ELUCIDATING THE PROPERTIES OF EPIZOOTIC EPITHELIOTROPIC DISEASE VIRUS (SALMONID HERPESVIRUS-3) TRANSMISSION TO FACILITATE IMPROVED DISEASE CONTROL

By

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ABSTRACT

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Epizootic epitheliotropic disease virus (EEDV, Family Alloherpesviridae) emerged in the 1980's as a significant mortality source in hatchery-reared lake trout (LT; *Salvelinus namaycush*) in the Great Lakes basin and continues to impede hatchery-based LT conservation and rehabilitation efforts today. Little is known about EEDV disease ecology, a matter hampering EED prevention/control. To address unknowns surrounding EEDV host range, transmission, and susceptibility to hatchery disinfectants, three studies were conducted. In the first, I clarified the EEDV host range, whereby the virus was detected in LT, splake (LT x brook trout, *Salvelinus fontinalis*), and mottled sculpin (*Cottus bairdii*), but not the other 8 experimentally challenged fish species. Susceptibility to EEDV varied within two LT strains (e.g., Lake Superior and Seneca strains), but the virus was able to replicate to high titers in both, as well as rarely in splake. In contrast, there was no evidence for virus replication in mottled sculpin. In study 2, I examined virus shedding in microchipped and EEDV challenged LT, which showed that shedding into water began at 9 days post-infection (pi), was greatest ~three weeks pi (i.e., $<10^8$ virus copies/fish/hour), and continued for at least 9 weeks pi. In study 3, I found Virkon® Aquatic to be effective at preventing EEDV contagion on hatchery tools at the manufacturer recommended concentration/duration. Collectively, findings provide fishery managers with valuable knowledge on the biological properties of EEDV so that improved strategies can be devised to prevent and control EEDV in Great Lakes hatcheries and beyond.

This thesis is dedicated to my father, mother and my sister for their pray, love and support that always become my spirit.

I also dedicate this work to all my teachers and friends who supported, mentored and helped me very patiently to face with the challenges of graduate school life.

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INTRODUCTION

The lake trout (*Salvelinus namaycush*) is an important native fish in the North American Laurentian Great Lakes. As an apex predator, lake trout is important for the stability of the Great Lakes' ecosystem (Lepak et al., 2006) and is considered an indicator of its health status (Edwards et al., 1990). Lake trout is also a popular recreational fish species in the United States of America (USA). Historically, lake trout faced near extinction in the 1950's due to multiple factors, including the invasion of the sea lamprey (*Petromyzon marinus*) and overfishing activities (Lawrie and Rahrer, 1972). Stock enhancement efforts being performed by the departments of natural resources in the Great Lakes states that involve stocking of millions of hatchery-raised juvenile lake trout in the basin allowed lake trout population to reach safe levels and, in some instances, self-sustaining populations were attained. Catch surveys also showed an upward trend in Lake Huron lake trout with fish age often reaching 22 years (Riley et al., 2007; He et al., 2012; Johnson et al., 2015; MDNR, 2016). Fish health status is one of the limiting factors that can impinge on the quality and survival of stocked fish in rehabilitation programs. Several diseases that devastate hatchery-reared lake trout in hatcheries or even after stocking in the Great Lakes basin have been described, including early mortality syndrome (EMS) (Wolgamood et al., 2005), *Flavobacterium psychrophilum*, epizootic epitheliotropic disease virus (EEDV) infections, infectious pancreatic necrosis virus (IPNV) infections, and bacterial kidney disease (BKD; Faisal et al., 2013). In particular, EEDV has caused the demise of millions of hatchery-reared lake trout in the 1980s and 2010s (Bradley et al., 1988; Shavalier, 2017). This herpesvirus cannot propagate in any of the primary tissue cultures or established fish cell lines and therefore, most of its biological characteristics remain largely unknown (McAllister and Herman, 1989). Knowledge of the biological characteristics of the virus is necessary for the development of effective control strategies. To this end, I chose to investigate three important

properties of this virus; 1) its host range, 2) its ability to be shed from infected fish into the surrounding environment, and 3) its sensitivity to Virkon®, a commonly used disinfectant in aquaculture worldwide.

1. Herpesviruses

Herpesviruses are a very large group of viruses, ubiquitous in the terrestrial and aquatic environments, and are recently grouped in the order Herpesvirales (Davison et al., 2009). Herpesviruses infect an array of organisms, including mollusks, fish, reptiles, birds and mammals, including humans (reviewed in Wozniakowski and Salamonowicz, 2015). To date, there are more than 100 herpesviruses identified within the order Herpesvirales, which are divided into three families, three subfamilies, and 19 genera, according to the International Committee on Taxonomy of Viruses (Davison et al., 2009; ICTV, 2011). The revised family Herpesviridae includes herpesviruses of mammals, birds, and reptiles; the new family Alloherpesviridae includes herpesviruses of fish and frogs; and the new family Malacoherpesviridae contains herpesviruses of bivalves (Davison et al., 2009). In fish, 14 herpesvirus have been identified with some of them associated with disease outbreaks (reviewed in Hanson et al., 2011).

Five Alloherpesviruses have been identified in salmonid fish species that are grouped in the genus Salmoniherpesvirus (Waltzek et al., 2009; Glenney et al., 2016b). The first salmonid herpesvirus was originally isolated from steelhead trout (*Oncorhynchus mykiss*) in Washington, USA (Wolf et al., 1978) and later recognized as Salmonid herpesvirus-1 (SalHV-1) (Waltzek et al., 2009). Salmonid herpesvirus-2 (SalHV-2) infects *Oncorhynchus masou* in Japan (Yoshimizu et al., 2005). The third salmonid herpesvirus is recognized as the most pathogenic to its host, the

lake trout, and was given the name epizootic epitheliotropic disease virus (EEDV, SalHV-3). EEDV was initially described in 1989 and was incriminated as the causative agent of mortality of 15 million hatchery-reared juvenile lake trout (Bradley et al., 1989; McAllister and Herman, 1989). Salmonid herpesvirus-4 (SalHV-4) was recognized in Russia in 2012 associated with papilloma in the Atlantic salmon (*Salmo salar*; Doszpoly et al., 2013). Namaycush herpesvirus (SalHV-5) was identified in apparently healthy lake trout (Glenney et al., 2016b).

2. Epizootic epitheliotropic disease virus (EEDV)

2.1. EEDV historical perspectives and biological properties

EEDV was first recognized as a serious lake trout pathogen in the late 1980s, when it was associated with the demise of over 15 million fish in 7 hatcheries in 3 states within the Great Lakes region (Bradley et al., 1988). The research conducted by McAllister and Herman (1987) and Bradley et al. (1988) led scientists to speculate that the etiologic agent was either epitheliocystis or chlamydia infection. However, investigations using transmission electron microscopy provided evidence that a putative herpesvirus was involved in these mass kills (McAllister and Herman, 1989). Moreover, Bradley et al. (1989) were able to reproduce the infection by collecting EEDV-infected lake trout from a natural outbreak, generating infected homogenate from the external and internal tissues that was passed through a 0.45 μm filter, and then exposing healthy fish to the filtrate. The authors were able to purify a herpesvirus from tissues of affected fish by isopycnic centrifugation and examined its morphological characteristics. The EEDV virion is anatomically similar to those of other herpesviruses, with a linear double stranded DNA (dsDNA), icosahedral capsid, tegument, and an envelope Bradley et al. (1989). The enveloped virus measures 220-235 nm and a capsid diameter of 100-105 nm.

Kurobe et al. (2009) developed a polymerase chain reaction (PCR)-based assay that amplifies a stretch of the EEDV terminase gene in fish tissues. This assay detected EEDV in hatchery raised and wild lake trout in Midwest states despite the absence of mortality episodes.

After an apparent disappearance of the virus for several years, EEDV re-emerged and caused mortality episodes in 2012 and 2017 in lake trout in a fish hatchery in Michigan. Shavalier (2017) investigated these two events and correlated the disease onset to stress and that water temperature plays an important role in disease onset. Using a series of experimental infections, the author was able to determine the median lethal dose of the virus to be $4.7x10^4$ virus DNA copies/ml water, a matter that led to the development of a standardized protocol for water-borne infection. She then described the disease course of EEDV and determined its relatively long incubation time (~21 days). Using an *in situ* hybridization assay that is based on the terminase gene sequence, it was possible to determine the target host tissue of the virus (i.e., tropism). EEDV targets skin and gill epithelial cells, endothelial cells lining blood vessels, and mononuclear cells (Shavalier, 2017). Histopathological observation revealed that EEDV causes necrosis of the cornea and skin epithelium (Shavalier, 2017).

Shavalier (2017) also noticed that in the case of EEDV eruption in the Michigan fish hatchery, lake trout was the only species affected with EEDV within the hatchery, despite the presence of other species; namely brook trout (*Salvelinus fontinalis*) and splake, a hybrid between lake trout and brook trout. However, when she examined free-roaming fish belonging to multiple species (brown trout (*Salmo trutta*) splake, brook trout, and mottled sculpin (*Cottus bairdii*)) receiving effluents from the hatchery, only the mottled sculpin was positive by both quantitative PCR (qPCR) and qLAMP assays.

Because EEDV does not replicate in tissue culture, Shavalier (2017) developed a standard immersion experimental infection protocol in lake trout. Initially, she maintained an *in vivo* continuous infection by exposing naïve juvenile lake trout with skin and gill homogenate from an infected lake trout that succumbed to EEDV. When the newly infected fish became moribund, a homogenate of its skin and gills was prepared and used in the infection of a new group of naïve lake trout. When this process was repeated seven times, a large pool of infected tissue homogenate supernatant was prepared and its number of viral DNA copies determined. Using this pool, Shavalier (2017) determined the median lethal dose (LD_{50}) of EEDV to juvenile lake trout to be $4.7x10^4$/ml of water, which she used to study the virus course and tissue distribution.

Using the standardized immersion infection protocol, Shavalier (2017) followed up the virus distribution in infected lake trout and estimated EEDV incubation period prior to observation of clinical signs to be three weeks post exposure. She also reported that the skin, fin, and ocular tissues were the first targets of EEDV based upon virus detection at 18 days pi in these but not other tissue. Similarly, skin, fin, and eye tissues yielded the highest viral loads throughout the course of infection, as determined qPCR. Using in situ hybridization assay (ISH), Shavalier (2017) reported that during early stages of the disease course, intense labeling for EEDV DNA was identified in epithelial cells of the epidermis, with subsequent labeling detected in the epithelial lining of primary and secondary gill lamellae. During advanced disease course, EEDV-positive staining was observed in endothelial and dendritic cells as well as blood monocytes.

Shavalier (2017) also examined the pathological and histopathological alterations that take place in experimentally infected juvenile lake trout (by immersion) as the disease

progresses. Infected fish develop exophthalmia, ocular hemorrhage, and fin congestion. As the disease advances, affected skin foci coalesce and ulcerate. Microscopically, skin lesions were characterized by localized cellular degeneration of epidermal epithelial cells. As the disease advanced further, skin lesions progressed to erosions and focal necrosis that was associated with dermatitis and perivasculitis. She concluded that progression of lesions is consistent with the cutaneous epithelium representing the primary target of viral infection.

The studies of Bradley et al. (1988; 1989), McAllister and Herman (1987; 1989) and Shavalier (2017) constitute the only information we currently have on the biological properties and disease course of EEDV. Unfortunately, despite the previous intriguing studies, wide gaps of knowledge regarding this virus still exist and fisheries managers are overwhelmed with the devastation this virus can cause in lake trout. In particular, managers are eager to find answers to the following questions:

1. What is the exact host range of EEDV? Although Bradley et al. (1988; 1989) did a very limited experimental infection study with a few species whose endpoints were morbidity and mortality, a more comprehensive, standardized experimental infection study is needed to identify whether other fish species can harbor EEDV without exhibiting clinical disease signs.
2. How is EEDV transmitted horizontally? In specific, is the virus shed from infected fish into the surrounding water; and if it is shed, what is the time range when EEDV is shed the most? Answering these questions is integral towards better understanding the disease ecology of this virus.
3. Because experience proved that EEDV is difficult to control, the efficacy of Virkon®, the most commonly used disinfectant used in hatcheries, in inactivating this virus became

questionable. As a result, hatchery personnel became eager to know if indeed Virkon® can attenuate the ability of EEDV to infect naïve susceptible fish.

To this end, I am devoting my research in this study to address these three unknowns.

2.2.The host range of EEDV

It has been established that herpesviruses recognize target cells and attach to them through a process that requires compatibility between the virus and the outer surface of the target cell. This process of recognition and attachment was studied extensively using the herpes simplex virus as a model (WuDunn and Spear, 1989). Initially, herpesvirus envelope glycoproteins attach and interact with molecules on the host cell membrane that allows for virus internalization. For example, in herpes simplex virus-1 and 2, glycoprotein B and/or C binds with heparan sulfate, a receptor located on the surface of mammalian cells (WuDunn and Spear, 1989), which then allows the viral capsid to enter into the internal environment of the host cell (Herold et al., 1991). Several envelope glycoproteins of herpesviruses have been identified as essential to the recognition of, and binding to, target cells, the most conserved of which are gB and gH/gL. Receptor binding then triggers fusion and virus entry inside the cell. Among herpesviruses, the variety of receptor binding glycoproteins determines the host species range of the virus and cell types within the host that can support viral replication (Agelidis and Shukla, 2015).

The host range of herperviruses has always been a matter of debate. Originally, herpesvirologists believed a herpesvirus infects a single species; however, later observations provided evidence that there are exceptions to this rule. On one hand, there are herpesviruses that infect only a single species, such as herpes simplex virus 1 and 2 (HSV, genus Simplexvirus,

subfamily Alphaherpesvirinae, family Herpesviridae, ICTV, 2011), which both infect humans (*Homo sapiens*) but cause two different diseases (reviewed in Corey et al., 1983; Pourchet et al., 2017). Equine herpesvirus-1 (genus Varicellovirus, subfamily Alphaherpesviridae, family Herpesviridae) infects horses (*Equus ferus*) only (Thorsen and Little, 1975; Carrol and Westbury, 1985). Another herpesvirus, the elephant endotheliotropic herpesvirus (genus Proboscivirus, subfamily Betaherpesvirinae, family Herpesviridae) infects African elephants only (*Loxodonta Africana*), (McCully et al., 1971; Jacobson et al., 1986; ICTV, 2011).

In contrast, there are other herpesviruses that can infect more than one host species, such as gallid herpesvirus-2 (genus Mardivirus, subfamily Alphaherpesvirinae), the causative agent of Marek's Disease in domestic chickens (*Gallus gallus domesticus*), turkeys *(Meleagris gallopavo*), and common quail (*Coturnix coturnix*; Calnek, 1992, Colwell et al., 1973; Wakenell et al., 2010). Marek's Disease Virus targets the bird's T cells, thereby causing deadly lymphomas (Calnek, 2001). Other herpesviruses can infect multiple species within a host genus or family. One such example is the macaque herpesvirus 1 (genus Simplexvirus, subfamily Alphaherpesvirinae, family Herpesviridae, ICTV, 2011), which was originally isolated from a population of long tailed macaques (*Macaca fascicularis*) in Asia (Lee et al., 2015), but has since been identified in a number of primates, including humans (Holmes et al., 1990). Another example of interspecies host infection by herpesviruses is the otarine herpesvirus-1 (OtHV-1), a member of the family Gammaherpesviridae, which is known to infect California sea lions (*Zalophus californianus*; Buckles et al., 2007). In 2013, OtHV-1 was reportedly identified in South American fur seals (*Arctocephalus australis*) as well, causing urogenital carcinomas similar to those described in the California sea lion (Dagleish et al., 2013). Hinojosa et al. (2016) reported that the same virus also infects northern fur seals (*Callorhinus ursinus*). It was also

found that some herpesviruses can naturally jump to a new host after years of being restricted to a single host. For example, equine herpesvirus 9, the natural host of the Thomson's gazelle (*Gazella thomsoni*; Fukushi et al., 1997), was identified in a polar bear (*Ursus maritimus*) with meningoencephalitis, 10 years after the initial description of the virus (Schrenzel et al., 2008).

Similar examples of host range variation were observed in members of the family Alloherpesviridae. For example, koi herpesvirus (i.e., cyprinid herpesvirus-3) infects only two varieties of the same species; the mirror carp (*Cyprinus carpio carpio*) and the koi carp *(Cyprinus carpio koi)* (Hedrick et al., 2000; Yuasa et al., 2008; Cheng et al., 2011). Conversely, escoid herpesvirus-1 is known to infect both northern pike (*Esox lucius*) and muskellunge (*Esox masquinongy*) (Margenau et al., 1995), whereas the channel catfish virus (ictalurid herpesvirus-1) was initially thought to infect channel catfish (*Ictalurus punctatus*) only (Plumb, 1977) but can also cause mortalities in farmed black bullhead catfish (*Ameiurus melas*; (Alborali et al., 1996). Interspecies transmission also occurs in malacoherpesviruses. Ostreid herpesvirus-1 (OsHV-1), which was first identified in eastern oysters (*Crassostrea virginica*; Farley et al., 1972), and was found three decades later to also infect several other bivalve mollusks, such as the Pacific oyster (*Crassostrea gigas*), European flat oyster (*Ostrea edulis*), Palourde clam (*Ruditapes decussatus*), and the Manila clam (*Ruditapes philippinarum*) (Arzul et al., 2001).

Lake trout (*Salvelinus namaycush*) were the only species affected during the natural outbreaks of Salmonid herpesvirus 3 (epizootic epitheliotropic disease virus, EEDV) in the Great Lakes fish hatcheries in the 1980's (Bradley et al., 1989). When these authors exposed naïve lake trout to filtered (450 nm pore diameter) supernatants of affected fish tissue homogenates, clinical signs consistent with those observed during the natural outbreak were observed. The presence of intranuclear inclusion bodies in the skin epithelium of experimentally exposed fish led the

authors to believe that EEDV was replicating in the skin cells. Further, Bradley et al. (1989) screened the susceptibility of other salmonid species by cohabitating them with infected lake trout. The species tested included rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), and Chinook salmon (*Oncorhynchus tshawytscha*). In their first trial, juvenile fish of each of three species (rainbow, brook and brown trout) and naïve lake trout were placed in a cage inside tanks together with EEDV-infected juvenile lake trout for three months. Meanwhile, juvenile Atlantic salmon and Chinook salmon were kept together with EEDV-infected lake trout for 60 days. The result of these trials revealed that cumulative mortality of naïve juvenile lake trout exposed to EEDV-infected lake trout reached up to 60% in less than 30 days, whereas mortality rates in the other salmonid species never exceeded 5% and were likely not due to active EEDV infection. McAllister and Herman (1989) did a similar experiment using cohabitation with EEDV infected lake and four salmonid species (i.e., rainbow trout, brown trout, brook trout and Atlantic salmon). The authors reached a similar conclusion in that non-lake trout salmonid species seem to be resistant to EEDV infection.

Despite the thoroughness with which these experimental infection studies were performed, several questions remained unanswered. First, both studies used mortality rate as the only endpoint to assess susceptibility, not addressing the fact that many herpesvirus infections can run subclinical courses. Second, both studies were unable to follow up on the presence of EEDV in tissues of infected fish since in the 1980s, as molecular diagnostic assays for EEDV had not yet been developed (Kurobe et al, 2009; Glenney et al., 2016a). The presence of inclusion bodies in infected lake trout skin epithelium, though suggestive of an active EEDV infect, could have been caused by a number of other viruses. Glenney et al. (2016b) reported that

lake trout in the Great Lakes are infected with a novel herpesvirus, Salmonid herpesvirus 5. Additionally, neither study tested the susceptibility of non-salmonid fish species. This is important in light of the recent 2012 EEDV episode (Shavalier, 2017), during which EEDV was detected in the tissues of mottled sculpin near the hatchery experiencing the EED outbreak. Furthermore, neither study used a known virus dose to examine susceptibility; both relied on tissue homogenates or virus shedding from naturally infected fish. Lastly, variations in susceptibility among the different strains of lake trout, as well as its hybrid with brook trout, the splake, were never assessed.

To this end, the first objective of the present study is to use a standardized experimental infection protocol to:

a. compare the susceptibility of the two lake trout strains commonly raised in the Great Lakes federal and state fish hatcheries toward EEDV,
b. determine if splake (lake trout x brook trout) is susceptible to EEDV,
c. screen the susceptibility of representative Great Lakes salmonid and non-salmonid fish species.

2.3.EEDV transmission

The ability of a virus to disseminate in the surrounding environment relies primarily on its ability to be shed from the body of infected animals. In general, water is an ideal vehicle for virus transmission, even for viruses pathogenic to terrestrial animals and humans. For example, in a study by Jones et al. (2014), it was demonstrated that in the case of the zoonotic influenza virus A (H7N9), which infects several species of songbirds, the virus is shed from the oropharynx of infected birds into water where its titer in the water column remains high for an

extended period of time. The authors concluded that water can serve as a primary reservoir for this virus, not only for birds, but also for humans. Similar observations were reported on the herpes simplex virus (HSV-1) which maintains its infectivity even after being in hot tub water in spa facilities for several hours (Nerurkar et al., 1983). Most gastroenteritis viruses such as enterovirus, rotaviruses, noroviruses and astroviruses are waterborne (Díaz et al., 2009). Herpesviruses seem to be very stable in the aquatic environment. In a study performed by Dayaram et al. (2017), it was demonstrated that the equine herpesvirus-1 (EHV-1) can maintain its infectivity in distilled water for 14 days and in water with a pH range 8-10, which normally degrades nucleic acids of most viruses, for up to 6 days.

Water is believed to play a major role in the transmission of fish-pathogenic viruses. An investigation by Kim and Faisal (2012) followed the shedding of the viral hemorrhagic septicemia virus (VHSV, novirhabdovirus, Rhabdoviridae) in muskellunge (*Esox masquinongy*) infected by immersion in VHSV tainted water. VHSV shedding from infected fish started as early as 7 days post-infection (pi) and the virus continued to shed until day-116 pi. The authors demonstrated that surviving muskellunge can resume shedding of VHSV if exposed to handling stress. In the same context, El-Matbouli and Soliman (2011) reported that transmission of fish-pathogenic herpesviruses also occurs through water. In their experiment, the authors were able to infect naïve common carp (*Cyprinus carpio*) with the koi herpesvirus (KHV, Cyprinid herpesvirus-3) after cohabitation with infected goldfish (*Carassius auratus auratus*). A year later, Kempter et al. (2012), demonstrated that cyprinid herpesvirus-3 can be transmitted from infected to naïve specific pathogen free (SPF) through cohabitation. Authors of both studies underscored water as the main vehicle of infection of this virus.

In an elegant study, Yuasa et al. (2008) found that the kinetics of virus shedding following immersion infection with cyprinid herpesvirus-3 is temperature-dependent; starting as early as day-7 pi at 16 °C, day-1 pi at 23 °C, and day-3 pi at 28 °C. The duration of shedding, however, differed, being the longest (34 days pi) at the lowest water temperature (16 °C), while never exceeding 14 days pi at the higher temperatures. The shedding of another fish herpesvirus; the channel catfish virus (CCV), a serious pathogen that causes devastation to the catfish industry, was studied by Kancharla and Hanson (1996). Their results suggest that CCV is shed into water by experimentally infected channel catfish as early as 2-4 days pi at high virus titers and continued throughout the experiment observation period (12 days).

It is believed that because herpesviruses replicate primarily in external tissues, horizontal transmission takes place by either skin-to-skin contact or through the sloughing of dead infected cells into the surrounding environment. For example, feather follicle cells where the gallid alphaherpesvirus-2, the infection of Mareks's disease virus (MDV), replicate is a potential source for virus dissemination (Calnek, 2001) and so are the papules and vesicles caused by HSV-1 (Bernstein et al., 2013), or the nodular lesions on elephant trunks caused by the elephant endotheliotropic herpesvirus-1 (EEHV-1) (Stanton et al., 2010).

Regarding EEDV, there is very little information available on virus transmission; however, it has been reported that the disease can be successfully transmitted by cohabitation and water immersion (Bradley et al., 1989; McAllister and Herman, 1989). Bradley et al. (1989) collected tissue samples from various organs (brain, liver, spleen, kidney, pyloric caeca, stomach, intestine, skin, and gill) of lake trout naturally infected with EEDV. Following homogenization and resuspension in tissue culture medium, they filtered the supernatants using 220 and 450 nm filters. The filtrates were then added to the aquaria containing uninfected

juvenile lake trout. The authors reported mortalities in all fish groups exposed to the 450 nm-filtered homogenate of infected fish by the 39th day post-exposure. All dead fish found with clinical signs consistent with those occurring in the EEDV natural event. In contrast, there was no mortality in the group with filtrate 220 nm.

Another EEDV transmission trial was conducted by McAllister and Herman (1989). In this study, infection took place by cohabitation and flow-through cascade. In the cohabitation method, healthy fingerling lake trout were exposed to EEDV infected lake trout in static and flow-through water systems. The flow-through cascade was done by holding infected lake trout in upper tanks and non-infected lake trout below them, and water was allowed to flow from the upper tanks into the lower tanks. In another group of fish, the authors added infected tissue filtrate into aquaria containing healthy fingerling lake trout. Mortalities occurred in all treatment groups with fish exhibiting clinical signs consistent with EEDV.

Most recently, Shavalier (2017), using an *in-situ* hybridization assay, was able to determine that EEDV replicates in skin and gill cells and it is, therefore, highly likely that the virus is shed by infected fish. Despite the availability of this information, it is unknown how much an infected lake trout can shed EEDV and for how long. Knowledge of this information is critical for the design of management strategies to control EEDV and its spread within the hatchery. Therefore, the second objective of this study is to follow EEDV shedding into the water by infected fish, both temporally and quantitatively.

3. Disinfectant use to inactivate virus in aquaculture

Disinfection by physical or chemical methods is integral to prophylaxis and control of pathogens in aquaculture (Danner and Merril, 2006; OIE, 2017). The search for disinfectants

with proven efficacy at a reasonable cost is one of the challenges that keeping fish under intensive aquaculture conditions is facing. The work by Verner-Jeffreys et al. (2009) allowed, for the first time, the development of bactericidal and virucidal testing standards for aquaculture disinfectants. In general, virucidal effects are tested by observing a significant decrease in virus titer following exposure to the effective dilution of the disinfectant. Unfortunately, EEDV cannot replicate in an *in vitro* system, a matter that makes disinfectant efficacy testing a challenge.

Previous studies have proven that many chemical disinfectants (Lysol®, Listerine®, bleach, alcide, and alcohol) were reliable for inactivating herpesviruses (Croughan and Behbehani, 1988). Regarding the use of disinfectants for the control of fish herpesvirus, an investigation was performed on chemical disinfectant efficacy against koi herpesvirus (KHV) by Kasai et al. (2005). The authors exposed supernatants containing KHV to commonly used disinfectants in aquaculture and found that all disinfectants used had a dose-dependent efficacy against KHV.

Among common aquaculture disinfectants, Virkon® (potassium peroxymonosulfate, PPMS) occupies a central position. PPMS is a strong oxidant (Venkatesh and Karunakaran, 2013) that is proven safe upon contact with fish at the dose recommended by the manufacturer (Stocton-Fiti and Moffitt, 2017). In aquaculture facilities, Virkon® Aquatic is heavily used for the disinfection of vehicles, nets, boots, waders, brushes, and hard surfaces. Virkon® Aquatic is preferred because of its fast acting effects (10 minutes is the contact time needed), non-irritating nature on skin and eyes, and its quick degradation within the environment.

Virkon® Aquatic was first made available in the USA in 2007 and was specifically designed for use in fish culture facilities, while Virkon®-S is designed for use in the production of poultry, swine, and other types of agriculture. At a 1% concentration, Virkon® Aquatic was

found to be very effective against a variety of bacteria and viruses in as little as 5 minutes of exposure (Hernández et al., 2000). In 2015, Tsujimura et al. (2015) investigated the susceptibility of equine herpesvirus type 1 (EHV-1) to some chemical disinfectants (anionic surfactant-based disinfectant) which Virkon® was one of them. The results demonstrated that Virkon® Aquatic worked well to eliminate EHV-1 under room temperature.

Despite the fact that Virkon® Aquatic has been heavily used in hatcheries, its efficacy against EEDV has not been tested. This is in part due to the absence of an *in vitro* system for EEDV that would allow the virus titration before and after exposure to the disinfectant. Most recently, Shavalier (2017) developed a reliable, reproducible *in vivo* system that will allow for the testing of the effects of Virkon® Aquatic on the infectivity of EEDV to its natural host, the lake trout. To this end, the third objective of this study is to determine the ability of Virkon® Aquatic at its commonly used concentration (1%) to totally eliminate the pathogenicity of EEDV.

4. Thesis purpose and objectives

Working with EEDV is a challenge that is facing scientists and managers alike. The virus is extremely hard to work with and is a threat to the valuable indigenous lake trout. This study is tackling three important questions whose answers can shed light on some of the unknown biological characteristics of EEDV, as well as of importance to everyday practices in hatcheries. In specific, objectives of this study are:

1. Determine the difference in susceptibility to EEDV between two strains of lake trout (*Salvelinus namaycush*) commonly used in stock enhancement programs in Michigan, to determine if the splake (lake trout x brook trout) and another congener (i.e., *Salvelinus*

fontinalis, brook trout) are susceptible to EEDV infection, and to determine if other Great Lakes salmonid and non-salmonid fish species are susceptible to EEDV.

2. Investigate whether EEDV infected fish shed the virus into the water.
3. Determine whether Virkon® Aquatic has virucidal effects against EEDV at the recommended dose by the manufacturer.

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CHAPTER 1.

Elucidating the host range of epizootic epitheliotropic disease virus (Salmonid herpesvirus-3)

1. Abstract

Epizootic epitheliotropic disease virus (EEDV, salmonid herpesvirus-3; Family Alloherpesviridae) emerged in the 1980's as a significant mortality source in lake trout (*Salvelinus namaycush*) reared in multiple hatcheries within the Great Lakes basin. However, EEDV-associated losses were not reported again until the virus re-emerged in 2012 and 2017, at which time the source of the virus remained unknown. During these and the earlier EED outbreaks, questions surfaced regarding the intraspecific susceptibility to EEDV among Great Lakes lake trout strains that are raised for conservation and stock enhancement purposes, as well as whether EEDV was truly a lake trout-specific virus. Herein, the susceptibility of eleven Great Lakes fish species/strains, representing four families (i.e., Salmonidae, Cottidae, Centrachidae, and Esocidae) were intra-coelomically injected with two concentrations of EEDV corresponding to $4.75x10^3$ (low dose, LD) or $4.74x10^5$ (high dose, HD) virus copies per fish (as determined by an EEDV-specific SYBR Green qPCR assay). No mortality, EED disease signs, and/or virus were detected in brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), coho salmon (*O. kisutch)*, lake herring (*Coregonus artedii)*, largemouth bass (*Micropterus salmoides)*, or muskellunge (*Esox masquinongy*). However, the same HD EEDV concentration led to clinical EED and 80% mortality in Lake Superior strain lake trout, as well as virus loads that in one case exceeded $1.9x10^9$ virus copies/mg of skin tissue. The same infectious dose did not induce any mortality in Seneca strain lake trout, but nevertheless resulted in clinical EEDV infections, whereby EED signs were observed and virus loads in a subset of fish at 100 days post-infection ranged from $1.6x10^7 – 7.2x10^7$ virus copies/mg skin tissue. Moreover, EEDV was detected in one of ten HD-challenged splake (lake trout x brook trout hybrid) that died 10 days post-infection at titers

substantially higher than the initial inoculum ($3.8x10^7$ viral copies/mg skin), indicating EEDV replication had occurred. EEDV DNA was also detected in one mottled sculpin (*Cottus bairdii*), although the role this species plays in supporting EEDV replication and serving as EEDV reservoir appears unlikely. Collectively, study results show that lake trout strains vary in their susceptibility to EEDV, that in some cases, splake may serve as a short term EEDV reservoir, and overall affirm the complexities of EEDV disease ecology.

2. Introduction

In the 1980's, Great Lakes fishery managers were confronted with the emergence of a deadly disease of lake trout (*Salvelinus namaycush*). These outbreaks resulted in the death of > 15 million lake trout in multiple hatcheries within the Laurentian Great Lakes region (Bradley et al., 1988, 1989), and were of particular concern due to the reliance of the basin-wide Lake Trout Rehabilitation Program on these hatchery fish. Despite the initial difficulties in identifying the etiological agent responsible for these mortality events, it was eventually determined that a herpesvirus was the cause; namely, the Epizootic Epitheliotropic Disease Virus (EEDV; Bradley et al., 1988, 1989) that was later identified as the Salmonid Herpesvirus-3 (SalHV-3) and placed in the family Alloherpesviridae (Waltzek et al., 2009). To date, EEDV has yet to be cultured under *in vitro* conditions.

Herpesviruses are thought to have emerged approximately 400 million years ago, and have since been associated with infections across an array of animal phyla. The fish herpesviruses make up a monophyletic family, the Alloherpesviridae (Waltzek et al., 2009). Several alloherpesviruses have been associated with severe economic losses in a variety of cultured fish species (lake trout (Bradley et al., 1988), catfish (Alborali et al., 1996) and common

carp (*Cyprinus carpio*, Garver et al., 2010)). As is the case with terrestrial herpesviruses, those affecting fish are known to have a very narrow host range, typically causing disease in only one fish species (Hanson et al., 2011). Following this trend, to date, EEDV epizootics have only been reported in lake trout (Bradley et al., 1989), but the ability of other fish species to harbor EEDV in the absence of clinical disease signs is not well understood.

In efforts to examine whether other salmonid species were susceptible to EEDV, McAllister and Herman (1989) housed fingerling brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) with EEDV-infected lake trout. They also immersed the same four species in water containing filtered skin homogenate collected from EEDV-infected lake trout. No deaths attributable to EEDV were observed in any non-lake trout salmonids through either exposure method during the 60 day experiments (McAllister and Herman, 1989); however, the authors acknowledged that this "does not preclude the possibility that these species could develop an inapparent infection…". Bradley et al. (1989) came to a similar conclusion, in that brook, brown, and rainbow trout, Atlantic salmon, and Chinook salmon cohabitated with EEDV-infected lake trout did not develop characteristic EED signs, despite the fact that mortality exceeded 60% in the challenged lake trout. EEDV infection status in these fish could not be assessed due to an inability to culture EEDV *in vitro* and the lack of EEDV-specific molecular diagnostic tests at that time.

In 2009, a PCR based diagnostic assay was developed and proved to be capable of detecting EEDV viral DNA in infected fish tissues (Kurobe et al., 2009). Unpublished reports using this assay have suggested that some non-lake trout salmonids may be capable of harboring EEDV, but the sensitivity of this assay has since been questioned (Glenney et al., 2016a) and others have suggested that specificity problems may be at the core of these positive results. As a

result, Glenney et al. (2016b) developed a new sensitive and specific quantitative PCR (qPCR) assay, which has since been employed to investigate EEDV infection status in Great Lakes fishes. In 2017, Shavalier (2017) found that mottled sculpin (*Cottus bairdii*) harbored EEDV DNA.

Armed with the new sensitive and specific qPCR assay of Glenney et al. (2016a), a series of experiments were designed to: a) determine if susceptibility to EEDV infection and mortality vary in lake trout strains; and b) assess the ability of EEDV to infect a range of Great Lakes fish species, including mottled sculpin, as well as the lake trout x brook trout hybrid known as splake, with the overarching goal of improving EEDV prevention strategies by investigating the potential for non-lake trout Great Lakes fish species to serve as reservoirs of EEDV infection.

3. Materials and methods

3.1.Fish maintenance

Eleven fish species currently residing within the Great Lakes basin, representing four families (i.e., Salmonidae, Cottidae, Centrachidae, and Esocidae) were selected for species susceptibility experiments (Table 1.1). This included two strains of lake trout (Lake Superior and Seneca Lake strains) that are a primary focus of hatchery-based lake trout rehabilitation efforts in the Great Lakes basin, as well as splake, a lake trout x brook trout hybrid. Fish were either obtained from the Michigan Department of Natural Resources State Fish Hatcheries, the Little Traverse Bay Bands of Odawa Indians Hatchery, or were collected from the wild (Table 1.1). All experimental animals were maintained at the Michigan State University Research Containment Facility (MSU-URCF). At the time of experimental challenge, fish ranged from 4 to 14 months old and from 11.5 to 16.5 cm length in Seneca (SE) strain lake trout, 9.5 – 16.9 cm in Lake

Superior (LS) strain lake trout, 6.4 – 12.5 cm in splake, 10.5 – 14.8 cm in brook trout, 6.5 – 10.2 cm in rainbow trout, 10.2 – 13.0 cm in brown trout, 10.4 – 13.0 in Atlantic salmon, 8.0 – 13.0 in coho salmon, 5.4 – 7.1 cm in lake herring, 5.7 – 9.1 cm in mottled sculpin, 7.0 – 12.3 cm in muskellunge, and 5.4 – 9.4 in largemouth bass (Table 1.1). Fish were housed in 680 L flow-through fiber glass tanks supplied with oxygenated, dechlorinated water (9 to 14 °C), and fed commercial pellets twice per day. All fish handling and maintenance was performed in accord with the Michigan State University Institutional Animal Care and Use Committee (IACUC) committee approval.

3.2.Infectious inoculum preparation

To date, all efforts to culture EEDV *in vitro* have been unsuccessful. Therefore, infectious inoculum was prepared from EEDV-infected lake trout skin collected during a natural EED outbreak in 2012 following the protocol of Shavalier (2017). In brief, the skin was homogenized via manually trimming until reaching a size of 1-2 mm in diameter, to which sterile sample diluent (pH 7.525 ± 0.025) containing 458 mL Minimal Essential Medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts), 7 mL of 1 M tris buffer, 1 mL gentamycin sulfate (Sigma-Aldrich, St. Louis, Missouri), 5 mL penicillin/streptomycin (Invitrogen), and 5 mL Amphotericin B (Thermo Fisher Scientific) was added at a 1:3 (w/v) ratio. The suspension was then homogenized (Seward Stomacher 80, Biomaster Lab System) for 120 seconds at high speed, centrifuged at 368 x g (20 minutes; 4 °C), and then frozen at -80 °C until used for infections.

3.3.Infection challenges

Prior to infection challenge experiments, three groups of 10 fish each per fish species/strain were randomly assigned to 42 L fiberglass tanks (flow through and concurrently supplied with aeration) and acclimatized over 15 days from a water temperature of 14 to 9 °C. The three tanks for each species corresponded to a negative control (NC) group, a low dose group (LD), and a high dose (HD) group. After acclimation, fish were anesthetized with tricaine methanesulfonate (MS-222; Western Chemical Inc., Ferndale, Washington) at dose 0.1 mg/ml buffered with sodium bicarbonate (Church & Dwight Co., Inc., Ewing, New Jersey) at dose of 0.2 mg/mL and then intraceolomically (IC) injected with 100 µl of either sample diluent as previously described (NC groups) or EEDV infectious inoculum corresponding to $4.75x10^3$ (LD) or $4.74x10^5$ (HD) virus copies per fish (as determined by qPCR; see below). The IC injection in this study was performed to induce a systemic infection with a limited virus stock, as EEDV cannot currently be cultured *in vitro.* The two doses were selected based upon median lethal doses experiments of Shavalier (2017).

After injection, fish were returned to their respective tanks, monitored for recovery, and then maintained for 30 – 100 days (challenge period modified based upon the observation of disease signs; Table 1.2). Daily fish care was done to include feeding and monitoring water flow, air flow, water temperature, fish behavior and clinical signs. Any severely moribund fish were euthanized using a lethal dose of MS-222 (0.25 mg/mL, buffered with sodium bicarbonate at dose of 0.5 mg/mL). Complete external and internal examinations were performed on all dead or moribund fish. During the necropsy, clinical signs were noted and skin samples collected from the caudal peduncle area and stored at -20 °C for molecular analysis. At the end of the study period, surviving fish were euthanized and analyzed as described above.

3.4.DNA extraction

A maximum of 10 mg (as recommended by in the Mag-Bind® Blood & Tissue DNA HDQ 96 Kit Tissue Protocol) of skin tissue was transferred into a sterile 1.5 ml tube for DNA extraction. In this extraction, skin tissues were the primary samples to be investigated because previous EEDV studies revealed skin was a primary site of virus replication (Bradley et al., 1989; McAllister et al., 1989; Shavalier, 2017). All extractions were performed following the protocol outlined by Glenney et al. (2016a). Each tissue sample was digested using Proteinase-K at 55 °C overnight following the protocol in Mag-Bind® Blood & Tissue DNA HDQ 96 Kit (OMEGA Bio-tek), then added with 250 µl TL buffer (OMEGA Bio-tek) and 20 µl Proteinase-K (OMEGA Bio-tek) then incubated in the shaker at 55 °C overnight. The next day, the compound (samples, Proteinase-K, and TL buffer) were vortexed and pelleted in the centrifuge at 14,000 rpm for 10 minutes. The supernatant was then transferred into Lysate Clearance Plates (OMEGA Bio-tek) and clarified in the centrifuge at 1000 rpm for 3 minutes. The filtrate was received in 96-well round-bottom plates (Costar 3799, Corning Incorporation, Corning, New York) and the DNA extraction completed using the Mag-Bind® Blood & Tissue DNA HDQ 96 Kit (OMEGA Bio-tek) following the manufacturer's protocol. Extracted DNA was then quantified (Qubit™ fluorometer, Invitrogen, Eugene, Oregon). Samples with greater than 50 ng of nucleic acid were diluted with sterile DNase-free water.

3.5.Molecular detection of EEDV

All qPCR reactions were carried out in a Mastercycler ep realplex^2 real-time PCR machine (Eppendorf, Hauppauge, New York) and were performed as described by Glenney et al. (2016a) using the primers 5' – TGG GAG TCC GTC GTC GAA – 3' (SalHV3_23F) and 5' –

TCC ACA CAG GAG CTC ACG AA – 3' (SalHV3_23F). The 20 µl reaction contained 10 µl of SYBR® Select Master Mix, 2 µl of nuclease-free water (Promega), 2 µl of forward primer, 2 µl of reverse primer, and 4 µl of template containing 50 nmol total DNA. The qPCR cycling parameters consisted of step at 50 °C for 2 minutes; 95 °C followed for 10 minutes; and 40 cycles of 95 °C for 15 seconds, 60 °C for 60 seconds and were carried out in a Mastercycler Pro Thermal Cycler (Eppendorf, Hamburg, Germany). Samples were considered positive if the cycle threshold (Ct) value was < 35 (Shavalier, 2017). Utilized controls included previously confirmed EEDV-positive tissue homogenate (positive extraction control, PEC), sample diluent (negative extraction control, NEC), EEDV-positive DNA extract positive reaction control, PRC), and nuclease-free water (negative reaction control, NRC). The quantification of EEDV copies present in a sample was determined based upon comparison to the standard curve generated by 8 serial 10-fold dilutions of EEDV PRC (Shavalier, 2017) that were run with each 96 plate.

3.6. Data analysis

The prevalence of EEDV infection in exposed fish was estimated by dividing the number of positive skin tissue samples by the total number of samples (i.e., positive fish / total fish exposed).

4. Results

4.1. Cumulative mortality, gross disease signs, and EEDV detection

Throughout the course of this study, no Seneca strain lake trout, brook trout, brown trout, rainbow trout, Atlantic salmon, coho salmon, lake herring, or largemouth bass died in any of the challenge groups, nor was EEDV detected in any of these species (Table 1.2). Three

muskellunge died in the LD group and one muskellunge died in the HD group; in all cases, however, these were due to aggression (LD group) or cannibalism (HD group) and EEDV was never detected in any muskellunge (Table 1.2). The number of mortalities and/or gross disease signs for the remaining fish species/strains are presented in Table 1.2 and discussed in sections 4.1.1-4.1.4.

4.1.1. Lake Superior strain lake trout

In the HD LS strain lake trout group, disease signs consistent with EED were observed in all fish and included mild to severe ocular hemorrhage, mild to severe corneal opacity, mild to severe exophthalmia, mild to severe skin erosion of the caudal peduncle, congestion at the base of the fins, mild petechial hemorrhage on the caudal peduncle, and gill pallor with concurrent mucous accumulation. Mortality in the HD group began 21 days post-infection (pi) and continued to day 66 (Table 1.3). The two surviving fish were euthanized on day 66. All 10 fish in the HD group were EEDV-positive according to qPCR (Table 1.2), whereby EEDV loads ranged from $1.3x10^4 – 2.0x10^9$ virus copies/mg of skin tissue taken from the caudal peduncle area in each individual fish (Table 1.3).

No disease signs consistent with EED were observed in the LD group and all fish survived until the end of the study period (Table 1.2). Likewise, EEDV was not detected in any LD fish (Table 1.3). Four fish died in the negative control group and were attributed to aggression; however, a low EEDV load (i.e., 8.9×10^3 virus copies/mg of skin tissue) was detected in one negative control fish (Table 1.2).

4.1.2. SE strain lake trout

No mortality occurred in the HD SE lake trout group; however, exophthalmia, ocular hemorrhage, congestion at the base of the fins, mild hemorrhage within the caudal peduncle, and multifocal skin pallor were observed, albeit less severe when compared to the LAT-LS HD group. EEDV was detected in three of ten fish in this group via qPCR at the end of the experiment (Table 1.2), with loads ranging from $1.6x10^7$ – $7.2x10^7$ virus copies/mg of skin tissue (Table 1.3). No mortality occurred in the LD or NC groups, nor was EEDV detected via qPCR in either group (Table 1.2).

4.1.3. Splake

Three splake died in the HD group on days 5, 7 and 10 p.i. (Table 1.2), whereby severe lethargy, erythema along the lateral line, gill pallor, and congestion/hemorrhage at the base of the fins were observed. However, EEDV was only detected in one of these three fish, but at a load of $3.8x10^7$ viral copies/mg skin (Table 1.3). In the 7 HD fish surviving until the end of the experiment, no EEDV was detected. No disease signs consistent with EED were observed in the LD or NC groups, nor was EEDV detected in either group. However, three fish died in the LD group, and one fish died in the NC group (Table 1.2).

4.1.4. Mottled sculpin

Six of ten mottled sculpin in the HD group died (Table 1.2) during days 4 – 40 pi. Observed disease signs varied and included combinations of moderate exophthalmia, congestion at the base of the fins, and/or hemorrhage on the isthmus. Despite this, EEDV was not detected in the six fish succumbing to death, nor in any of the four survivors. Five fish died in the LD group (Table 1.2); EEDV was detected in one of the five mortalities and at a low load (i.e.,

$1.1x10^2$ viral copies/mg skin; Table 1.3). Four fish died in the NC group, but EEDV was not detected in these fish, nor in the survivors (Table 1.2).

5. Discussion

Since EEDV was first described in the 1980's, the virus has been considered specific to lake trout even though no sensitive and specific diagnostic tools were available to test this hypothesis. Thus, despite the thorough studies that were designed by Bradley et al. (1989) and McAllister and Herman (1989) to examine the host range of EEDV in non-lake trout salmonids, questions surrounding EEDV host range remain. However, the development of a highly sensitive and specific SYBR Green qPCR assay by Glenney et al. (2016b) that targets the glycoprotein gene of EEDV, differentiates the virus from all other currently recognized salmonid herpesviruses, and detects as few as 10 viral copies/mg of skin tissue, enabled this knowledge gap to be addressed in the current study.

After ten fish species and one hybrid (representing Salmonidae, Coregonidae, Cottidae, Centrachidae, and Esocidae) were injected with two different doses of EEDV (i.e., $4.75x10^3$ or $4.74x10^5$ virus copies per fish), at least eight (i.e., brook trout, brown trout, rainbow trout, Atlantic salmon, coho salmon, lake herring, largemouth bass, and muskellunge) were found to be refractory to EEDV infection under laboratory conditions. Despite the fact that the HD EEDV infectious inoculum led to 100% infection prevalence in the EEDV susceptible lake trout LS strain, these findings strongly suggest that these other salmonid and non-salmonid fish species do not support the replication of EEDV, nor do they develop clinical disease under the utilized experimental conditions. Of particular note was the lack of EEDV detection in the coregonid representative, the Great Lakes indigenous lake herring, as Glenney et al. (2016b) recently

detected very low EEDV titers in the skin of this species. Whether the previous detection of EEDV in lake herring was a result of virus/viral DNA being superficially present on the skin or whether the species can harbor EEDV infections under conditions not examined in this study remain to be determined.

Following the 2012 EEDV epizootic in a hatchery in northern Michigan, yearly routine surveillance continued both within the hatchery as well as in surrounding waters (Shavalier, 2017). In 2013, EEDV DNA was detected in wild mottled sculpin collected from Cherry Creek (Shavalier, 2017), which is the water source that feeds and drains the EEDV-affected hatchery. For this reason and to determine their potential as an EEDV reservoir, mottled sculpin was evaluated for their ability to support EEDV replication in the current study. Although 40 – 60% of the mottled sculpin died in the three challenge groups (e.g., NC, LD, and HD, respectively), daily observations revealed significant aggression between tank mates, as well as substantial competition for feed. Moreover, among the mortalities and survivors, EEDV was detected in only one mottled sculpin from the LD group at the lowest viral titer of this study (i.e., 110 viral copies/mg of skin), which notably also died early in the experiment at eight days PI. These findings, in conjunction with the lack of characteristic EED signs in sculpin, suggest that this fish species likely cannot support EEDV replication and that the single detection in this study, as well as that of Shavalier (2017), may have resulted from inactivated EEDV genetic material. However, exploration of EEDV loads in the internal organs of EEDV-challenged mottled sculpin warrant further investigation.

Splake, the lake trout x brook trout hybrid, were also assessed for susceptibility to EEDV/the ability to support virus replication. Although three fish died in each of the LD and HD EEDV-challenged groups, EEDV was only detected in one of three HD mortalities and was

not detected in any splake surviving to the end of the experiment in either challenge group. However, a non-peer reviewed report indicates that splake can be experimentally infected with EEDV (McAllister, 1991) and in this study, some gross pathological changes consistent with EED were observed in a portion of EEDV-challenged splake. Moreover, EEDV was detected in the kidney/spleen homogenate of the same fish with an EEDV positive skin sample, as well as in the skin at a virus load of $3.8x10^7$ viral copies/mg skin, which shows the EEDV replication did occur in this fish (i.e., fish was injected with $4.74x10^5$ virus copies). Thus, it appears that, in some instances, splake can support EEDV replication over the short term. When designing a fish health management plan and/or EEDV surveillance efforts, splake should warrant attention, as their role as a potential EEDV reservoir cannot be currently excluded.

When EEDV re-emerged in a state fish hatchery in Michigan in 2012, Shavalier (2017) observed differences in cumulative percent mortality within the rearing units housing the SE and LS lake trout strains. However, differences in rearing unit densities, first vs. second pass water, and other epidemiological variables made any conclusive determination of differential strain susceptibility to EEDV all but impossible at that time. In this study, lake trout from both strains were challenged with identical EEDV doses and maintained under the same rearing conditions so that EEDV susceptibility within two lake trout strains could be assessed, a matter of importance for Great Lakes lake trout conservation and stock enhancement efforts. Surprisingly, no mortality occurred in the SE lake trout strain (HD group) compared to 80% mortality in the comparable LS lake trout strain. Likewise, EEDV loads were orders of magnitude higher in most LS strain lake trout compared to the SE strain (Table 1.3), but interestingly were higher in the SE strain lake trout that survived until the end of the study compared to the two surviving LS lake trout (Table 1.3). This is even more interesting considering that the SE lake trout were

maintained for 100 days compared to 66 for the LS lake trout, perhaps indicating some SE lake trout can serve as a relatively high titer EEDV reservoir for extended periods of time. Apparent intraspecies differences in virus infection susceptibility have also been reported in other fish species. For example, multiple koi carp strains showed differential susceptibility to koi herpesvirus (KHV) infection (Shapira et al., 2005). Among the five koi strains used in that study (NxS, NxD, NxN, DxD, and DxS) that were infected with KHV, the NxN strain experienced mortality sooner and reached the highest cumulative mortality rate, whereas the DxS strain had the lowest cumulative mortality rate with delayed onset of mortality (Shapira et al., 2005).

In conclusion, results from this study revealed that the SE and LS lake trout strains vary in their susceptibility to EEDV, whereby infection-induced mortality under laboratory conditions only occurred in LS lake trout. However, EEDV loads reached as high as 10^9 viral copies/mg skin tissue in LS lake trout but were 10^3 viral copies/mg skin tissue in survivors, compared to ~10^7 virus copies/mg skin tissue in SE lake trout surviving to 100 days pi, suggesting that some SE lake trout may serve as higher titer EEDV reservoirs that may be less likely to succumb to death. Study results also suggest that some splake have the potential to serve as a short term EEDV reservoir.

APPENDIX

Table 1.1: Origin and median length/weight of the fish species that were assessed for Epizootic epitheliotropic disease virus (EEDV) susceptibility in this study. (*) (LS) Lake Superior strain lake trout; (SE) Seneca strain lake trout; (MSFH) Marquette State Fish Hatchery; (OSFH) Oden State Fish Hatchery; (PRSFH) Platte River State Fish Hatchery; (WLSFH) Wolf Lake State Fish Hatchery; (LTBBFH) Little Traverse Bay Bands of Odawa Indians Fish Hatchery.

Common name	**Species/Strain**	**Median Length (cm)**	**Median Weight (g)**	**Origin**
Lake trout (LS)*	*Salvelinus namaycush*, LS strain	13.36 ± 6.4	18.72 ± 7.6	MSFH*
Lake trout (SE)*	*Salvelinus namaycush*, SE strain	13.87 ± 1.5	22.40 ± 7.5	MSFH*
Brook trout	*Salvelinus fontinalis*	12.31 ± 1.1	17.35 ± 4.7	MSFH*
Mottled sculpin	*Cottus bairdii*	6.75 ± 1.1	4.69 ± 3.9	wild captured
Brown trout	*Salmo trutta*	11.68 ± 1.9	17.05 ± 8.9	OSFH*
Rainbow trout	*Oncorhynchus mykiss*	8.57 ± 0.9	5.82 ± 2.1	OSFH*
Atlantic salmon	*Salmo salar*	12.58 ± 3.2	12.9 ± 3.6	PRSFH*
Largemouth bass	*Micropterus salmoides*	5.7 ± 2.1	6.42 ± 1.6	wild captured
Coho salmon	*Oncorhynchus kisutch*	11.38 ± 1.8	11.55 ± 1.9	PRSFH*
Muskellunge	*Esox masquinongy*	7.9 ± 3.4	8.6 ± 3.4	WLSFH*
Splake	*Salvelinus namaycush* x *Salvelinus fontinalis* crosses	10.14 ± 2.6	10.90 ± 2.3	MSFH*
Lake Herring	*Coregonus artedii*	1.7 ± 1.7	3.9 ± 3.3	LTBBFH*

Table 1.2: The number of mortalities and prevalence of Epizootic epitheliotropic disease virus (EEDV; as determined by qPCR) in the fish treatment groups of this study. Results reported as number of EEDV positive skin samples out of number of skin samples tested. (*) mortality not associated with EEDV; (LS) Lake Superior strain lake trout; (SE) Seneca strain lake trout. Lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), mottled sculpin (*Cottus bairdii*) brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), largemouth bass (*Micropterus salmoides*), coho salmon (*Oncorhynchus kisutch*), muskellunge (*Esox masquinongy*), splake (*Salvelinus namaycush* x *Salvelinus fontinalis*), lake herring (*Coregonus artedii*). A pool of kidney and spleen tissues was also PCR tested from representative splake, with results: 0/2 (negative control), 0/2 (low dose), 1/5 (high dose).

Species tested	Experiment length (days)	Negative control (NC)		Low dose (LD)		High dose (HD)	
		Mortalities	PCR result	Mortalities	PCR result	Mortalities	PCR result
Lake trout (LS)	66	4/10	1/10	0/10	0/10	8/10	10/10
Lake trout (SE)	100	0/10	0/10	0/10	0/10	0/10	3/10
Brook trout	53	0/10	0/10	0/10	0/10	0/10	0/10
Mottled sculpin	62	4*/10	0/10	*5/10	1/10	*6/10	0/10
Brown trout	35	0/10	0/10	0/10	0/10	0/10	0/10
Rainbow trout	36	0/10	0/10	0/10	0/10	0/10	0/10
Atlantic salmon	35	0/10	0/10	0/10	0/10	0/10	0/10
Largemouth bass	33	0/10	0/10	0/10	0/10	0/10	0/10
Coho salmon	33	0/10	0/10	0/10	0/10	0/10	0/10
Muskellunge	33	0/9	0/9	3*/10	0/10	1*/10	0/9
Splake	36	1*/10	0/10	3/10	0/10	3/10	1/10
Lake herring	36	0/10	0/10	0/10	0/10	0/10	0/10

Table 1.3: Days to death post- epizootic epitheliotropic disease virus (EEDV) infection, whether EED disease signs were present, and the estimated EEDV loads that were detected in the skin of fish that were EEDV positive throughout the course of this study. (*) Euthanized; (+) present; (-) absent; (PI) post-infection; (LS) Lake Superior strain lake trout; (SE) Seneca strain lake trout. Lake trout (*Salvelinus namaycush*), Mottled sculpin (*Cottus bairdii*), Splake (*Salvelinus namaycush* x *Salvelinus fontinalis*).

Species	**Mortality Day (pi)**	**Presence of EED Disease Signs**	**Viral copies per mg skin**
LS strain lake trout	21*	+	$1.95x10^9$
	35	+	$4.18x10^7$
	46	+	$4.08x10^8$
	46	+	$5.29x10^8$
	50	+	$1.05x10^9$
	54	+	$2.22x10^8$
	54	+	$1.55x10^8$
	66	+	$5.83x10^6$
	66*	+	$5.90x10^4$
	66*	+	$1.33x10^4$
SE strain lake trout	100*	+	$1.59x10^7$
	100*	+	$3.31x10^7$
	100*	+	$7.18x10^7$
Mottled sculpin	8	-	$1.10x10^2$
Splake	10*	+	3.84×10^7

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CHAPTER 2.

Shedding of Salmonid herpesvirus-3 by infected lake trout (*Salvelinus namaycush*)

1. Abstract

Salmonid herpesvirus-3, commonly known as the epizootic epitheliotropic disease virus (EEDV), causes a serious disease of the lake trout (*Salvelinus namaycush*) that killed millions of fish over the last four decades. Currently, most aspects of EEDV disease ecology are unknown. In this study, we investigated virus shedding into water in pit tag microchipped Lake Superior strain trout that were intraperitoneally (IP) injected with EEDV-infected tissue homogenate. A negative control group was injected with sample diluent alone. To assess virus shedding, each infected fish was housed individually in a static aerated aquarium for 8 hours, then water was assessed for the presence of EEDV DNA using quantitative PCR assay. Water sampling was conducted every seven days for up to 93 days post-infection (pi). Our results demonstrated that lake trout started to shed EEDV into the water as early as 9 days pi and shedding was greatest ~three weeks pi and continued for up to ~9 weeks pi. Mortalities started occurring at day 40 pi, and viral shedding ceased ~ 70 days pi. Although mortality reached 73.9%, surviving fish ceased shedding and continued to grow. Findings of this study demonstrated that EEDV is shed in the water by infected hosts for extended periods of time, a matter that favors the dissemination of the virus.

2. Introduction

Herpesvirus are ubiquitous pathogens that infect a wide range of hosts that extends from mollusks to mammals (Calnek, 1992, Lee et al., 2015, Schrenzel et al., 2008, Bradley et al., 1989, Waltzek et al., 2009; Corey et al., 1983). The mechanisms used by herpesviruses to disseminate from one host to another vary considerably. Skin-to-skin contact is essential for the transmission of some herpesviruses, such as Marek's disease virus of chicken, which replicates

in feather follicles (Calnek, 2001), or the elephant endotheliotropic herpesvirus-1 (EEHV-1), which replicates in external trunk cells (Stanton et al., 2010). Herpesviruses that affect the respiratory system, such as the equine herpesvirus-1, are shed from infected bronchi into the air directly (Slater et al., 1994). In the case of alloherpesviruses, infecting primarily fish and amphibians, water appears to be the main vehicle for virus transmission (Kancharla and Hanson, 1996; Yuasa et al., 2008). Most of our current knowledge on alloherpesvirus transmission stem from two pilot studies. When Yuasa et al. (2008) cohabitated common carp (*Cyprinus carpio carpio*) that were previously experimentally infected with koi herpesvirus (KHV; cyprinid herpesvirus-3) with naïve koi carp (*Cyprinus carpio koi*), the koi carp contracted the deadly virus, suggesting that KHV was transmitted by water. Kancharla and Hanson (1996) attempted to estimate the duration and level of shedding of the channel catfish virus (ictalurid herpesvirus-1) by experimentally infected catfish. The authors demonstrated that virus shedding remained throughout the observation period (12 days) at levels that reached up to $4.5x10^7$ viral DNA copies/fish.

Among the fish-pathogenic alloherpesviruses, salmonid herpesvirus-3, commonly known as epizootic epitheliotropic disease virus (EEDV), causes serious losses in its natural host; the lake trout (*Salvelinus namaycush*). Since the late 1980s, the disease devastates lake trout populations in North America and caused the demise of millions of fish (McAllister and Herman, 1987; 1989; Bradley et al., 1988; 1989; Shavalier, 2017). Despite its high pathogenicity, relatively little is known about the virus ecology; its transmission in particular. Experimental infection was successful by cohabitation and/or exposure to filtered (450 nm) homogenate of infected fish tissues, suggesting water as a possible transmission route (Bradley et al. 1989; McAllister and Herman, 1989, Shavalier 2017). Further, using an *in-situ* hybridization assay,

Shavalier (2017) demonstrated that EEDV targets skin cells, where it replicates more than in any of the other external or internal tissues tested. How much of the virus is shed in the water is currently unknown. Equally unknown is the time period post-infection (pi) in which infected fish constitute a high risk to other naïve lake trout. To this end, the aim of the present study is to assess and follow up the shedding levels and duration of EEDV by experimentally infected lake trout.

3. Materials and methods

3.1. Fish maintenance

Twenty-nine Lake Superior (LS) strain lake trout (*Salvelinus namaycush*), age 25 months post-hatch were used in this study. The fish were provided by the Marquette State Fish Hatchery (MSFH; Marquette, Michigan, USA) and maintained at the Michigan State University Research Containment Facility (URCF). Prior to their use, fish were housed in a 680-L flow-through fiberglass tank supplied with dechlorinated water at 14 °C. The fish were fed AquaMax® Fingerling Starter 300 (Purina®, Gray Summit, Missouri) *ad libitum*, and detritus was removed daily. All fish handling and maintenance was performed in accordance with the Michigan State University Institutional Animal Care and Use Committee (IACUC) standards.

Prior to the start of the study, fish were randomly divided into two groups: negative control group (NC) or experimental group (EEDV group). Those groups were assigned two separate 42-L fiberglass cylindrical tanks, with one tank consisting of 23 fish to be infected with EEDV, and a second tank consisting six fish to receive a sham inoculation. The water temperature during this study was 10 ± 1°C (i.e., close to the water temperature at which natural

EEDV outbreaks occur; Bradley et al., 1989; Shavalier, 2017), to which the fish were allowed to acclimate to over a period of 15 days.

3.2. Fish tagging

Fish tagging was performed to identify individual fish, and group water samples based on individual fish throughout the study using a 9 mm-pit tag microchip full duplex (FDX) (HPT9; Biomark®, Boise, Idaho) that was placed into the body cavity of each fish. Before the tagging was conducted, all pit tag microchips were disinfected by soaking in 70% ethanol for a minimum of 10 minutes. Fish were anesthetized with tricaine methanesulfonate (MS-222; Western Chemical Inc., Ferndale, Washington) at dose of 0.1 mg/mL, buffered with sodium bicarbonate (Church & Dwight Co., Inc., Ewing, New Jersey) at dose of 0.2 mg/mL. When the fish showed signs of adequate anesthesia, a pit tag microchip was removed from the 70% ethanol using sterile forceps, rinsed with a sterile phosphate buffered saline (PBS; pH 7.5 ± 0.5; Sigma-Aldrich, St Louis, Missouri) solution for 10 seconds, and placed into the N125 needle (Biomark®). Microchip insertion into the body cavity of each fish was performed following the manufacturer's instructions for fish >55 mm in length. In order to confirm successful pit tag implantation, the identification number of each fish was read and recorded using the pit tag reader (Pocket Reader 098494; Destron-Fearing™, Eagen, Minnesota), after which the fish were transferred back into their respective tanks and observed to ensure proper post-anesthesia recovery. In their respective tanks, fish were maintained and observed for 15 days before infection challenges.

3.3. Infection challenges

A frozen stock of EEDV confirmed as EEDV positive and containing $1x10^6$ viral copies/mL using qPCR (Shavalier, 2017) was prepared through homogenization of skin tissue collected from naturally infected lake trout (Shavalier, 2017). For this study, the infectious inoculum was prepared by combining 1.6 mL EEDV stock with 1.4 mL of sample diluent as previously described (*Chapter 2*), resulting in a final working virus concentration of $4.5x10^5$ viral copies/mL.

Fish were anesthetized as described above and then injected intraperitoneally (IP) with 100 µL/fish of either sample diluent (pH 7.525 ± 0.025) containing 458 mL Minimal Essential Medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts), 7 mL of 1 M tris buffer, 1 mL gentamycin sulfate (Sigma-Aldrich, St. Louis, Missouri), 5 mL penicillin/streptomycin (Invitrogen), and 5 mL Amphotericin B (Thermo Fisher Scientific) was added at a 1:3 (w/v) ratio (NC group) or EEDV infectious inoculum (EEDV group). After injection, fish were returned to their respective experimental tanks and monitored daily. If a fish became severely moribund during the study observation period (93 days), it was euthanized using an overdose dose of MS-222 (0.25 mg/mL, buffered with sodium bicarbonate at dose of 0.5 mg/mL) and a clinical examination performed.

3.4. Assessment of shedding

The twenty-three fish in the EEDV group were assigned to one of three sub-groups (1, 2, and 3) for the duration of the study, which consisted of eight, eight, and seven fish, respectively. Water sampling was conducted every seven days, on 13 sampling periods, starting at day-7 post-infection (pi) for sub-group 1, day-8 pi for sub-group 2 and day-9 pi for sub-group 3 (Table 2.1).

The NC group was also assigned to three sub-groups that consisted of two fish each. Fish within a sub-group shared the same water collection day. Each sampling day utilized the fish of one sub-group from the EEDV group and the corresponding sub-group of the NC group.

On each sampling day, ten 11.4-L glass aquariums (two for the NC group and eight for the EEDV group) were filled with 3.4 L of water (10 ± 1 °C, static water system) and placed inside ten separate 42-L fiberglass cylindrical tanks filled to 18.9 L of chilled water (continuous flow-through system). This arrangement of a static aquarium within a flow-through system ensured a constant water temperature throughout the sampling period. Clean and disinfected air-lines and air-stones were equipped in each of glass aquarium and adjusted to supply appropriate oxygen in the static water system. Two fish from the NC group were selected (from the appropriate cohort, based on pit tag code) and carefully placed into individual glass aquarium. For the EEDV group, fish from the corresponding sub-group were selected using pit tag reader to identify the pit tag code and placed into individual aquariums in the same manner as the NC group.

Fish remained in the glass aquarium for eight hours, under continuous supervision, after which 40 mL water was collected from the aquarium and stored at -20 °C until DNA extractions could be performed (maximum 2 months after the sample collection date). After the collection, all fish in the glass aquariums were transferred back into their respective tanks (NC tank or EEDV tank). All aquariums, air-lines and air-stones were uninstalled, and thoroughly disinfected using 10% bleach (Clorox®, Oakland, California) or Nolvasan® (Zoetis Inc., Kalamazoo, Michigan), prior to their use with the next cohort the following day. It is noteworthy that none of the fish showed signs of discomfort or hypoxia during the period spend in the static aquarium.

3.5. DNA extraction

Herpesviruses can persist within host cells (Smith et al., 2001); therefore, DNA extraction of herpesvirus from water samples required a method that can disrupt both host cells and viral capsid to yield satisfied result. All DNA extractions were performed following the Alternative PowerSoil Protocol for Low Bacterial Biomass Fluids from the Qiagen DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen, Hilden, Germany) with minor modifications that also included mechanical disruption via bead-beating. Frozen water samples were thawed to room temperature and vortexed briefly. 500 µL bead solution, 200 µL phenol (isoamyl alcohol; AMRESCO, Solon, Ohio), and 60 µL C1 solution were added into the supplied bead tubes. 250 µL of the water sample (as recommended in the protocol) was then added into this mixture, vortexed briefly, and loaded into the bead beater (Mini-Beadbeater-16; Biospec Inc., Bartlesville, Oklahoma) for 30 seconds twice with a 20-second period between the two bead beatings. The mixture was then centrifuged at 10,000xg for 1 minute at 4 °C. The supernatant was collected and transferred into a new tube provided by the manufacturer, and 1 µL of RNase A was added. The C6 solution was heated to 60 °C before being used to elute the DNA. The remaining steps were followed using the manufacturer's instructions. Extracted DNA was then quantified using Qubit™ fluorometer (Invitrogen, Eugene, Oregon), and samples diluted with sterile DNase-free water to a maximum of 12.5 ng/uL qPCR template DNA.

3.6. Quantification of EEDV DNA in water samples

The SYBR Green qPCR assay of Glenney et al. (2016a) that targets the glycoprotein gene of EEDV and differentiates the virus from all other currently recognized salmonid herpesviruses was utilized in this experiment. All qPCR reactions were carried out in a

Mastercycler ep *realplex*^{2} real-time PCR machine (Eppendorf, Hauppauge, New York) and were performed as previously described (Glenney et al., 2016a; *Chapter 1*). EEDV-positive tissue homogenate was used as a positive extraction control (PEC) and sample diluent was used as the negative extraction control (NEC). Further controls included EEDV-positive purified DNA and nuclease-free water, which served as the positive reaction control (PRC) and negative reaction control (NRC), respectively. Samples were considered EEDV positive if the fluorescence exceeded 10% of the maximum florescence within 35 amplification cycles as determined with the Mastercycler ep *realplex*^{2} S accompanying software and the manufacturer's default settings (Shavalier, 2017). Shedding rates (viral copies per hour per fish) were calculated using the Mastercycler ep *realplex*^{2} S accompanying software via comparison to a standard curve that was generated via 8 serial 10-fold dilutions of EEDV positive standards (Shavalier, 2017).

4. Results

4.1.Mortalities and clinical signs

Infected fish developed typical signs of EEDV infection in the form of patches of skin pallor, hemorrhage in the lower canthus of the eye (Figure 2.1 A), and skin erosions in the trunk (Figure 2.1 B) and around the nares. Mortalities in the EEDV-infected group started by day-40 pi and cumulative mortality rate peaked by Day-68 pi and remained steady at ca 74% (17 of 23 fish) through the end of the observation period (Day-93). There were no further mortalities through the end of the study period (day-93 pi) and 6 fish survived the infection (Figure 2.2, Table 2.1). External lesions of surviving fish healed, and the fish resumed feeding and growth by the end of observation period. On the contrary, none of the negative control fish developed clinical signs, with one of them died 11 pi due to causes not related to EEDV.

4.2. Levels of EEDV DNA in the water

The shedding of EEDV from infected lake trout into the water, as indicated by the presence of EEDV DNA, started from the first sampling period and continued into the 9th sampling period (Table 2.1, Figure 2.3). Only one fish shed EEDV at day-9 pi and the number of shedding fish increased to four at the second sampling period. Shedding reached its maximal levels during the sampling periods 3 and 6 when all fish shed EEDV DNA into the surrounding waters at high titers. The majority of infected fish shed EEDV DNA during the 4th, 5th, 7th, 8th, and 9th sampling period. From the 10th sampling period through the end of observation period, shedding ceased as the number of alive fish decreased due to EEDV-mortalities (Table 2.1, Figure 2.3).

The levels of EEDV DNA shed from each fish per hour varied greatly from one sampling period to the other. While EEDV was shed at 5.1x10^6 viral copies/hour (n = 1/23 fish) at the first sampling period, viral shedding increased to an average of 1.92x10^7 viral copies/fish/hour at the second sampling period (n = 4/23). The levels of EEDV DNA copies shed increased significantly at the third sampling period when all fish shed EEDV DNA with an average copy number of 2.47x10^8 copies/fish/hour with one of the fish shedding as much as 9.66x10^8 viral copies/hour (Figure 2.3). A decrease in shedding was noticed as of the fourth sampling period (3.34x10^7 viral copies/fish/hour; n = 19/23), and again at the fifth sampling period (8.33x10^6 viral copies/fish/hour; n = 21/23) after which there were no significant changes in the level of virus shed through sampling period 9 (Table 2.1). Likewise, the number of infected individuals (as determined by viral shedding) increased from sampling periods one (4.3% positive) to three (100% positive) and remained high through sampling period eight. As surviving fish began to recover from the infection, viral shedding was detected from only 66% of fish at the ninth

sampling period (n = 6/9), and from no fish at sampling periods 10-13 (n = 0/6). Also, of note was the observation of three shedding "trends" in this experiment characterized by shedding at relatively high titers until they died, intermittent shedding, and shedding followed by survival

5. Discussion

Findings of this study unravel some details on an important aspect of EEDV disease ecology; virus dissemination. Although the virus was injected IP, shedding of EEDV DNA took place in the water and in relatively high titers that far exceeded the initial challenge dose. This implies that initial virus replication took place in the visceral organs, followed by the development of a generalized infection with the virus reaching its target tissue as previously reported by Shavalier (2017); the skin. In a study by Shavalier (2017), virus potentially reach targeted tissue through blood stream (viremia) as the author detected EEDV genetic material in mononuclear cells in the spleen through *in situ* hybridization. Closer examination of Figure 2.3 and Table 2.1 clearly demonstrates that EEDV needs up to three weeks to reach to the target tissue of all infected fish, where it attains high levels of replication that led to skin cell destruction, with sloughed host cells likely facilitating shedding of the virus into the surrounding environment. However, the role of urine, feces, and/or fish body fluids in EEDV shedding may also have contributed to the observed EEDV shedding loads and warrants further study.

A simple mathematical calculation demonstrates the extraordinary amplification of EEDV by infected fish. The levels of viral DNA shed per one hour is several hundred folds higher than the number of virus copies injected per fish, a matter that can overwhelm the immune system of naïve fish population. In fact, the hourly virus loads that were shed by individual infected lake trout in this study were substantially higher than the estimated EEDV

median lethal dose via immersion (i.e., $4.7x10^4$ virus copies/mL; Shavalier 2017), demonstrating the number of subsequent infections one infected individual can lead to over several weeks of infection. The matter is further complicated by the relatively long time of high levels shedding of EEDV that extended for six additional weeks. Studies done on other fish pathogenic viruses, such as the novirhabdovirus, viral hemorrhagic septicemia virus (VHSV), also indicated high levels of shedding that extended up to 15 weeks (Kim and Faisal, 2012). However, the degree of virus amplification by infected fish that is shed in the water seems to be much higher in the case of EEDV. Whether other Alloherpesviruses have shedding patterns that are similar to EEDV is currently unknown since earlier studies were performed using different virus doses, observation periods, endpoints measured, and water temperature. Of interest, water temperature can have a strong effect on shedding rates of at least one fish-pathogenic herpesvirus, cyprinid herpesvirus-3 (CyHV-3). Yuasa et al. (2008) showed that CyHV-3 shedding from infected fish began as early as day-7 pi at 16 °C, day-1 pi at 23 °C, and day-3 pi at 28 °C. However, the duration of shedding varied with water temperature, being the longest (34 days pi) at the lowest water temperature (16 °C) and less than half as long at the higher temperatures (e.g., 14 days pi). Water temperature is also important for reactivation of CyHV-3 infections (St-Hillaire et al., 2005) but its effect on EEDV reactivation and/or recrudescence are unknown.

The ability of a virus to disseminate in the surrounding environment relies primarily upon its ability to be shed from the body of infected animals and water provides an ideal vehicle for virus transmission. In this context, some herpesviruses are relatively stable in aquatic systems, whereby Dayaram et al. (2017) showed that equine herpesvirus-1 (EHV-1) maintains infectivity for 14 days in distilled water. Clearly, further studies investigating the length of time EEDV

remains active and/or infective in water, both in controlled laboratory environments and under variable field conditions, are needed and will aid future EED prevention and control strategies.

A portion of infected lake trout (~25%) in this study seemed capable of combating EEDV to some degree, whereby they eventually ceased shedding and continued to grow. However, EEDV surviving fish seem to continue to harbor the virus, since Shavalier (2017) demonstrated the recrudescence of EEDV in a lake trout population that survived an EEDV outbreak upon exposure to the stress of high rearing density. Similar observations were reported by Eide et al. (2011), who failed to detect koi herpesvirus (KHV) in survivor koi fish, yet when these fish were exposed to temperature-induced stress, KHV DNA was detected in gill swabs. Thus, culling of fish surviving an EEDV infection in hatchery populations is likely warranted so as to minimize infection spread.

Pit tagging of EEDV-infected lake trout allowed the identification of individual variations in shedding levels, as well as shedding trends. A trend of shedding peaked at ca 3 weeks pi, followed by a decrease in shedding levels that may be due to the demise of most target ectodermal cells and that ultimately ended with host death. Another trend showed fish seemed to better resist the infection and very high titers of the virus and survive. The reason for this resistance is currently unknown, primarily because the host immune responses of lake trout to EEDV has not been adequately studied.

In conclusion, findings of this study prove that EEDV is indeed shed from infected lake trout into the water column in high quantities ($<10^8$ virus copies/fish/hour) over an extended period of time ($\leq$9 weeks pi). Additionally, individual fish vary in EEDV shedding loads and patterns, whereby some survive initial infection and have the potential to serve as long-term virus reservoirs.

APPENDIX

Table 2.1: EEDV DNA shedding by experimentally infected lake trout. Data are expressed as viral copies/fish/hour. Each sampling period consists of three days and sampling periods are a week apart. (*) fish died; AVERAGE: the average of fish that were shed the virus only.

Group	Fish #	Sampling period												
		1 (D7, 8, 9)	**2 (D14, 15, 16)**	**3 (D21, 22, 23)**	**4 (D28, 29, 30)**	**5 D35, 36, 37)**	**6 (D42, 43, 44)**	**7 (49, 50, 51)**	**8 (D56, 57, 58)**	**9 D63, 64, 65)**	**10 (D70, 71, 72)**	**11 (D77, 78, 79)**	**12 (D84, 85, 86)**	**13 (D91, 92, 93)**
Sub-group 1	1	0	0	$2.18x10^8$	$3.75x10^7$	$2.90x10^7$	*	*	*	*	*	*	*	*
	2	0	0	$3.49x10^7$	$2.36x10^7$	$9.95x10^6$	$7.91x10^7$	*	*	*	*	*	*	*
	3	0	0	$2.37x10^8$	$2.87x10^7$	$1.95x10^7$	$1.51x10^7$	0	*	*	*	*	*	*
	4	0	0	$8.17x10^8$	$6.95x10^7$	$5.87x10^6$	$3.03x10^7$	$2.45x10^6$	*	*	*	*	*	*
	5	0	0	$1.40x10^8$	$4.82x10^7$	$7.65x10^6$	$1.39x10^8$	$1.76x10^7$	$1.73x10^6$	$5.74x10^6$	*	*	*	*
	6	0	0	$1.13x10^7$	$6.72x10^7$	$1.18x10^7$	$2.57x10^7$	$7.61x10^7$	*	*	*	*	*	*
	7	0	0	$2.20x10^7$	$4.15x10^7$	$9.08x10^6$	$2.12x10^7$	$8.98x10^6$	*	*	*	*	*	*
	8	0	$4.53x10^7$	$1.79x10^8$	$9.09x10^7$	$3.54x10^6$	$3.93x10^7$	*	*	*	*	*	*	*
Sub-group 2	9	0	0	$5.64x10^7$	$3.22x10^7$	$4.39x10^6$	$5.90x10^8$	$3.58x10^7$	*	*	*	*	*	*
	10	0	0	$1.09x10^8$	$7.32x10^6$	$4.21x10^6$	$3.36x10^7$	$1.27x10^7$	$7.03x10^7$	$1.24x10^7$	*	*	*	*
	11	0	0	$1.63x10^8$	$1.97x10^7$	$8.72x10^6$	$4.95x10^6$	0	$1.04x10^8$	$1.61x10^6$	0	0	0	0
	12	0	0	$1.98x10^8$	$6.36x10^7$	0	$1.62x10^6$	$1.63x10^6$	0	$1.29x10^6$	0	0	0	0
	13	0	0	$4.86x10^8$	0	$5.41x10^6$	$5.13x10^6$	$5.84x10^6$	$2.04x10^6$	$2.83x10^6$	0	0	0	0
	14	0	0	$6.61x10^7$	$2.09x10^7$	$2.96x10^6$	$4.62x10^6$	$3.09x10^6$	0	0	0	0	0	0
	15	0	0	$2.70x10^7$	0	$8.42x10^6$	$9.74x10^6$	*	*	*	*	*	*	*
	16	0	0	$2.24x10^7$	0	$5.61x10^6$	$1.36x10^7$	$3.26x10^6$	$5.02x10^6$	$2.05x10^7$	*	*	*	*
Sub-group 3	17	0	0	$2.62x10^8$	$8.03x10^6$	$3.93x10^6$	$1.60x10^6$	$3.60x10^6$	*	*	*	*	*	*
	18	0	0	$8.66x10^7$	$9.79x10^6$	$6.83x10^6$	$6.53x10^6$	$1.12x10^7$	*	*	*	*	*	*
	19	0	0	$1.32x10^7$	0	$2.18x10^6$	$3.32x10^6$	$2.93x10^6$	$3.09x10^6$	0	0	0	0	0
	20	0	$1.56x10^7$	$4.88x10^8$	$6.20x10^6$	$1.95x10^6$	$1.68x10^6$	$2.22x10^6$	$9.51x10^6$	0	0	0	0	0
	21	0	0	$2.36x10^8$	$2.34x10^7$	$2.35x10^6$	$1.53x10^7$	$1.59x10^6$	*	*	*	*	*	*
	22	$5.10x10^6$	$1.34x10^7$	$9.66x10^8$	$8.93x10^6$	$6.45x10^6$	$1.19x10^7$	$2.88x10^6$	$3.42x10^6$	*	*	*	*	*
	23	0	$2.55x10^6$	$8.39x10^8$	$2.85x10^7$	$2.35x10^7$	$9.84x10^6$	*	*	*	*	*	*	*
AVERAGE		$5.1x10^6$	$1.92x10^7$	$2.47x10^8$	$3.34x10^7$	$8.33x10^6$	$4.83x10^7$	$1.20x10^7$	$2.49x10^7$	$7.38x10^6$	0	0	0	0
STDEV		$0.0x10^0$	$1.59x10^7$	$2.76x10^8$	$2.38x10^7$	$6.89x10^6$	$1.22x10^8$	$1.86x10^7$	$3.70x10^7$	$6.95x10^6$	0	0	0	0
# fish died		0	0	0	0	0	1	4	8	1	3	0	0	0
# fish shed		1	4	23	19	22	22	16	8	6	0	0	0	0
% fish shed		4.35	17.39	100.00	82.61	95.65	95.65	69.57	34.78	26.09	0	0	0	0

Figure 2.1: Representative clinical signs of lake trout experimentally infected with EEDV. A: hemorrhage in the lower canthus of the eye and B: areas of skin pallor and erosion.

Figure 2.2: Cumulative mortality of lake trout (*Salvelinus namaycush*) in the EEDV group (n=23) injected with $4.5x10^5$ EEDV DNA copies/mL.

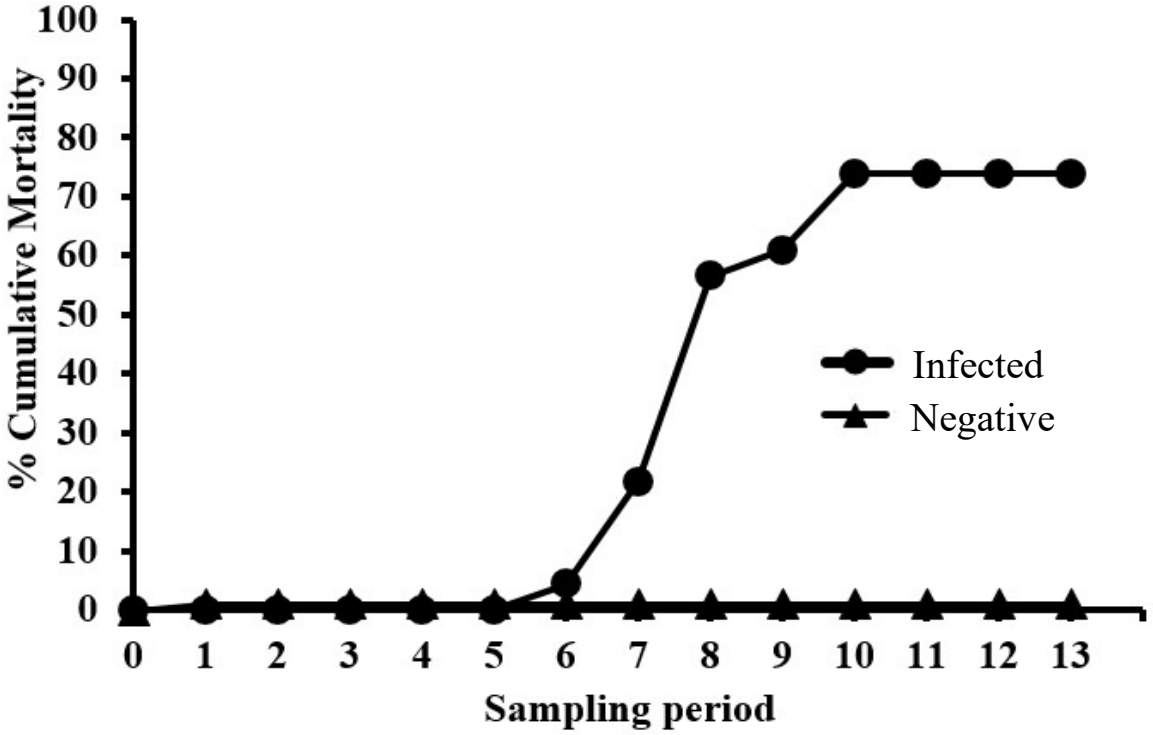
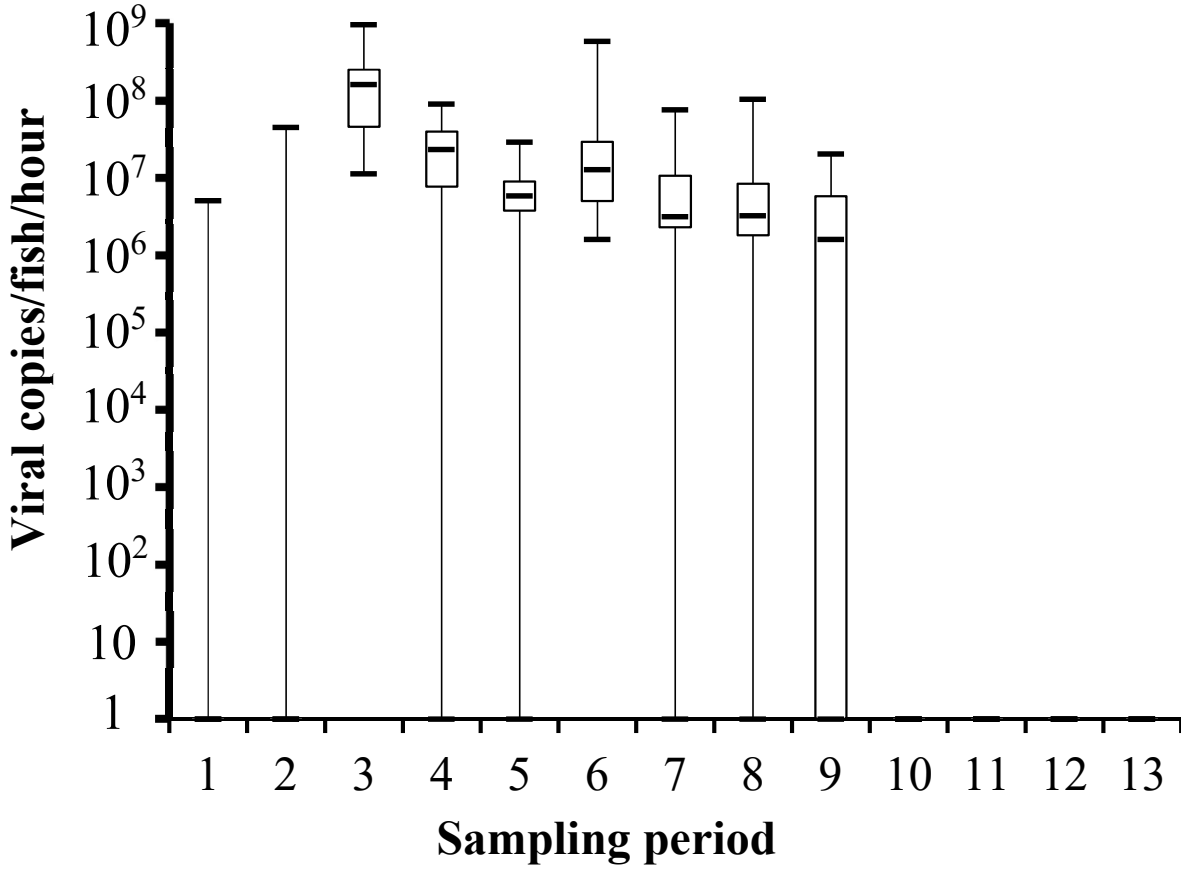


Figure 2.3: Shedding rate of lake trout in the EEDV group exposed EEDV at a dose of $4.5x10^5$ viral copies/mL. The box plots showing minimum, 1st quartile, median, 3rd quartile and maximum viral copies/fish/hour by sampling period in water samples.



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CHAPTER 3.

The efficacy of a commonly used commercial hatchery disinfectant against epizootic epitheliotropic disease virus (EEDV)

1. Abstract

Epizootic epitheliotropic disease virus (EEDV) causes substantial losses in hatchery-reared lake trout (*Salvelinus namaycush*) in the Great Lakes Basin. Due in part to the inability to culture EEDV *in vitro*, almost nothing is known about effective means for inactivating the virus using disinfectants that are approved for use in hatcheries/aquaculture facilities. One disinfectant that is widely used in hatcheries is Virkon® Aquatic (active ingredient, potassium peroxymonosulfate; Syndel, Ferndale, Washington), as previous experiments have found it to be efficacious against multiple microbial fish pathogens. Thus, our study aimed to investigate the efficacy of Virkon® Aquatic against EEDV. Positive control lake trout were challenged with EEDV by soaking a net in $1.77x10^5$ virus copies/mL of water and then transferring naïve fish into the EEDV-laden net. The Virkon® Aquatic treatment groups were EEDV challenged analogously, with the exception that prior to fish transfer, the virus laden net was soaked in a 1% Virkon® Aquatic suspension for 20 minutes (i.e, the manufacturer recommended disinfection concentration/duration). Beginning at 26 days post-infection (pi), characteristic signs of EED were observed in positive control fish but were never observed in the Virkon® Aquatic treatment groups throughout the 148 days challenge period. At the end of the study period, 80% mortality occurred in the positive control groups compared to 3.3% in the Virkon® Aquatic treatment group. Similarly, EEDV was detected in 90% of the positive control fish but was never detected in the Virkon® Aquatic treatment group. Results from this study suggest that Virkon® Aquatic is effective at preventing EEDV contagion on hatchery tools at the manufacturer recommended concentration/duration and highlights a promising tool for improving lake trout hatchery biosecurity.

2. Introduction

Lake trout (*Salvelinus namaycush*) are indigenous to the Laurentian Great Lakes and have been the focus of substantial hatchery conservation and rehabilitation efforts to address massive previous population declines (Lawrie and Rahrer, 1972). Like many other hatchery-based fishery conservation efforts, infectious diseases are an impediment to the rearing of lake trout in Great Lakes hatcheries. In particular, epizootic epitheliotropic disease virus (EEDV) has directly killed millions of juvenile lake trout in multiple Great Lakes hatcheries since the 1980's and led to further losses due to subsequent hatchery depopulation efforts to prevent virus perpetuation and spread (Bradley et al., 1989). To date, there are no means for treating EEDV-infected fish, limiting EED prevention and control to avoidance, culling, and/or depopulation. Biosecurity is a primary means of infectious disease prevention and control within hatchery environments, including the use of hatchery disinfectants to not only prevent the introduction of fish pathogens into hatchery systems via contaminated fomites, but also to control their spread within hatcheries themselves (reviewed in Tørgesen and Hårsein, 1995). Virkon® Aquatic (Syndel, Ferndale, Washington) is a potassium peroxymonosulfate (PPMS) based disinfectant that has viricidal and bactericidal effects (Hernández et al., 2000) and is commonly used commercially in aquaculture facilities to disinfect fish husbandry tools (Paetzold and Davidson, 2011). Additionally, Virkon® Aquatic is relatively safe for fish when used according to manufacturer protocols (Stockton-Fiti and Moffitt 2017).

PPMS-based disinfectants have proven to be efficacious at inactivating a range of viruses. For example, an investigation by Rohaim et al. (2015) showed that 1% Virkon® completely inactivated the Egyptian H5N1 avian influenza virus at a 5 to 30 minutes contact time. Another study reported that 1% Virkon® could inactivate the H7N2 strain of avian

influenza virus as well (Bieker, 2006). The use of Virkon® Aquatic has also been reported to eliminate viruses by immersing common carp in 3 ppm of the soluble disinfectant for 2 hours before experimental treatment was conducted (Sunarto et al., 2012). In addition, PPMS inactivates some dsDNA enveloped viruses, as demonstrated with Virkon® No Foam against the hepatitis B virus (Scioli et al., 1997). Lastly, PPMS was experimentally studied to investigate its efficacy against a herpesvirus *in vitro*, which revealed that even at low temperatures, Virkon® inactivated equine herpesvirus-1 (EHV-1; Tsujimura et al. 2015).

Nevertheless, in herpesviruses that infect fish, especially EEDV, specific knowledge on the efficacy of Virkon® Aquatic has yet to be determined. Therefore, this study was designed to investigate the efficacy Virkon® Aquatic against EEDV, thereby providing hatchery managers with scientific evidence to hatchery managers with the goal of establishing proper biosecurity measures targeting EEDV.

3. Materials and methods

3.1.Fish maintenance

Juvenile Lake Superior strain lake trout (*Salvelinus namaycush)* were obtained from Marquette State Fish Hatchery (MSFH; Marquette, Michigan) at 4 months of age while still raised on a closed (i.e., deep well) water source and were maintained at the Michigan State University – University Research Containment Facility (URCF) until experimental challenge (at 22 months of age) and for the duration of the study. Prior to EEDV-challenge, fish were housed in a 680-L flow-through fiberglass tank supplied with dechlorinated water (temperature at 14 °C) and fed AquaMax® Fingerling Starter 300 (Purina®, Gray Summit, Missouri) *ad libitum* with detritus/feces siphoned and removed once per day. A subset of lake trout was examined for the

presence of EEDV via qPCR (section 2.6 below) to ensure freedom from infection. All fish handling and maintenance during the study period (148 days) was in accordance with Institutional Animal Care and Use Committee (IACUC) standards.

3.2.Disinfectant solution preparation

One hour prior to the infection challenge, a 1% Virkon® Aquatic solution was prepared following the manufacturer's protocol. In brief, 8.5 grams Virkon® Aquatic was dissolved into 946 mL clean water. Then, 600 mL of this solution was transferred into an 11.4-L glass aquariums. These steps were repeated for each replicate.

3.3.Infection challenges

Because EEDV has yet to be successfully cultured *in vitro*, infectious inoculum was prepared from EEDV-infected lake trout skin collected from a natural EED epizootic as previously described (Shavalier, 2017). The skin was homogenized and trimmed to ~ 1-2 mm in diameter, to which sterile sample diluent (pH 7.525 ± 0.025) containing 458 mL Minimal Essential Medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts), 7 mL of 1 M tris buffer, 1 mL gentamycin sulfate (Sigma-Aldrich, St. Louis, Missouri), 5 mL penicillin/streptomycin (Invitrogen), and 5 mL Amphotericin B (Thermo Fisher Scientific) was added at a 1:3 (w/v) ratio. Afterwards, the suspension was homogenized (Seward Stomacher 80, Biomaster Lab System) for 120 seconds at high speed, centrifuged at 368 x g (20 minutes; 4 °C), and then frozen at -80 °C for use in infection challenges.

Prior to EEDV challenge, fish were randomly divided into five flow-through 42-L fiberglass cylindrical tanks filled with 18.9 L chilled water and categorized into three treatment

groups: one tank for the negative control (NC) group that consisted of 20 fish, three replicate tanks for the Virkon® Aquatic group consisting of 20 fish each, and one tank for the positive control (PC) group that consisted of 20 fish. All fish were acclimatized for 15 days in the water at a temperature of 10 ± 1.0 °C, and all tanks were equipped with air-lines and air-stones in a flow-through water system. On day-0 of the infection challenge, three treatments were conducted as follows.

1. Negative control (NC) treatment group. A clean and disinfected net (net-A) was soaked for 5 minutes in 600 mL water containing 7 mL sterile sample diluent (section 2.3). A second clean net (net-B) was used to transfer all 20 fish from their holding tank into net-A, where they were held for 20 seconds, and then transferred back into the NC tank.
2. The Virkon® Aquatic treatment group. A clean and disinfected net (net-A) was soaked in 600 mL water mixed with 7 mL of the EEDV solution, making the final EEDV concentration $2.25x10^5$ virus copies/mL of the water EEDV suspension (Table 3.1), for 5 minutes. Net-A was then transferred into 600 mL of the 1% Virkon® Aquatic solution for 20 minutes (manufacturer recommended duration). A second clean net (net-B) was used to transfer all 20 fish from their holding tank into net-A, where they were held for 20 seconds and then returned to their respective aquaria. This protocol was repeated for each of the three replicates and each replicate used two different nets.
3. Positive control (PC) treatment group. Fish in the positive control treatment were treated identically as the Virkon® Aquatic groups, with the exception that net-A was soaked in a 1% sample diluent water suspension for 20 minutes instead of a 1% Virkon® Aquatic solution.

Following experimental infection, all fish were observed daily for the development of disease signs and daily fish care was performed. Fish were fed *ad libitum* twice a day using

AquaMax® Fingerling Starter 300. Any moribund fish during the study period were removed from the tanks and euthanized using a lethal dose (0.25 mg/mL) of tricaine methanesulfonate (MS-222; Western Chemical Inc., Ferndale, Washington) buffered with 0.5 mg/mL of sodium bicarbonate (Church & Dwight Co., Inc., Ewing, New Jersey). A necropsy was performed, eye, gill, and skin tissues were collected and frozen at -20 °C. The same protocol was followed for any mortalities. At the end of the study (140 days post infection (pi) for PC group, 148 days pi for Virkon® Aquatic and NC groups), all surviving fish were euthanized using a lethal dose of MS-222 at a dose of 0.25 mg/mL, buffered with sodium bicarbonate (0.5 mg/mL). Then, Pooled samples were taken from the eye, gill, and skin and frozen at -20 °C.

3.4.Water and suspension sampling

To assess EEDV loads, a 1 mL sample was collected from: a) the water used to prepare the net solutions; b) the water/sample diluent suspension before net soaking (NC group); c) the water/sample diluent suspension after net soaking (NC group); d) the water/EEDV suspension before net soaking (1% Virkon® Aquatic treatment group); e) the 1% Virkon® Aquatic solution after EEDV-laden net immersion (1% Virkon® Aquatic treatment group); f) the water/EEDV suspension before net soaking (PC group); and g) the sample diluent/water suspension after EEDV-laden net immersion (PC group; Table 3.1).

3.5.DNA extraction

Skin (for the PC group) and pooled eye, gill, and skin tissues (for the NC and Virkon® Aquatic group) were thawed and a maximum of 10 mg of tissue was transferred into a sterile 1.5 mL tube for DNA extraction. All extractions were performed following the protocol outlined by

Mag-Bind® Blood & Tissue DNA HDQ 96 Kit (OMEGA Bio-tek, Inc, Norcross, Georgia) as previously described (*Chapter 1*). The DNA extractions of water and solution samples were performed following the Alternative PowerSoil Protocol for Low Bacterial Biomass Fluids from the Qiagen DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen, Hilden, Germany) as previously described (*Chapter 2*). Extracted DNA was then quantified using the Qubit™ fluorometer (Invitrogen, Eugene, Oregon), and samples were diluted with sterile DNase-free water to obtain a maximum of 12.5 ng/uL qPCR template DNA.

3.6.Molecular detection of EEDV

The SYBR Green qPCR assay of Glenney et al. (2016a) that targets the glycoprotein gene of EEDV and differentiates the virus from all other currently recognized salmonid herpesviruses was utilized in this experiment. All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} real-time PCR machine (Eppendorf, Hauppauge, New York) and were performed as previously described (Glenney et al., 2016a; *Chapter 1*). EEDV-positive tissue homogenate was used as a positive extraction control (PEC) and sample diluent was used as the negative extraction control (NEC). Further controls included EEDV-positive purified DNA and nuclease-free water, which served as the positive reaction control (PRC) and negative reaction control (NRC), respectively. Samples were considered EEDV positive if the fluorescence exceeded 10% of the maximum florescence within 35 amplification cycles (Shavalier, 2017). Viral loads (copies/mg and copies/mL) were calculated using the Mastercycler ep realplex2 S accompanying software via comparison to a standard curve that was generated via 8 serial 10-fold dilutions of EEDV positive standards (Shavalier, 2017).

4. Results

4.1.Gross disease signs and cumulative mortality

At the time of necropsy, lake trout in the three treatments ranged from 10.2 - 29.5 cm (average 18.2 ± 2.98 cm) in length and 8.6 – 110.1 grams (average 54.9 ± 27.1 grams) in weight. Disease signs consistent with EED were observed in the positive control group beginning at day 26 pi. Initially, these disease signs included lethargy and exophthalmia/corneal opacity, which continued to progress and become more severe. In addition to increasing lesion severity, ocular hemorrhage, severe congestion at the base of the fins, and secondary water mold invasion of the fins, body, and eyes were observed as infections progressed. The first mortality in the PC group occurred on day 29 pi and continued on days 31 (n=3), 34 (n=4), 38 (n=1), 39 (n=1), 40 (n=1), 42 (n=1), 44 (n=1), 47 (n=1), 56 (n=1), and 63 pi (n=1) until stabilizing at 80% cumulative mortality (Figure 3.1). In contrast, no EED disease signs were observed in any of the Virkon® Aquatic treatment replicates. However, one fish in replicate 1 and one fish in replicate 2 died during the study period (Figure 3.1; overall mean cumulative percent mortality of 3.3%). No mortality, nor EED disease signs, were observed in any negative control fish throughout the course of this study (Figure 3.1).

4.2.Molecular detection of EEDV

The estimated EEDV loads in the water/EED suspension prior to net soaking for the positive control and Virkon® Aquatic treatment groups ranged from $1.77x10^5$ - $2.25x10^5$ virus copies/mL and from $1.85x10^4$ - $7.29x10^4$ virus copies/mL after net soaking (Table 3.1). In contrast, EEDV was not detected in negative control water or sample diluents (Table 3.1), nor was it detected in lake trout prior to infection challenge. Likewise, EEDV was not detected in

the pooled eye, gill, and skin samples of any NC fish, nor was it detected in any of the fish within the Virkon® Aquatic treatment (Table 3.1). In the positive control group, however, 18/20 fish were EEDV positive, whereby estimated virus loads exceeded the initial challenge concentration and ranged from $2.16x10^6$ - $3.64x10^{11}$ virus copies/mg skin tissue (Table 3.1). Among the EEDV positive individuals, 16 died and 2 were euthanized at 140 days pi.

5. Discussion

Contaminated equipment has been implicated in the transmission of numerous microbial fish pathogens (reviewed in Woo and Cipriano, 2017). In this context, disinfection is one of several tools that can be successfully used for preventing pathogen transmission into and within hatchery systems. Prior to this study, however, there was a complete lack of knowledge on the efficacy of hatchery disinfectants against EEDV, a matter hampering hatchery biosecurity efforts to prevent and control EEDV-associated losses. Herein, we provide evidence that under laboratory conditions, Virkon® Aquatic can prevent EEDV contagion to a highly susceptible host species/strain (i.e., Lake Superior strain lake trout) on contaminated fomites. Indeed, despite the development of severe and characteristic EED disease signs (Bradley et al. 1989), 80% cumulative mortality, and 90% EEDV infection prevalence with concomitantly high virus loads in lake trout that were netted with an EEDV-contaminated net, no signs of disease, EED-associated mortality, or the virus itself were detected in lake trout netted with an EEDV contaminated but then 1% Virkon® Aquatic treated net.

Although this study was the first to empirically assess EEDV susceptibility to Virkon® Aquatic, previous studies have examined the efficacy of Virkon® against other enveloped dsDNA viruses. For example, Gasparini et al. (1994) found that Virkon® damaged the surface

antigen of the hepatitis B virus (Family Hepadnaviridae) and Scioli et al. (1997) similarly found that Virkon® modified the morphology of hepatitis B, thereby causing lost infectivity. Likewise, Bryan et al. (2009) found Virkon® was effective at inactivating a ranavirus (Family Iridoviridae) isolate, whereas McCormick and Maheshwari (2004) showed that Virkon® was also capable of inactivating adenovirus 5 and 6 (Family Adenoviridae). Specifically, against herpesviruses, Tsujimura et al. (2015) found that Virkon® effectively inactivated equine herpesvirus type 1 (Family Herpesviridae) and Hick et al. (2016) reported it inactivated ostreid herpesvirus-1 (Family Malacoherpesviridae). Results from the current study show that the use of Virkon® Aquatic holds promise against a fish-pathogenic herpesvirus belonging to the Family Alloherpesviridae, EEDV.

One of the limitations of the current study, primarily brought about by the inability to culture EEDV *in vitro*, was the relatively low concentration of EEDV used for experimental challenge of the fish. Although the utilized virus concentration in the net soak suspension (i.e., $1.77x10^5$ - $2.25x10^5$ virus copies/mL) exceeded the estimated median lethal dose for EEDV via immersion (i.e., $4.7x10^4$ virus copies/mL; Shavalier 2017), the exact concentration the fish were exposed to on the virus-contaminated net is unknown and may have been substantially lower than the suspension concentration. Nevertheless, EEDV loads in 90% of the positive control fish exceeded that of the virus suspension concentration, thereby showing EEDV infection and subsequent virus replication was achieved. Likewise, the current experimental challenge model led to initial mortality (day 29 pi) that was similar to what was observed via immersion challenge by Shavalier (2017; day 28 pi) and thus shows promise for future disinfection studies that mimic common hatchery practices.

Although not the focus of this study, it is of interest that two of the four-lake trout that survived to 140-day pi still harbored relatively high EEDV loads ($2.16x10^6$ - $3.35x10^6$ virus copies/mg skin). In comparison, EEDV loads ranged from $1.33x10^4$ – $5.83x10^6$ virus copies/mg skin in Lake Superior strain lake trout at the end of the day 66 experimental challenge period (*Chapter 1*) and $1.59x10^7$ – $7.18x10^7$ virus copies/mg skin in Seneca strain lake trout at 100 days pi (*Chapter 1*). Even though virus delivery varied between this study and Chapter 1 (i.e., net exposure vs. intraperitoneal injection), both studies further demonstrate the extended periods that lake trout can harbor relatively high EEDV loads in their skin.

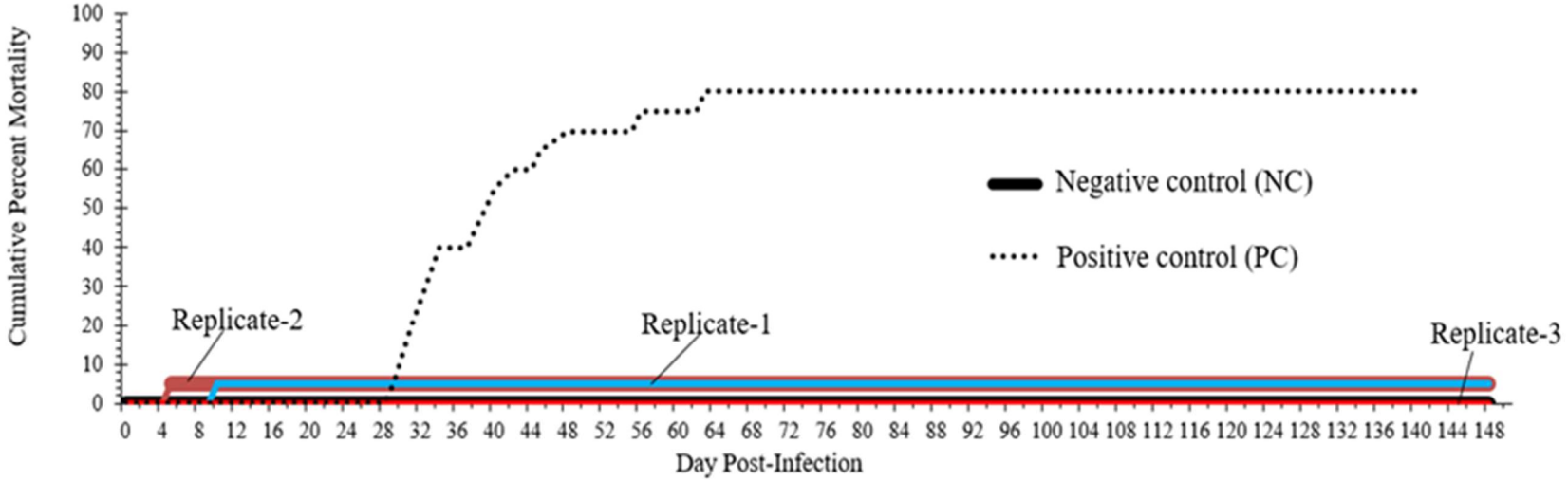
In conclusion, herein we provide the first evidence that Virkon® Aquatic is effective at reducing the risk of EEDV contagion on contaminated hatchery equipment. Although Virkon® Aquatic is marketed for effective disinfection in the presence of organic material, further studies should evaluate its capacity to prevent EEDV transmission on hatchery equipment under field conditions.

APPENDIX

Table 3.1: The EEDV qPCR result of the samples of the negative control (NC), the Virkon® Aquatic, and the positive control (PC) group as well water and treated water.

Group	Number of samples	qPCR result	Virus load (virus copies/mg skin or mL water)
▪ Negative control (NC)	20	0/20	-
▪ Virkon® Aquatic			
Replicate-1	20	0/20	-
Replicate-2	20	0/20	-
Replicate-3	20	0/20	-
▪ Positive control (PC)	20	18/20	$2.16x10^{6}$ - $3.64x10^{11}$ /mg
▪ Sample of water that was used in this study	1	0/1	-
▪ Sample of water containing sample diluent before soaking it with net (NC)	1	0/1	-
▪ Sample of water containing sample diluent after soaking it with net (NC)	1	0/1	-
▪ Water sample collected from EEDV-laden water prior to soaking net in 1% Virkon® Aquatic	1	1/1	$2.25x10^{5}$ /mL
▪ Sample collected from 1% Virkon® Aquatic solution after EEDV-laden net immersion	1	1/1	$1.85x10^{4}$ /mL
▪ Water sample collected from EEDV-laden water prior to soaking net in the PC group	1	1/1	$1.77x10^{5}$ /mL
▪ Sample collected from sample diluent treated water after EEDV-laden net immersion	1	1/1	$7.29x10^{4}$ /mL

Figure 3.1: The cumulative mortalities that occurred in the positive control (PC), negative control (NC) and Virkon® Aquatic (Replicate-1, 2, and 3) group during the study period.



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OVERALL CONCLUSIONS AND FUTURE RESEARCH

1. Overall Conclusions

Epizootic epitheliotropic disease virus (EEDV) emerged as a threat to Great Lakes hatchery-based lake trout conservation efforts in the 1980's and has since re-emerged as a source of lake trout mortality decades later. Despite EED-associated losses and concomitant concerns from Great Lakes fishery managers and biologists, much remained unknown regarding the host-range of EEDV, which fish species can serve as infection reservoirs, how and for how long this virus can be shed by infected fish, and how the risk of EEDV transmission can be reduced on contaminated hatchery equipment. In this context, the three studies presented herein shed light on these challenges facing fishery managers in the Laurentian Great Lakes.

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

results showed that two lake trout strains (e.g., Seneca and Lake Superior) not only varied in EED-associated mortality, but possibly also in the length of time that the EEDV infections persist.

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

Findings from the third study provided evidence that a potassium peroxymonosulfate-based hatchery disinfectant (Virkon® Aquatic) effectively prevented EEDV transmission on contaminated fomites (e.g., nets) to a highly susceptible lake trout strain (i.e., Lake Superior strain). In fact, EEDV was not detected in a single lake trout that was handled with an EEDV-contaminated but then Virkon® Aquatic disinfected net, compared to a 90% EEDV infection prevalence in lake trout handled with an undisinfected, EEDV-contaminated net.

Despite the significant challenges posed by working with a virus that has yet to be cultured *in vitro*, the collective findings presented herein provide fishery managers, fishery biologists, and fish health professionals with valuable knowledge on the biological properties of EEDV so that improved strategies can be devised to prevent and control EEDV in Great Lakes hatcheries and beyond.

2. Future Research

Although this thesis has uncovered important knowledge regarding EEDV transmission, reservoirs of infection, and practical means for prevention of EEDV contagion within hatchery environments, much more remains unknown. For example, I showed that lake trout strains vary in their susceptibility to EEDV-associated mortality and also possibly in virus persistence. Whether shedding rates, tissue tropism, and/or virus incubation time also vary between these and other lake trout strains, however, remains to be elucidated and warrants further study. Likewise, it may be possible to focus lake trout conservation efforts on strains that are less EEDV-susceptible, but any effects this might have on conservation goals would need to be evaluated. Another remaining unknown surrounds EEDV latency and recrudescence, whereby EEDV shedding in Chapter 2 suddenly stopped at approximately nine weeks post-infection. However, whether surviving fish that did not shed the virus for three more weeks eventually shed the virus again (i.e., latent carriers) needs to be determined, as does the site of virus latency within the host. Similarly, knowing now that lake trout strains vary in EEDV susceptibility, do they also vary in shedding rates and shedding periods? Moreover, the fact that individual fish varied in their EEDV shedding rates and/or patterns demonstrates the importance of future studies examining the host immunological factors that are involved in combatting EEDV, as well as how such factors are affected by rearing density and environmental factors. Regarding Virkon® Aquatic, it's efficacy against EEDV under field conditions (i.e., fish slime, heavy organic loads) is an important next step towards improved hatchery biosecurity. Additionally, the efficacy of other hatchery disinfectants against EEDV, including those used for egg surface disinfection (e.g., iodophor), warrants further attention, as does the ability to inactivate EEDV via treatment of the water (i.e., ultra violet water disinfection).

REFERENCES

REFERENCES

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