment groups (5 viral doses and 1 negative control group). Viral doses and the negative control dose were prepared as described above using 7^{th} pass EEDV stock. The highest infected group was exposed to $9.5x10^7$ viral copies/mL water with subsequent doses being 1:10 dilutions made using sterile sample diluent (MEM). Exact viral doses were determined using qPCR to test water samples and calculate viral copies per mL of water for each dose (Table 3.2).

Immersion exposure was achieved by transferring experimental fish into static, aerated glass aquaria where the infectious or control dose had been added. Fish were monitored for 1 hour during which time the immersion water was held at a constant temperature consistent with the flow-through experimental tanks (9 ± 0.5°C) by submerging the experimental aquaria in larger flow-through vessels. After one hour, fish were transferred back to their flow-through aquaria and monitored daily for a period of 30 days for mortalities or development of clinical morbidity. Any moribund fish displaying severe clinical signs such as altered behavior, inability to maintain balance, gasping for air or significantly pale gills was euthanized. Skin and gill tissues were collected from each fish immediately following death. These tissues were tested for the presence of EEDV using qPCR as described above and viral loads in water samples were used to calculate projected viral dose ranges and mortality percentage pairings based on the calculations of Reed and Muench (1938) (*127*) (Table 3.3).

3.4.2. Repeatability

Following establishment of a minimum viral dose required for development of clinical disease, we wished to determine whether these morbidity and mortality rates could be reproduced across multiple infections groups within the target dose range. This study involved a total of 95 naïve juvenile lake trout and four treatment groups (high and low doses of EEDV via immersion, negative control sample diluent via immersion, and positive control virus intraperitoneal injection). For the positive control, five fish were intraperitoneally injected with 300 µL of 7^{th} pass stock using sedation and recovery as described above. The remaining fish were divided into three treatment groups (i.e., high dose, low dose and negative control) and the experiment run in triplicate (10 fish per group) (Table 3.4). Viral doses were created using 7^{th} pass EEDV stock ($9.5x10^6$ copies/µL). The high dose fish were exposed to $9.5x10^6$ viral copies/mL water while the low dose fish were exposed to $9.5x10^3$ viral copies/mL water and the negative control fish were exposed to sample diluent (MEM) containing no virus.

4. Results

4.1. EEDV in vivo serial passages

Consecutive rounds of intraperitoneal and immersion challenge of naïve juvenile lake trout with clarified tissue homogenate from previously EEDV-infected fish consistently produced morbidity and mortality, from which EEDV genetic material was recovered (Table 3.1). Additionally, clinical signs were comparable with those seen in natural infections and included multifocal skin erosions (Figure 3.1a), petechial ocular hemorrhage (Figure 3.1b), fin erosion and congestion (Figure 3.1c), pale gills and visceral organs, renal congestion, and hemorrhagic enteritis. Initiation of mortality ranged from 6 to 36 days following viral exposure

with the majority of trials seeing the first mortality 2-3 weeks after viral exposure. A similar range was seen regarding cessation of mortalities with 0-2 fish surviving in most trials after approximately 1 month after initial infection (Table 3.1). Viral titers in EEDV stocks increased approximately 1,000-fold from the initial stock to the 6th pass (Table 3.1).

4.2. Morbidity and mortality response to varying doses of EEDV

Fish were monitored for a period of 35 days following viral exposure to the 10-fold dilutions of EEDV, after which time all survivors were euthanized. During that time, the negative control group had 2 mortalities (no evidence of EEDV infection) while mortalities (out of 10) in dilution groups from low dose to high dose ranged as follows: 3, 1, 0, 8, and 10 (Figure 3.2). Clinical findings were consistent with those seen in previous experimental challenges and detailed above. Using qPCR to test skin samples collected from all fish, EEDV was detected in all 10 of the high dose fish, one of the fish in the first dilution group and none of the remaining study specimens (Table 3.2). Using the calculation method developed by Reed and Muench (1938) (*127*), doses were calculated to theoretically represent a range of projected mortality percentages (Table 3.3, Figure 3.3).

4.3. Immersion model reproducibility

Out of the ten treatment groups (3 immersion high dose, 3 immersion low dose, 3 immersion negative control, 1 intraperitoneal positive control), no EEDV related mortalities were seen in either the low dose or negative control fish. Replicate 1 of the high dose had 9/10 mortalities, replicate 2 had 6/10 mortalities and replicate 3 had no mortalities (Table 3.4). All five positive control fish succumbed and had detectible levels of EEDV in their tissues. No

evidence of an EEDV infection was detected in any of the fish from the negative control or low dose groups nor were there any clinical signs suggestive of an EEDV infection.

On the other hand, disease signs in the high dose fish were overt and included substantial mortalities along with fin erosions, generalized pallor, epidermal erosions, ocular petechial hemorrhage, pale gills, pale and swollen spleen, pale liver, congested kidney, hyperemia of enteric vessels, and a swollen vent. Water mold growth was often noticed over skin erosions of infected fish. Surprisingly, fish in high dose replicate 3 had no clinical signs of disease except one fish with mild splenomegaly and one with mild hemorrhagic enteritis and fin erosions. Skin, gill and a kidney/spleen pool were tested for presence of EEDV from all 95 fish. No EEDV was detected in any of the negative control or low dose tissues. The three high dose replicates had 10/10 fish positive, 9/10 fish positive and 1/10 fish positive for EEDV (Table 3.4).

5. Discussion

Recent mortality events have highlighted the continued presence of EEDV within the Great Lakes basin and more importantly, within the hatchery system (*Chapter 2*), representing a dangerous threat to recently established populations and ongoing rehabilitation efforts for one of the region's most economically prized and ecologically vital native fish species, the lake trout. With the vast remaining knowledge to uncover regarding this deadly virus and a current lack of a susceptible cell line, it is of great importance to develop a repeatable *in vivo* challenge model that as closely as possible mimics a natural EEDV infection. Such a model is important both in producing a uniform, infectious EEDV stock for future studies and also in order to more thoroughly examine the biologic and pathologic properties of this virus. Without an *in vitro* method of viral replication available, serial *in vivo* passage is the only way to ensure continuous

infectious viral stocks are available for future studies. What we have proven here is that while direct intraperitoneal injection is capable of producing clinical disease, so too is immersion challenge, supporting our hypothesis. With the close association of fish and their water environment, plus an epitheliotropic virus such as EEDV, an immersion model is not only more accurate in terms of mimicking a natural infection, but also has the potential to produce more severe clinical disease.

Challenges outlined in Table 3.1 served multiple purposes: to maintain virus infectivity as some herpesviruses are known to lose their virulence following extensive frozen storage (*128*), to serve as a guide for future experimental challenge dose range choices, to increase viral titers in available stocks, and to expand our knowledge about the disease course and associated clinical signs following exposure to EEDV. With these trials we were able to successfully maintain an active and virulent stock of EEDV over the span of 12 months, increase the relative viral loads within each stock batch, and reproduce clinical EED consistent with natural outbreaks in a controlled laboratory environment.

Based on the results of the dose-dependent challenges, for future *in vivo* experiments with EEDV, in order to produce clinical morbidity and mortality in juvenile lake trout, we would recommend an immersion challenge model as follows: virus dose at or above the $4.7x10^4$ copies/mL water, exposure of at least 1 hour, and constant maintenance during and after viral exposure of water temperature at 9 ± 1°C. The use of these conditions with EEDV has demonstrated the ability to cause juvenile LS strain lake trout to develop clinical disease. Finally, we would recommend a monitoring period of at least 60 days rather than the 30 used here, as this virus appears to be rather slow growing and may take additional time to develop an active infection.

In order to test our own theories, additional immersion challenges were implemented using juvenile lake trout exposed to EEDV doses either above or below the proposed minimum level. As evidenced herein, a dose of 10^3 viral copies per mL of water is not capable of producing infection and clinical disease while 10^6 is, although in only 2/3 trials. While high dose fish were exposed to $9.5x10^6$ copies per mL immersion water, calculated copies per mg tissue ranged from $4.1x10^3$ to $1.3x10^9$. With an average total body weight per fish of 6,500 mg, this suggests an overall increase in viral load and lends support to viral replication within the high dose exposed fish.

The experimental infections presented herein resulted in a few inconsistencies in cumulative mortalities as well as disease development and virus identification. For example, while two of the high dose replicates had >50% mortalities and ≥90% viral recovery, EEDV was detected from only a single fish in the third replicate and there were no mortalities. This demonstrates the challenging nature of working with a slowly propagating virus, where multiple factors (e.g., water temperature, fish density, relative health of other fish in the environment) must be precisely accounted for in order for a disease to develop. One explanation for the differences in mortality percentages in the high dose challenge groups could be individual variations in fish susceptibility to the virus, as density, water temperature and all other conditions remained the same among trials. As was demonstrated during the 2012 EEDV mortality event (*Chapter 2*), there appears to be intra-species variation in susceptibility to this virus. In 2012, mortalities originated in the individual raceways over a span of approximately eight weeks. Combined with the results of our immersion replicates herein, it is reasonable to surmise that in some trials, EEDV will take longer to induce morbidity than initially accounted for, and if the present studies had been extended for a period of 60 days rather than 30, more consistent

cumulative mortalities may have been observed. The inter-replicate variation in day to first death could be explained by individual fish variation in susceptibility as stated above.

At relatively low fish densities (i.e., low stress), it may be that most fish are able to fight off the EEDV virus at the viral doses used herein, however if a single fish fails to do so, and begins not only showing clinical signs but also shedding additional active virus, it may be enough to lead to more fish becoming infected in a snowballing effect. This is particularly apparent when comparing the relatively low density of natural populations of salmonids to the higher densities in aquaculture facilities where fish are more likely to pass pathogens among themselves. One aspect of experimental challenges that we have altered for later studies is adjusting the fish density in challenge tanks following exposure to the virus to more closely mimic that seen in a hatchery setting. It is well known that high density leads to increased stress and that high stress levels lead to decreased immune function (*129*, *130*). Hatchery settings are typically very dense, particularly with juvenile, production-aged fish (*129*), making hatcheries an ideal environment for infectious diseases that can be shed and subsequently picked up by fish in close association with one another. In our experiments, it was also apparent that in earlier infection challenges when the water temperature was allowed to reach 11 or 12°C, clinical disease was not observed (as quickly, as severely or at all), indicating the importance of maintaining a constant cold (<10°C) water temperature in order to study this finicky virus.

Using this acquired information we can proceed in further studies examining the disease course, tissue tropism and more specific effects of stressors on development of clinical disease as a result of EEDV infection. Determination of optimal lethal dose ranges will allow for the infection of a large number of fish with a viral dose that allows for deliberate monitoring throughout development of clinical disease and potential recovery from infection. Based on these

studies above, we can predict that this virus has a relatively long incubation period (up to several months in some situations) requiring the ability to implement an extensive study to monitor development of disease. The procedures developed here have opened the doors and will allow for limitless additional studies into the mysteries surrounding this highly destructive virus.

APPENDIX

Virus Stock Passage #	Method of Infection	Dose Received	Day of First Mortality	Day of Last Mortality	Total Mortalities	Virus Titer (per mg tissue)
1	IP	$1.22x10^{7}$	6	29	4/5	$5.39x10^{5}$
1	IP	$1.50x10^{7}$	22	66	10/10	$6.4x10^{4}$
1	IP	$1.50x10^{7}$	36	116	10/10	$2.21x10^{4}$
2	IP	$8.85x10^{7}$	113	134	8/10	$1.07x10^{6}$
2	IP	$1.23x10^{4}$	26	30	4/5	$6.64x10^{4}$
2	Imm	$6.18x10^{4}$	25	45	9/10	$5.49x10^{6}$
3	IP	$7.32x10^{8}$	24	41	10/10	$2.99x10^{5}$
3	IP	$7.32x10^{8}$	11	26	2/3	$5.49x10^{6}$
4	IP	$3.55x10^{6}$	7	13	2/3	$2.66x10^{4}$
5	Imm	$7.09x10^{3}$	19	23	4/5	$2.13x10^{4}$
6	Imm	$1.46x10^{4}$	11	33	9/10	$4.38x10^{7}$

Table 3.1 Mortality data following *in vivo* passage of EEDV stocks. Virus stock passage # represents stock batch used to infect specified group of fish. Method of Infection: (IP) = intraperitoneal injection; (Imm) = Immersion bath. Dose received is presented in viral copies per fish (IP) or viral copies per mL water (Imm). First and last mortalities presented as days post virus exposure. Viral titers calculated per mg skin tissue from infected fish following mortality.

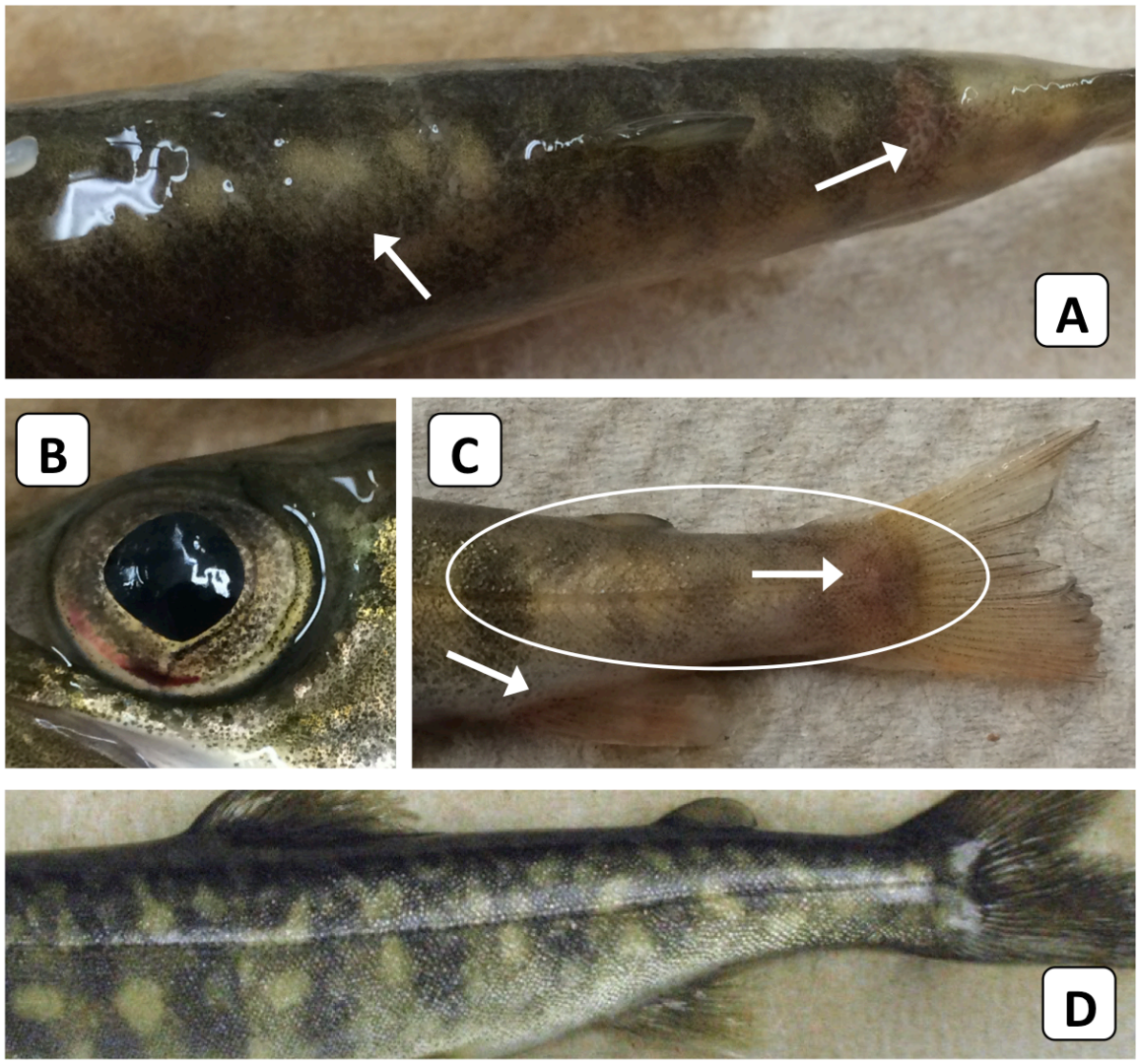

Figure 3.1 Typical gross pathology observed following experimental infection of juvenile lake trout (*Salvelinus namaycush*) with EEDV. A) multifocal skin erosions with erythema and excess mucous accumulation; B) ocular hemorrhage; C) anal and caudal fin erosion with congestion, caudal peduncle erosion with erythema, generalized pallor; D) normal skin.

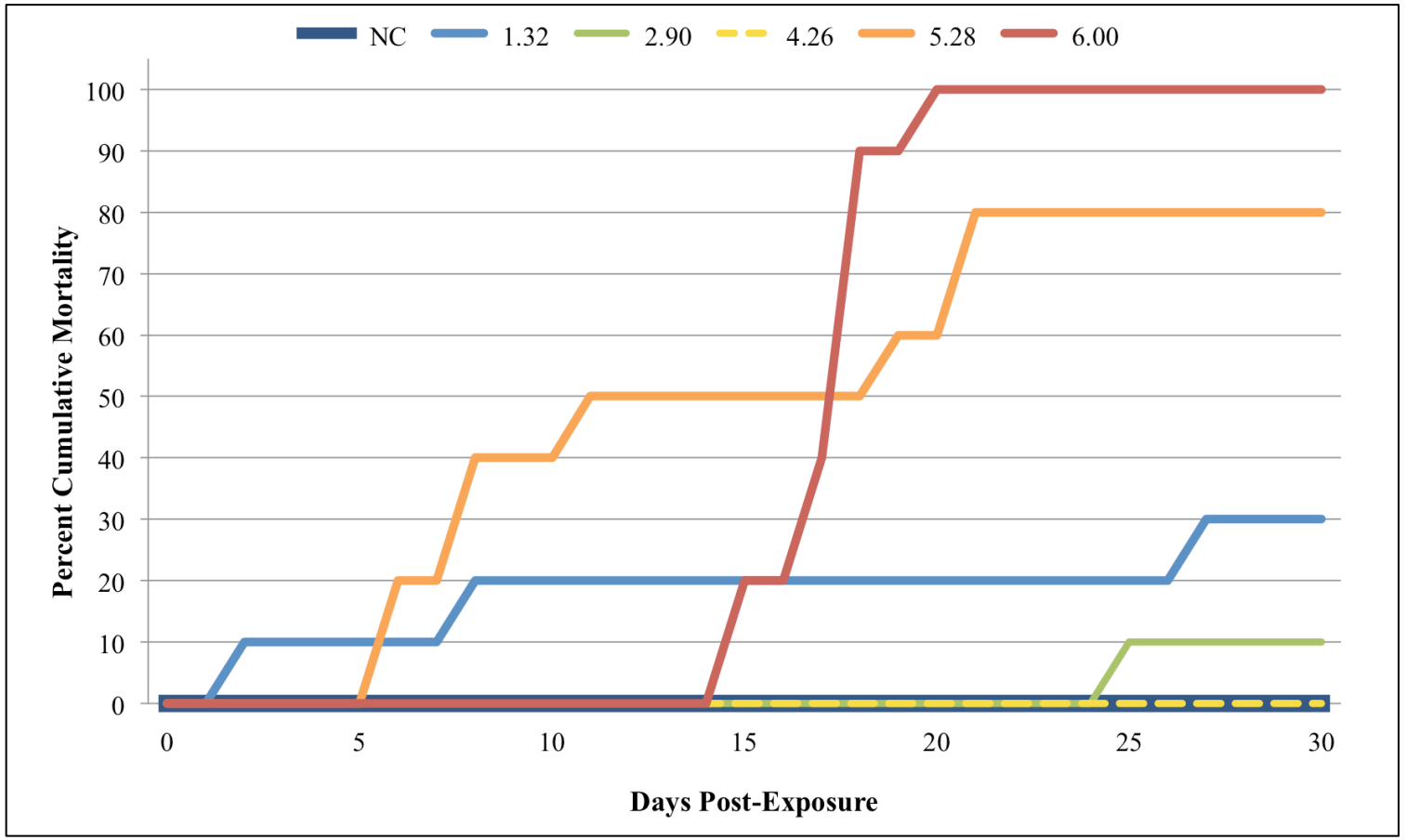

Figure 3.2 Dose-dependent percent cumulative mortality of juvenile lake trout (*Salvelinus namaycush*) following immersion bath exposure to EEDV. Doses presented as a base-10 log of the number of copies per mL immersion bath water.

Viral Dose (copies/ml water)	Cumulative Mortalities	qPCR Positives
0	2/10	0/10
$2.08x10^{1}$	3/10	0/10
$7.95x10^{2}$	1/10	0/10
$1.82x10^{4}$	0/10	0/10
$1.92x10^{5}$	8/10	1/10
$1.01x10^{6}$	10/10	10/10

Table 3.2 Dose-dependent mortalities and qPCR identification of EEDV following experimental exposure of naïve, juvenile lake trout (*Salvelinus namaycush*). Negative control mortalities showed no evidence of EEDV infection.

% Mortality	Viral copies per ml immersion water
10	2.30×10^{1}
25	1.07×10^{3}
50	4.70×10^{4}
75	1.21×10^{5}
90	3.83×10^{5}

Table 3.3 Calculated lethal immersion doses of EEDV based on dose dependent cumulative mortalities and calculated following methods developed by Reed and Muench (1938).

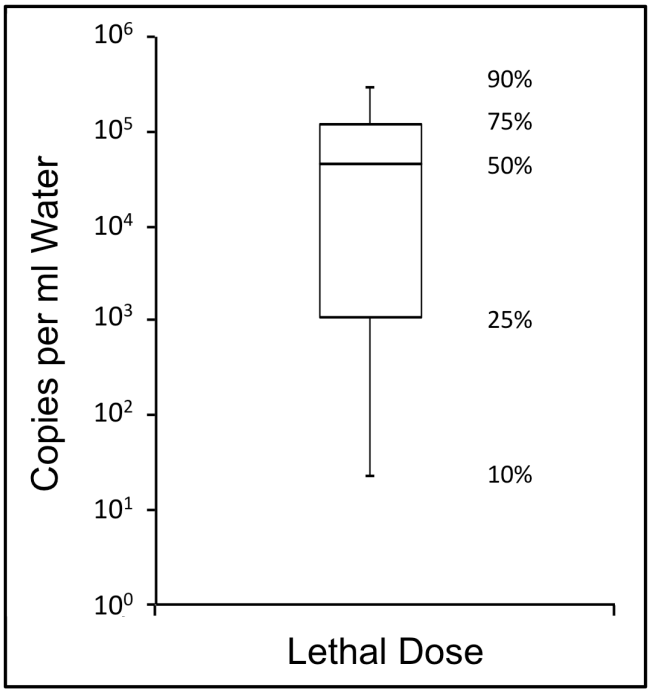

Figure 3.3 Box plot highlighting calculated lethal immersion doses of EEDV based on dose dependent cumulative mortalities and calculated following methods developed by Reed and Muench (1938) (*127*). Lower hash mark represents LD_{10} (i.e., dose required to cause 10% mortality), lower box margin represents LD_{25}, middle box bar represents LD_{50}, upper box bar represents LD_{75}, and upper hash mark represents LD_{90}.

Dose (Replicate)	Mortalities	PCR Positive	Copies/mg (skin)
Negative Control (1)	0/10	0/10	0
Negative Control (2)	2/10	0/10	0
Negative Control (3)	0/10	0/10	0
Low Dose (1)	0/10	0/10	0
Low Dose (2)	0/10	0/10	0
Low Dose (3)	0/10	0/10	0
High Dose (1)	9/10	10/10	$2.4x10^{7}$-$1.2x10^{9}$
High Dose (2)	6/10	9/10	$4.1x10^{3}$-$6.5x10^{7}$
High Dose (3)	0/10	1/10	$2.3x10^{2}$
Positive Control	5/5	5/5	$2.04x10^{5}$-$1.47x10^{8}$

Table 3.4. Mortalities and EEDV qPCR results following immersion challenge for repeatability. All high dose fish were exposed to $9.5x10^{6}$ viral copies per mL immersion water. Positive control fish received $2.85x10^{9}$ viral copies per fish. Viral titers presented as a low-high range of viral copies per mg of skin tissue.

Chapter 4

Progression of Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) in target tissues and cells of its host, the lake trout (*Salvelinus namaycush*)

1. Abstract

Salmonid Herpesvirus-3 (commonly known as the Epizootic Epitheliotropic Disease Virus; EEDV) is an Alloherpesvirus (Order *Herpesvirales*) responsible for the deaths of millions of hatchery-raised lake trout (*Salvelinus namaycush*) in the Laurentian Great Lakes Basin (GLB) over the past three decades. Despite being recognized as a deadly virus for decades, there is little known about the tissue and cellular tropism of EEDV and the associated pathology of the affected tissues. In this study, we immersion-challenged naïve, juvenile lake trout with a predetermined moderately lethal dose of EEDV. Experimentally infected fish were monitored daily for morbidity and mortality and were euthanized on pre-determined days over the course of six weeks. EEDV viral load was determined in individually collected tissues using quantitative real-time PCR (qPCR). Oligoprobes targeting the EEDV glycoprotein were designed for the use in an *in situ* hybridization (ISH) assay to visualize the virus tropism in infected fish and to associate viral infection with tissue pathology. The epidermis of the skin and fins was the first tissues targeted by the virus and yielded higher viral loads compared to other tissues examined, such as gills or kidney. ISH labeling corroborated qPCR findings. During the early stages of disease manifestation, intense labeling for viral nucleic acid was identified in degenerated and necrotic epithelial cells of the epidermis. Subsequent labeling was detected in the epithelial lining of primary and secondary gill lamellae. After initial viral replication in surface epithelium associated with epithelial cell degeneration and necrosis, EEDV next infected endothelial cells and dendritic cells as well as blood monocytes. EEDV infection of endothelial cells and white blood cells was followed by viremia that resulted in disseminated infection of multiple visceral organs including the spleen, heart, omentum, liver, kidney and intestine. Severe lymphoid necrosis and lymphohistiocytic perivasculitis were most commonly associated with detection of

large amounts of viral nucleic acid by *in-situ* hybdridization and qPCR of affected tissues. Our study characterized EEDV tissue tropism and associated pathology for the first time, and sheds light on the pathogenesis of this unusual Alloherpesvirus. Our results will serve to guide future research aimed at understanding EEDV disease ecology, as well as helping to improve strategies for diagnostic sampling and disease control.

2. Introduction

The family *Alloherpesviridae* is comprised of a group of highly pathogenic aquatic viruses that often result in devastating mortality events in populations of their fish hosts, such as is the case with the OIE-reportable Koi Herpes Virus (KHV; Cyprinid Herpesvirus-3) (*16*). While KHV can cause significant morbidity and mortality in common and ornamental carp, Ictalurid Herpesvirus-1 and -2 cause severe disease in catfish (*80*, *131*), and Salmonid Herpesvirus-2 and -3 have caused mass mortalities in trout and salmon (*2*, *87*) (*Chapter 2*). Despite the losses caused by each of these viruses, for long periods of time, little was known about their pathogenesis, a step that is vital in understanding or predicting infection outcome. Recently, however, the development of sensitive and specific molecular and serologic assays has expedited research aimed at determining the tropism and dissemination of a few related viruses within their hosts' bodies.

For example, using quantitative PCR (qPCR), and *in situ* hybridization (ISH) assays, Miwa et al. (2015) (*132*) demonstrated that in experimentally challenged Koi and Common Carp (*Cyprinus carpio*), skin is the major entry point of KHV, followed by the gills within an additional 1-4 days and internal organs after that. ISH positive labeling was particularly intense in the epithelial cells of both skin and gills of these fish (*132*). Similarly, a fluorescence ISH

assay, paired with conventional PCR, was used to identify the gill, kidney and spleen as the target tissues of Cyprinid Herpesvirus-2 (the causative agent of Goldfish Hematopoietic Necrosis Virus) in Prussian carp (*Carassius auratus gibelio*) (*108*). These molecular advances have allowed for the elucidation of viral targets of other herpesviruses outside the Alloherpesvirus family, including the localization of Ostreid Herpesvirus-2 (Family *Malacoherpesviridae*) DNA, RNA and viral proteins in the gills, mantle, heart, adductor muscle, and labial palps of the host by 28 hours post viral exposure (*133*).

What is highlighted by this subset of studies, is that herpesviruses target a wide range of host tissues and cells and while specific interactions between virus and host have been identified for some of these pathogens, many more require further scrutiny to pinpoint the pathogenesis and targets at a cellular level.

Of particular concern to fishery managers in the Midwestern United States is the Alloherpesvirus Salmonid Herpesvirus-3, (Epizootic Epitheliotropic Disease Virus; EEDV), which targets lake trout (*Salvelinus namaycush*) (*2–4*). A highly prized, indigenous species in the Laurentian Great Lakes Basin (GLB), the lake trout is of high economic and recreational importance in addition to being a key apex predator in many ecosystems (*6*). Since its primordial emergence more than 30 years ago, this virus has appeared and disappeared among various state and federal fish hatcheries, seemingly without warning, and often resulting in the death or destruction of millions of fish (*2*, *3*, *5*, *6*) (*Chapter 2*). To date, EEDV has not been successfully replicated *in vitro*, making the study of the pathogenesis of this viral disease especially difficult. With the recent development of an *in vivo* model for replication and propagation of EEDV in a controlled laboratory environment, studies aimed at uncovering the target cells of viral infection and viral spread within the host species can be implemented. Herein, we identify and track

EEDV viral targets throughout a course of infection using quantification of viral load by real time quantitative PCR (qPCR), and visualization of viral DNA by *in situ* hybridization (ISH) conjointly. Specifically we hypothesized that EEDV would first and primarily target external tissues such as the skin and gills.

3. Materials and Methods

3.1. Fish and maintenance

Juvenile, Lake Superior strain lake trout (6 months post-hatch) collected from Marquette State Fish Hatchery (Marquette, Michigan) were used for experimental infections with EEDV. The lot from which these fish was obtained was determined to be free of pathogens of interest at a 95% confidence level based on recommendations by the American Fisheries Society Fish Health Section blue book (*40*) and the Model program for fish health management in the Great Lakes (*39*). Additionally, the presence of EEDV was excluded from these fish with the use of qPCR as described below.

All experiments were performed at the Michigan State University –Research Containment Facility (East Lansing, Michigan) in accordance with the Institutional Animal Care and Use Committee. Fish were allowed to acclimate to laboratory conditions for a minimum of one month prior to the start of experimental challenges while being held in a 680-liter fiberglass aquarium with continuous, oxygenated well water (12.0 ± 1.0°C), and fed *ad lib* with 1.0 mm sinking trout feed (BioOregon, Westbrook, Maine, USA).

All experimental challenges were performed in fiberglass aquaria receiving flow-through, chilled, oxygenated well water. Studies were performed at a water temperature of 9.0 ± 0.5°C,

and fish were allowed to acclimate to colder water temperatures for a minimum of 48 hours prior to the start of experimental challenges.

3.2. Infectious virus stock

As EEDV has not been successfully replicated *in vitro*, a stock of infectious virus for use in experimental challenges was produced from the skin of lake trout collected during a natural outbreak and stored at -80^{o}C. Skin was homogenized in a sterile phosphate buffered saline solution, (pH 7.5±0.5; Sigma-Aldrich, St Louis, Missouri, USA) at a ratio of 1:3 (w/v), and clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C. This supernatant was then used to infect naïve juvenile lake trout via an intraperitoneal injection. Fish were anesthetized using tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.1 mg/mL) then injected with 300 µl of virus stock and allowed to recover from sedation prior to return to flow-through aquaria for the duration of the study. Following virus exposure, fish were monitored daily for development of morbidity or mortality, and upon death or development of severe clinical disease, the fish were collected or euthanized with an overdose of MS-222 (0.25 mg/mL), and their skin sampled and processed as described above to create a new batch of EEDV stock. After the initial stock production, skin samples were homogenized with an Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen) rather than PBS. This process of infection and stock production was repeated with new groups of naïve fish until an adequate volume of 7th passage virus stock was produced for use in the current study (*Chapter 3*).

3.3. Experimental challenge

For this study, 84 lake trout were immersion challenged with the previously determined moderately lethal dose of EEDV (*Chapter 3*) while 48 lake trout were exposed to a sham suspension of MEM as a negative control group. Immersion exposure was achieved by transferring experimental fish to aerated glass aquaria where the infectious or control dose was added. Fish were maintained and monitored for 1 hour during which time the water was held at a constant temperature (9 ± 0.5°C). After one hour, fish were transferred back to their flow-through aquaria and monitored daily for mortalities or development of clinical disease for the duration of the study.

3.4. Sample collection

Seven infected fish and four negative control fish were collected in parallel and euthanized on Days 0, 1, 3, 6, 9, 12, 15, 18, 21, 28, 35, and 42 post-infection (p.i.), focusing on minimizing stress for both sampled and remaining fish throughout the sampling event. On these days, one fish from each group was preserved whole in 10% neutral buffered formalin following creation of a ventral midline incision to allow for improved fixation. External and internal examinations were performed on the remaining 6 infected and 3 control fish at which time individual portions of skin, fin, gill, eye, brain, spleen, heart, liver, intestine, and kidney were collected from each fish. Each tissue was divided, one portion to be frozen at -20°C for quantification of EEDV DNA while the other portion was fixed in 10% neutral buffered formalin for viral DNA visualization. Eyes were collected whole, utilizing both right and left rather than attempting to split, portions of both anterior and posterior kidney were collected for “kidney” samples, and “intestine” tissues consisted of a portion of the intestine approximately 1 cm oral to

the vent. Fixed tissues were processed for paraffin embedding, sectioned and applied to glass slides.

3.5. Quantification of EEDV DNA in tissues

Tissues collected for viral DNA quantification were individually digested and DNA extractions performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) based on the protocol outlined by Glenney et al. (2016) (*14*). After individual digestion, negative control tissues were extracted from in pools of 3, by tissue type. Eluted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All PCR reactions were carried out in a Mastercycler ep *realplex*^{2}S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with a total reaction volume of 20 µL. Each reaction contained 10 µL SYBR Select Master Mix (2x; Life Technologies, Grand Island, New York, USA), 1.0 µM of forward and reverse primers (*14*) and 50 nmol total DNA template. Positive control standards were produced using known positive skin samples following the method outlined in Glenney et al. (2016) (*14*). Viral loads (copies/mg) were then calculated using resulting reaction copy number following qPCR and original digested tissue weights (mg).

3.6. Statistical analysis

Statistical analyses were performed in order to evaluate the relationships between the number of positive samples or the viral DNA load with respect to organ, days post viral exposure

and external vs. internal tissue groups. These comparison analyses were generated using a generalized linear mixed model in SAS software, Version 9.4 of the SAS System (Copyright © 2017 SAS Institute Inc.). For viral loads, analyses were performed on log-transformed copies per mg tissue in order to increase normality of distribution. Statistical significance was determined based on a probability level of 1% or 5% as indicated below.

3.7. Design and preparation of ISH probes

An EEDV specific oligonucleotide probe was designed following a previously described algorithm (*134*), using the computer program Oligo 6 and based on the glycoprotein gene sequence published in GenBank (JX886027.1). This oligonucleotide probe (5'-GCT CAA TTT ATC GTG CTC AAA TGG TTC ACT GGC CAG CTC CAT GTC CAT CG-3') is labeled with digoxigenin at the 5' end (IDT). This specific probe was developed to differentiate EEDV from the other four salmonid herpesviruses, and use of the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast.cgi) demonstrated no cross-reactivity with salmonid herpesvirus-1, -2, -4, or -5. The probe was purified by high performance liquid chromatography (HPLC) (IDT).

3.8. Performance of ISH on fixed tissue sections

In order to maximize the sensitivity and specificity of this ISH assay, preliminary tests were performed in order to identify the optimal protocol and reagent concentrations as previously described (*135*). Briefly, 5 μm thick sections were cut from paraffin-embedded tissues previously collected and placed onto positively charged slides, which were then deparaffinized and fixed using the Discovery XT automated slide-processing system (Ventana Medical Systems, Inc., Tucson, Arizona) as programed in the protocol for the RiboMap *in situ*

hybridization reagent system (Ventana Medical Systems). Protease 3 (0.02 units/mL alkaline protease; Ventana Medical Systems) was used for 12 minutes at 37°C for a proteolytic treatment followed by a mild cell conditioning step using the citrate buffer-based RiboCC reagent (Ventana Medical Systems) for 4 minutes at 95°C. The slides were then denatured for 4 minutes at 37°C, followed by hybridization for 1 hour at 37°C with the antisense oligonucleotide probe for EEDV suspended in hybridization buffer (RiboHybe; Ventana Medical Systems). The concentration used for the EEDV probe was 1.59 ng/mL (1:10,000 dilution). Four stringency washing steps were performed at 42°C using 0.1× RiboWash (equivalent to 0.1× saline sodium citrate; Ventana Medical Systems) for 4 minutes for the first three and for 8 minutes for the fourth washing step. After the stringency washes, the slides were incubated with a rabbit monoclonal antidigoxigenin antibody (Invitrogen Corporation, Frederick, MD) at a dilution of 1:10,000 for 32 minutes at 37°C. Slides were then incubated in streptavidin-alkaline phosphatase conjugate (UMap anti-Rb AP; Ventana Medical Systems) for 16 minutes at 37°C and the signal was detected automatically using the BlueMap nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate kit (Ventana Medical Systems) for 2 hours at 37°C. The final step involved conterstaining the slides with nuclear fast red-equivalent reagent Red Counterstain II (Ventana Medical Systems) for 4 minutes before adding a coverslip. Skin and gill tissues collected from naïve lake trout raised in a bio-secure containment facility were used as negative controls while experimentally infected lake trout with qPCR confirmed EEDV-positive tissues were used as positive controls.

4. Results

4.1. Clinical disease, morbidity and mortality

Following exposure to either EEDV or sterile medium, all infected and negative control lake trout were monitored daily while maintained at a water temperature of 9 ± 0.5°C for 42 days. Gross disease signs in experimentally challenged fish were consistent with those seen during natural EEDV outbreaks. Clinical signs observed as early as three days p.i. included petechiae to ecchymoses in the lower quadrant of the eyes as well as hyperemia or engorgement of enteric blood vessels. By Day 15 p.i., multifocal to coalescing erosions and ulcerations of the skin were observed along with proximal congestion and distal erosion of all fins. Abnormalities in visceral organs ranged from mild pallor to congestion, and hyperemia of both hepatic and enteric vessels was commonly observed after Day 6. The only mortalities occurred on Day 28 p.i. (n = 4 fish), despite the continuously decreasing fish density within the tank caused by sampling as outlined above. No clinical signs were observed and no mortalities occurred in the negative control group.

4.2. Quantification of EEDV DNA

A quantitative analysis of viral load in tissues of experimentally infected lake trout was performed using a SYBR Green qPCR assay. EEDV DNA was detectible from a single fish on Day 9 post-infection (p.i.) as well as from multiple fish, beginning on Day 18 p.i. and through the end of the study. No EEDV DNA was detected by qPCR in any tissues collected from infected fish on Days 0-6 or 12-15 p.i., as summarized in Table 4.1. No EEDV DNA was detected in any of the tissues sampled from the negative control fish.

Ocular tissue contained high loads of EEDV DNA as early as 9 days post exposure to the virus (Table 4.2). By 18 days p.i., EEDV DNA was also detectible in the fin and skin, followed on Day 21 by the kidney, spleen, liver and brain. EEDV DNA was not identified within heart or intestinal tissues until Day 28, at which time the virus was detected in all 10 tissues from all six fish sampled that day. In comparison to other tissues, eye, skin and fin consistently had the highest EEDV loads, often 100 to 1,000 fold higher than the load in internal organs (Table 4.2).

When examining differences between tissue types across the entire study, eye, skin, fin and gill were EEDV-positive most commonly, however, the only statistically significant pairings were between heart (n = 13) and eye, skin or fin (n = 25 each; $p < 0.05$) (Figure 4.2). However, when amounts of viral DNA were examined, eye, skin and fin each had significantly higher loads than the gills as well as each of the internal organs ($p < 0.01$) (Figure 4.2). Interestingly, while the upper range of EEDV copy numbers detected in gill tissues equaled that in the other external tissues, the median viral load more closely matched that of the internal tissues.

Comparisons of the number of EEDV-positive samples per tissue type, by day post-infection (p.i.) are presented in Figure 4.3. On Day 18 p.i., the number of positive skin and fin tissues (n = 2 each) was statistically significantly different from the number of positive gill, kidney, spleen, heart, liver, intestine, or brain tissues (n = 0 each; $p < 0.01$). On Day 21 p.i., in pairwise comparisons, the number of positive eye, skin, fin, or gill tissues (n = 5 each) was statistically significantly different from the number of positive kidney (n = 3), spleen (n = 2), heart (n = 2), intestine (n = 3), or brain (n = 2) tissues ($p < 0.01$). On Day 35 p.i., in pairwise comparisons, the number of positive eye, skin, fin, or gill tissues (n = 6 each) was statistically significantly higher than the number of positive spleen (n = 3) or heart tissues (n = 4) ($p < 0.01$). Finally, on Day 42 p.i., the number of positive eye, skin, fin, or gill tissues (n = 6 each) was

statistically significantly higher than the number of positive kidney (n = 2), heart (n = 1), liver (n = 4) or intestine (n = 3) tissues.

At the apparent peak of infection, on Day 28 p.i., EEDV DNA was detectible in all 60 tissues collected, with viral loads in external tissues ranging from 10^6 to 10^9 copies per mg host tissue while the viral loads in internal tissues ranged from 10^4 to 10^7 copies per mg host tissue (Table 4.2). As mentioned above, while certain gill tissues contained a viral load equal to those of the eye, skin and fin, as a whole, viral loads in gill tissues were more similar to viral loads of internal tissues as is evidenced in Figure 4.4. When analyzing pairwise comparisons of viral loads, the eye, skin, and fin tissues had statistically significantly higher viral loads than the internal organs on Day 21 (kidney, liver, and brain; $p < 0.05$), Day 28 (kidney, spleen, heart, liver, intestine, and brain; $p < 0.05$), Day 35 (all other tissue types; $p < 0.01$), and Day 42 p.i. (liver, intestine, and brain; $p < 0.05$).

An additional statistical analysis was performed comparing the number of positive samples, as well as the average viral load, by sampling day, from all external tissues combined versus all internal organs combined on Days 21-42 (Figure 4.5). On all four sampling days, the external tissues had a statistically significantly higher viral load compared to internal organs ($p < 0.01$) and except for Day 28 when EEDV was detected in all 60 tissues, the external tissues also harbored the virus in a higher number of tissue samples than the internal organs ($p < 0.01$).

4.3. Design and verification of ISH assay

Initially, skin and gill tissues with high EEDV loads based on qPCR (i.e., ct values < 20) were used as positive controls and compared to tissues from negative control fish in order to standardize the ISH procedure as well as to confirm a lack of non-specific reaction. Using this

standardized procedure, no signal was detected in any negative control tissues, while specific intranuclear labeling was detected in positive tissues.

4.4. Visualization of EEDV-infected tissues and cells

Following quantification of viral loads, tissues were examined for pathologic alterations using an H&E stain, and *in situ* hybridization was performed on tissues with specific lesions. Positive labeling was observed in the skin, gills, and spleen as well as endothelial cells and monocytes of vessels in different organs from fish sampled on Days 28, 35, and 42 post infection. The number of positive cells varied between days with the largest number of positive cells correlating to the most advanced stages of disease and the highest viral load based on qPCR. Positive labeling was not evident in any of the negative control tissues tested.

In skin tissues collected from fish in early stages of disease, positive ISH labeling confirmed the presence of EEDV in the nuclei of degenerating epithelial cells as well as in infiltrating lymphocytes and dendritic cells (Figure 4.6). Early skin lesions (Figure 4.6a and 4.6b) were characterized by individual epithelial cell necrosis and sloughing of degenerated cells, and viral nucleic acid was detected in nuclei of individual necrotic epithelial cells. Advanced cutaneous lesions (Figure 4.6c and 4.6d) had more widespread epithelial cell necrosis that caused focal erosions, and sloughing of degenerate epithelial cells was common. Viral nucleic acid was readily detected in large numbers of nuclei of degenerate and necrotic epithelial cells that commonly slough off. The most severe skin lesions (Figure 4.6e and 4.6f) were characterized by widespread erosions and full thickness necrosis with only a few necrotic and degenerate cells clinging to the basement membrane. Viral nucleic acid was detected in the nuclei of the vast majority of epithelial cells throughout all layers prior to epithelial loss. In the gills, early gill

lesions were characterized by mild thickening of the primary lamellae, infiltration of mononuclear cells into the propria, swelling of epithelial cells, congested blood vessels and few degenerated epithelial cells (Figure 4.7a). Viral nucleic acid was detected in nuclei of morphologically unremarkable epithelial cells (Figure 4.7b). More advanced gill disease (Figure 4.7c-f) was characterized by loss of secondary lamellae and massive infiltration of monomuclear cells (Figure 4.7c and 4.7e). Viral nucleic acid was detected in nuclei of attenuated epithelial cells and nuclei of infiltrating mononuclear cells (Figure 4.7d and 4.7f).

During the later disease stages, lesions in internal organs most likely developed secondary to viremia, as is supported by the sudden detection of large amounts of virus in internal organs by qPCR. Depletion of lymphoid cells and multifocal necrosis in the spleen was the most prominent finding (Figure 4.8a) indicating widespread viral replication in hematopoietic cells at the height of viremia. Viral nucleic acid was detected in nuclei of large numbers of mononuclear cells in the spleen (Figure 4.8b). A severe lymphohistiocytic perivasculitis also developed secondary to viremia and was most severe in the omentum (Figure 4.8c), but was also found in other organs. This perivasculitis was most likely secondary to viral infection of endothelial cells (Figure 4.8d). Viremia was most likely caused by large numbers of monocytes being infected as evidenced by significant nuclear labeling of mononuclear cells (Figure 4.8d) in the vessels of different organs.

5. Discussion

The reemergence of highly pathogenic and deadly Salmonid Herpesvirus-3 in the Laurentian Great Lakes Basin in 2012 as well as 2017 (*Chapter 2*) has highlighted the fact that EEDV remains a significant threat to lake trout populations throughout the GLB and continues to

represent a major hurdle in the species' rehabilitation throughout the Midwestern United States. The present study reports the development of an *in situ* hybridization probe capable of detecting the EEDV glycoprotein gene within both external and internal tissues that have been formalin-fixed and paraffin-embedded. Additionally, this is the first account of the tracing of EEDV within target cells and tissues of lake trout throughout the course of disease. This study utilized both the ISH assay as well as a quantitative SYBR Green PCR assay to identify EEDV target tissues and cells at a wide range of disease progression time points following experimental exposure to the virus.

Previous studies conducted immediately after the initial EEDV mortality episodes, while lacking these novel techniques, were capable of determining infectivity of EEDV and identification of target species (*3*, *4*). In order to expand our knowledge of the pathogenesis of EEDV, juvenile lake trout were exposed to a previously determined dose of the virus capable of causing morbidity via immersion challenge (simulating a natural route of infection) (*Chapter 3*). The first six weeks of infection were investigated by quantifying viral load within specific target tissues as well as visualizing the virus within microscopic lesions.

In the present study, samples were collected at 1-7 day intervals from 10 separate tissues. For the first week of sampling, EEDV levels remained below the detection limit of both the qPCR and the ISH assays. While first detectible in ocular tissues on Day 9, it wasn't until Day 18 when the virus became consistently detectible from multiple tissues and multiple fish. However, until Day 21, the virus was only identified in external tissues, and throughout the study, the external tissues had consistently higher viral loads, particularly the eyes, skin, and fins. This apparent delayed or prolonged spread of virus to visceral organs (e.g., kidney, spleen, and liver) after initial detection within external tissues is evidence of EEDV first targeting and establishing

an infection in the epithelium, followed after a few days by viremia that leads to development of systemic disease. This pattern of an initial infection site in an external tissue followed by systemic spread has also been observed in the herpesviruses of cyprinids, catfish, and eels (*136*).

While occasionally viral loads in the gills were comparable to those in the other external tissues, the median viral load in the gills was more comparable to those of the internal organs across all time points (Figure 4.1), suggesting the gills are a less preferred viral target and thus a less ideal diagnostic target than the skin, fins or eyes. This is in opposition to trends seen with Ictalurid Herpesvirus-1, Cyprinid Herpesvirus-2, and Anguillid Herpesvirus-1 in their hosts where the gills are preferentially and persistently infected (*54*, *57*, *137*).

Of particular note is the consistently lower viral load in the kidney, spleen, and heart, tissues that are commonly used when attempting to diagnose unknown pathogenic aquatic viruses or suspected viruses such as Viral Hemorrhagic Septicemia Virus or Infectious Pancreatic Necrosis Virus (*40*). Surprisingly, while the viral titers in brain tissue were comparable to those of other internal organs, the virus was detectible in brain tissue on the same day as all tissues other than eye, skin and fin, despite the presence of a blood-brain barrier. We speculate that infected monocytes may be responsible for carrying the virus across the blood brain barrier functioning as a Trojan horse as has been reported for other viral diseases. While the data herein suggests a sequential spread of EEDV from external to internal tissues, additional sampling efforts focusing at and around the time of first detection through development of systemic disease should be carried out to further characterize this process.

Visualization of EEDV DNA using *in situ* hybridization within epithelial cells of both the skin and gills, associated with degeneration and sloughing, indicates these cells are likely the primary targets of viral infection, after which, cell to cell transmission allows EEDV to infect

infiltrating dendritic cells. This infection of mononuclear cells along with the endothelial cells lining blood vessels both in the gills and in visceral organs indicates a source of viremia and widespread infection of internal organs in later stages of disease. In particular hematopoietic cells seem to be the primary target of viral infection at advanced disease stages. These findings correlate with the qPCR data in support of development of systemic disease beyond 21 days post infection.

Identification of viral targets throughout a course of disease is important for many reasons, one of which is diagnostics. As mentioned previously, kidney and spleen are commonly used for diagnosis of pathogenic aquatic viruses. However, as is also the case with some of the other aquatic herpesviruses such as KHV (*132*), these are not appropriate tissues for diagnosis of EEDV. When compared to external tissues (e.g., eye, skin, fin), kidney and spleen carry consistently lower viral loads not detectible as early in the course of infection. While internal tissues collected and tested from a highly infected individual may have detectible levels of EEDV, in order to maximize chances at detecting low-level carriers of the virus, external tissues should be screened instead. This study has vastly improved our understanding of the pathogenesis of EEDV, allowing for the tailoring and focusing of diagnostic efforts.

In conclusion, the data provided herein establishes that EEDV primarily targets epithelial cells, supporting our hypothesis, with later stages developing into a systemic disease through the infection of blood and endothelial cells. Additionally, the newly developed ISH assay has been established as a viable confirmatory tool in support of positive qPCR results or histopathologic lesions. This information can be used to alter screening efforts of GLB lake trout populations as well as to focus future research into the location and establishment of latency as an explanation for the long periods of undetection in this virus' history.

APPENDIX

	Day Post Viral Exposure								
Tissue	0−6	9	12	15	18	21	28	35	42
Brain	------	------	------	------	------	++----	++++++	-+++++	++++-+
Eye	------	---+--	------	------	--+---	+++++-	++++++	++++++	++++++
Fin	------	------	------	------	++----	++-+++	++++++	++++++	++++++
Gill	------	------	------	------	------	++-+++	++++++	++++++	++++++
Heart	------	------	------	------	------	------	++++++	++-+-+	+-+---
Intestine	------	------	------	------	------	------	++++++	++++++	+-++--
Kidney	------	------	------	------	------	+-+--+	++++++	++++-+	+-+---
Liver	------	------	------	------	------	+++---	++++++	++++++	++++--
Skin	------	------	------	------	----++	++++-+	++++++	++++++	++++++
Spleen	------	------	------	------	------	+-+---	++++++	-++--+	+++++-

Table 4.1 Identification of EEDV DNA in various tissues of experimentally challenged lake trout (*Salvelinus namaycush*) sampled in parallel on pre-determined days (n = 6 fish/day) after exposure to the virus using qPCR. "**+**" indicates identification of EEDV DNA in designated fish and tissue; "−" indicates no detectible EEDV DNA.

Day‡	Fish	Brain	Eye	Fin	Gill	Heart	Intestine	Kidney	Liver	Skin	Spleen
18	1	--	--	$6.59x10^{3}$	--	--	--	--	--	--	--
	2	--	--	$8.56x10^{3}$	--	--	--	--	--	--	--
	3	--	$1.37x10^{3}$	--	--	--	--	--	--	--	--
	4	--	--	--	--	--	--	--	--	--	--
	5	--	--	--	--	--	--	--	--	$2.93x10^{5}$	--
	6	--	--	--	--	--	--	--	--	$3.45x10^{4}$	--
21	1	$4.25x10^{2}$	$2.50x10^{4}$	$2.65x10^{7}$	$3.32x10^{4}$	--	$4.81x10^{4}$	$1.82x10^{4}$	$3.25x10^{5}$	$2.23x10^{7}$	$7.48x10^{4}$
	2	$5.37x10^{4}$	$1.41x10^{6}$	$6.95x10^{7}$	$1.27x10^{5}$	--	$1.89x10^{3}$	--	$4.26x10^{3}$	$1.23x10^{8}$	--
	3	--	$2.69x10^{6}$	--	--	--	$3.94x10^{5}$	$1.83x10^{4}$	$3.43x10^{4}$	$1.54x10^{5}$	$1.51x10^{5}$
	4	--	$2.98x10^{6}$	$5.27x10^{5}$	$1.67x10^{4}$	$1.07x10^{4}$	--	--	--	$1.87x10^{3}$	--
	5	--	$2.32x10^{7}$	$1.29x10^{7}$	$2.80x10^{5}$	--	--	--	--	--	--
	6	--	--	$1.79x10^{4}$	$2.52x10^{5}$	$1.33x10^{5}$	--	$1.30x10^{4}$	$2.46x10^{3}$	$8.07x10^{4}$	--
28	1	$1.44x10^{5}$	$4.11x10^{7}$	$9.27x10^{7}$	$1.01x10^{7}$	$1.39x10^{6}$	$1.33x10^{7}$	$9.26x10^{5}$	$1.57x10^{5}$	$1.29x10^{8}$	$1.02x10^{6}$
	2	$2.70x10^{5}$	$8.09x10^{8}$	$1.82x10^{9}$	$1.65x10^{7}$	$9.99x10^{5}$	$6.88x10^{6}$	$4.67x10^{6}$	$1.68x10^{6}$	$1.64x10^{8}$	$6.68x10^{5}$
	3	$1.98x10^{5}$	$1.14x10^{8}$	$5.70x10^{8}$	$1.29x10^{6}$	$1.46x10^{7}$	$1.78x10^{6}$	$2.83x10^{6}$	$3.45x10^{6}$	$4.96x10^{8}$	$4.18x10^{6}$
	4	$1.43x10^{4}$	$1.62x10^{8}$	$4.30x10^{8}$	$2.12x10^{6}$	$8.61x10^{7}$	$1.51x10^{5}$	$1.32x10^{5}$	$3.58x10^{4}$	$1.40x10^{8}$	$9.01x10^{4}$
	5	$9.62x10^{4}$	$2.20x10^{6}$	$9.56x10^{7}$	$3.03x10^{6}$	$1.15x10^{5}$	$1.38x10^{5}$	$5.88x10^{5}$	$5.21x10^{5}$	$3.91x10^{8}$	$3.01x10^{6}$
	6	$6.48x10^{4}$	$1.08x10^{8}$	$1.83x10^{9}$	$1.62x10^{7}$	$1.91x10^{6}$	$5.26x10^{6}$	$2.13x10^{6}$	$4.62x10^{6}$	$2.07x10^{9}$	$3.42x10^{7}$
35	1	--	$1.78x10^{8}$	$8.97x10^{8}$	$2.63x10^{6}$	$1.25x10^{6}$	$8.50x10^{5}$	$3.80x10^{5}$	$2.49x10^{5}$	$4.74x10^{8}$	--
	2	$7.97x10^{6}$	$1.72x10^{8}$	$7.58x10^{8}$	$1.67x10^{4}$	$6.08x10^{4}$	$2.22x10^{5}$	$2.30x10^{5}$	$3.86x10^{4}$	$5.57x10^{8}$	$8.85x10^{4}$
	3	$3.20x10^{5}$	$3.82x10^{6}$	$4.06x10^{8}$	$2.91x10^{4}$	--	$2.96x10^{4}$	$1.35x10^{4}$	--	$8.53x10^{7}$	$1.85x10^{4}$
	4	$1.21x10^{5}$	$9.76x10^{7}$	$5.27x10^{6}$	$2.00x10^{4}$	$3.42x10^{3}$	$9.84x10^{4}$	$1.91x10^{4}$	$1.83x10^{4}$	$6.00x10^{8}$	--
	5	$3.43x10^{5}$	$1.01x10^{8}$	$6.45x10^{7}$	$2.57x10^{4}$	--	$1.69x10^{5}$	--	$6.95x10^{4}$	$1.68x10^{9}$	--
	6	$4.74x10^{3}$	$1.81x10^{8}$	$9.33x10^{8}$	$4.22x10^{6}$	$2.11x10^{5}$	$7.91x10^{5}$	$1.10x10^{5}$	$7.18x10^{3}$	$5.97x10^{6}$	$6.32x10^{4}$
42	1	$1.34x10^{6}$	$2.03x10^{8}$	$5.82x10^{8}$	$9.68x10^{7}$	--	$1.70x10^{4}$	$3.30x10^{5}$	$2.36x10^{4}$	$3.13x10^{8}$	$2.09x10^{4}$
	2	$2.36x10^{6}$	$8.61x10^{7}$	$6.53x10^{7}$	$1.95x10^{4}$	--	--	--	$5.20x10^{4}$	$3.91x10^{7}$	$5.06x10^{5}$
	3	$6.29x10^{4}$	$1.75x10^{8}$	$3.54x10^{8}$	$1.92x10^{9}$	$2.39x10^{6}$	$9.26x10^{5}$	$1.22x10^{5}$	$1.05x10^{6}$	$1.75x10^{9}$	$1.56x10^{5}$
	4	$9.90x10^{3}$	$1.49x10^{8}$	$3.45x10^{6}$	$9.50x10^{4}$	--	$5.03x10^{4}$	--	$1.57x10^{5}$	$3.29x10^{7}$	$2.33x10^{6}$
	5	--	$1.19x10^{5}$	$2.93x10^{4}$	$5.74x10^{4}$	--	--	--	--	$4.87x10^{7}$	$1.79x10^{7}$
	6	$2.05x10^{6}$	$4.13x10^{7}$	$3.09x10^{4}$	$1.15x10^{4}$	--	--	--	--	$1.25x10^{7}$	--

Table 4.2 EEDV glycoprotein gene copies per mg tissue by day post-infection following experimental exposure of lake trout to the virus via immersion bath as calculated using SYBR qPCR. Data points marked with a "--" indicate no virus detected. ‡All tissues from all fish tested prior to Day 18 showed no detectible levels of EEDV except the eye of a single fish on Day 9 ($3.81x10^{4}$ copies/mg).

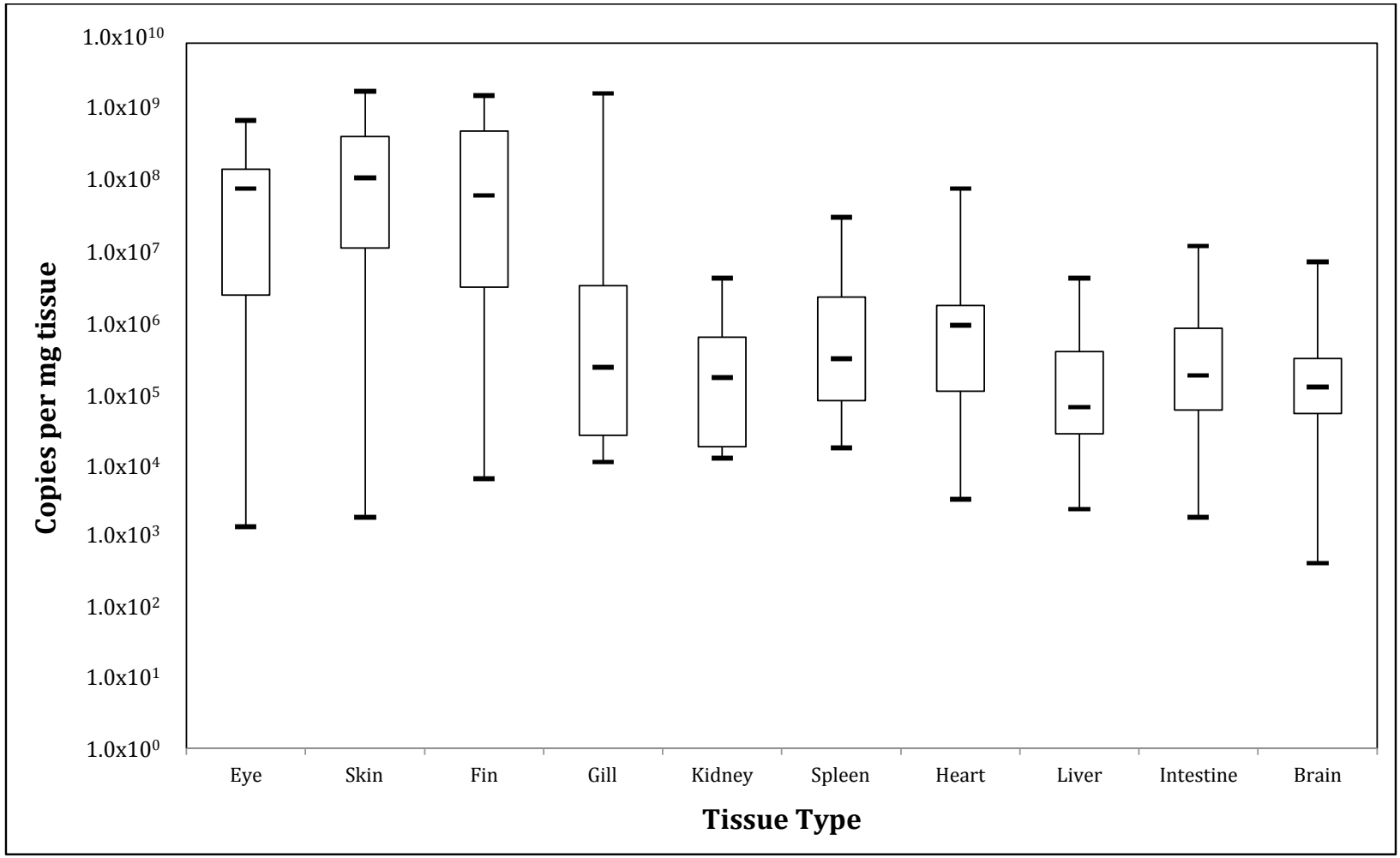

Figure 4.1 Box plots showing minimum, 1st quartile, median, 3rd quartile and maximum viral glycoprotein gene copies per mg of tissue by tissue type across all EEDV-positive samples on all sampling days.

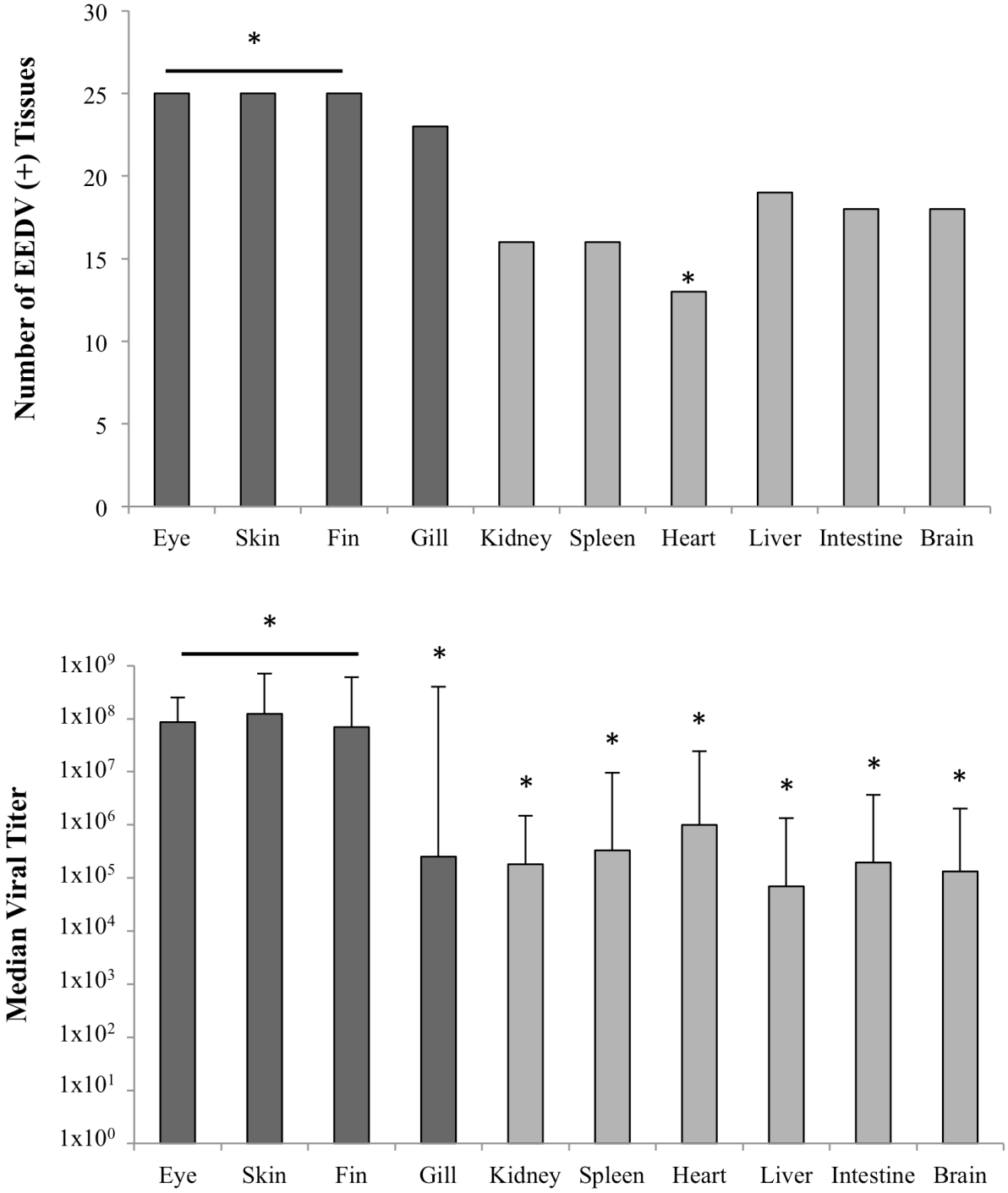

Figure 4.2 Top: Total number of EEDV-positive samples by tissue type across all sampling days. "*" indicates statistical significance compared to tissues below the horizontal bar; ($p < 0.05$). Bottom: Median EEDV viral titer by tissue type across all sampling days. "*" indicates statistical significance compared to tissues below the horizontal bar; ($p < 0.01$). Error bars signify one standard deviation.

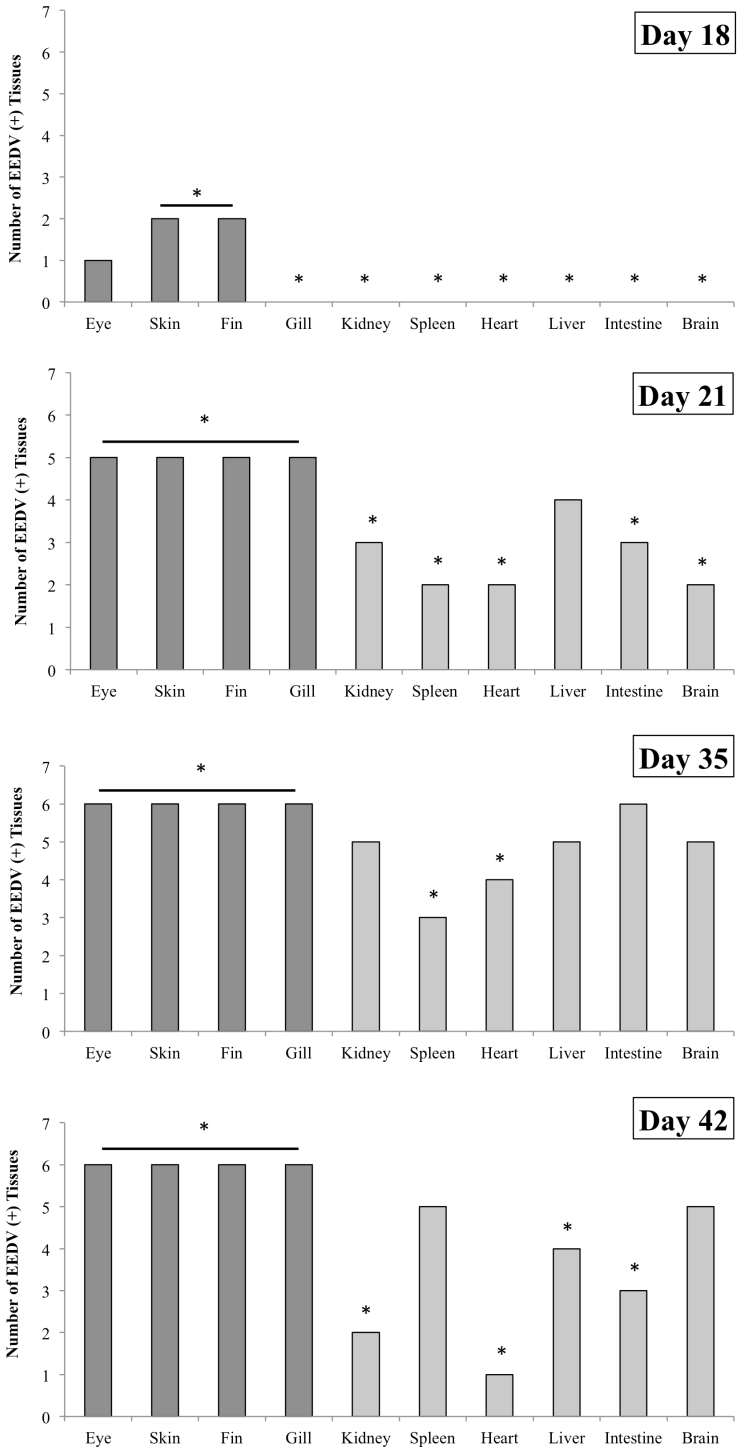

Figure 4.3 Total number of EEDV-positive samples by tissue type and by sampling day. "*" indicates statistical significance compared to tissues below the horizontal bar; ($p < 0.01$). Additional significant pairwise comparisons not pictured include: liver vs. spleen, heart and brain on Day 21; spleen vs. kidney, liver, intestine and brain plus heart vs. intestine on Day 35; and kidney vs. spleen, liver, and brain, plus heart vs. spleen, liver, intestine and brain, plus intestine vs. spleen and brain on Day 42.

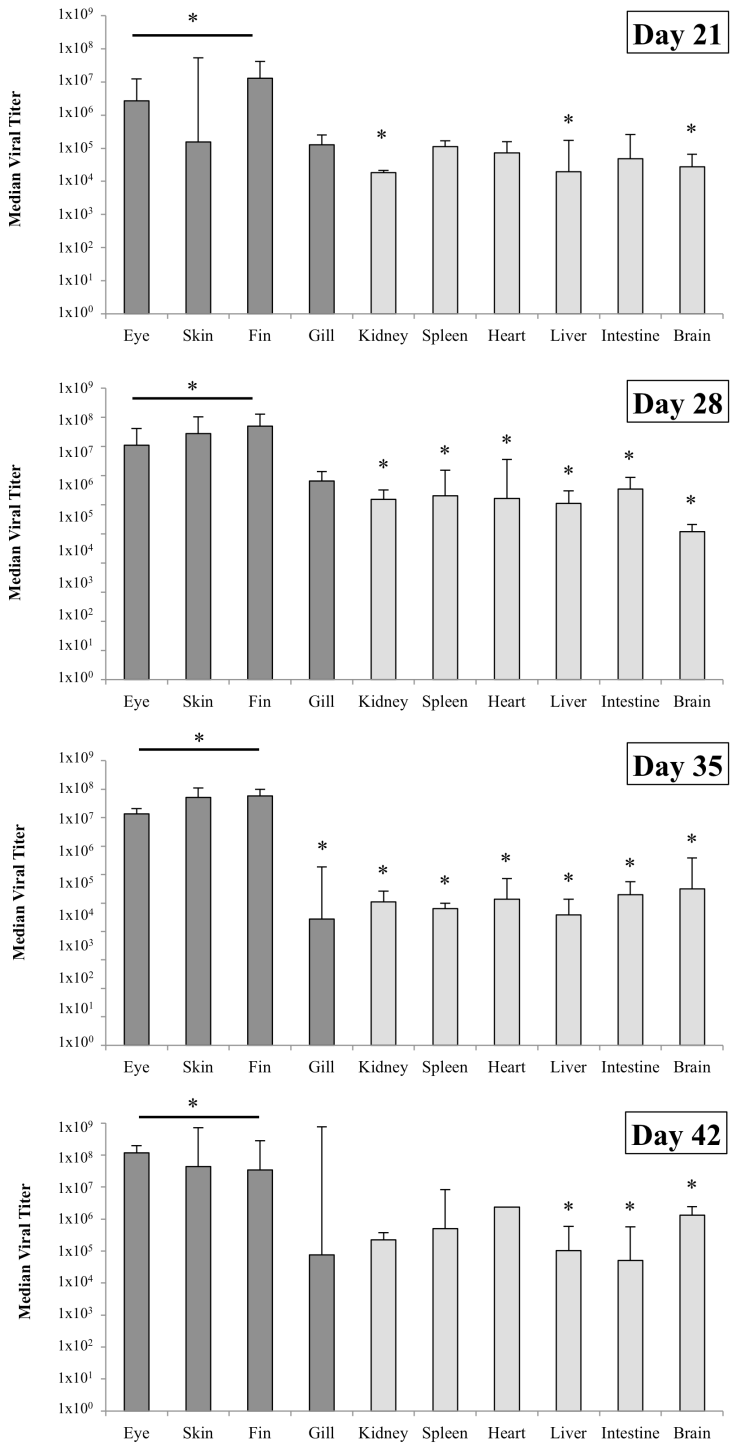

Figure 4.4 Median EEDV viral titer by tissue type and sampling day. "*" indicates statistical significance compared to tissues below the horizontal bar; Day 21: $p < 0.01$ for brain vs. eye and fin, liver vs. eye and fin and kidney vs. fin; $p < 0.05$ for all remaining combinations. Day 28: $p < 0.01$ for all pictured comparisons with the exception of eye vs. heart ($p < 0.05$); Additional significant pairings include brain vs. heart, intestine and spleen ($p < 0.05$). Day 35: $p < 0.05$. Day 42: $p < 0.01$ for all skin and eye comparisons, $p < 0.05$ for all fin comparisons. Error bars signify one standard deviation.

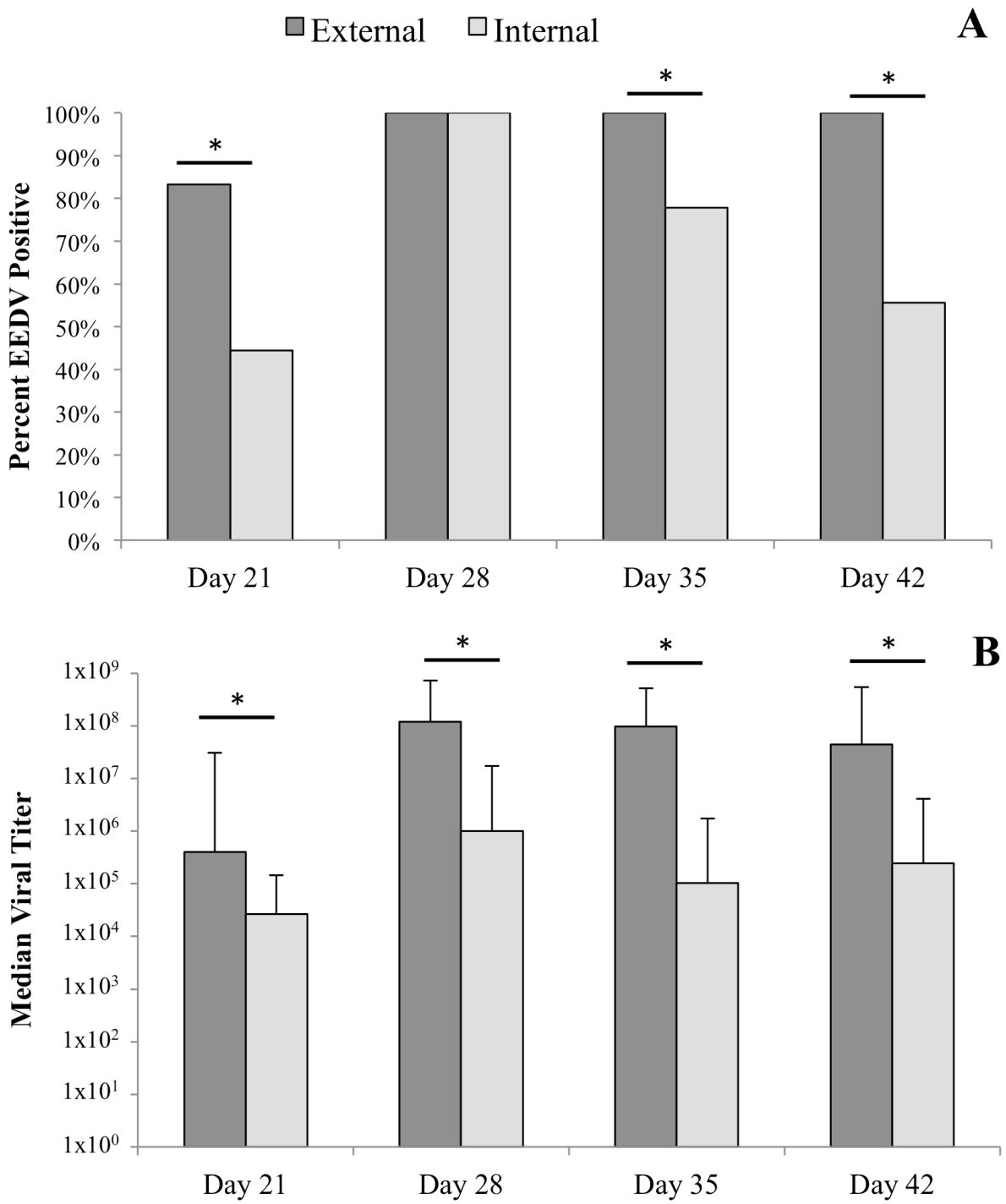

Figure 4.5 A) Total number of EEDV positive tissues by day and external vs. internal tissue type. "*" indicates statistical significance compared to tissues below the horizontal bar; $p < 0.01$ on Days 21, 35 and 42. B) Median EEDV viral titer by tissue type (external vs. internal) and sampling day. "*" indicates statistical significance compared to tissues below the horizontal bar; $p < 0.01$ on all sampling days.

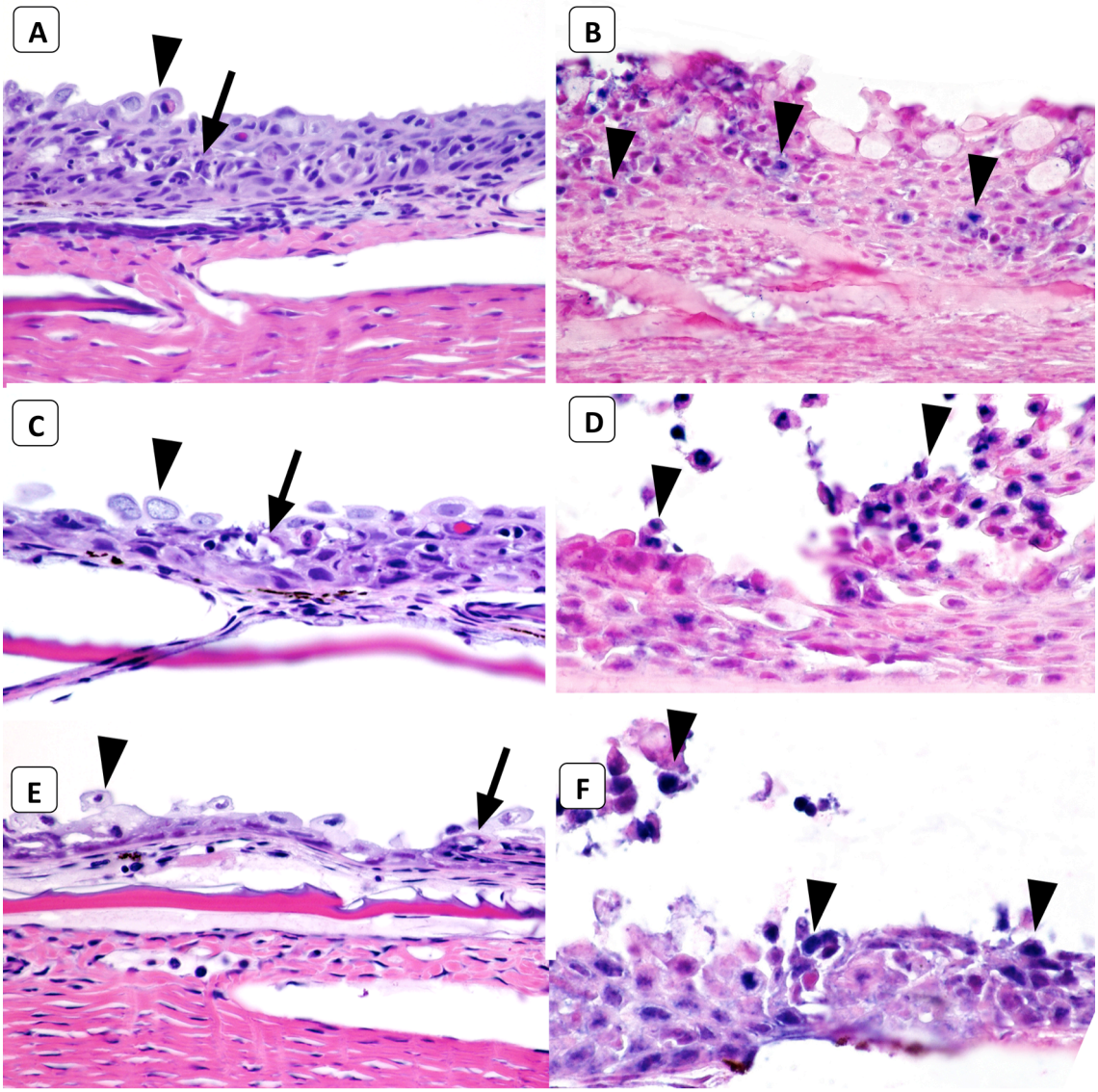

Figure 4.6 Distribution of EEDV within skin lesions of lake trout during early and late stages of experimental infection. Magnification 400X, Hematoxylin and eosin (A, C, E) and *in situ* hybridization for EEDV (B, D, F) with NBT labeling (blue) and nuclear fast red counterstaining. Early skin lesions (A, B) are characterized by individual epithelial cell necrosis (A: arrow) and sloughing of degenerated cells (A: arrowhead). Viral nucleic acid is detected in individual necrotic epithelial cells (B: arrowhead). At advanced stages (C, D) more widespread epithelial cell necrosis causes focal erosions (C: arrow) and sloughing of degenerate cells is common (C: arrowhead). Viral nucleic acid is readily detected in large numbers of degenerate and necrotic cells that commonly slough off (D: arrowheads). The most severe lesions (E, F) are characterized by widespread erosions and full thickness necrosis with only a few necrotic (E: arrow) and degenerate (E: arrowhead) cells clinging to the basement membrane. Viral nucleic acid can be detected in the vast majority of epithelial cells throughout all layers (F: arrowheads) prior to epithelial loss.

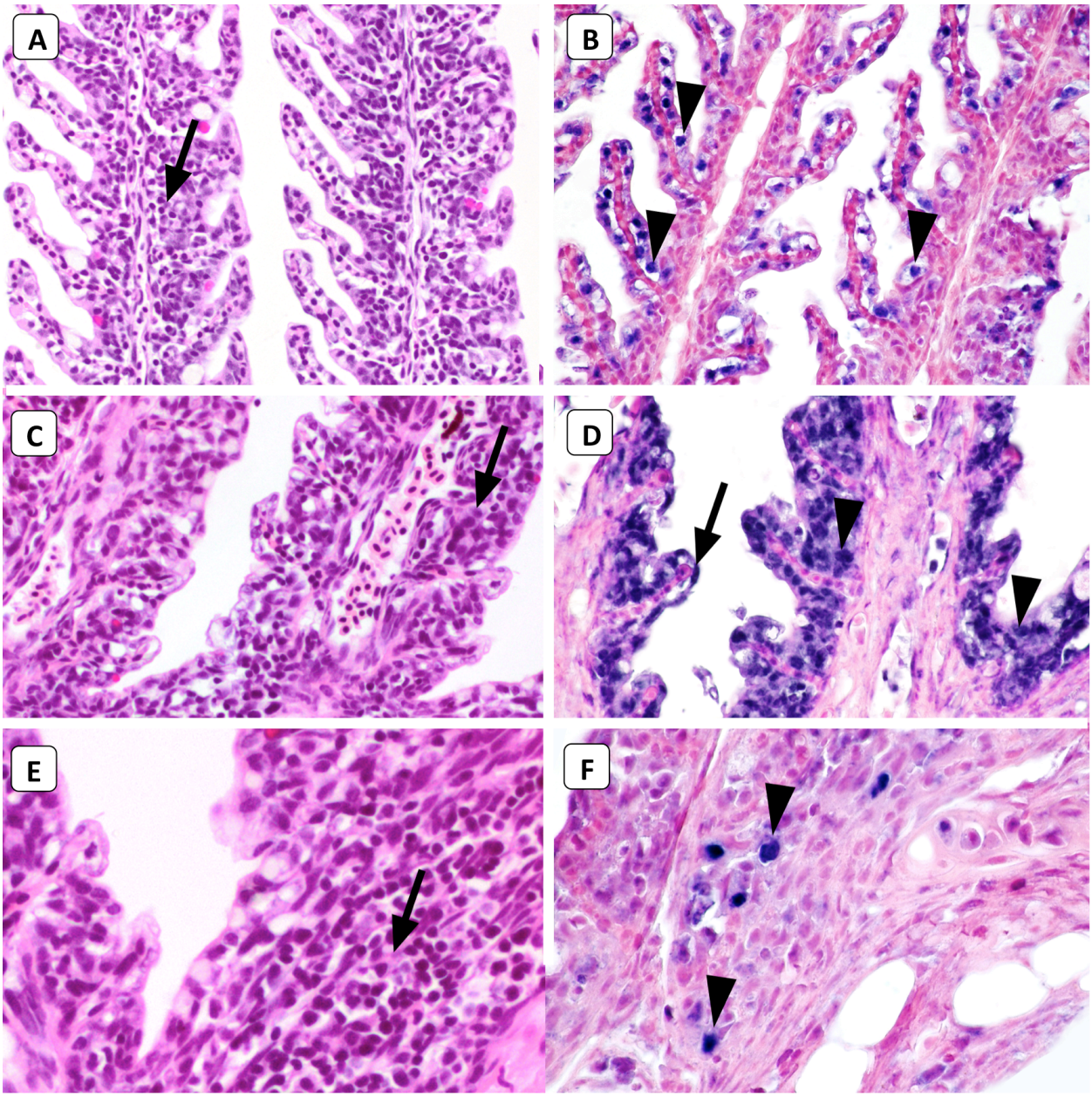

Figure 4.7 Distribution of EEDV within gill lesions of lake trout during early and late stages of experimental infection. Magnification 400X, Hematoxylin and eosin (A, C, E) and *in situ* hybridization for EEDV (B, D, F) with NBT labeling (blue) and nuclear fast red counterstaining. Early gill lesions (A, B) are characterized by mild thickening of the primary lamellae, infiltration of mononuclear cells into the propria (A: arrow), swelling of epithelial cells, congested blood vessels and few degenerated epithelial cells. Viral nucleic acid is detected in nuclei of morphologically unremarkable epithelial cells (B: arrowheads). More advanced gill disease (C, D, E, F) is characterized by loss of secondary lamellae and massive infiltration of mononuclear cells (C and E: arrows). Viral nucleic acid is detected in attenuated epithelial cells (D: arrow) and nuclei of infiltrating mononuclear cells (D and F: arrowheads).

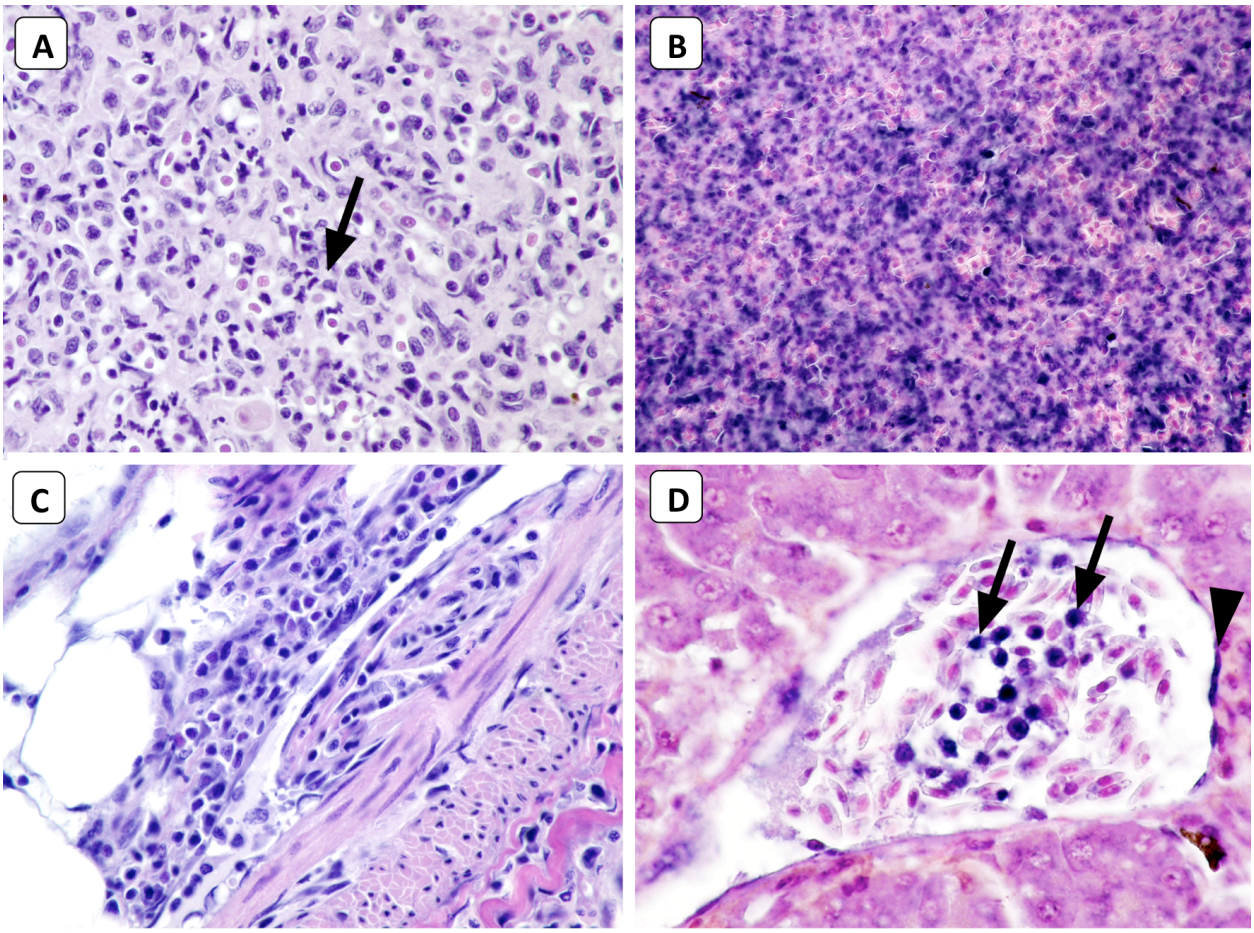

Figure 4.8 Distribution of EEDV within lesions of internal organs in lake trout during late stages of experimental infection. Magnification 400X, Hematoxylin and eosin (A, C) and *in situ* hybridization for EEDV (B, D) with NBT labeling (blue) and nuclear fast red counterstaining. During the later diseases stages lesions develop in internal organs and depletion of lymphoid cells and multifocal necrosis (arrow) in the spleen (A) is the most prominent finding. Viral nucleic acid can be detected in large numbers of mononuclear cells in the spleen (B). A severe lymphhistiocytic perivasculitis (C) also develops secondary to viremia and is most severe in the omentum, but can also be found in other organs. This perivasculitis is most likely secondary to viral infection of endothelial cells (D: arrowhead). Viremia is caused by large numbers of monocytes being infected as evidenced by significant nuclear labeling of mononuclear cells (D: arrows) within this hepatic vessel.

Chapter 5

Development and progression of gross and microscopic lesions in lake trout (*Salvelinus namaycush*) experimentally infected with Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3)

1. Abstract

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) is a lethal disease of lake trout (*Salvelinus namaycush*) currently threatening the rehabilitation efforts of this indigenous fish species. Virus biology, pathology and host interactions remain mostly unknown, thereby preventing the design of effective control strategies. Our current knowledge on the pathology caused by EEDV stems from the examination of severely moribund fish collected at the peak of natural mortality events when many external lesions were obscured by secondary bacterial agents and water mold contaminants invading lacerated tissues. This study aims at examining the progression of disease and associated pathology following exposure of naïve juvenile lake trout to EEDV via bath immersion. Groups of six fish were randomly collected and euthanized following a pre-determined schedule over the course of 6 weeks with individual tissues analyzed for gross and histopathologic changes. Based on our data, there was an incubation period of three weeks prior to observation of clinical signs and morbidity in EEDV infected fish, which is significantly longer than that of other pathogenic fish viruses. Early gross pathology included exophthalmia and ocular hemorrhage as well as fin congestion, and hyperemia of enteric and hepatic blood vessels. Advanced disease manifested as the previous gross pathology with the addition of multifocal to coalescing erosions and ulcerations of the skin and fins as well as congestion of visceral organs. The earliest microscopic lesions developed at 21 days post-exposure in the skin and fins, and were characterized by localized cellular degeneration of epidermal epithelial cells that progressed to erosions and ultimately focally extensive necrosis and associated dermatitis and perivasculitis. Early signs of systemic disease were observed at Day 28, characterized by multifocal to confluent necrosis in the spleen, multifocal intestinal necrosis and lymphohistiocytic perivasculitis of multiple internal organs

including the omentum and the epicardium. In addition, fish presented with focal necrosis of interstitial hematopoietic cells in the anterior kidney and rare apoptotic cells in the liver. The progression of lesions is consistent with the cutaneous epithelium representing the primary target of viral infection with hematopoietic organs and vessels undergoing pathology at the later viremic phase of the disease.

2. Introduction

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) has been implicated in multiple mortality events throughout the Midwestern United States over the past three decades (*2*, *5*) and continues to be a concern in Great Lakes fisheries management today (*Chapter 2*). EEDV has been shown to be particularly pathogenic to juvenile lake trout (*Salvelinus namaycush*) based both on viral identification during mortality events (*2*) (*Chapter 2*) as well as through experimental challenges (*3*, *4*) (*Chapter 3*). The clinical manifestation of EEDV in experimental challenges was consistent with that seen in natural outbreaks which included catastrophic mortalities in juvenile hatchery raised lake trout, with fish exhibiting ocular hemorrhage, corneal opacity, gill pallor, skin lesions ranging from pallor to erosions and ulcerations, water mold overgrowth, erosion and congestion of the fins and erythema of the oral cavity, isthmus and ventrum (*Chapter 2*). Additional detection of EEDV was reported from the reproductive fluids and kidneys of mature spawning lake trout (*5*, *14*). Unfortunately, specific information regarding virus biology, pathology and host interactions remains largely unknown in part due to the lack of an EEDV susceptible cell line and inability to culture this virus *in vitro*. This knowledge gap hampers the design and implementation of effective disease control strategies in the Laurentian Great Lakes Basin. A recently developed *in vivo* model of immersion

challenge and virus replication (*Chapter 3*) has provided a crucial step in the ability to study the pathogenesis of EEDV, allowing for examination of EEDV pathology and disease progression under controlled laboratory conditions.

Elucidating the sequential pathology and disease progression following EEDV infection is of vital importance as this virus continues to threaten lake trout rehabilitation efforts throughout the Midwestern United States. While many questions remain about the pathology of EEDV, this is not the case for all Alloherpesviruses. Evaluation of samples collected during natural outbreaks and experimental challenges aided in describing the pathology associated with infection of rainbow trout (*Oncorhynchus mykiss*) with *Oncorhynchus masou* virus (OMV; Salmonid Herpesvirus-2) (*87*). In both naturally and experimentally infected fish, severe pathology was observed in the intestine, spleen and kidney including necrotic and hemorrhagic foci in the splenic pulp and hematopoietic tissue. Some fish also displayed necrotized gill filaments and hepatocytes, yet surprisingly, carcinoma development was not noted in surviving fish as is typical with other salmonids infected with OMV (*87*).

Similar experimental studies performed with Salmonid Herpesvirus-1 resulted in gross lesions including alterations of pigmentation, exophthalmia and abdominal distension as well as hemorrhages in the fins, pale gills and yellow to red tinged ascites (*101*). Microscopic pathologic lesions were noted in the heart, musculature, liver, kidney and pancreas (*101*). Salmonid herpesvirus-4 is the causative agent of Atlantic salmon papillomatosis (ASP), a proliferative disease seen primarily in juvenile Atlantic salmon (*Salmo salar*) undergoing the smolting process (*109*). Reported ASP lesions consist of epithelial cell hyperplasia and karyomegaly with a loss of goblet cells and disruption of the basement membrane (*90*).

Previous histopathologic examination of EEDV-infected tissues has been limited to primarily severely diseased fish collected during an epizootic (*2*) (*Chapter 2*). In such severe cases, epithelial lesions have often been obscured and contaminated by the invasion of opportunistic and pathogenic bacteria and water mold into lacerated tissues, leading to difficulties in accurate assessment of underlying disease pathology. In order to alleviate the inherent challenges of attributing severe lesions during natural infections to EEDV, a study was designed to characterize the progression of gross and histopathologic disease after experimental exposure of naïve host fish to a moderately lethal dose of EEDV. The data generated herein will provide researchers, diagnosticians and fisheries managers with crucial information regarding the pathogenesis of EEDV.

3. Materials and Methods

3.1. Fish and maintenance

EEDV experimental challenges were performed using juvenile, Lake Superior strain lake trout (6 months post-hatch) supplied by the Marquette State Fish Hatchery (Marquette, Michigan). All experimental fish were randomly collected from a lot certified to be free of any reportable pathogens as per the AFS-FHS Bluebook (*40*). Certification was achieved following normal clinical examination of 60 randomly collected fish. Additionally, the absence of EEDV in the lot was confirmed using qPCR as detailed below. Experimental challenges were performed at the Michigan State University – Research Containment Facility (East Lansing, Michigan) in accordance with the Institutional Animal Care and Use Committee guidelines and approval. Upon receipt, all fish were housed in a 680-liter fiberglass aquarium and allowed to acclimate to standard laboratory conditions for a minimum of one month prior to experimental challenges. At

all times fish received continuous, oxygenated well water and were fed 1.0mm sinking feed *ad lib* (BioOregon, Westbrook, Maine, USA). During the experimental studies, fish were housed in 42 L fiberglass aquaria receiving continuous, flow-through, oxygenated well water at a temperature of 9.0 ± 0.5°C.

3.2. Virus

A stock of infectious EEDV for use in experimental challenges was produced through *in vivo* serial passage. Skin was initially collected from lake trout experiencing a natural EEDV outbreak in 2012 and stored at -80 °C. This skin was homogenized in a sterile phosphate buffered saline solution (pH 7.5 ± 0.5; Sigma-Aldrich, St Louis, Missouri, USA) at a ratio of 1:3 (w/v), clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C and used to infect naïve juvenile lake trout via intraperitoneal injection (IP). Prior to IP injections, naïve fish were anesthetized using tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.1 mg/mL). After an IP injection of 300 µL virus stock, fish were allowed to recover from sedation and returned to flow-through aquaria for the duration of the study. Fish were monitored daily, collected immediately upon death or development of morbidity (euthanized with an overdose of MS-222; 0.25 mg/mL), and a necropsy performed. Skin was again collected, processed as previously with the substitution of Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen) rather than PBS, and used to infect a new group of naïve lake trout. This process

continued until an adequate volume of 7th passage stock was created for use in this and future experimental studies.

3.3. Experimental challenge

Fish were divided into an EEDV group (n = 84) and a negative control group (n = 48). All fish were challenged via the immersion method previously described (*Chapter 3*). Briefly, fish were transferred to static, aerated glass aquaria to which experimental dose (virus or sham control) was added directly to the water. Fish were monitored in these holding aquaria for a period of one hour during which water temperature was maintained at 9.0 ± 0.5°C by utilizing a separated, larger surrounding bath of continuous water flow. The EEDV group was exposed to a moderate lethal dose of EEDV (*Chapter 3*) while the negative control group was immersed in a sham suspension of MEM diluent. After completion of the exposure period (1 hour), fish were transferred back to their experimental flow through aquaria for the duration of the study where they were monitored daily for development of clinical disease.

3.4. Sample collection and preparation

Sampling occurred in parallel from the two experimental groups on Days 0, 1, 3, 6, 9, 12, 15, 28, 21, 28, 35, and 42-post infection (p.i.) at which time seven EEDV infected fish and four negative control fish were collected and euthanized in a manner which minimized the stress for both sampled and remaining fish. On each sampling day, one infected fish and one control fish were preserved whole in 10% neutral buffered formalin; a ventral midline incision was created in order to allow for improved internal fixation. Full external and internal exams were performed on the remaining six infected and three negative control fish and portions of skin, fin, gill, eye,

brain, spleen, heart, liver, intestine, and kidney were collected from each fish. Each tissue was divided for: 1) detection of EEDV by PCR (frozen at -20°C) and 2) microscopic examination (fixed in 10% neutral buffered formalin). Portions were collected from both anterior and posterior kidney, one whole eye was collected for each assay, and the "intestine" sample consisted of a segment approximately 1 cm oral to the vent.

3.5. Virus detection

The presence of EEDV in sampled tissues was confirmed using quantitative PCR. DNA extractions and PCR reactions were performed following the protocol outlined by Glenney et al (2016) (*14*). DNA extractions were performed manually using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) after tissue digestion. Eluted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All PCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with a total reaction volume of 20 µL and 50 nmol total DNA template.

3.6. Histopathology and lesion scoring

After fixation, tissues were processed for routine paraffin embedding. Paraffin blocks were sectioned at 5 µm, and slides were routinely stained with hematoxylin and eosin (H&E) for histopathologic examination (*117*). All slides were examined by an American College of Veterinary Pathologists board-certified pathologist who was blinded to the presence of gross

lesions or results of EEDV detection in individual tissues. All identifiable lesions were scored on a scale of 0 to 3 with 0 being no lesion (normal), 1 being mild lesion, 2 being moderate lesion and 3 being severe lesion.

4. Results

4.1. Clinical disease and mortalities

Infected fish exhibited exophthalmia and ocular hemorrhage (Figure 5.1a) as early as one day post infection, increasing from 33% of fish sampled on Day 1 (n = 2/6) to 100% of fish sampled on Day 28 (n = 6/6). Other early signs of clinical disease (i.e., first observed in a minimum of 2 fish/day prior to Day 18 p.i.) included congestion at the base of pectoral and pelvic fins (Figure 5.1b) and along the isthmus, mild splenic pallor, and hyperemia or engorgement of enteric and hepatic blood vessels (Figure 5.1c). During later stages of disease (i.e., Days 21-42 p.i.), fish consistently exhibited moderate to severe congestion of multiple fins (Figure 5.1d), ocular hemorrhage and exophthalmia, multifocal to coalescing erosions and ulcerations of the skin, fins and caudal peduncle (Figure 5.1d) as well as congestion of visceral organs such as kidney, liver and spleen. The number of fish with clinical disease increased each sampling day from 33% of fish being affected at Day 6 to 100% being affected by Day 15 through the end of the study. Likewise, the total number of observed gross lesions each day increased from ≤ 6 on Days 0-12 (average of < 1 per fish per day) to averaging > 5 lesions per fish per day from Day 21 through the end of the study. Mortalities occurred in the infected group on Day 28 post-infection (n = 4) while no mortalities or gross lesions were noted in the negative control group. A detailed account of gross pathology is presented in Table 5.1.

4.2. Virus identification

In order to confirm the presence of EEDV in experimentally challenged fish, external tissues (e.g., skin) and internal organs (e.g., kidney) were tested via qPCR as described above. EEDV DNA was detected in the skin (n = 2/6 fish) as early as Day 18 p.i., and from Days 21-42 p.i. the virus was detected in the skin of 96% of fish sampled (n = 23/24) (Table 5.2). In comparison, the virus was not detected in the kidney until Day 21 p.i., and in only 67% of fish from Days 21-42 p.i. (n = 16/24). Throughout the experiment, no EEDV nucleic acid was detected from control fish.

4.3. Microscopic lesions and scoring

A total of 754 tissues from 12 control fish and 67 infected fish were examined for microscopic lesions. A small number of tissues (< 5%) were unable to be appropriately examined due to preservation, preparation artifact, or sectioning angle which led to insufficient tissue available for analysis. A detailed chart of lesions by day and tissue type, along with the number of fish from which lesions were observed is presented in Table 5.3.

Hepatic lipidosis was nearly ubiquitous among control fish throughout the study (Figure 5.2a, 5.2b), and among infected fish during the early weeks of the experiment (Figure 5.2c, 5.2d) yet was observed in only 30% of infected fish examined during the last three sampling weeks (Figure 5.2e, 5.2f). Fixation artifact was observed in many intestinal sections from fish in both the EEDV group and the negative control group where distal villi became mild to heavily, multifocally vacuolated which was likely caused by superficial epithelial cells swelling due to contact with buffered formalin prior to complete fixation. There was a mild, patchy proliferative branchitis in the gills of many fish in both groups throughout the study. This was characterized

by a loss of secondary lamellae coupled with a thickening epithelial layer and infiltration of mononuclear cells. There was no evidence of an increasing severity over time, and there were no differences in distribution and severity of branchitis between fish in the negative control group and the EEDV group.

The primary microscopic lesions noted in this current study were observed in the skin and fins (Figure 5.3). The earliest lesions were observed in the epidermis at Day 21 and consisted of individual epithelial cell degeneration and single acantholytic cells. In multiple areas there were intracytoplasmic eosinophilic vacuoles in few epithelial cells as well as rare intraepithelial inflammatory cells (Figure 5.3b). Lesions progressed in some areas to epithelial erosions with sloughing of degenerated epithelial cells (Figure 5.3c). By Day 28 epithelial erosions were more severe with extensive intraepithelial inflammatory infiltrates and cellular degeneration (Figure 5.3d) and in the most advanced lesions epithelial cells were undergoing degeneration and necrosis throughout all layers of the epidermis (Figure 5.3e). The most severe lesions were observed at Day 35 and characterized by massive epithelial ulceration and complete epidermal loss (Figure 5.3d). Cutaneous lesions were not observed prior to Day 21, but were observed in all fish examined at later time points except for a single fish on Day 35 and one fish on Day 42.

As early as Day 3 post-exposure, focal areas of single cell necrosis were observed in the liver (Figure 5.2c), characterized by cells with deeply basophilic, shrunken, pyknotic hepatocellular nuclei and hypereosinophilic, contracted cytoplasm. Such changes were observed with increasing severity as evidenced by increasing numbers of foci of pyknotic cells and in more fish per sampling day. Beginning on Day 9, $\geq$ 66% of fish were affected by similar lesions with 100% of fish sampled over the final three weeks showing at least mild changes. Starting at Day 18, single cell necrosis focally expanded to small foci of hepatocellular necrosis and there

was also lymphohistiocytic perivascular inflammation affecting both hepatocellular arteries and veins (Figure 5.2c). Lesions were most severe in fish collected on days 21 and 28, but similar lesions were found in fish examined at later days (Figure 5.2d).

Also at 28 days post exposure, both examined fish had severe lymphoid necrosis of the spleen (Figure 5.4a and 5.4b) and lymphohistiocytic perivasculitis affecting multiple organs, but being most severe in the omentum (Figure 5.4c and 5.4d), heart (Figure 5.4e) and as previously discussed liver. Lymphohistiocytic perivasculitis was most severe in the omentum of one fish euthanized on Day 28 and there were rare intranuclear inclusion bodies in monocytes in the lumens of affected blood vessels (Figure 5.4d). While proliferative branchitis with focal lymphohistiocytic inflammatory cell infiltrates (Figure 5.4f) was observed in numerous EEDV infected fish, similar lesions were also found in control fish.

Microscopic lesions in the kidneys were not observed until Day 28 post-exposure, however, once these changes began to occur, they were noted in every fish examined from that point through the end of the study. Renal pathology was characterized by depletion and multifocal necrosis of interstitial hematopoietic cells. At higher magnifications, pyknotic and degenerate nuclei were found in hematopoietic cells (Figure 5.5c, 5.5d), while at low magnifications, lesions were characterized by a marked reduction in the number of interstitial cells (Figure 5.5e, 5.5f). While these changes were observed in both anterior and posterior kidney sections, no significant pathology was noted in the renal tubules at any time points.

Epithelial lesions in the skin and fins were not appreciable until Day 21 p.i., and represented the first evidence of viral disease. While single cell necrosis in the liver was described at an earlier stage of the experiment, these lesions were less specific and multi organ

involvement with lymphohistiocytic perivasculitis and lymphoid necrosis was not reported until Day 28 p.i.

5. Discussion

In this study, we have demonstrated that infection with Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) leads to development of a lethal, systemic disease in lake trout and have further elucidated the pathogenesis of EEDV in its primary host species. Clinical signs and gross and microscopic lesions induced through experimental immersion challenge were consistent with those seen observed in natural EEDV outbreaks (*2*) (*Chapter 2*). The incubation period was significantly longer for EEDV infected fish compared to other viral diseases of fish and the earliest lesions were observed in the eyes, skin and fin. While exophthalmia and ocular hemorrhage were described grossly, no ocular lesions were reported microscopically. This discrepancy may simply reflect our inability to track individual fish with gross lesions across subsequent sampling days. The grossly observed skin lesions corresponded with the reported degenerative and necrotic epidermal lesions described microscopically and represent the first manifestation of EEDV in lake trout. Early cutaneous lesions were followed by viremia that resulted in severe lesions in internal organs and mortality at Day 28. The severity of gross lesions, histopathologic lesions, and mortalities also coincided with peak viral identification around 28 days p.i. via qPCR in the previous study (*Chapter 4*). These results demonstrate the prolonged and delayed development of clinical disease following exposure to EEDV. This is ecologically and epidemiologically important in terms of identifying potential viral sources following a mortality event. The point of exposure may have been up to a month prior to observation of diseased fish given the extended incubation period in the present study.

Interestingly, while EEDV nucleic acid was not identified in the liver until Day 21, pathological changes were observed among hepatocytes soon after exposure. One hypothesis is that the virus may rapidly lead to irreversible hepatic damage prior to establishment of appreciable systemic disease. Alternatively, the single cell necrosis in the liver may represent a response to the actual inoculum and not true viremia and hepatocellular infection by EEDV. Additionally, it is important to highlight the fact that while hepatic lipidosis is common in captive reared fish due to the limited amount of work required for the fish to receive high volumes of nutrient dense food, identification of such lesions in the present study revealed an interesting trend. Hepatic lipidosis was observed in nearly all negative control fish as well as infected fish sampled early in the study, however by the end of the challenge period, prevalence had dropped to less than a third of the fish over the final three sampling weeks. This can be used as an indicator that these fish, while surviving the EEDV infection were ill and had reduced feed intakes as a result. While hepatic lipidosis in many instances is an abnormal finding, in hatchery-raised fish such as these, it is in fact the absence of such a lesion that is the noteworthy finding.

Despite previous reports of pathologic changes in the gills and more specifically the lamellar epithelium (*3*), in this study we were unable to attribute the observed gill lesions directly to EEDV infection. There was no difference in gill lesions between infected and control fish and no progression of branchitis was observed in EEDV infected fish throughout the course of infection. Further studies using *in situ* hybridization were carried out to determine a potential association between EEDV and gill lesions and the results are presented in Chapter 4. The cause of proliferative branchitis in control fish remains unclear, but may be due to a number of reasons. Many of the fish used for this experiment had shortened opercula, a not uncommon developmental abnormality seen in captive reared salmonids. While not particularly life

threatening to the fish, this shortening allows for an increase in gill exposure to environmental conditions. This could have led to increased exposure to waterborne infectious agents, but also increased exposure of the sensitive gill lamellae to non-infectious debris and particles in the environment, leading to thickening and increased cellular infiltrates as described. So while the EED virus may cause degenerative and inflammatory changes within the gills, variation in individual fish anatomy may cause similar changes that could obscure such lesions. Additionally, an oblique angle when sectioning gill arches can have a great affect on the ability to interpret lamellar changes. In order to more definitively identify EEDV-related lamellar changes, multiple gill arches from each fish should be examined.

This study also provides evidence for the potential cause of death of EEDV infected fish. During the last three weeks of the experiment, cutaneous lesions were observed in 80-100% of infected fish and epithelial necrosis as observed here is known to lead to hypo-osmotic shock and death in fish infected with other viruses such as Koi Herpesvirus (Cyprinid Herpesvirus-3) (*132*). Additionally, by Day 28 p.i., infected fish had developed lesions in multiple organs that were primarily centered on vessels. Observation of intranuclear inclusion bodies in blood monocytes indicates transmission of virus from affected surface epithelium through infected monocytic cells causing viremia and secondary infection of internal organs. Such lesions are most likely indicative of an overall poor systemic health of viremic fish. As renal tubular damage was not appreciated throughout the study, renal function was likely unaffected. However, the combination of lymphoid necrosis causing immune suppression, perivasculitis most likely resulting in a systemic cytokine response, and epithelial damage coupled with the general stress caused by high densities and standard operating procedures within salmonid hatcheries is enough to tip the scales, resulting in the observed mortalities.

Finally, when comparing the relatively minor mortality rates observed in the current study with the high viral titers demonstrated in the previous chapter, a concern arises that lake trout are able to not only survive an EEDV infection, but such fish may in fact be able to act as a viral reservoir and subsequently pose a substantial risk to younger, more susceptible populations of fish. While we have demonstrated that EEDV causes a lethal disease in experimentally challenged lake trout, we have also shown that fish are able to survive despite high viral loads and advanced pathological lesions. This highlights the need for aggressive and persistent screening for EEDV in captive lake trout in order to rapidly identify and quarantine any potentially infected populations before the virus can spread throughout the hatchery. Recent mortality events (*Chapter 2*) have highlighted the fact that EEDV remains present and a threat within the Great Lakes basin, and this current study has provided us with key information regarding the host-virus interactions.

APPENDIX

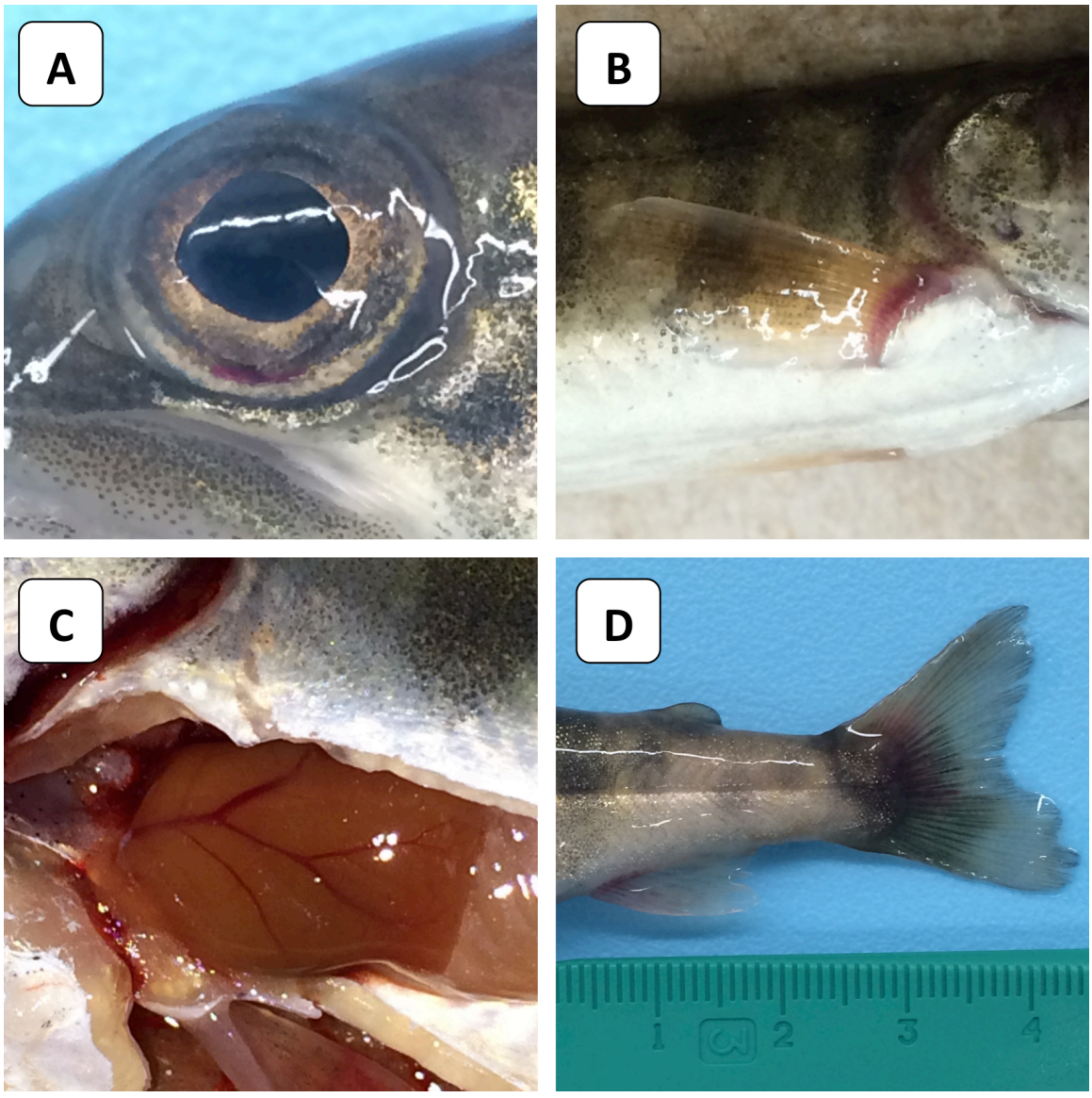

Figure 5.1 Gross pathology following experimental exposure of naïve juvenile lake trout to EEDV by bath immersion. A) ocular hemorrhage; B) pectoral fin congestion; C) engorgement or hyperemia of major hepatic vasculature; D) caudal and anal fin congestion, generalized pallor of the caudal peduncle, erosion of caudal peduncle and caudal fin.

Fish #	Day 0	Day 1	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 28	Day 35	Day 42
1	Mildly short right operculum. Mildly congested, melanotic, darkened kidney.	Normal external. Normal internal.	Mildly short operculum, bilateral.	Normal external. Mildly pale heart.	Mild bilateral exophthalmia. Mildly short operculum bilateral. Mild splenomegaly.	Normal external. Mildly pale kidney.	Normal external. Mildly hyperemic hepatic vessels.	Normal external. Mild hyperemic enteric vessels.	Mildly short operculum bilateral. Normal internal.	Occular hemorrhage 6:00 bilateral. Congestion base pectoral and pelvic fins. Mildly swollen vent. Moderate autolysis. Congested kidney.	Swollen vent. Occular hemorrhage 6:00 bilateral.	Mild erosion caudal fin. Multifocal skin palor and erosion. Mildly swollen spleen. Mildly pale kidney.
2	Mildly short operculum, bilateral. Normal internal.	Normal external. Normal internal.	Normal external. Normal internal.	Hemorrhage 6:00 left eye. Mild hyperemia hepatic vessels.	Mild exophthalmia left eye. Mildly short operculum bilateral. Normal internal.	Mildly short right operculum. Normal internal.	Normal external. Mildly pale liver	Mild congestion base pectoral fin & isthmus. Mild erosion around dorsal opercular margin (left side). Mild hyperemia enteric vessels.	4x4 mm pale patch, mild erosion, between dorsal and adipose fins. Severe congestion base of right pectoral fin. Mild erosion caudal, dorsal, anal fins. Mild hyperemia enteric vessels.	Hemorrage 6:00 left eye. Congestion base all fins. Moderate autolysis.	Normal external. Mild congestion liver. Moderate congestion kidney.	Multifocal skin erosion and swelling. Mild erosion all fins. Multifocal hemorrhagic staining in abdominal adipose tissue around spleen. Congested and friable spleen. Congested kidney.
3	Normal external. Normal internal.	Moderate congestion base of right pectoral fin. Normal internal.	Mild short right operculum. Normal internal.	Mild exophthalmia left eye. Mildly short/eroded right operculum. Mild hyperemia enteric vessels.	Normal external. Normal internal.	Hemorrhage 6:00 left eye. Normal internal.	Mild exophthalmia left eye. Mildly congested kidney.	Midlly pale gills. Normal internal.	Mild erosion caudal/anal fins. Mild erosion around nares. Mildly pale spleen. Mild hyperemia enteric vessels.	Hemorhage 6:00 left eye. Generalized palor. Mild erosion/ragged all fins. Moderate autolysis.	Multifocal mucous accumulations along dorsum and on left eye. Mildly swollen spleen. Hyperemia hepatic and enteric vessels. Moderate congestion kdiney.	Multifocal erosion and swellingin the skin. Mild erosion all fins. Moderately pale gills. Mildly swollen spleen. Hyperemia hepatic/enteric vessels. Mildly congested kidney.
4	Normal external. Normal internal.	Mild short right operculum. Mild congestion isthmus. Hemorrhage 6:00 left eye. Normal internal.	Hemorrhage 6:00 left eye. Mildly pale spleen. Mild hyperemia enteric vessels.	Normal external. Green distended gall bladder.	Normal external. Normal internal.	Hemorrhage 6:00 left eye. Swollen spleen.	Hemorrhage 6:00 left eye. Mild erosion between right nare and eye. Hyperemic hepatic vessels. Midly congested kidney.	Hemorrhage 6:00 left eye. Mildly pale liver.	Mild erosion caudal fin. 3x3 mm erosion/pale patch caudal to head (dorsal). Mild generalized palor and scale loss. Mild exophthalmia. Moderately pale spleen. Hyperemia enteric vessels. Mildly congested kidney.	Mort. Hemorrhage 6:00 left eye. Swollen vent. Congestion base pectoral/pelvic fins. Generalized palor. Moderate autolysis.	Multifocal skin erosion along dorsum. Mildly short right operculum. Mild generalized palor. Mildly swollen spleen.	Multifocal skin erosion/swelling/palor. Mildly pale gills. Swollen spleen. Congested kidney
5	Mild congestion base of left pectoral, bilateral pelvic fins. Mildly pale spleen.	Normal external. Mild hyperemia hepatic vessels	Moderate congestion base of left pectoral fin. Mildly pale spleen.	Normal external. Normal internal.	Mild congestion base of pelvic fins. Mildly short operculum bilateral. Normal internal.	Normal external. Mild hyperemia enteric vessels.	Mild erosion dorsal margin opercular opening bilaterally. Mild bilateral exophthalmia. Hyperemic enteric vessels.	Mild generalized palor. Mild hyperemia enteric vessels.	Normal external. Mild hyperemia enteric vessels.	Moribund. Hemorrhage 6:00 left eye. Swollen vent. Congesiton base of anal fin/caudal fin. Moderately ragged caudal fin. Multifocal generalized palor and epidermal erosion. Green distended gallbladder. Mild congestion kidney. Mild hyperemia enteric vessels.	Hemorrhage 6:00 left eye. No right eye (chronic/healed). Mildly pale gills. Mild congestion liver/kidney. Hyperemia enteric vessels.	Generalized palor. Mild bilateral exophthalmia. Mildly pale gills. Pale liver.
6	Mildly short operculum, bilateral. Mildly small spleen.	Mild generalized melanosis, mildly prominent lateral line. Normal internal.	Normal external. Normal internal.	Normal external. Normal internal.	Normal external. Mildly hyperemic enteric/colonic vessels.	Hemorrhage 6:00 left eye. Normal internal.	Hemorrhage 6:00 left eye. Mild congestion base pelvic/anal fins.	Mild development deformity pectoral fins bilateral. Mild congestion kidney.	Mild erosion/scale loss caudal peduncle and left lateral side cranial to dorsal fin. Moderate hyperemia enteric vessels.	Hemorrhage 6:00 left eye. Mild erosion all fins and caudal peduncle. Multifocal generalized palor and mild erosion skin. Mild hyperemia enteric vessels. Moderate congestion kidney.	Generalized palor. Mild congestion liver. Mild hyperemia enteric vessels. Mildlly swollen spleen.	Mild erosion caudal peduncle. Multifocal skin erosoin/scale loss.

Table 5.1 Summary of gross pathologic changes observed following immersion challenge of naïve juvenile lake trout with EEDV.

EEDV Positive Samples												
Day P.I.	Skin						Kidney					
	Fish Number						Fish Number					
	1	2	3	4	5	6	1	2	3	4	5	6
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	+	+	-	-	-	-	-	-
21	+	+	+	+	-	+	+	-	+	-	-	+
28	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+	-	+
42	+	+	+	+	+	+	+	-	+	-	-	-

Table 5.2 Identification of EEDV genomic material in tissues of experimentally challenged lake trout (*Salvelinus namaycush*) collected in parallel on predetermined days using a SYBR Green qPCR assay. "-" indicates no EEDV genetic material; "+" indicates presence of EEDV DNA.

Tissue		Day 0	Day 1	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 28	Day 35	Day 42
Fin	# of Fish	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/2	1/4	4/5
	Lesion Description	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Moderate epidermal erosion amd ulceration	Mild epidermal erosion and necrosis.	Mild epidermal erosion and ulceration with necrosis.
Gill	# of Fish	3/4	4/5	5/5	5/6	5/6	1/4	5/5	1/3	4/5	1/2	3/5	3/6
	Lesion Description	Mild proliferative branchitis	Mild proliferative branchitis	Mild-moderate proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis	Mild proliferative branchitis
Intestine	# of Fish	3/6	2/6	1/6	3/6	0/6	2/6	3/6	5/6	2/6	2/2	0/6	0/6
	Lesion Description	Mild-moderate distal villar vacuolation	Moderate distal villar vacuolation	Mild distal villar vacuolation	Mild-moderate distal villar vacuolation	n/a	Mild-moderate distal villar vacuolation	Mild distal villar vacuolation	Mild distal villar vacuolation	Mild-moderate distal villar vacuolation	Lymphohistiocytic perivasculitis. Mild distal villar vacuolation	n/a	n/a
Kidney	# of Fish	0/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/2	5/5	6/6
	Lesion Description	n/a	Mild multifocal hemorrhage, posterior kidney	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Mild-moderate hematopoietic cell depletion and necrosis, anterior and posterior kidney	Mild-moderate hematopoietic cell depletion and necrosis, anterior and posterior kidney	Mild-moderate hematopoietic cell depletion and necrosis, anterior and posterior kidney
Liver	# of Fish	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	2/2	5/5	6/6
	Lesion Description	Hepatic lipidosis	Hepatic lipidosis	Hepatic lipidosis; Mild multifocal single cell necrosis	Hepatic lipidosis; Mild multifocal single cell necrosis	Hepatic lipidosis; Mild multifocal single cell necrosis	Hepatic lipidosis; Mild multifocal single cell necrosis	Hepatic lipidosis; Mild-moderate multifocal single cell necrosis and lymphohistiocytic perivascular inflammation.	Hepatic lipidosis; Mild-severe multifocal single cell necrosis and lymphohistiocytic perivascular inflammation.	Hepatic lipidosis; Mild-moderate multifocal single cell necrosis and lymphohistiocytic perivascular inflammation.	Hepatic lipidosis; Mild-moderate multifocal single cell necrosis and lymphohistiocytic perivascular inflammation.	Hepatic lipidosis; Mild-moderate multifocal single cell necrosis and lymphohistiocytic perivascular inflammation.	Hepatic lipidosis; Mild-moderate multifocal single cell necrosis.
Skin	# of Fish	0/4	0/3	0/3	0/6	0/6	0/6	0/5	0/6	2/6	2/2	4/5	4/5
	Lesion Description	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Individual epithelial cell necrosis and intracytoplasmic eosinophilic vacuoles, focal erosins.	Mild epidermal erosion and focal ulceration	Severe epidermal erosions and ulcerations with focally extensive necrosis	Moderate epidermal erosions and focal ulceration.
Spleen	# of Fish	0/6	0/6	0/6	0/6	0/6	0/5	0/6	0/6	0/5	2/2	4/5	5/6
	Lesion Description	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Multifocal to diffuse lymphoid necrosis	Multifocal lymphoid necrosis	Multifocal lymphoid necrosis
Heart	# of Fish	0/4	0/3	0/3	0/6	0/6	0/6	0/5	0/6	0/6	2/2	0/6	0/6
	Lesion Description	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Lymphohistiocytic perivasculitis and epicarditis	n/a	n/a

Table 5.3 Summary of histopathologic changes on all sampling days. No appreciable lesions were observed in the eyes or brains, on any days.

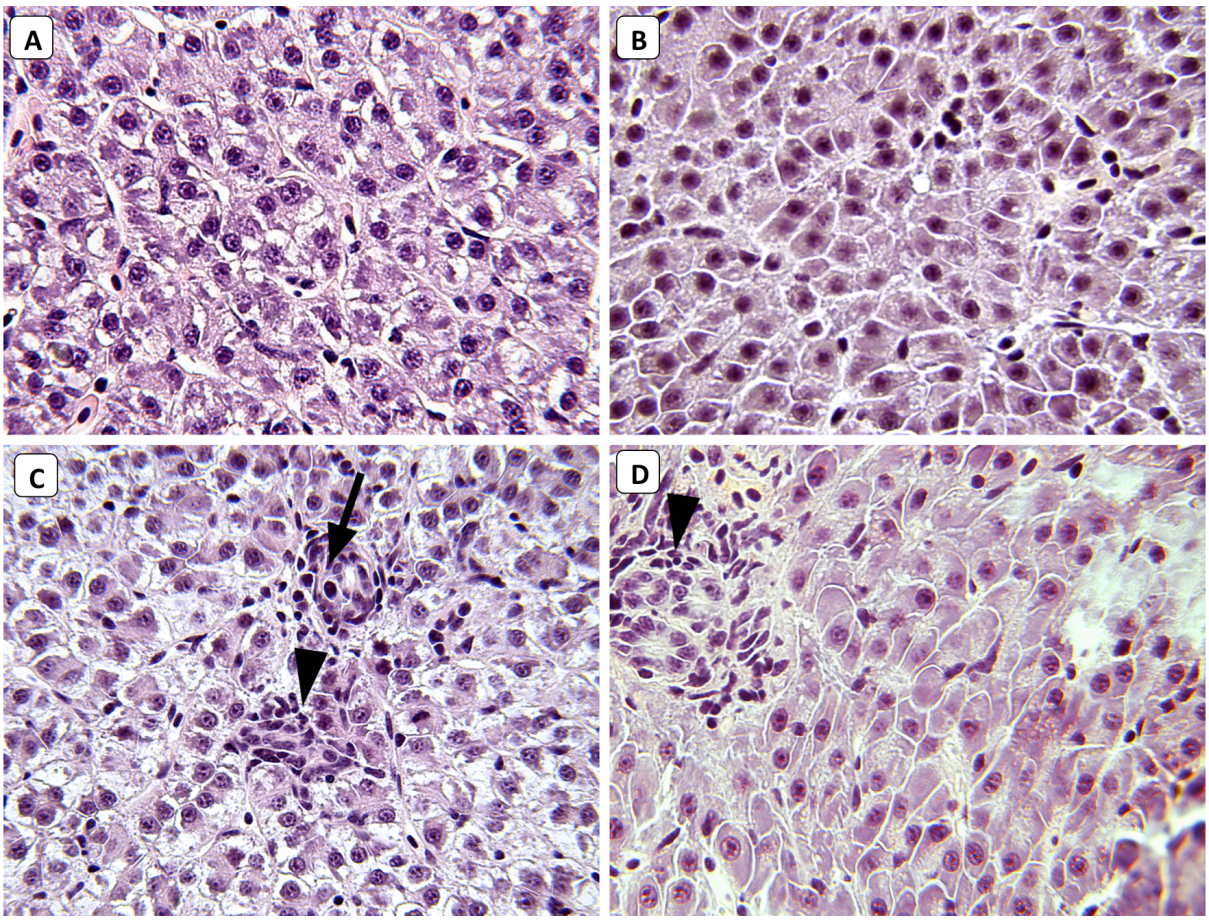

Figure 5.2 Liver collected from negative control fish and lake trout experimentally infected with EEDV. Magnification 400X. A) negative control, Day 1, severe hepatic lipidosis; B) negative control, Day 9, moderate hepatic lipidosis; C) infected, Day 18, focal hepatocellular necrosis (arrow) and lymphohistiocytic perivascular inflammation (arrowhead), severe hepatic lipidosis; D) infected, Day 42, focal hepatocellular necrosis and lymphohistiocytic inflammation (arrowhead), mild hepatic lipidosis.

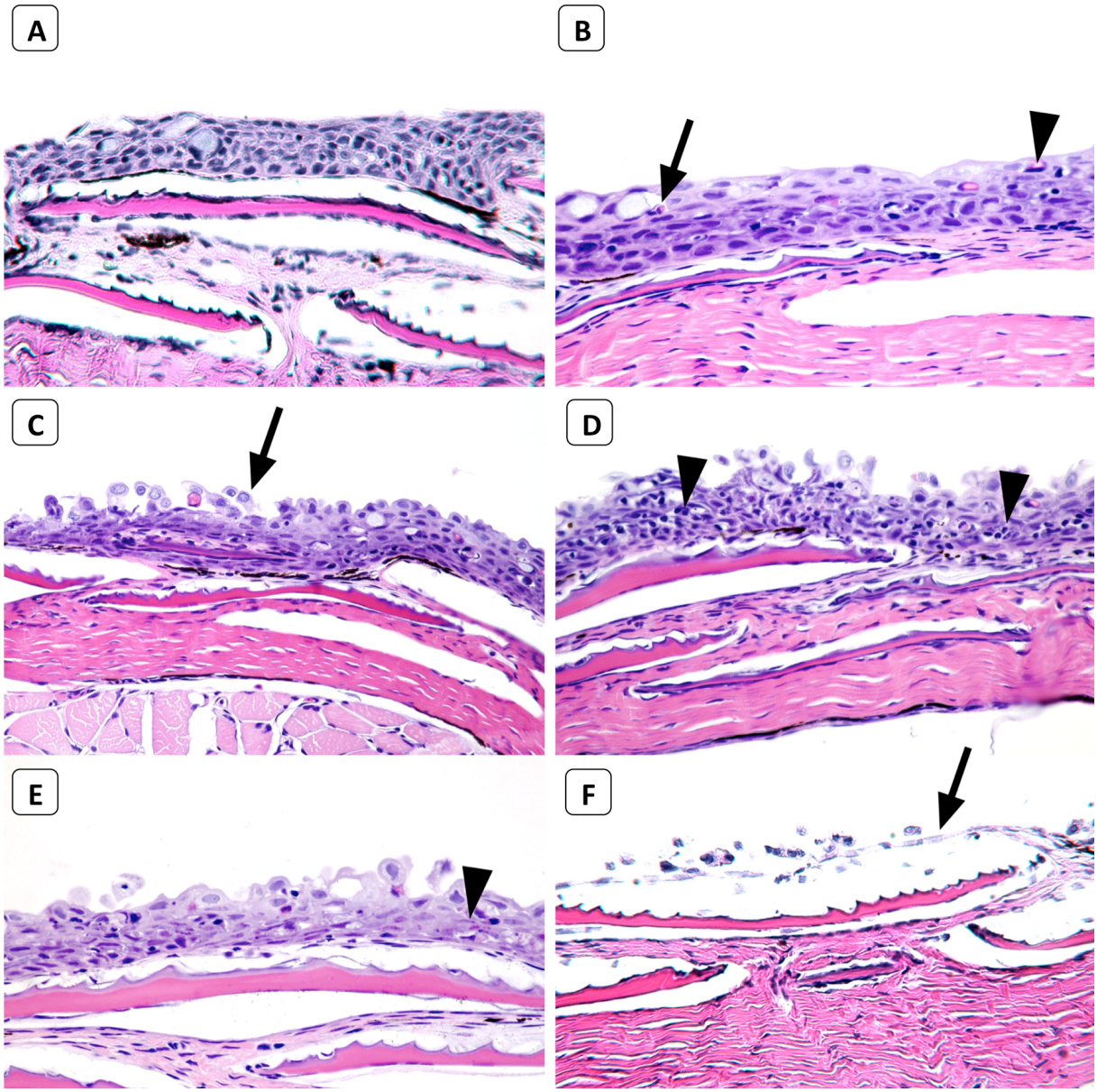

Figure 5.3 Skin collected from negative control fish and lake trout experimentally infected with EEDV. Magnification 200X, hematoxylin and eosin staining. A) negative control, Day 35; B) infected, Day 21, individual epithelial cell necrosis (arrow) and intracytoplasmic eosinophilic vacuoles in few cells (arrowhead) as well as rare intraepithelial inflammatory cells are the earliest lesions; C) infected, Day 21, early epithelial erosion with sloughing of degenerated epithelial cells (arrow); D) infected, Day 28, more severe epithelial erosions with extensive intraepithelial inflammatory infiltrates and cellular degeneration (arrowheads); E) infected, Day 28, advanced stage with epithelial cells undergoing degeneration and necrosis throughout all layers of the epidermis (arrowheads); F) infected, Day 35, late stage with massive epithelial ulceration and complete epidermal loss.

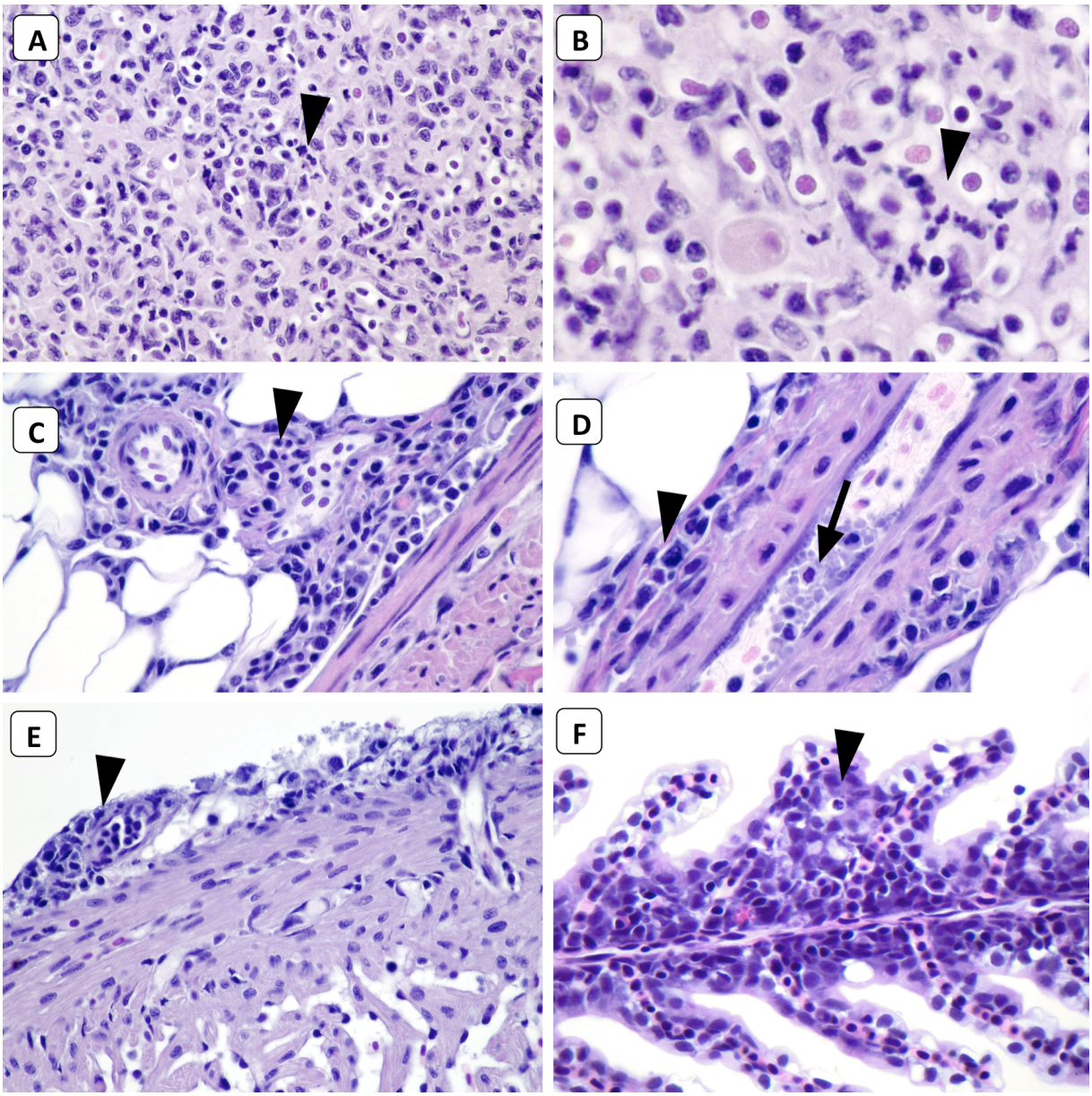

Figure 5.4 Tissues collected from lake trout experimentally infected with EEDV. Magnification 200X (except B; magnification 400X), hematoxylin and eosin staining A) spleen, Day 28, diffuse lymphoid depletion with focal areas of necrosis (arrowhead); B) spleen, Day 28, focal areas of necrosis (arrowhead); C) omentum, Day 28, severe lymphohistiocytic perivasculitis (arrowhead); D) omentum, Day 28, lymphohistiocytic perivasculitis (arrowhead) with intranuclear inclusion body in blood monocyte (arrow); E) heart, Day 28, lymphohistiocytic perivasculitis (arrowhead) and epicarditis; F) gill, Day 28, proliferative branchitis with focal lymphohistiocytic inflammatory cell infiltrate (arrowhead).

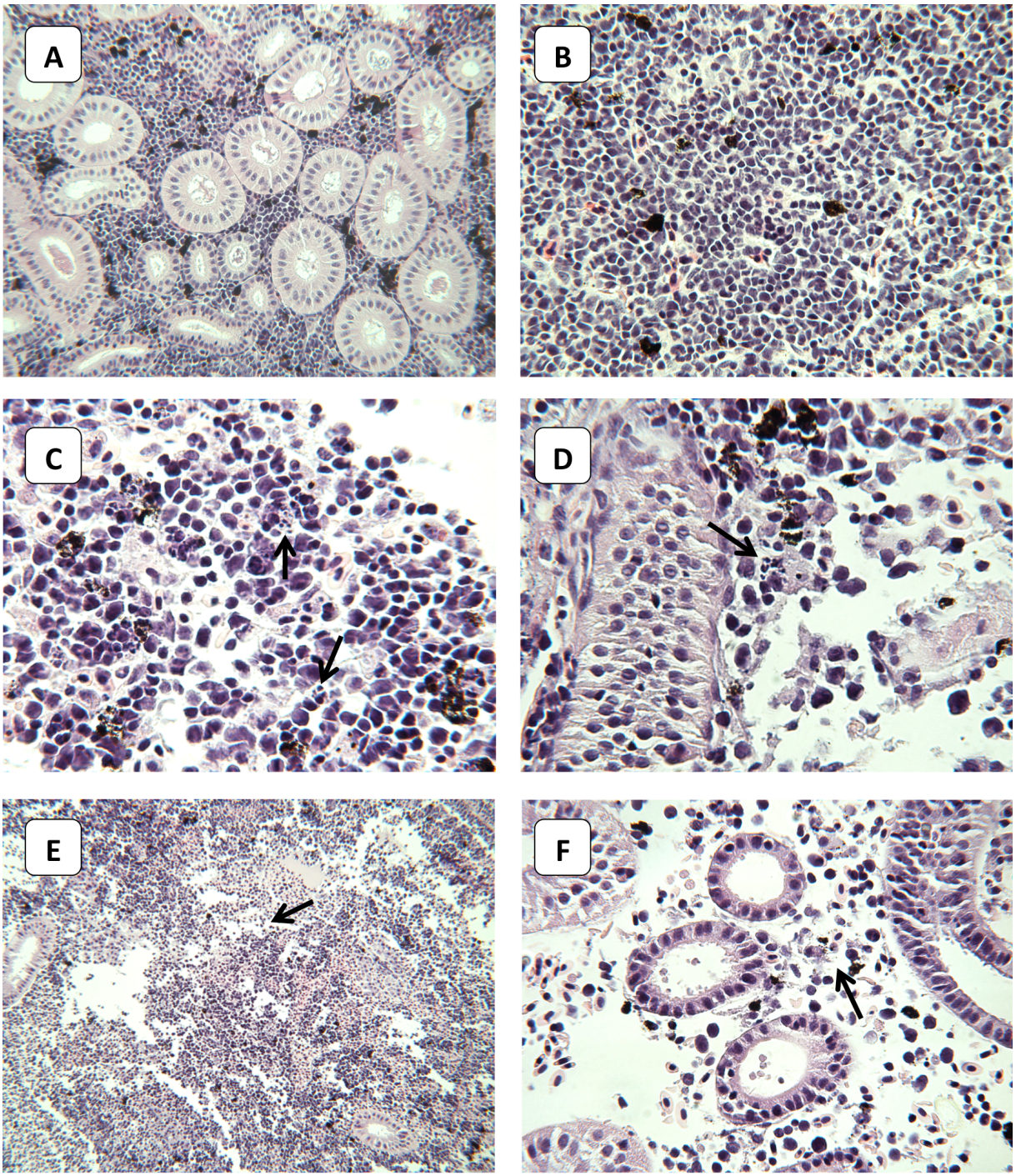

Figure 5.5 Kidney collected from negative control fish and lake trout experimentally infected with EEDV. Magnification 100X (E), 200X (A), 400X (B, C, D, F). A) negative control, Day 35; B) negative control, Day 35; C) infected, Day 28, hematopoietic cellular necrosis and depletion; D) infected, Day 28, hematopoietic cellular necrosis; E) infected, Day 28, hematopoietic cellular depletion; F) infected, Day 28, hematopoietic cellular depletion.

Chapter 6

Establishment and characterization of two novel cell lines from lake trout (*Salvelinus namaycush*)

1. Abstract

In this study, we establish two novel lake trout (*Salvelinus namaycush*) cell lines, produced from yearling gonad tissue and sac fry bodies with species origin confirmed using DNA barcoding. These cell lines were each produced following manual and enzymatic digestion of tissue and incubation at 15°C with Earle's salt-based minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS). Primary cultures of both cell types were readily established and subculturing occurred within 2-4 weeks of initial seeding. Repeated passaging of cells has resulted in gonad cells reaching subculture number 35 and fry cells reaching 52. Additional primary cell cultures were produced from yearling fin and broodstock liver tissues, however these cultures were unable to be reliably subcultured. Morphologically, both fry and gonad cells started out as mixed populations with a substantial percentage of fibroblast-like cells, however as passages went on, cells became increasingly epithelial-like. Infection of novel lake trout cell lines with pathogenic aquatic viruses VHSV, IPNV and EEDV suggested the cells are capable of supporting viral replication. The lake trout is ecologically and economically important in the Great Lakes basin, and the production of novel lake trout cell lines will provide fish health professionals and natural resource managers with an additional diagnostic and research tool.

2. Introduction

The lake trout (*Salvelinus namaycush*) is an extremely important native fish species in the Laurentian Great Lakes Basin (GLB), and in fact, Lake Michigan once held the world's largest population of this species (*13*). This coldwater, apex predator is well adapted to life in the Great Lakes, has a steadying effect on local ecosystems and is prized by the sports and commercial

fishing industries alike (*6*, *13*). Tragically, fishery managers and enthusiasts have been confronted with significant population threats and declining numbers over the past 60 years due to a conglomerate of ecological and anthropogenic factors including invasive species invasion (i.e., sea lamprey (*Petromyzon marinus*) and alewife (*Alosa pseudoharengus*)), habitat destruction, over fishing, and emerging infectious diseases (*7–13*, *95*). Rehabilitation programs focused on the recovery of this important fish rely heavily on the use of captive breeding programs, unfortunately, the intensive nature of salmonid aquaculture serves as a ripe location for the eruption of infectious diseases (*6*, *13*).

Lake trout, particularly in the Great Lakes, are susceptible to a number of viral diseases. In the mid 2000s, Infectious Pancreatic Necrosis Virus (IPNV) was detected in the Allegheny National Fish Hatchery in Warren, Pennsylvania, leading to the culling of all lake trout and brook trout (*Salvelinus fontinalis*) on site (*111*). Lake trout are also especially sensitive to infection with the Epizootic Epitheliotropic Disease Virus (EEDV), which led to the death or culling of more than 15 million lake trout in the mid 1980s (*2*, *4*, *5*) and has recently re-emerged as a pathogen of particular interest in the Great Lakes (*Chapter 2*). Lake trout are also susceptible to the OIE reportable pathogen Viral Hemorrhagic Septicemia Virus (VHSV) which has been detected throughout the Great Lakes basin and led to mortality events in multiple species of fish (*41*, *138*, *139*).

One key aspect of aquatic animal health programs is the diagnosis, prevention and study of viruses such as these, tasks that often utilize cell culture techniques and assays. Cell culture has become ubiquitous in many fields of study, including toxicology, immunology and others (*96*, *97*), and tissue culture *in vitro* models often also serve as an acceptable alternative to whole animal models in research study design (*96*). Unfortunately, while American Type Culture

Collection (ATCC) carries over 3,400 commercially available distinct cell lines, less than 20 of those are derived from fish tissues, leaving many researchers and diagnosticians no choice but to develop their own cell lines for particular projects. The development of primary cell cultures has inherent difficulties as normal somatic cells are not immortal and will eventually trigger senescence if not immortalized (*96*). Additionally, as primary cells are passaged and subcultured, original characteristics may be altered over time, affecting their usefulness to virological, pathological or toxicological studies (*96*). Attempts were made several decades ago following the initial outbreak of EEDV, to produce a cell line of lake trout origin, unfortunately, the cell cultures were not preserved (*15*). To date, there are no established cell lines originating from lake trout tissues. However, primary cultures have been successfully created from rainbow trout gills and head kidney for use in immunology and pharmacology studies (*140*, *141*).

We hypothesized that cell cultures originating from lake trout tissues would demonstrate an increased susceptibility to EEDV as compared to other established salmonid cell lines. In the present study we describe the establishment and characterization of two novel cell lines from lake trout tissues, which can be used in virology, toxicology or immunology studies to improve the health and maintenance of this treasured species.

3. Materials and Methods

3.1. Fish and tissue collection

For this study, three different groups of lake trout were obtained for tissue collection and production of primary cell cultures: 1) a single sexually mature fish (>12 years of age), which had been spawned and housed its entire life at the University Research Containment Facility (Michigan State University, East Lansing, Michigan); 2) yearling fish collected from the

Marquette State Fish Hatchery (MSFH, Marquette, Michigan); and 3) sac fry collected from the Marquette State Fish Hatchery (MSFH, Marquette, Michigan).

The adult lake trout was removed from its holding tank, and euthanized with an overdose of tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.25 mg/mL) prior to tissue collection. After dissection each tissue was placed in sterile cell culture media (MEM-0) of Earle's salt-based minimal essential medium (MEM; Invitrogen, Carlsbad, California) and supplemented with 10% tryptose phosphate broth (BD Biosciences, San Jose, California), 29.2 mg/mL L-glutamine (Invitrogen), penicillin (100 IU/mL) (Invitrogen), streptomycin (0.1 mg/mL) (Invitrogen), amphotericin B (250 μg/mL; Invitrogen) and sodium bicarbonate (7.5% w/v) (Sigma) for temporary holding and to prevent trying during transport between facilities.

Blood was drawn directly from the ventricle following a cut down dissection of the heart and diluted 1:2 with MEM-0 containing heparin at a minimum of 30 IU/mL blood. External tissues (i.e., skin and fin) were briefly flame sterilized to remove external pathogens and then dissected and placed into a solution of MEM-3x, which contained triple antibiotic/antifungal (penicillin (300 IU/mL), streptomycin (0.3 mg/mL) amphotericin B (550 μg/mL)), for 30 minutes, after which time tissue were transferred to MEM-0 while remaining tissues were collected. The opercular cavity was briefly dried with sterile gauze after which an entire gill arch was dissected and placed in MEM-3x for 30 minutes and transferred to MEM-0 for transport. Finally, portions of liver, testes, anterior kidney and posterior kidney were aseptically collected and placed directly into MEM-0.

Tissues were collected similarly from yearling lake trout with the exclusion of blood. For lake trout sac fry, fish were dissected in order to remove the yolk sac and the head (cranial to

opercular margin) with the remaining body processed as a singular tissue sample. Multiple individual fry were processed together until an adequate amount of tissue was collected.

3.2. Isolation and in vitro culture of primary cells

Individual tissues collected from all three groups of fish were transferred to sterile petri dishes where they were manually digested using scissors until they reached a size of approximately 1-2 mm diameter. Next, enzymatic digestion was performed using 0.25% Trypsin-EDTA (Gibco, Life Technologies, Carlsbad, California). Minced tissue was combined with 10 mL trypsin in an Erlenmeyer flask and placed on a stir plate. After three minutes, the initial trypsin was removed and replaced with a fresh 20 mL of trypsin. This solution was left to stir for 20-60 minutes or until tissues visually determined to have reached complete digestion. The tissue-trypsin suspension was then filtered through sterile gauze to remove any remaining tissue pieces, after which, MEM-10 (10% fetal bovine serum; Hyclone Laboratories Inc.) was added to the resulting filtrate at a ratio of 2:1 (medium/trypsin) in order to deactivate the trypsin. The single cell suspension was then centrifuged at 190 x *g* for 5 minutes at 15°C. Following centrifugation the supernatant was discarded and the cell pellet resuspended in 10 mL of MEM-10. This rinsing step was repeated a total of three times. The final cell suspension was seeded into 25cm^2 cell culture flasks (Corning) and incubated at 15°C. This process was repeated for all tissue types with the exception of the blood. Heparinized blood diluted with MEM-0 was centrifuged at 4,700 x *g* for 10 minutes at 4°C. Next, the visible buffy coat plus one half mL red cells were collected, mixed with sterile water 1:1, then washed with MEM-10 and seeded into culture flasks as above.

3.3. Routine subculture

Primary cell flasks were monitored daily for evidence of attachment and replication. Unattached cells and spent media were removed from the flask every 2-3 days, replaced (75%) by fresh MEM-10 and unattached cells checked for viability using Trypan Blue (Sigma-Aldrich, St. Louis, Missouri) exclusion staining. If greater than 20% viable cells were observed in stained sample, remaining cell suspension was pelleted (centrifuged at 190 x *g* for 5 minutes), resuspended in 10 mL of MEM-10, and seeded into a new culture vessel. If no viable cells were detected either attached to the culture surface, or in suspension via exclusion staining, flasks were discarded.

In flasks where primary cell growth occurred, once cultures reached >90% confluence, or if replication rate slowed, they were subcultured per standard laboratory protocols. Growth media was removed from the flask and cells rinsed briefly with 1 mL 0.25% trypsin-EDTA (<10 seconds). After first trypsin rinse was removed, 2 mL fresh trypsin was added to the flask and very gently rocked until cells had released from flask, at which time MEM-10 growth media was added at a ratio of 3:1 (medium/trypsin) in order to deactivate the trypsin. Cell suspension was centrifuged at 190 x *g* for 5 minutes at 15°C after which the supernatant was removed, cell pellet resuspended in MEM-10 and centrifuged a second time. Final cell suspension was reseeded into culture flask with approximately 40-60% of original cells. Subculturing continued in the same manner once flasks reached near 100% confluence with select cultures being cryopreserved in liquid nitrogen (180 µL dimethyl sulfoxide (DMSO) per 1 mL cell suspension).

3.4. Optimization of culture conditions

3.4.1. Influence of temperature on cell growth

The effect of incubation temperature on cell growth was evaluated for the two most promising cell lines: yearling gonad tissue (subculture 4) and fry tissue (subculture 11). Gonad and fry cells were seeded into 12.5 cm^2 culture flasks (CELLTREAT Scientific Products, Pepperell, Massachusetts) at a density of $4x10^5$ and $1.5x10^5$ cells per flask respectively. Flasks were incubated at 15, 21, and 25°C while all other culture conditions remained consistent as described above. Flasks were monitored daily for a subjective assessment of percent confluence. Every 2-5 days, cells were detached using trypsin, from $n = 2$ flasks per day, and counted using a hemacytometer in order to assess relative cell growth and density (cells per cm^2).

3.4.2. Influence of serum type and concentration on cell growth

The effect of serum supplementation type and concentration on cell growth was also evaluated for the same two cell cultures, seeded as with the temperature trial. In this experiment, all flasks were incubated at 15°C but contained growth medium supplemented with either 10% fetal bovine serum (FBS), 15% FBS, or 15% FBS plus 1% heat inactivated lake trout serum. All other culture conditions remained identical. Lake trout serum was collected from mature lake trout such as was used to produce primary cultures and heat inactivated at 56°C for 30 minutes. Flasks were monitored and assessed as described above.

3.4.3. Influence of growth medium base on cell growth

The effect of two different growth medium bases was evaluated for the yearling gonad and fry cell cultures as well. All cells were seeded as above in growth medium containing 15%

FBS and incubated at 15°C. Growth medium was produced using either Earle's salt-based minimal essential medium as described above, or Leibovitz's L-15 medium. All other culture conditions remained identical. Cells were monitored and counted as above.

3.5. Confirmation of species of origin

In order to establish that these novel cell lines were indeed of lake trout origin, a DNA barcoding technique was employed to amplify and sequence the cytochrome *c* oxidase 1 (CO1) gene (*142*, *143*). An early and late passage cell sample from both the fry and yearling gonad cell lines were used, with lake trout skin tissue serving as a positive control and *Epitheliosum papulosum cyprini* (EPC; ATCC) cells as a negative control. DNA extractions were performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions for extractions from cell cultures. All PCR reactions were carried out in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The COI-3 primer cocktail designed by Ivanova et al. (2007) (*142*) was used to amplify a 631 bp fragment of the COI gene. Each 25 µL reaction mixture was comprised of 12.5 µL 2x Go-Taq Green Master Mix (Promega, Madison, Wisconsin, USA), 0.8 µM of each primer, and 4.5 µL DNA template. Cycling parameters were as described by Ivanova et al. (2007) (*142*) for the COI-3 primer cocktail. Amplicons and a 1 kb Plus molecular ladder (Roche Applied Science, Penzberg, Germany) were, electrophoresed through a 2% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) at 100V for 30 minutes, and visualized under ultraviolet light.

Amplicons were prepared for sequencing using ExoSAP-IT (Thermo Fisher Scientific). 1 µL of each PCR product was combined with 3 µL 1x $MgCl_2$ Buffer and 0.25 µL ExoSAP-IT

reagent. The ExoSAP-IT mixture was placed in the thermocycler with a program of 37°C for 20 minutes followed by 95°C for 10 minutes. After clean up, amplicons were Sanger sequenced at the Michigan State University Research Technology Support Facility using M13 forward and reverse primers (*142*). Sequences and chromatograms provided by the Michigan State University Research Technology Support Facility were visually examined using 4Peaks software (http://nucleobytes.com/4peaks/; Version 1.8) and contigs were assembled and aligned using ClustalW in the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0) (*119*). Resulting contigs were then entered into the Barcode of Life Data System (BOLD) (*144*) search function where each sequence was compared against the ID System to identify nearest neighbors using a global alignment of more than 3,000,000 barcode sequences from 180,000 animal species.

3.6. Viral susceptibility

The susceptibility of our newly established lake trout cells to three different aquatic viruses was evaluated. Flasks of fry cell cultures were exposed to isolates of viral hemorrhagic septicemia virus (VHSV), and infectious pancreatic necrosis virus (IPNV), while both fry and gonad cells were inoculated with EEDV. Viral stocks of VHSV and IPNV were inoculated into 25cm^2 flasks of fry cells at and incubated at 15°C. After inoculation, cells were monitored via light microscopy for development of cytopathic effect (CPE) at 48 and 72 hours post infection.

As a current *in vitro* model of replication does not exist for EEDV, our lake trout cells were exposed to virus-positive tissue homogenate supernatant (first passage on cells), followed by a second passage on cells of either first pass supernatant or first pass cells. As optimal

incubation temperature for EEDV *in vitro* is unknown, infected cells were incubated at a range of temperatures (i.e., 4, 9, and 15°C).

3.6.1. Quantification of viral DNA

A TaqMan quantitative PCR (qPCR) described by Glenney et al. (2016) (*14*) was used to compare viral titers in tissue samples to those in cells and supernatant following inoculation of lake trout cells. In this manner, a relative increase in viral loads would suggest replication by active virus rather than merely the presence of viral genetic material. For DNA extractions, the MagMax^{TM} 96 Viral RNA isolation kit (Life Technologies, Grand Island, New York, USA) was used manually, following manufacturer's instructions, after which, extracted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with qPCR protocols as previously described (*14*).

4. Results

4.1. Primary culture and routine subculture

Out of the eight tissues collected from the lake trout broodstock, within two days of seeding, attached cells were observed from the gills (few, <1% confluence), and the liver (moderate number, ~5% confluence). No attached cells were observed in the skin, testes, anterior kidney, posterior kidney, fin or blood cultures and flasks were discarded on Day 5 post-seeding. The flasks of gill cells improved to contain a few small clusters of attached cells on Day 3, however by Day 5, almost all of these had detached and on Day 8 the flasks were discarded. The

number of attached liver cells decreased between days 3 and 5, at which time a media change was performed. By the following day, small clusters of cells had begun to develop (Figure 6.1a). The number and size of these small clusters increased through Day 10, however by Day 15 the cells were beginning to detach, and the flask was subcultured. Following subculture, the flask was monitored, with media changes performed once weekly until the flask reached 50% confluence around Day 85 at which time a second subculture was performed. A total of 9 subcultures were performed before cell growth began to significantly decrease, with the flask reaching 100% confluence (Figure 6.1b) within an average of 3-4 weeks from subcultures 3-8.

Of the tissues collected from yearling lake trout, no cell attachment or growth was observed from either the anterior or posterior kidney. Occasional attached cells were observed over the first few days following seeding of the skin, gill and liver tissues, however only a single liver flask produced replicating cells, and these ceased to grow following the second subculture. Fin cells proceeded to grow (Figure 6.2) and reached 90% confluence by Day 20, were successfully subcultured and again reached 100% confluence in a second 20 days, however following the second subculture, no growth was recovered. Yearling gonad cells on the other hand had a moderate number of attached cells and a few small clusters by Day 2 after primary seeding (Figure 6.3a) with large areas of up to 50% confluence by Day 3 (Figure 6.3b). Subculturing occurred as early as two weeks after primary seeding with subsequent passages occurring approximately every month for the first five months and every 1-2 weeks after that (Figure 6.3c). To date, gonad cells have reached 35 subcultures and continue to grow (Figure 6.3d).

Primary cultures established from fry tissues (Figure 6.4a) reached 50% confluence within the first 3 days after seeding (albeit with patchy growth), and 75-100% confluence by Day

14 (Figure 6.4b) at which time they were subcultured. The next 3 subcultures occurred 3-4 weeks apart (flasks reaching 75-80% confluence), and after the 4th subculture the flasks were reaching 100% confluence within 2 weeks (Figure 6.4c). In later passages (e.g., >20), the fry cells could be subcultured weekly. Fry cells have been successfully cultured out to 52 subcultures (Figure 6.4d).

4.2. Optimization of culture conditions

4.2.1. Influence of temperature on cell growth

Both fry and gonad cells grew extremely poorly at 25°C (Figure 6.5, light grey lines). While some level of growth was achieved in both cell types at 21 and 15°C the trend was for best growth at the coldest temperature (although statistical strength is low due to the size). In fact, in the fry, both the percent confluence as well as the number of cells per cm^2 was higher at all time points beyond 3 days post seeding. As such, all further growth of lake trout cells was performed at 15°C.

4.2.2. Influence of serum type and concentration on cell growth

When comparing the three different serum supplement concentrations, it was clear that a 15% FBS concentration was preferred over 10% FBS (Figure 6.6). When grown in media containing only 10% FBS, fry cells only reached a final percent confluence level of just under 30%, while the other two trials resulted in more than 80% confluence by the end of two weeks. A similar although less defined trend was observed in the gonad cells. The addition of the lake trout serum did not appear to have a positive affect on cell growth and in fact, for both the fry cells and the gonad cells, the ultimate percent confluence was approximately 20% lower in the flasks

receiving media with the lake trout serum. As such, 15% FBS was used in all future medium preparations.

4.2.3. Influence of growth medium base on cell growth

A comparison of the two main growth medium bases (i.e., MEM vs. L-15) showed mixed results. While 100% confluence was achieved in the fry cells with both media types (Figure 6.7a), relatively poor growth was observed in the gonad cells grown in MEM (Figure 6.7b), which was uncharacteristic compared to all previous gonad cell growth. In spite of the inconsistencies in this single trial, all cells continued to be grown in MEM rather than changing to L-15.

4.3. Morphologic characteristics

Primary cultures of the gonad cells (Figure 6.3a, 6.3b) displayed fibroblast-like morphologic characteristics. Cells appeared to be bipolar with a length > 2x cell width. However, by the 6^{th} subculture, a more mixed population of fibroblast-like and epithelioid cell were observed, with the epithelial-like cells appearing more polygonal and in discrete patches between the other cells. This trend toward an epithelial-like cell morphology continued through the later subcultures as pictured in Figure 6.3d.

Morphologically, the fry cells appeared to be a mixture of fibroblast-like and epithelial-like cells in the primary cultures (Figure 6.4a, 6.4b). However, through passages, they became consistently more epithelioid with regular dimensions growing in discrete patches.

4.4. Confirmation of species of origin

Origin of both fry and gonad cell cultures was verified through DNA barcoding. Resulting barcode sequences for fry cells (early and late subcultures), gonad cells (early and late subcultures), lake trout skin and EPC cells were entered into the BOLD ID System. This analysis returned a 100% probability that all four lake trout cell cultures and the lake trout skin tissue were in fact lake trout (*Salvelinus namaycush*) while the EPC cells were confirmed to be of fathead minnow (*Pimephales promelas*) origin.

4.5. Virus susceptibility

Clear changes were observed in the fry cells infected with both VHSV and IPNV within 48 hours post infection (Figure 6.8). Fry cells began to round, shrink and release from the growing surface, disrupting the monolayer. This lysis and rounding of cells is comparable with the typical CPE seen in EPC cells infected with VHSV.

Both gonad and fry cells were infected with various samples known to be EEDV-positive. While cytological changes were observed following initial inoculation, including cell rounding (Figure 6.9a, 6.9b), piling of cells, vacuolation (Figure 6.9c), and mild areas of cell lysis (Figure 6.9b, 6.9d), upon subsequent passages such changes were no longer observed. In order to account for the potential necessity of cell to cell contact for *in vitro* infection as is seen with Marek's Disease Virus (*145*) EEDV infectivity trials were completed using both supernatant and cell suspensions for second passages. However, while mild cytotoxicity was observed from the previous cells being introduced onto new cultures, no overt CPE was observed. Cellular changes were more severe when infected flasks incubated at 9°C than at 4°C or 15°C.

The viral loads from four separate infectivity trials with fry cells are highlighted in Figure 6.10. This data highlights how the viral load detected from fry cells or supernatant following infection with EEDV tissue homogenate is consistently substantially lower than in the original samples. The exception was in the fourth group where it appeared there might in fact be some viral replication (evidenced by an increase in viral load from sample to P1 supernatant), however upon passage to a P2 using both supernatant and cells, once again viral titers decreased substantially.

5. Discussion

Cell lines originating from aquatic species are important for a variety of scientific fields, but unfortunately, the number of well established fish cell lines is substantially lacking compared to that of mammals. In this study, we established primary cultures of multiple different lake trout tissues, and subcultured, expanded and characterized two: one from yearling gonads and one from fry. As demonstrated by Hedrick et al. (1991), there are times when situations require the availability of host species specific cell lines, such as was the case when in the 1980s significant mortalities were seen in juvenile white sturgeon (*Acipenser transmontanus*) caused by what was later determined to be three separate viruses (an adenovirus, an iridovirus, and a herpesvirus), but at the time were unculturable in the only two established sturgeon cell lines, originating from different species (*98*).

The methods described herein resulted in the creation of primary cell cultures from all three age groups of lake trout tested: liver from the broodstock, fin and gonads from the yearling fish and whole sac fry. While neither the yearling fin nor the adult liver cells survived beyond 10 subcultures, the establishment of primary cultures from both of these tissues indicates that they

remain potential options for future studies utilizing primary cultures rather than established cell lines. Both yearling gonad and fry cells on the other hand readily established monolayers and produced stable subcultures. These cells were subcultured with relative ease, suggesting that they will be suitable for use in standard laboratory assays.

We determined that both gonad and fry cells were well adapted for growth in either MEM or L-15 growth media, supplemented with 15% fetal bovine serum and incubated at 15°C. These conditions are comparable to those required by other salmonid cell lines (*97*), however differ slightly from those used in previous attempts at producing lake trout cell cultures which grew best at a higher temperature (18-21°C) (*15*).

Morphologically, in early passages, all cultures contained mixed populations of cells, both epithelial- and fibroblast-like. This was particularly clear in the fry cells, which was not surprising as these cells originated from whole body tissues as opposed to a single organ. However, as passage number increased, proportions of fibroblast-like and epithelioid cells changed with both cell types becoming more epithelial-like. In many individual flasks, it became clear that with mixed cell populations, the fibroblast-like cells were out competing the epithelioid cells, however through regular subculturing and splitting of flasks, certain cultures of epithelial-like fry cells were able to prevail.

A crucial component of cell line characterization is definitive identification of species origin. Historically, methods such as karyotyping, and isoenzyme analysis have been popular (*146*), however recent advancements in molecular diagnostics have helped cement a new protocol for cell line species identification, DNA Barcoding, which has been used successfully to determine the species of origin of a wide range of cell lines from all animal kingdoms (*143*). By sequencing a stretch of the cytochrome *c* oxidase 1 (COI) gene in early and late passages from

both the fry and gonad cell lines, and comparing the sequence to the BOLD database, we established that these cells were indeed of lake trout origin.

Following inoculation of fry cells with VHSV and IPNV we demonstrated the development of cytopathic effect (CPE) including rounding and lysis of cells (Figure 8). This is crucial, as it suggesting the susceptibility of lake trout cells to two key lake trout pathogens in the Great Lakes Basin. If further investigation reveals that these cultures are truly capable of becoming infected with VHSV and IPNV, the have the potential to serve as a diagnostic tool in the detection and identification of these important aquatic pathogens. Inoculation of both fry and gonad cells with EEDV resulted in the development of mild, inconsistent CPE that included some lysis and rounding of cells, as well as vacuolation of cells exposed to the virus, indicating a decreased health of the cells. However, qPCR data indicates a substantial difference in viral titers in tissue samples compared to that recovered from cell cultures. With the exception of one inoculation on fry cells (trial number 4 in Figure 6.10), the identification of EEDV in these cell cultures is consistent with the detection of genetic material rather than active and replicating virus. While these results with EEDV are inconclusive, and do no fully support our hypothesis, we have demonstrated the ability to produce lake trout cell cultures and further attempts can be made with additional cell types and culture conditions to improve changes at supporting EEDV replication *in vitro*.

The lake trout is a commercially, recreationally and ecologically important fish species throughout the Great Lakes Basin (GLB). Unfortunately, lake trout populations in the Midwestern United Stated have faced continued threats due to invasive species, overfishing and infectious diseases throughout the past half century (*6*, *13*). In order to combat these threats to species rehabilitation, we have established two novel cell lines of lake trout origin that have the

potential to be used in future diagnostic assays and research studies on viral diseases of lake trout as well as other fields such as genetics, toxicology and medicine.

APPENDIX

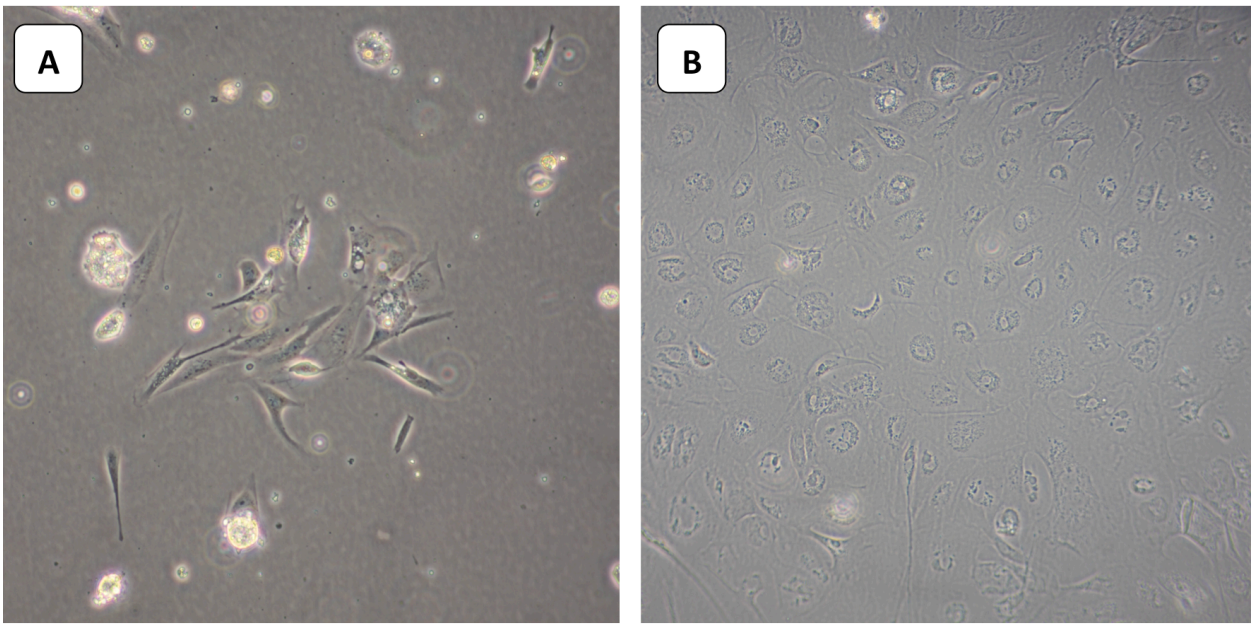

Figure 6.1 Liver cells cultured from an adult lake trout (*Salvelinus namaycush*). A) primary culture, 6 days after seeding; B) 6th subculture, confluent.

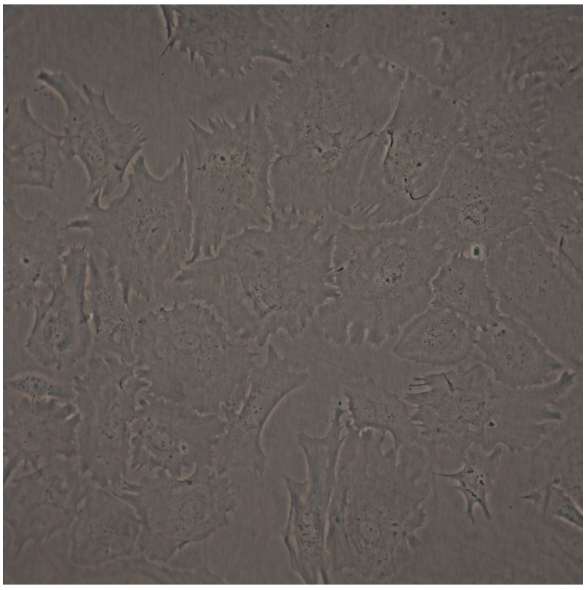

Figure 6.2 Fin cells cultured from yearling lake trout (*Salvelinus namaycush*), primary culture.

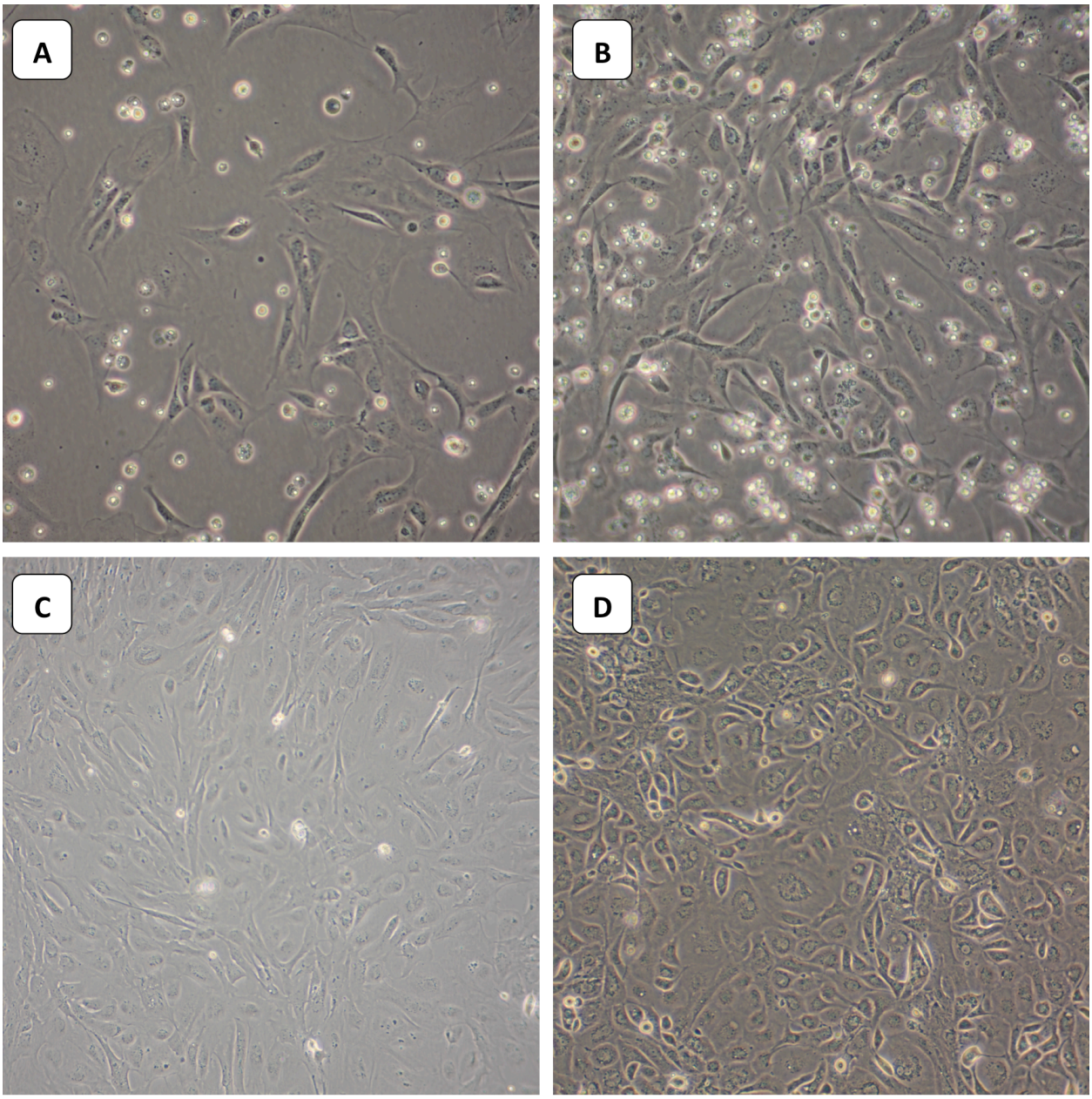

Figure 6.3 Gonad cells cultured from yearling lake trout (*Salvelinus namaycush*). A) primary culture, 3 days after seeding; B) primary culture, 5 days after seeding, ready for subculture; C) 6th subculture; D) 32nd subculture.

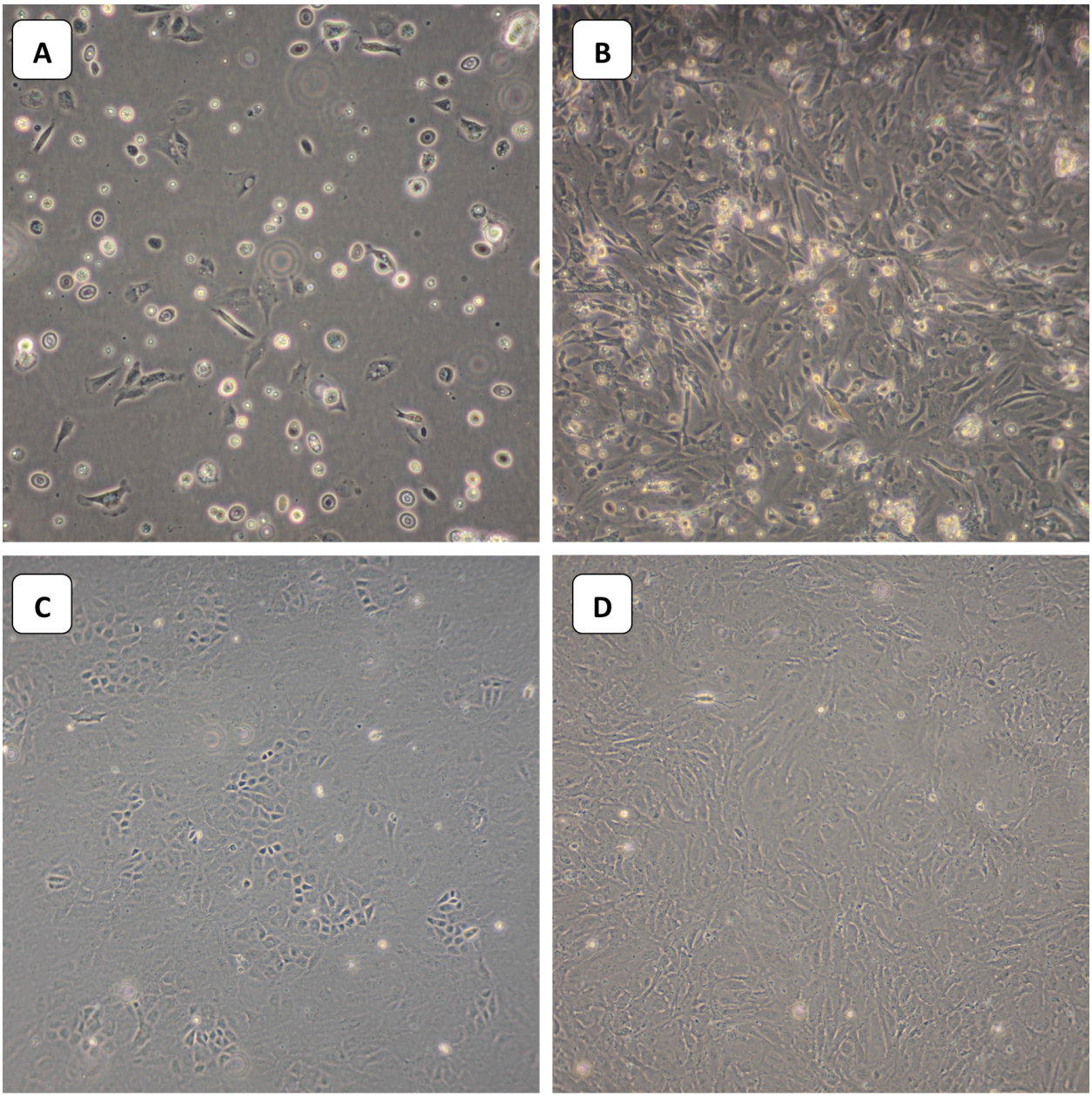

Figure 6.4 Cell cultures established from body tissue of lake trout (*Salvelinus namaycush*) sac fry. A) primary culture, 2 days after seeding; B) primary culture, ready for subculture; C) 14th subculture; D) 47th subculture.

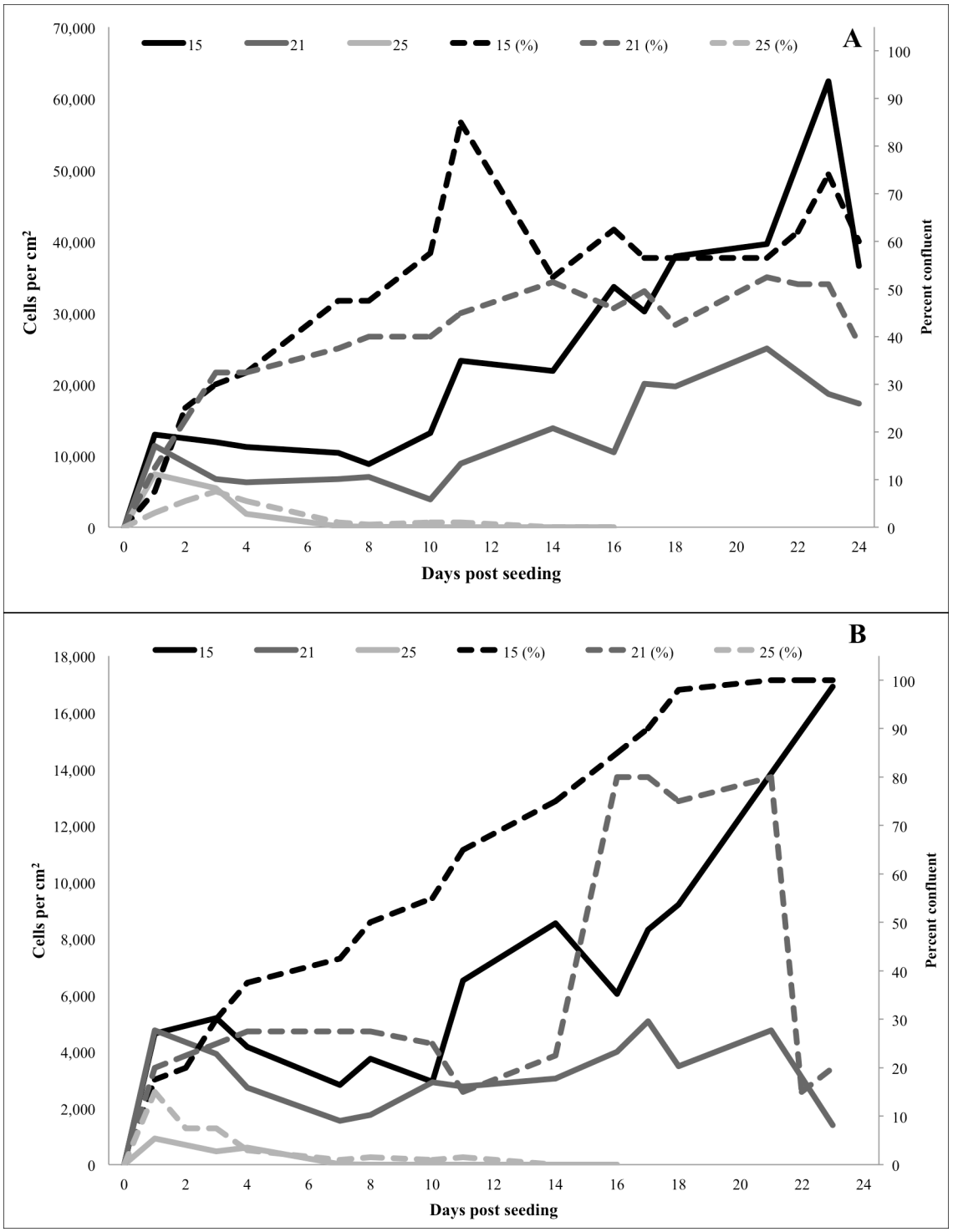

Figure 6.5 Influence of incubation temperature on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

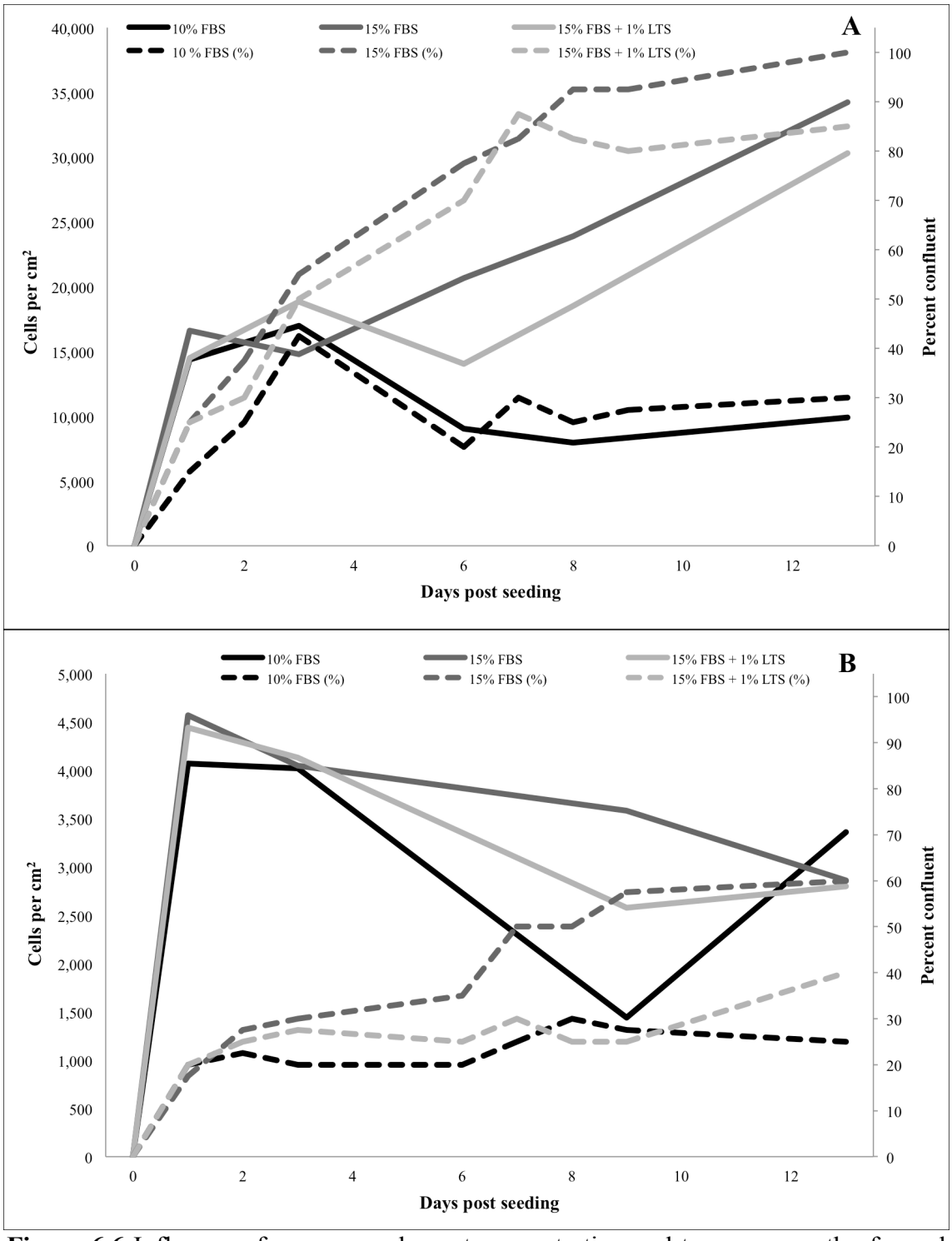

Figure 6.6 Influence of serum supplement concentration and type on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

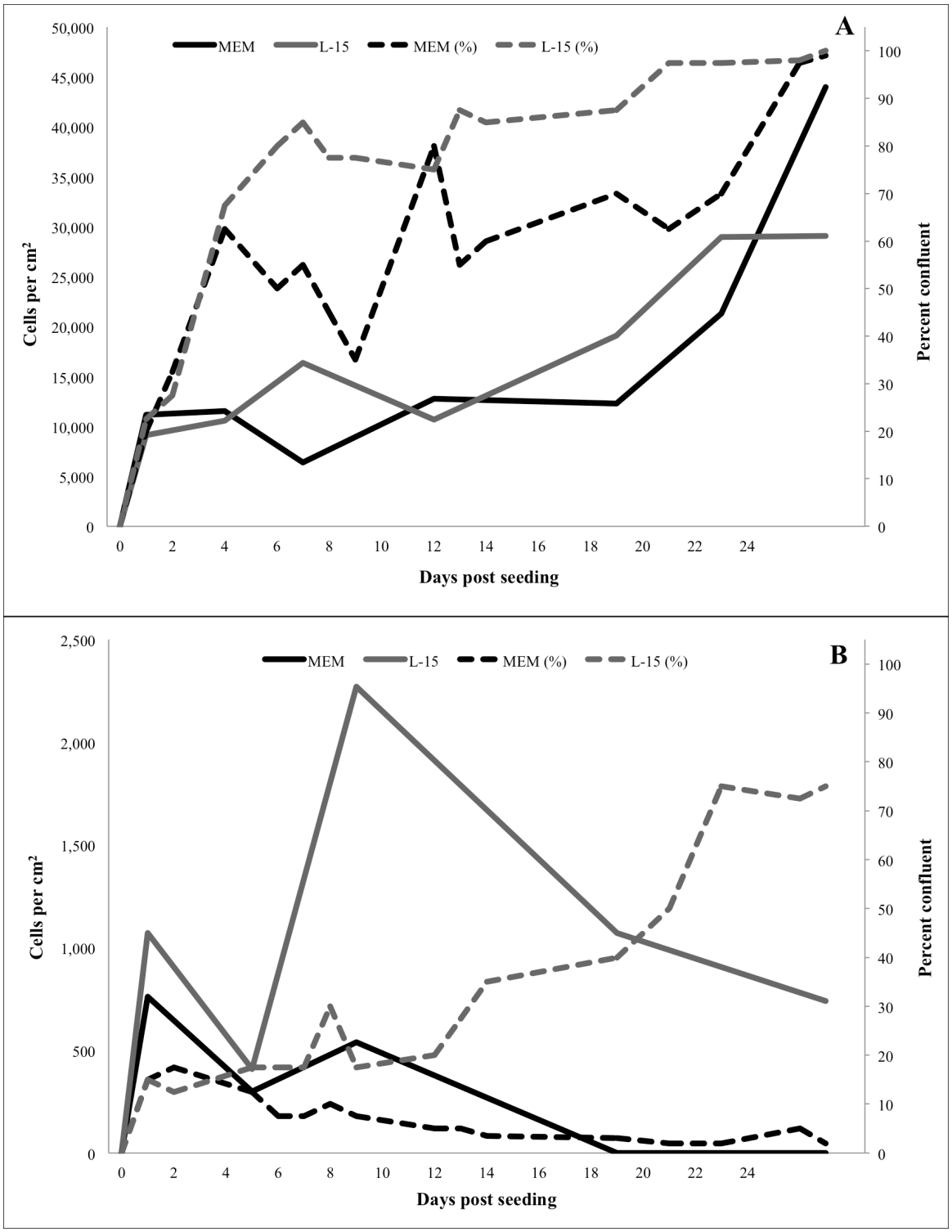

Figure 6.7 Influence of growth medium base on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

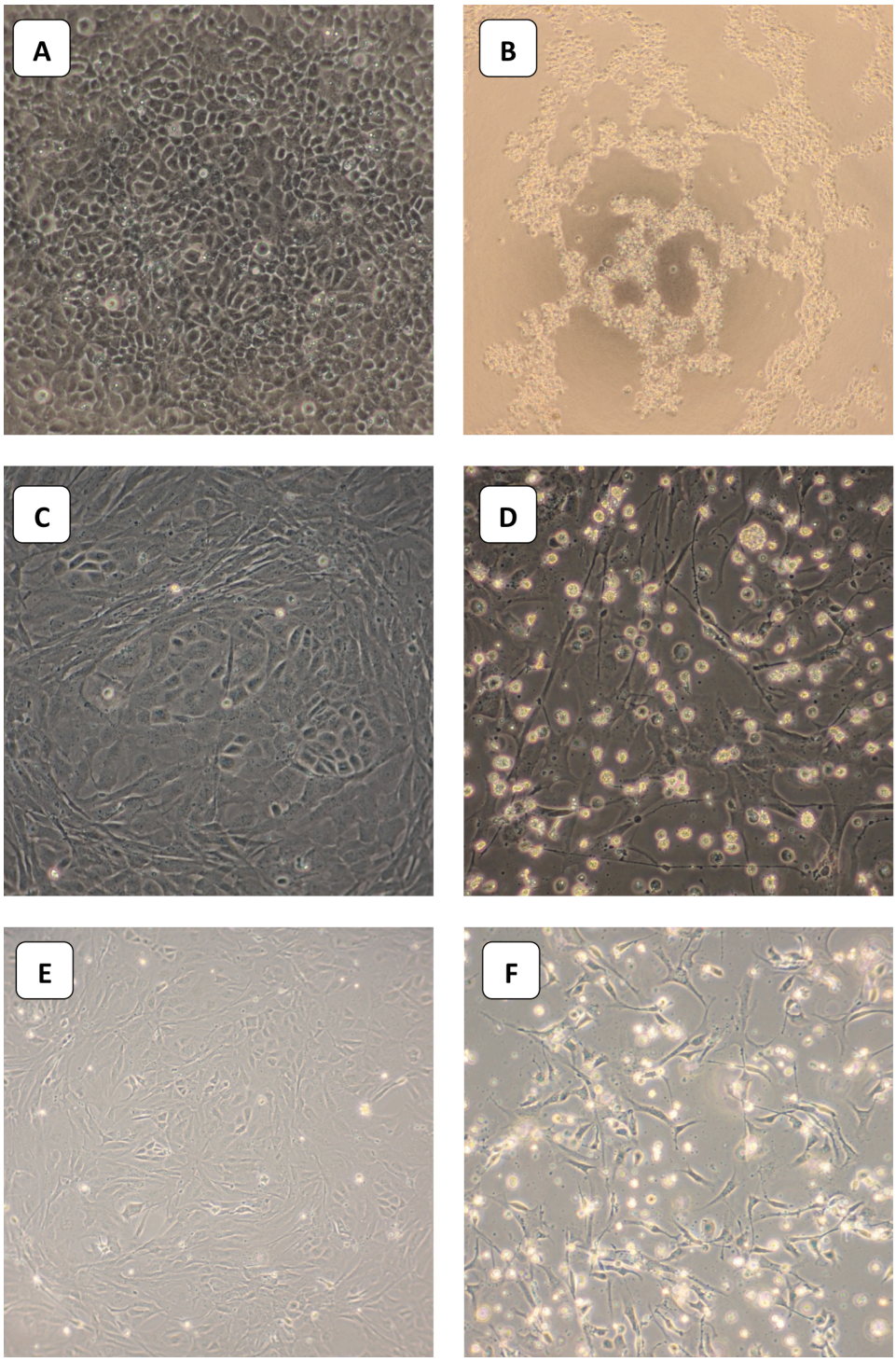

Figure 6.8 Cell cultures infected with VHSV or IPNV. A) EPC negative control; B) EPC cells infected with VHSV, displaying typical CPE; C) fry cell negative control; D) fry cells infected with VHSV, cell lysis; E) fry cell negative control; F) fry cells infected with IPNV, cell lysis.

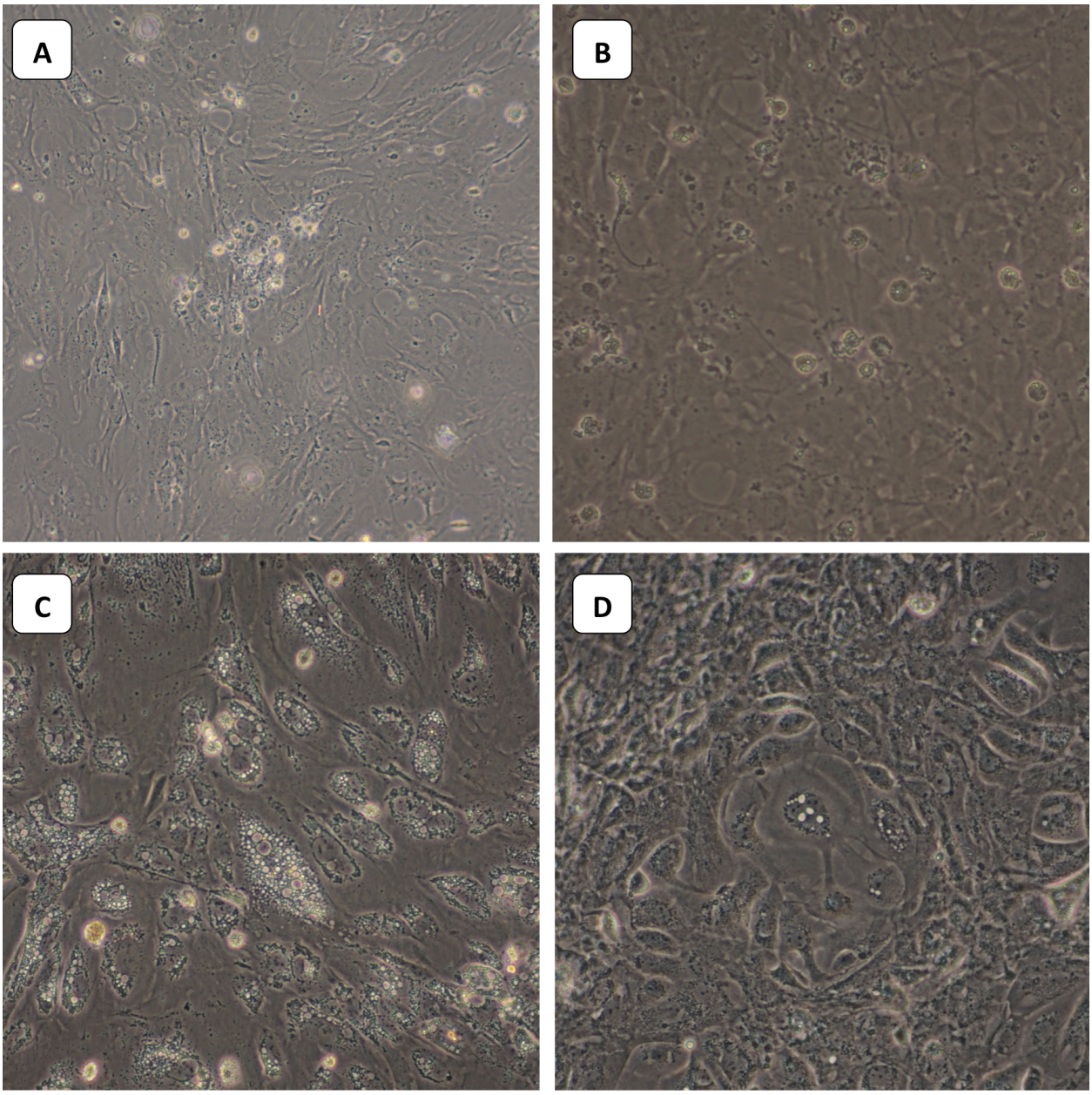

Figure 6.9 Lake trout cells inoculated with EEDV. A) fry cells (47th subculture), cell rounding; B) fry cells (47th subculture), cell rounding, some lysis; C) gonad cells (32nd subculture), vacuolation, some rounding; D) gonad cells (32nd subculture), vacuolation, early lysis.

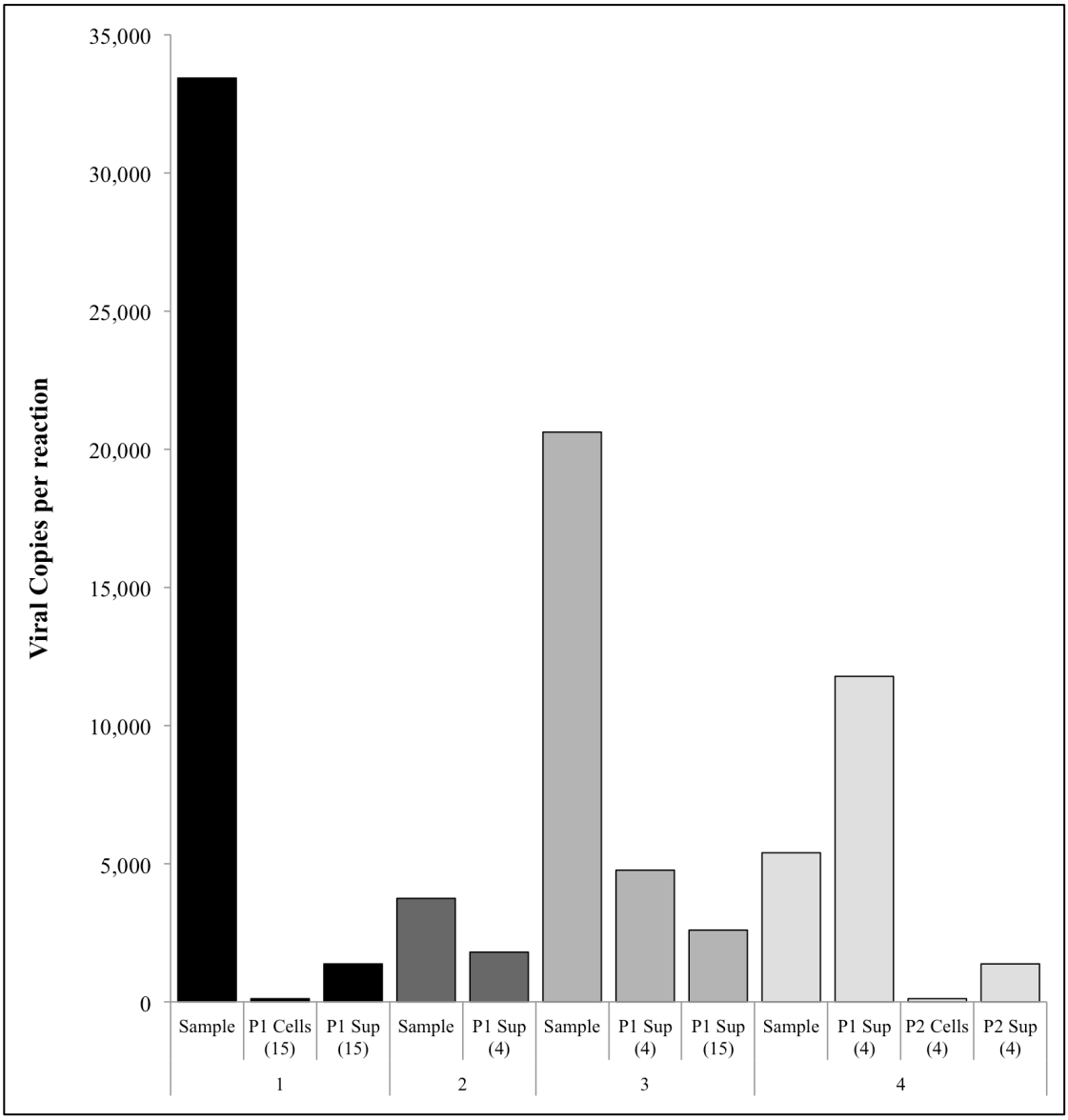

Figure 6.10 Relative viral loads following inoculation of lake trout fry cells with EEDV positive tissue sample homogenate; represented as number of viral copies per qPCR reaction. P1 = 1st pass infection, P2 = 2nd pass infection, Cells = cell pellet tested for presence of EEDV, Sup = flask supernatant tested for presence of EEDV. Number in parentheses indicates temperature of incubation. Numbers at bottom indicate separate infection trials, but have no sequential significance.

Chapter 7

Development of a loop-mediated isothermal amplification (LAMP) assay for the detection and quantification of Epizootic Epitheliotropic Disease Virus (Salmonid Herpesvirus-3) in lake trout (*Salvelinus namaycush*)

1. Abstract

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) causes a serious disease of lake trout (*Salvelinus namaycush*) that threatens the restoration efforts of this species in North America. The current inability to replicate EEDV *in vitro* necessitates the search for a reproducible, sensitive, and specific diagnostic assay that allows for accurate diagnosis that is both time and cost effective. Herein, we describe a loop-mediated isothermal amplification (LAMP) assay that we developed for the rapid and quantifiable detection of EEDV in infected fish tissues. The newly developed LAMP reaction was optimized in the presence of calcein, and the best results were produced using 2 mM $MgCl_2$, 1.8 mM dNTPs and an incubation temperature of 67.1ºC. The analytical sensitivity of the LAMP method was estimated to be as low as 7.8 pg extracted DNA from lake trout tissues. The diagnostic sensitivity and specificity of the newly developed LAMP assay compared to the SYBR Green qPCR assay were 84.3% and 93.3%, respectively. The quantitative LAMP for EEDV had a high correlation coefficient (R^2 = 0.990), and when compared to the SYBR Green quantitative PCR for validation, no statistical difference found between the two assays ($p > 0.05$). Thus, it is anticipated that the developed LAMP and quantitative LAMP methods will be instrumental in the future reliable diagnosis of EEDV.

2. Introduction

Viruses in the *Alloherpesviridae* family (Order *Herpesvirales*) cause a variety of diseases in amphibians and teleost fish, often with severe economic consequences (*45*, *147*). Within the *Alloherpesviridae* is the genus Salmonivirus, which currently contains five viruses: the Salmonid Herpesvirus-1 (Herpesvirus salmonis), Salmonid Herpesvirus-2 (*Oncorhynchus masou* virus),

Salmonid Herpesvirus-3 (Epizootic Epitheliotropic Disease Virus; EEDV), Salmonid Herpesvirus-4 (Atlantic salmon papillomatosis virus), and Salmonid Herpesvirus-5 (Namaycush herpesvirus) (*90*, *94*, *148*).

Among the five salmonid herpesviruses, EEDV causes one of the more lethal diseases in its host, leading, for example, to the morality of over 15 million hatchery-reared juvenile lake trout in the early 1980s (*2–4*). Recently, after 30 years of minimal mortalities associated with EEDV, the virus reappeared in Wisconsin and Michigan hatcheries, resulting in morbidity and mortality in hundreds of thousands of lake trout (*5*) (*Chapter 2*). In the absence of other available control measures to combat this virus, the implementation of stringent biosecurity measures and use of avoidance strategies remain our only tools to prevent EEDV spread to additional lake trout rearing units or facilities should another outbreak occur. A sensitive and specific diagnostic tool that is rapid and reasonably inexpensive is needed in order to perform testing of wild gamete donor fish as well as periodic testing of hatchery-reared fish throughout their growth. Early detection of EEDV prior to the start of a mortality episode, would allow for more rapid disease control and perhaps prevention of such devastating losses as previously seen.

Endpoint and quantitative PCR-based detection assays for EEDV have been developed that target stretches of the EEDV terminase gene (*5*, *14*). After the molecular characterization of Salmonid Herpesvirus-4 and -5, it was determined however, that the current EEDV qPCR assay was unable to distinguish between Salmonid Herpesvirus-3, -4, and -5, as the viruses share high sequence identity in the terminase gene. This led Glenney et al. (2016) (*14*) to design three primer sets based on the glycoprotein gene; and using a SYBR Green qPCR assay, was able to amplify each virus individually. Herein, we report on the development of a loop-mediated isothermal amplification (LAMP) assay for the detection and quantification of EEDV in infected

lake trout tissues, which we hypothesized would be faster, more cost effective and yet equally as specific and sensitive as previously established assays.

3. Materials and Methods

3.1. Virus and template DNA

Tissues used in this study for the development and testing of the EEDV LAMP assay were obtained from juvenile naïve lake trout experimentally infected with EEDV-positive tissue homogenate by either intraperitoneal injection or immersion bath (*Chapter 3*).

For the purpose of this study, tissues of selected fish were collected, and enzymatically digested with Proteinase K. Viral DNA extractions were performed manually using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) after tissue digestion (24). Following all nucleic acid extractions, DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA) and diluted to a standard concentration using nuclease free water.

3.2. Primers and LAMP design

A partial sequence of the Salmonid Herpesvirus-3 glycoptorein gene (GenBank accession number JX886027.1) was used as a template to design the EEDV LAMP primer set with the Primer Explorer software, version 4.0 (http://primerexplorer.jp/elamp4.0.0/index.html). The details of the primers are displayed in Table 7.1. Following alignment of the EEDV primer target sequences on the glycoprotein gene with the same segment of Salmonid Herpesvirus-4

(GenBank accession number JX886028) and Salmonid Herpesvirus-5 (GenBank accession number KP686091), the *in silico* analysis guided the selection of primer sets that are strictly specific to Salmonid Herepesvirus-3 and hence used in this study.

The LAMP reaction was carried out in a 25 µL reaction mixture containing 1.6 µM of each of the forward inner primer (FIP) and backward inner primer (BIP); 0.8 µM of each of the LF and LB primers; 0.2 µM of each of the F3 and B3 primers; 1X isothermal amplification buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8); 2 mM $MgCl_2$; 1 M betaine; 1.6 mM deoxynucleoside triphosphates (dNTPs); 0.2 mM $MnCl_2$; 20 µM calcein; 8 U *Bst* DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA) and 1 µL template DNA. Calcein was used as a fluorescent indicator which yields strong fluorescence by forming complexes with divalent magnesium ions in LAMP reactions as reported by Tomita et al. (2008) (*149*).

The mixture was incubated for 50 minutes (one cycle per minute) in an Eppendorf mastercycler real/plex ep gradient S PCR machine (Eppendorf, Hauppauge, New York). Changes in fluorescence were monitored every min at 520 nm. Three separate assay factors were optimized: 1) temperature, assay run at 58.3, 60.3, 62.6, 64.9, 67.1, 69.1, and 70.7ºC, (each followed by 80°C for 20 min to terminate the reaction); 2) $MgCl_2$ concentration; and 3) dNTP concentration. $MgCl_2$ and dNTP concentrations were optimized by Taguchi's L16 (2(4)) orthogonal design with two elements (dNTPs and $MgCl_2$) at four concentration levels (Table 7.2).

3.3. Analytical specificity of the EEDV LAMP assay

The specificity of the LAMP primer set was tested by performing the assay under the optimized conditions as described above. Nucleic acids were extracted from a number of DNA and RNA fish pathogenic viruses such as Salmonid Herpesvirus-1, -2, -4, and -5, Infectious Pancreatic Necrosis Virus (IPNV), Spring Viremia of Carp Virus (SVCV), Infectious Hematopoietic Necrosis Virus (IHNV), Golden Shiner Reovirus (GSRV), Fathead Minnow Nidovirus, and Viral Hemorrhagic Septicemia Virus (VHSV) and used as templates in this analysis. Additionally, the 212 bp target sequences of glycoprotein gene of Salmonid Herpesvirus-3, -4, and -5 were aligned and compared using BLAST and the software BioEdit 7.0.

3.4. Analytical sensitivity of the EEDV LAMP assay

The detection limit of the EEDV LAMP assay was analyzed with two kinds of templates. One template was a plasmid vector (pCR®2.1-TOPO®) containing the target fragment from the EEDV glycoprotein gene (designated as pCR®2.1-EEDV). A 10-fold serial dilution of plasmid pCR®2.1-EEDV ($1.6x10^7$–10^1 copies) was used as the template for the LAMP under the predetermined conditions. The other template was gill tissue DNA extracted from infected lake trout and serially diluted ($7.8x10^6$–10^0 pg).

3.5. Quantitative EEDV LAMP assay

A quantitative LAMP assay was produced by using ten-fold dilutions of purified PCR product as standards. The end-point PCR assay for production of quantification standards consisted of a 50 µL reaction containing 25 µL GoTaq Green Mastermix, 0.25 µM each of F3

and B3 primers and 80 ng DNA template. The PCR reaction was 95ºC for 2 minutes followed by 40 cycles of 95ºC for 15 seconds, 50ºC for 15 seconds and 72ºC for 45 seconds and finished with a single cycle of 95ºC for 15 minutes. PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and copy number in each 10-fold dilution was calculated as described above for the plasmid.

For real-time monitoring, the qLAMP reactions were incubated at 67.1ºC for 50 cycles (one minute per cycle) with an Eppendorf realplex 2 (Eppendorf). For quantitative detection of samples, a standard curve was generated for EEDV qLAMP by plotting a graph between different concentrations of standards ranging from 10^1 to 10^7 copies to cycle threshold (*Ct*) value through real-time monitoring of the amplification.

3.6. Evaluation of the EEDV LAMP assay on samples

In order to validate the quantitative abilities of the EEDV LAMP assay, a group of 100 previously tested lake trout tissue samples with known viral load ranges (i.e., negative, low, medium, or high titers) were chosen in order to test a comprehensive range of virus loads in tissue. All samples came from experimentally infected or control group fish (*Chapter 3*). DNA was extracted from these tissue samples using the BioOregon kit described above after which the qLAMP was run in parallel with the SYBR Green qPCR assay as described by Glenney et al. (2016) (*14*). Resulting copy numbers from qLAMP and qPCR were analyzed using a paired t test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute Inc.).

The diagnostic sensitivity (DSe) and specificity (DSp), as defined by the World Organization for Animal Health (2011), of the qLAMP compared to the qPCR were calculated according to Zhang et al. (2013) (*42*, *150*).

4. Results

4.1. Optimization of the EEDV LAMP reaction

In order to determine the optimal reaction conditions, the LAMP assay was carried out for 50 minutes at 7 temperatures. As displayed in Table 7.2, the smallest average *Ct* value (17.35) was achieved when the reaction was incubated at 67.1ºC and resulted in a relatively small standard error of *Ct* value (0.45) compared to other incubation temperatures.

Concerning the optimization of $MgCl_2$ and dNTPs, the results indicated that the smallest average *Ct* value (17.19) was produced when the concentrations of $MgCl_2$ and dNTPs were 2.0 mM and 1.8 mM, respectively (Table 7.3). The smallest average *Ct* value was accompanied by a standard error of 0.34, indicating negligible fluctuation of amplification efficiency. Meanwhile, the second smallest *Ct* value (17.66) resulted in a higher standard error of 1.05, and was produced when the concentration of $MgCl_2$ and dNTP were 2.0 mM and 1.6 mM, respectively. Therefore, the optimal concentrations of $MgCl_2$ and dNTP were determined to be 2.0 mM and 1.8 mM, respectively. Based on these results, further LAMP assays were incubated for a total of 50 min at 67.1 ºC with 2 mM $MgCl_2$ and 1.8 mM dNTPs.

4.2. Analytical specificity of the EEDV LAMP assay

Alignment of the EEDV LAMP target sequence (212 bp) with the corresponding sequences from the closely related Salmonid Herpesvirus-4 and -5 indicated that the eight EEDV LAMP primers covered 35 or more mutation sites in the corresponding sequences of the other two Salmonid Herpesviruses (Figure 7.1). Positive results were obtained only when the template used contained the DNA from EEDV-infected fish tissue; no amplification was observed for the DNA or RNA extracted from stocks of Salmonid Herpesviruses-1, -2, -5, or -5, IPNV, SVCV,

IHNV, GSRV, Nidovirus or VHSV samples (Figure 7.2). Taken together, these results indicate that the LAMP primer set is specific for amplification of EEDV nucleic acid.

4.3. Analytical sensitivity of the EEDV LAMP assay

When the reaction was tested using 1 µL of 10-fold serial dilutions of plasmid pCR®2.1-EEDV DNA (7.2 ng/µL, equivalent to $1.6x10^9$ copies/µL), the analytical sensitivity of the EEDV-LAMP method was estimated to be as low as 16 copies of the plasmid per reaction while becoming more sporadic below 16 copies per reaction (Figure 7.3). When the reactions were tested using 1 µL of 10-fold serial dilutions of EEDV positive DNA from lake trout, the analytical sensitivities of the LAMP method were determined as 78 pg of DNA extracted from gill tissues (Figure 7.4).

4.4. Quantitative EEDV LAMP and validation against SYBR Green qPCR

DNA from 100 tissue samples collected from experimentally challenged lake trout were used to compare the newly developed qLAMP assay with the SYBR Green qPCR currently in use (*14*). A high correlation coefficient ($r^2 = 0.990$) was obtained by the EEDV qLAMP when the initial template was above 1000 copies (Figure 7.3). Quantification of viral copies in the experimental samples was extrapolated based on the *Ct* value of DNA samples using the generated standard curve. Positive qPCR samples ranged from 10.0 to $1.69x10^8$ copies per reaction while positive qLAMP samples ranged from 4.18 to $6.89x10^7$ copies per reaction (Table 7.4). Statistical analysis comparing the paired samples using a paired t test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute Inc.) revealed no significant difference between the quantifications recovered via the two assays ($p > 0.05$).

The qPCR results indicated that 70/100 samples were positive for EEDV. The qLAMP agreed that 59 of those qPCR positives were positive again. Meanwhile, of the 30 qPCR negative samples, the qLAMP agreed that 28 of those were negative again. Therefore, the DSe and DSp values for the qLAMP method compared to the SYBR Green qPCR method were 84.3% and 93.3% respectively.

5. Discussion

In light of the current absence of a cell line that can support the replication of EEDV, diagnostic tools are limited to endpoint PCR (*5*), real-time PCR (*14*), or electron microscopy (*3*). In the current study, we developed a time and cost effective LAMP assay for EEDV detection. This method amplifies EEDV DNA in fish tissue with high specificity and sensitivity, and therefore, represents a valuable diagnostic tool for the detection and quantification of this deadly virus.

The optimal reaction temperature was determined to be 67.1°C which is relatively higher than the optimal LAMP reaction temperatures reported for other viruses such as 62°C for the orf virus (*151*), 63°C for human papillomavirus (*152*), and 64°C for nervous necrosis virus (*153*). This anomaly could be explained due to the use of different primer sets for different viruses. Actually, the results of the temperature optimization showed that *Bst* DNA polymerase effectively amplified the nucleic acid templates at a relatively wide temperature range from 62.6 to 69.1ºC, which should greatly benefit the possible application of the method under field conditions. The *Ct* value of samples tested using the EEDV LAMP assay showed substantial variation when the concentration of $MgCl_2$ changed from 2 mM to 6 mM, and also when the

concentration of dNTPs changed from 1.2 mM to 1.4 mM, both of which are indications that the concentration of $MgCl_2$ and dNTPs are critical parameters in the EEDV LAMP reaction.

Testing the analytical specificity of the EEDV LAMP clearly demonstrated that amplification occurred only when DNA from EEDV was used as a template; no amplification occurred with the other fish pathogenic DNA viruses including the other closely related Salmonid Herpesviruses-4 and -5. The fact that the EEDV LAMP primers designed in this study cover gene stretches with greater than 35 mutation sites compared to the corresponding sequence stretch of Salmonid Herpesvirus-4 and -5, and did not cross react, attests to the high specificity of this newly developed assay for detection of EEDV.

The analytical sensitivity of the EEDV LAMP assay was determined to be 7.8 pg total DNA extracted from EEDV-positive lake trout gills, which is considerably higher than those reported by Chen et al. (2010) (*154*) for the swine transmissible gastroenteritis coronavirus, Li and Ling (2014) (*155*) for the tomato necrotic stunt virus, and Ma et al. (2016) (*156*) for the Eriocheir sinensis reovirus.

A standard curve was constructed using serial 10-fold dilutions of the pCR®2.1-EEDV plasmid with reference to Ct value. Based on the standard curve, an equation was calculated using regression analysis comparing Ct value to the standard copy number. In the range of 10^7 to10^3 plasmid copies, the correlation coefficient was high ($r^2 = 0.990$), which indicates that the LAMP is appropriate as a quantification tool. However, when numbers of plasmid decrease to less than 1000 copies, the correlation coefficient declines significantly (data not shown). Previous reports also demonstrated that it is difficult to determine the exact correlation of virus quantity and Ct value at very low concentrations of template (*157*, *158*).

When the developed EEDV LAMP assay was compared to the real-time SYBR Green qPCR (*14*), the diagnostic specificity was greater than 90%, however the diagnostic sensitivity was only 84.3%. While the qPCR identified 11 samples as positive that the qLAMP did not, all but two of them were less than 1,000 copies and as indicated above, accurate quantification below this level can be difficult.

When the viral loads determined by qLAMP were compared to those of the SYBR Green qPCR, both assays were capable of quantifying viral loads over a wide range (Table 7.4). While there were some discrepancies with identification of individual positive tissues between the two assays, when all samples were examined together, the paired t test demonstrated no significant difference between the results of the two different assays ($p > 0.05$). In total, these quantification results lend further support to the use of this qLAMP assay as a diagnostic tool, both in the laboratory and in field conditions.

In summary, a specific, sensitive LAMP assay was developed for the detection of EEDV in fish tissues, supporting our hypothesis. This novel assay has the advantage of being rapid and is promising to be an ideal surveillance tool for identification of EEDV. Moreover, the qLAMP established in this study provides a low-cost quantification method for EEDV loads in tissue samples, and the use of calcein as a fluorescent indicator, which can also be visualized by the naked eye, or under a UV light, provides a good platform for optimization of an assay that can be used in field conditions, such as at a fish hatchery.

APPENDIX

Primer	*Sequence*
F3	GGGGAGAGATCCCAGGTTC
B3	CGTGCTCAAATGGTTCACTG
FIP (F1c+TTTT+F2)	GCTCTCCGTGTCCCAACTGGTTTTTGAACGAGCGTCAACAGTG
BIP (B1c+TTTT+B2)	ACTTGGAGAAAATCAAGCGCGCTTTTCCAGCTCCATGTCCATCGA
LF	CCTCAAAGACGGTCTGGCAA
LB	TTTCGAGGAATACAGGATCACCT

Table 7.1 Primers used for EEDV loop-mediated isothermal amplification.

Temperature	**Primer set III**	
	Mean* of *Ct* value	SD* of *Ct* value
58.3	27.07	0.28
60.3	24.13	0.01
62.6	18.99	0.01
64.9	18.41	0.09
67.1	17.35	0.45
69.1	18.22	1.22
70.7	24.40	1.15

Table 7.2 Results of EEDV LAMP temperature optimization. Mean and standard deviation produced from duplicate repeats of LAMP assay.

$MgCl_2$ concentration	**dNTP** concentration	**Primer set**	
		Mean* of *Ct* value	SD* of *Ct* value
2mM	1.2 mM	21.39	0.74
2mM	1.4 mM	18.50	0.39
2mM	1.6 mM	17.66	1.05
2mM	1.8 mM	**17.19**	**0.34**
4mM	1.2 mM	36.93	0.68
4mM	1.4 mM	32.52	1.13
4mM	1.6 mM	28.24	1.00
4mM	1.8 mM	27.03	0.93
6mM	1.2 mM	-	-
6mM	1.4 mM	47.51	0.60
6mM	1.6 mM	45.02	1.15
6mM	1.8 mM	40.63	1.32
8mM	1.2 mM	-	-
8mM	1.4 mM	-	-
8mM	1.6 mM	-	-
8mM	1.8 mM	-	-

Table 7.3 Results of $MgCl_2$ and dNTP concentration optimization for EEDV LAMP. Mean and standard deviation produced from duplicate repeats of LAMP assay.

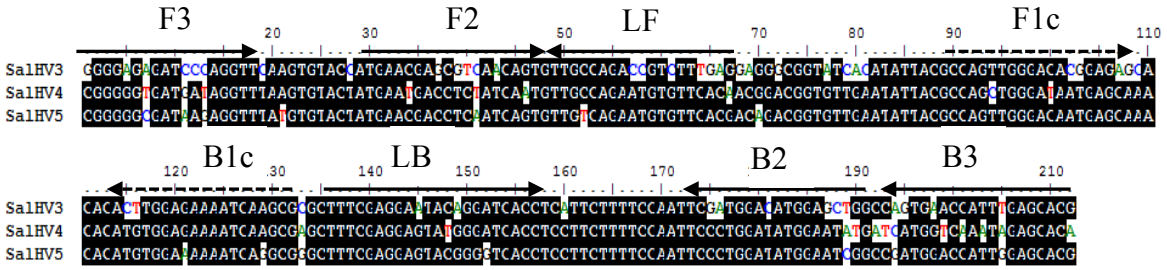

Figure 7.1 Alignments of the EEDV (Salmonid Herpesvirus-3) target gene region (GenBank JX886027) with the most related sequences of viruses available in GenBank including Atlantic salmon papillomatosis virus (Salmonid Herpesvirus-4; JX886028) and Namaycush herpesvirus (Salmonid Herpesvirus-5; KP686091). Notice that the eight EEDV LAMP primers cover 35 or more mutation sites in the corresponding sequences of the other two SalHV strains. F: forward primer, B: backward primer, LF: loop-forward primer, LB: loop-backward primer.

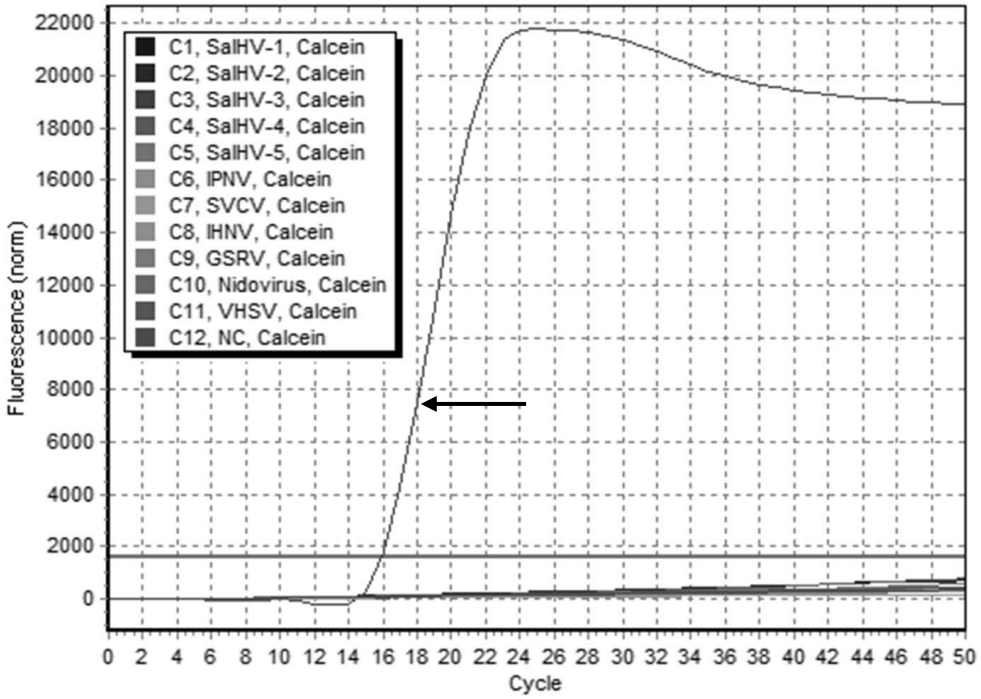

Figure 7.2 Ability of the EEDV LAMP assay to discriminate EEDV from other viruses (i.e., analytical specificity). The amplification plot of EEDV is indicated by the arrow and appears as expected.

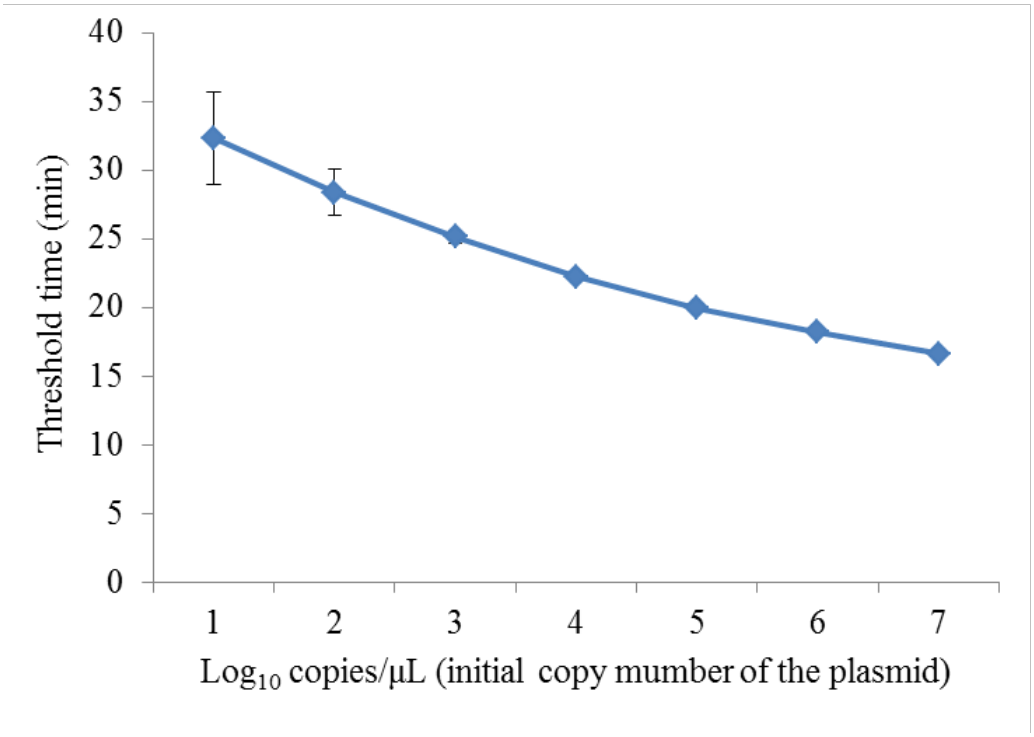

Figure 7.3 Analytical sensitivity shown in the standard curve generated from the amplification plots of the quantitative LAMP (qLAMP) assay for known concentrations of EEDV glycoprotein gene plasmid (pCR®2.1-EEDV plasmid). Plasmid was serially diluted 10-fold from $1.6x10^{7}$ to $1.6x10^{1}$ copies/reaction over three replicates.

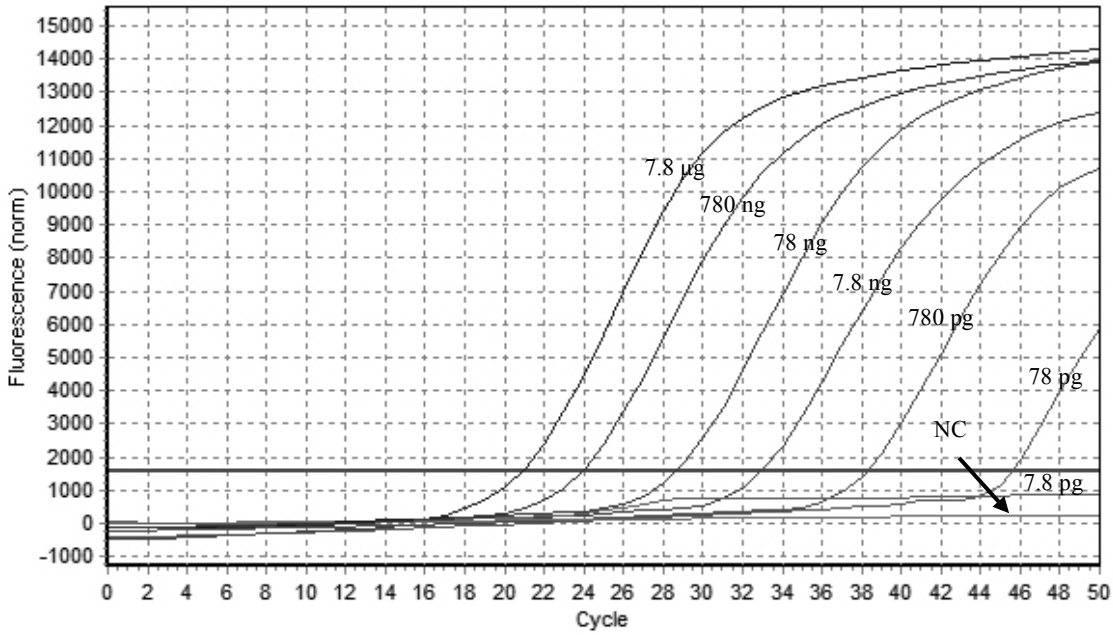

Figure 7.4 Analytical sensitivity or limits of detection of EEDV-positive lake trout gill DNA by the diagnostic LAMP assay for EEDV. Amplification plots 1–7 (from left to right): reaction conducted using 10-fold serial dilutions of DNA from lake trout: $7.8x10^6$, $7.8x10^5$, $7.8x10^4$, $7.8x10^3$, $7.8x10^2$, $7.8x10^1$, and 7.8 pg, respectively. Amplification plot 8 was the negative control (NC).

#	qPCR	qLAMP	#	qPCR	qLAMP	#	qPCR	qLAMP
1	-	-	35	$1.79x10^4$	$7.54x10^4$	69	$3.47x10^5$	$2.16x10^5$
2	-	-	36	960	820	70	$2.07x10^5$	$1.71x10^5$
3	-	-	37	122	-	71	$6.38x10^4$	$5.48x10^4$
4	-	-	38	-	-	72	$1.05x10^4$	$4.94x10^3$
5	-	-	39	159	-	73	$9.24x10^3$	635
6	-	-	40	$1.86x10^3$	$9.66x10^3$	74	$1.86x10^4$	$8.25x10^3$
7	-	-	41	347	-	75	$2.49x10^5$	$2.52x10^5$
8	-	-	42	$1.40x10^4$	$8.60x10^4$	76	$2.30x10^3$	144
9	-	-	43	$3.00x10^5$	$1.03x10^6$	77	$3.30x10^4$	$1.04x10^4$
10	-	-	44	$1.60x10^4$	$4.96x10^4$	78	$3.09x10^6$	$6.45x10^6$
11	-	-	45	$3.63x10^5$	$6.96x10^5$	79	$7.71x10^6$	$1.27x10^7$
12	-	-	46	$1.80x10^3$	$3.42x10^3$	80	$6.62x10^6$	$2.97x10^6$
13	-	-	47	220	-	81	$9.44x10^7$	$6.11x10^7$
14	-	-	48	495	$3.04x10^3$	82	$2.47x10^7$	$2.01x10^7$
15	-	-	49	527	95.3	83	$1.83x10^7$	$2.59x10^7$
16	-	-	50	$1.40x10^3$	$5.40x10^3$	84	$1.23x10^7$	$1.45x10^7$
17	-	-	51	$4.50x10^3$	579	85	$7.12x10^7$	$4.31x10^7$
18	-	-	52	937	4.18	86	$6.74x10^7$	$5.13x10^7$
19	-	-	53	$3.13x10^3$	267	87	$2.60x10^7$	$1.53x10^7$
20	-	-	54	-	566	88	$1.69x10^8$	$6.89x10^7$
21	-	-	55	$1.95x10^3$	119	89	$3.14x10^7$	$3.49x10^7$
22	-	$2.54x10^3$	56	825	-	90	$1.47x10^7$	$4.18x10^7$
23	202	-	57	$1.34x10^3$	283	91	$1.84x10^7$	$1.62x10^7$
24	256	-	58	$4.02x10^3$	205	92	$1.73x10^7$	$1.37x10^7$
25	166	-	59	$2.18x10^3$	-	93	$1.47x10^7$	$1.23x10^7$
26	-	-	60	$1.38x10^3$	-	94	$2.71x10^7$	$2.87x10^7$
27	-	-	61	$1.62x10^6$	$1.86x10^6$	95	$2.15x10^7$	$6.48x10^6$
28	-	-	62	$1.20x10^6$	$1.41x10^6$	96	$7.40x10^6$	$4.42x10^6$
29	-	-	63	$2.22x10^5$	$1.53x10^5$	97	$5.55x10^6$	$4.11x10^6$
30	84.9	-	64	$1.83x10^6$	$3.17x10^6$	98	$1.58x10^7$	$4.34x10^6$
31	102	18.3	65	$1.69x10^6$	$1.78x10^6$	99	$1.12x10^7$	$4.99x10^6$
32	-	-	66	$1.64x10^6$	$2.80x10^6$	100	$7.02x10^6$	$1.44x10^6$
33	-	-	67	$5.93x10^5$	$8.43x10^5$			
34	$1.41x10^3$	$1.14x10^4$	68	$3.44x10^4$	$1.53x10^3$			

Table 7.4 Comparison of SYBR Green qPCR assay (*14*) results and newly developed qLAMP assay results performed on 100 experimental samples of lake trout skin tissue. Data is presented as viral copies per reaction (50 ng template DNA added to each reaction, qPCR and qLAMP). There was no statistical difference between qPCR and qLAMP quantification ($p > 0.05$) using a paired t test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute Inc.).

Chapter 8

Conclusions and future research

1. Conclusions

While Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) was first identified as a serious pathogen of lake trout (*Salvelinus namaycush*) more than 30 years ago (*3*), and has been the causative agent in numerous mass mortality events in the Midwestern United States throughout the time since (*2*, *5*) (*Chapter 2*), there are many details of its pathogenesis and biology that remain unknown. Further, much of what we do know about this virus is either based on research from 30 years ago, or from retrospective case reports (*3–5*, *14*).

Herein I have presented work that serves to satisfy some of these unanswered questions as well as to set up a platform for future research. My work focused on understanding the epidemiological factors influencing a natural epizootic, developing a standardized experimental model, uncovering the sequential pathology and distribution of EEDV in its host from infection to death, and finally 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. The lake trout is an extremely important natural resource in the Great Lakes basin, prized by the fishing industry as well as for its intrinsic value as a native apex predator (*6*). Rehabilitation and management of this valuable species remain threatened today due to the continued presence of EEDV within hatchery-reared lake trout populations. Chapter 1 highlights not only what we currently know about EEDV, but also where the knowledge gaps remain.

In order to begin the discussion as to how to prevent future outbreaks of EEDV, we must first understand what led to the initiation and spread of past epizootics. In Chapter 2, I describe 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, Chapter 2 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.

Chapter 3 addresses the problem that in order to study this deadly virus, we need understand the dose dependent effects of EEDV infection and determine whether we are capable of reproducing clinical disease in a controlled laboratory environment. In this chapter, I answer two questions: 1) what is the necessary viral load or dose required to cause clinical disease? 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. Based on my results, I demonstrated that exposure to a viral dose of greater than or equal to $4.7x10^4$ viral copies per mL immersion bath water leads to development of clinical disease consistent with that seen in natural 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 I was able to reproduce clinical disease in experimentally challenged lake trout, however there may be additional factors such as individual fish variability and external stressors in a hatchery environment that contribute to development of disease.

Armed with a model of experimental disease challenge I was able to move onto my next two chapters, which address the temporal changes following an EEDV infection. In Chapter 4, I examined the sequential distribution of EEDV following viral exposure, in order to identify specific target tissues and cells. A quantitative PCR assay (*14*) 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. This study serves to widen our knowledge on the pathogenesis of EEDV, and identify specific viral targets throughout the course of disease. I show that the virus is first detectible in the eye, skin and fins and that consistently external tissues carry a higher viral load than visceral tissues. The

establishment of epithelial tissues as primary viral targets helps to not only better understand host-virus interactions, but also to guide the choice of diagnostic and surveillance tissues.

In Chapter 5, I present specific gross and histopathologic lesions throughout an EEDV infection. Primary lesions were observed in the skin and fins and correlated with qPCR data from the previous chapter 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. While 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. While I was unable to identify a specific point of entry, the severity of total epithelial damage of the skin by the end of the study likely resulted in hypo-osmotic shock and death. A final interesting finding form Chapters 4 and 5 is that I identified extremely high viral loads in tissues collected from fish that were only mildly affected grossly and microscopically. This highlights the importance of screening at risk populations of fish, as there is the potential for survivors to become a source of future exposure.

In Chapter 6, I shift my focus toward improving research and diagnostic tools and describe the development of two novel cell lines of lake trout origin. I established primary cell cultures from multiple lake trout tissues, and successfully expanded and subcultured cells from yearling gonads and sac fry bodies out beyond 35 and 50 subcultures respectively. Optimal growth conditions for these cells were established, and DNA barcoding was used to prove lake trout as the species of origin. Additionally, I demonstrated that these cells were capable of developing cytopathic effect following exposure to multiple aquatic 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.

Finally, in Chapter 7 I discuss the development of a novel molecular assay for quantitative diagnosis of EEDV. While endpoint and quantitative PCR assays have previously been developed, Loop Mediated Isothermal Amplification (LAMP) assays have several benefits including being both cost and time efficient as well as having the potential for commercialization and use in field conditions. I present a new quantitative method capable of amplifying EEDV DNA in fish tissue with relatively high specificity and sensitivity. There was no statistical difference between the viral loads obtained from this qLAMP and those of the SYBR Green qPCR (*14*).

The culmination of this dissertation shows that EEDV remains a significant threat to lake trout populations in the Great Lakes basin, but we are now better armed to combat its spread than ever before. Epidemiological analysis of the two recent EEDV outbreaks (as presented in Chapter 2) has demonstrated that not only is the virus still within the hatchery system, but also that it is capable of causing disease in both juvenile and older fish. In order to identify potential sources of infection before a mortality event erupts, continued surveillance for EEDV within the hatchery should continue among all age groups of lake trout on the property. Early identification of these infected fish would allow for more rapid implementation of control strategies such as decreasing densities (through culling or spreading out of fish lots) or increased biosecurity between infected and non-infected via establishment of a quarantine area. In order to accurately identify infected fish, appropriate diagnostic samples and assays must be run. The work within this dissertation has established that external tissues such as eyes, skin or fins are the primary viral targets, and thus should constitute primary diagnostic samples. Collection and analysis of

these tissues using the recently established qPCR (*14*), or the qLAMP assay described herein, will maximize detection of EEDV-positive fish and allow confidence in negative results.

2. Future Research

In this dissertation I have expanded our knowledge of the pathogenesis of EEDV and developed tools in the form of a disease model and diagnostic assays that can be put to use in future research studies.

While I have established herein that EEDV remains a threat to hatchery-reared lake trout in the Great Lakes basin, one key point that remains to be addressed is the current prevalence of EEDV among both hatchery and wild lake trout populations. Removal of infected individuals from the hatchery system as well as avoidance of gamete collection from bodies of water with high EEDV prevalence in wild fish may aid in the prevention of future mortality events. Comprehensive screening of all broodstock fish prior to the use of gametes for production purposes should continue. Direct molecular assays such as qPCR and qLAMP can be coupled with an ELISA for detection of EEDV antibodies in wild fish. It is clear that EEDV outbreaks are worsened by the relatively high fish densities in hatcheries, and that discovery of a mortality event in wild fish is unlikely, however, the detection of EEDV antibodies in wild fish can help to identify certain bodies of water, or locations where sampling of wild fish for gametes should be avoided. While it is likely that EEDV is fairly ubiquitous in Great Lakes lake trout populations, both hatchery and wild, every effort should be made to prevent the introduction of additional virus.

Next, the definitive identification of susceptible host species and potential viral reservoirs should be determined. The identification of epithelial viral targets including the eye, skin and fin

as described in Chapter 4, allows for more targeted sampling efforts in order to identify these "at-risk" fish populations. Of particular interest are splake (a lake trout x brook trout hybrid) and wild mottled sculpin. Past reports have stated that splake can be experimentally infected with EEDV (*105*), however in neither of the natural outbreaks did we see any evidence of clinical disease in the splake, even when housed directly downstream of diseased lake trout. One particular concern would be the capability of splake to act as a viral reservoir without development of clinical disease, representing a substantial biosecurity concern within hatcheries. The identification of EEDV DNA in wild mottled sculpin following the 2012 mortality event was extremely surprising. Susceptibility trials need to be performed in order to determine whether these findings can be reproduced in a controlled laboratory environment. Additionally, surveillance of the wild fish surrounding the Marquette State Fish Hatchery should continue in order to determine if mottled sculpin remain infected and to identify potential sources of EEDV before the water enters the hatchery.

Based on the history of EEDV outbreaks in hatcheries (*2*, *4*, *5*) (*Chapter 2*), they are most frequently preceded by a stressor event such as alterations in water quality or routine hatchery practices including tagging or transfer to a new raceway. This is a concept that can be put to use in the screening for infected individuals through the use of a stress test (*39*). I would recommend additional experimental studies examining the effect of different stressors both on the severity of disease as well as the eruption of disease in previously infected fish. Identification of stressors most likely to result in resurgence of EEDV can be useful to hatchery managers in planning to minimize stress and prevent disease outbreaks.

With the development of novel lake trout cell cultures, the next task will be to determine the necessary conditions in order to support replication of EEDV. In my dissertation I attempted

only a portion of potential viral culture techniques. Additional methods of viral replication are used to culture avian and mammal herpesviruses such as the cloning of Merek's Disease Virus into a Bac vector (*145*). This type of method requires the full genome sequence of the virus, something we don't yet have for EEDV, however were it to be completed, a method such as is used for Marek's disease, coupled with the established lake trout cells may result in an effective *in vitro* model of EEDV replication.

I would also recommend additional studies aimed at identification of latent infections and carrier hosts. The newly developed *in situ* hybridization assay can be utilized in combination with the experimental model described in this dissertation in order to localize EEDV genetic material in surviving fish. While I have established that the fin, skin and eye are prime viral targets at the start of and throughout a disease outbreak, it is still unknown where or if the EED virus establishes latency. Answering this question will provide an additional level of sensitivity when screening for infected or reservoir fish.

Finally, following the recent natural disease outbreaks as well as experimental challenge, a proportion of exposed or infected lake trout survived. As the immunologic status of these surviving fish is unknown, it would be of interest to experimentally determine if survivor status provides any degree of protection against re-exposure to EEDV. Should susceptibility of surviving fish be lower upon re-exposure to EEDV, and should a measureable and correlative antibody response occur, it could have significant value in terms of understanding the immunologic response to EEDV and vaccine development. Development of a modified live vaccine for Koi Herpesvirus (Salmonid Herpesvirus-3) has proven to be efficacious and safe when administered to koi fish (*Cyprinus carpio koi*) weighing more then 87 grams (*159*).

Preliminary genome sequencing data (unpublished) has identified the presence of potential virulence factor genes, which may serve as logical targets for vaccine design.

In conclusion, the study design and methods described herein can be readily extrapolated in order to address the remaining questions concerning EEDV in lake trout. The results of this dissertation demonstrate the significance of this virus to North American lake trout populations and the need for swift and decisive action toward preventing further losses due to EEDV. These research recommendations, coupled with the results of this dissertation will serve to provide a basis for an action plan regarding EEDV in the Great Lakes basin.

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