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

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

Megan Shavalier, Mohamed Faisal, Scott Fitzgerald, and Matti Kiupel

*Comparative Medicine and Integrative Biology, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA (MS)*

*Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA (MF, SF, MK)*

*Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI, USA (MF)*

*Corresponding author: Mohamed Faisal, Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, 1129 Farm Lane, Room 174, East Lansing, MI 48824. Phone: (517) 884-2018. Email: faisal@cvm.msu.edu

**Abstract**

Epizootic Epitheliotropic Disease Virus (salmonid herpesvirus-3) is a lethal disease of lake trout (*Salvelinus namaycush*) currently threatening rehabilitation efforts of this indigenous fish species in the Great Lakes Basin. Virus biology, pathology and host interactions remain mostly unknown, preventing the design of effective control strategies. This study examines the progression of disease and associated pathology following exposure of naïve juvenile lake trout via bath immersion (*n*=84 infected; *n*=48 control). Individual tissues (*n*=10 per fish) were analyzed for gross and histopathologic changes over the course of six weeks. A three-week incubation period occurred prior to observation of clinical signs and morbidity. Early gross pathology included exophthalmia, ocular hemorrhage, fin congestion, and hyperemia of visceral blood vessels. Advanced disease manifested with the addition of multifocal to coalescing erosions and ulcerations of the skin, and congestion of visceral organs. The earliest microscopic lesions developed in the skin and fins, and were characterized by localized cellular degeneration of epidermal epithelial cells that progressed to erosions and ultimately focally extensive necrosis and associated dermatitis and perivasculitis. Early signs of systemic disease were characterized by multifocal to confluent necrosis in the spleen, multifocal intestinal necrosis, and lymphohistiocytic perivasculitis of the omentum and the epicardium. Focal necrosis of interstitial hematopoietic cells in the anterior kidney and rare apoptotic cells in the liver were also observed. The progression of lesions is consistent with the cutaneous epithelium representing the primary target of viral infection with hematopoietic organs and vessels undergoing pathology at the later viremic phase of the disease.

Key words: epizootic epitheliotropic disease virus, fish, Great Lakes, lake trout, pathology, salmonid herpesvirus-3, skin, virology

Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) has been implicated in multiple mortality events throughout the Midwestern United States over the past three decades[2,8] and continues to be a concern in Great Lakes fisheries management today.[13] EEDV has been shown to be particularly pathogenic to juvenile lake trout (*Salvelinus namaycush*) based both on viral identification during mortality events[2] as well as through experimental challenges.[3,9] The clinical manifestation of EEDV in experimental challenges was consistent with that seen in natural outbreaks which included catastrophic mortalities in juvenile hatchery raised lake trout, with fish exhibiting ocular hemorrhage, corneal opacity, gill pallor, skin lesions ranging from pallor to erosions and ulcerations, water mold overgrowth, erosion and congestion of the fins and erythema of the oral cavity, isthmus and ventrum.[13] Additional detection of EEDV was reported from the reproductive fluids and kidneys of mature spawning lake trout.[6,8] Unfortunately, specific information regarding virus biology, pathology and host interactions remains largely unknown, in part due to the lack of an EEDV susceptible cell line and inability to culture this virus *in vitro*. This knowledge gap hampers the design and implementation of effective disease control strategies in the Laurentian Great Lakes Basin. A recently developed *in vivo* model of immersion challenge and virus replication (*data not shown*) has provided a crucial step in the ability to study the pathogenesis of EEDV, allowing for examination of EEDV pathology and disease progression under controlled laboratory conditions. Elucidating the sequential pathology and disease progression following EEDV infection is of vital importance as this virus continues to threaten lake trout rehabilitation efforts throughout the Midwestern United States.

While many questions remain about the pathology of EEDV, this is not the case for all Alloherpesviruses. Evaluation of samples collected during natural outbreaks and experimental challenges aided in describing the pathology associated with infection of rainbow trout (*Oncorhynchus mykiss*) with *Oncorhynchus masou* virus (OMV; salmonid herpesvirus-2).[5] In both naturally and experimentally infected fish, severe pathology was observed in the intestine, spleen and kidney including necrotic and hemorrhagic foci in the splenic pulp and hematopoietic tissue. Some fish also displayed necrotized gill filaments and hepatocytes, yet surprisingly, carcinoma development was not noted in surviving fish as is typical with other salmonids infected with OMV.[5]

Similar experimental studies performed with salmonid herpesvirus-1 (first isolated in healthy adult rainbow trout)[3,4] resulted in gross lesions including alterations of pigmentation, exophthalmia and abdominal distension, as well as hemorrhages in the fins, pale gills and yellow to red tinged ascites.[15] Microscopic pathological lesions were noted in the heart, musculature, liver, kidney and pancreas.[15]

Salmonid herpesvirus-4 is the causative agent of Atlantic salmon papillomatosis (ASP), a proliferative disease seen primarily in juvenile Atlantic salmon (*Salmo salar*) undergoing the smolting process.[7] Reported ASP lesions consist of epithelial cell hyperplasia and karyomegaly with a loss of goblet cells and disruption of the basement membrane.[4]

Previous histopathologic examination of EEDV-infected tissues has been limited to primarily severely diseased fish collected during an epizootic episode.[2,13] In such severe cases, epithelial lesions have often been obscured and contaminated by the invasion of opportunistic and pathogenic bacteria and water mold into lacerated tissues,

leading to difficulties in accurate assessment of underlying disease pathology. In order to alleviate the inherent challenges of attributing severe lesions to EEDV during natural infections, a study was designed to characterize the progression of gross and histopathologic disease after experimental exposure of naïve host fish to a moderately lethal dose of EEDV. The data generated herein will provide researchers, diagnosticians and fisheries managers with crucial information regarding early microscopic tissue alterations associated with an EEDV infection.

## Materials and Methods

### *Fish and maintenance*

EEDV experimental challenges were performed using juvenile, Lake Superior strain lake trout (6 months post-hatch) supplied by the Marquette State Fish Hatchery (Marquette, Michigan). All experimental fish were randomly collected from a lot certified to be free of any reportable pathogens as per the American Fisheries Society – Fish Health Section (AFS-FHS) Blue Book.[1] Certification was achieved following normal clinical examination of 60 randomly collected fish. Additionally, the absence of EEDV in the lot was confirmed using quantitative PCR (qPCR) as detailed below. Experimental challenges were performed at the Michigan State University – Research Containment Facility (MSU-URCF, East Lansing, Michigan) in accordance with the MSU Institutional Animal Care and Use Committee guidelines and approval. Upon receipt, all fish were housed in a 680 L fiberglass aquarium and allowed to acclimate to standard laboratory conditions for a minimum of one month prior to experimental challenges. At all times, fish received continuous, oxygenated well water and were fed 1.0 mm sinking feed *ad*

*lib* (BioOregon, Westbrook, Maine, USA). During the experimental studies, fish were housed in 42 L fiberglass aquaria receiving continuous, flow-through, oxygenated well water at a temperature of 9.0 ± 0.5°C.

*EEDV in vivo serial passages and stock production*

A stock of infectious EEDV was produced from the skin of naturally infected lake trout collected during a natural outbreak. Skin was homogenized in a sterile phosphate buffered saline solution, (pH 7.5 ± 0.5; Sigma-Aldrich, St. Louis, Missouri, USA) at a 1:3 (w/v) ratio, and clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C. The initial EEDV stock was then passed through multiple groups of naïve juvenile lake trout via either an intraperitoneal injection or an immersion bath challenge. For the intraperitoneal challenges, fish were anesthetized using tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, Washington, USA; 0.1 mg/mL) then injected with 300-400 µL of virus stock solution and allowed to recover from sedation prior to return to flow-through aquaria for the duration of the study. For the immersion bath challenges, fish were transferred to glass aquaria containing pre-determined and combined volumes of virus stock and water. Fish were held in these static aquaria at a constant water temperature (9 ± 0.5°C) with continuous aeration for 1 hour, after which time they were transferred back to their flow-through aquaria for the duration of the study. Following virus exposure, fish were monitored daily for development of morbidity or mortality, where upon death or development of severe clinical disease (e.g., loss of equilibrium, difficulties respiring), the fish were euthanized with an overdose of MS-222 (0.25 mg/mL), and their skin collected and processed as described above with the substitution

of Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL; Invitrogen), and amphotericin B (250 µg/mL; Invitrogen) rather than PBS. Skin homogenates created from multiple individual fish within each passage were pooled together to create new batches of EEDV stock from which a new group of naïve lake trout were in turn infected. This process continued until an adequate volume of 7$^{th}$ passage stock was produced for use in all future studies. Viral exposure route and dose are presented in Table 1.

*Experimental challenges – Dose range determination*

Following the challenges outlined in Table 1, our first experiment extrapolated on preliminary results in order to examine the dose range or viral load required to lead to infection and clinical EED. This study included a total of 60 fish and six treatment groups (5 viral doses and 1 negative control group). Viral doses and the negative control dose were prepared as described above using 7$^{th}$ pass EEDV stock. The highest infected group was exposed to 9.5x10$^{7}$ viral copies/mL water with subsequent doses being 1:10 dilutions made using sterile sample diluent (MEM). Exact viral doses were determined using qPCR to test water samples and calculate viral copies per mL of water for each dose (Table 2).

Immersion exposure was achieved by transferring experimental fish into static, aerated glass aquaria where the infectious or control dose had been added. Fish were monitored for 1 hour, during which time the immersion water was held at a constant

temperature consistent with the flow-through experimental tanks (9 ± 0.5°C) by submerging the experimental aquaria in larger flow-through vessels. After one hour, fish were transferred back to their flow-through aquaria and monitored daily for a period of 30 days for mortalities or development of clinical morbidity. Any moribund fish displaying severe clinical signs such as altered behavior, inability to maintain balance, gasping for air or significantly pale gills was euthanized. Skin and gill tissues were collected from each fish immediately following death. These tissues were tested for the presence of EEDV using qPCR as described above and viral loads in bath immersion water samples were used to calculate projected viral dose ranges and mortality percentage pairings based on the calculations of Reed and Muench (1938) (Table 3).

*Experimental challenges – Lesion progression*

Fish were divided into an EEDV group ($n$ = 84) and a negative control group ($n$ = 48). All fish were challenged via the immersion method described above. The EEDV group was exposed to the median lethal dose of EEDV calculated based on the previous challenges, while the negative control group was immersed in a sham suspension of MEM diluent. After completion of the exposure period (1 hour), fish were transferred back to their experimental flow through aquaria for the duration of the study where they were monitored daily for development of clinical disease signs.

Sampling occurred in parallel from the two experimental groups on Days 0, 1, 3, 6, 9, 12, 15, 28, 21, 28, 35, and 42-post infection (p.i.), at which time seven EEDV infected fish and four negative control fish were collected and euthanized in a manner which minimized the stress for both sampled and remaining fish. On each sampling day,

one infected fish and one control fish were preserved whole in 10% neutral buffered formalin; a ventral midline incision was created to allow for improved internal fixation. Full external and internal exams were performed on the remaining six infected and three negative control fish and portions of skin, fin, gill, eye, brain, spleen, heart, liver, intestine, and kidney were collected from each fish. Each tissue was divided for: 1) detection of EEDV by PCR (frozen at -20°C) and 2) microscopic examination (fixed in 10% neutral buffered formalin). Portions were collected from both anterior and posterior kidney, one whole eye was collected for each assay, and the "intestine" sample consisted of a segment approximately 1 cm oral to the vent.

*Virus detection and quantification*

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

total DNA template. Positive control standards for quantification were produced using known positive skin samples following the method outlined by Glenney et al. (2016). Positive extraction controls consisted of known positive skin samples; MEM was used for the negative extraction control and nuclease free water was used for a PCR negative control.

*Histopathology and lesion scoring*

After fixation, tissues were processed for routine paraffin embedding. Paraffin blocks were sectioned at 5 µm, and slides were routinely stained with hematoxylin and eosin (H&E) for histopathologic examination.[11] All slides were examined by an American College of Veterinary Pathologists board-certified pathologist who was blinded to the presence of gross lesions or results of EEDV detection in individual tissues. All identifiable lesions were scored on a scale of 0 to 3, with 0 being no lesion (normal), 1 being mild lesion, 2 being moderate lesion and 3 being severe lesion.

## Results

*EEDV in vivo serial passages*

Consecutive rounds of intraperitoneal and immersion challenges of naïve juvenile lake trout with clarified tissue homogenate from previously EEDV-infected fish consistently produced morbidity and mortality, from which EEDV genetic material was recovered (Table 1). Additionally, clinical signs were comparable with those seen in natural infections and included multifocal skin erosions (Figure 1), petechial ocular hemorrhage (Figure 2), fin erosion and congestion (Figure 3), pale gills and visceral

organs, renal congestion, and hemorrhagic enteritis. Initiation of mortality ranged from 6 to 36 days following viral exposure, with the majority of trials seeing the first mortalities 2-3 weeks after viral exposure. A similar range was seen regarding cessation of mortalities with 0-2 fish surviving in most trials approximately 1 month after initial infection (Table 1). Viral titers in EEDV stocks increased approximately 1,000-fold from the initial stock to the 6$^{th}$ pass (Table 1).

*Morbidity and mortality response to varying doses of EEDV*

Fish were monitored for a period of 35 days following viral exposure to the 10-fold dilutions of EEDV, after which time all survivors were euthanized. During that time, the negative control group had 2 mortalities (no evidence of EEDV infection) while mortalities (out of 10) in dilution groups from low dose to high dose ranged as follows: 3, 1, 0, 8, and 10 (Figure 4), respectively. Clinical findings were consistent with those seen in previous experimental challenges and detailed above. Using qPCR to test skin samples collected from all fish, EEDV was detected in all 10 of the high dose fish, one of the fish in the first dilution group and none of the remaining study specimens (Table 2). Using the calculation method developed by Reed and Muench (1938), doses were calculated to theoretically represent a range of projected mortality percentages (Table 3, Figure 5).

*Clinical disease and gross lesion progression*

Infected fish exhibited exophthalmia and ocular hemorrhage (Figure 6) as early as one day p.i., increasing from 33% of fish sampled on Day 1 (*n* = 2/6) to 100% of fish

sampled on Day 28 (*n* = 6/6). Other early signs of clinical disease (i.e., first observed in a minimum of 2 fish per day prior to Day 18 p.i.) included congestion at the base of pectoral and pelvic fins (Figure 7) and along the isthmus, mild splenic pallor, and hyperemia or engorgement of enteric and hepatic blood vessels (Figure 8). During later stages of disease (i.e., Days 21-42 p.i.), fish consistently exhibited moderate to severe congestion of multiple fins (Figure 9), ocular hemorrhage and exophthalmia, multifocal to coalescing erosions and ulcerations of the skin, fins and caudal peduncle (Figure 9), as well as congestion of visceral organs such as kidney, liver and spleen. The number of fish with clinical disease increased each sampling day from 33% of fish being affected at Day 6 to 100% being affected by Day 15 through the end of the study. Likewise, the total number of observed gross lesions each day increased from ≤ 6 on Days 0-12 (average of < 1 per fish per day) to averaging > 5 lesions per fish per day from Day 21 through the end of the study. Mortalities occurred in the infected group on Day 28 p.i. (*n* = 4) while no mortalities or gross lesions were noted in the negative control group. A detailed account of gross pathology is presented in Supplemental Table 1.

*Virus identification*

In order to confirm the presence of EEDV in experimentally challenged fish, external tissues (e.g., skin) and internal organs (e.g., kidney) were tested via qPCR as described above. EEDV DNA was detected in the skin (*n* = 2/6 fish) as early as Day 18 p.i., and from Days 21-42 p.i. the virus was detected in the skin of 96% of fish sampled (*n* = 23/24) (Table 4). In comparison, the virus was not detected in the kidney until Day

21 p.i., and in only 67% of fish from Days 21-42 p.i. (*n* = 16/24). Throughout the experiment, no EEDV nucleic acid was detected from control fish.

*Microscopic lesions and scoring*

A total of 754 tissues from 12 control fish and 67 infected fish were examined for microscopic lesions. A small number of tissues (< 5%) were unable to be appropriately examined due to preservation, preparation artifact, or sectioning angle which led to insufficient tissue available for analysis. A detailed chart of lesions by day and tissue type, along with the number of fish from which lesions were observed is presented in Supplemental Tables 2-3.

Hepatic lipidosis was nearly ubiquitous among control fish throughout the study (Figures 10, 11), and among infected fish during the early weeks of the experiment (Figure 12) yet was observed in only 30% of infected fish examined during the last three sampling weeks (Figure 13). Fixation artifact was observed in many intestinal sections from fish in both the EEDV group and the negative control group where distal villi became mild to heavily multifocally vacuolated, which was likely caused by superficial epithelial cells swelling due to contact with buffered formalin prior to complete fixation. There was a mild, patchy proliferative branchitis in the gills of many fish in both groups throughout the study. This was characterized by a loss of secondary lamellae coupled with a thickening epithelial layer and infiltration of mononuclear cells. There was no evidence of an increasing severity over time, and there were no differences in distribution and severity of branchitis between fish in the negative control group and the EEDV group.

The primary microscopic lesions noted in this current study were observed in the skin and fins (Figures 14-19). The earliest lesions were observed in the epidermis at Day 21 and consisted of individual epithelial cell degeneration and single acantholytic cells. In multiple areas, there were intracytoplasmic eosinophilic vacuoles in few epithelial cells as well as rare intraepithelial inflammatory cells (Figure 15). Lesions progressed in some areas to epithelial erosions with sloughing of degenerated epithelial cells (Figure 16). By Day 28, epithelial erosions were more severe with extensive intraepithelial inflammatory infiltrates and cellular degeneration (Figure 17) and in the most advanced lesions, epithelial cells were undergoing degeneration and necrosis throughout all layers of the epidermis (Figure 18). The most severe lesions were observed at Day 35 and characterized by massive epithelial ulceration and complete epidermal loss (Figure 19). Cutaneous lesions were not observed prior to Day 21, but were observed in all fish examined at later time points except for a single fish on Day 35 and one fish on Day 42.

As early as Day 3 post exposure, focal areas of single cell necrosis were observed in the liver (Figure 12), characterized by cells with deeply basophilic, shrunken, pyknotic hepatocellular nuclei and hypereosinophilic, contracted cytoplasm. Such changes were observed with increasing severity as evidenced by increasing numbers of foci of pyknotic cells and in more fish per sampling day. Beginning on Day 9, ≥ 66% of fish were affected by similar lesions with 100% of fish that were sampled over the final three weeks showing at least mild changes. Starting at Day 18, single cell necrosis focally expanded to small foci of hepatocellular necrosis and there was also lymphohistiocytic perivascular inflammation affecting both hepatocellular arteries and

veins (Figure 12). Lesions were most severe in fish collected on days 21 and 28, but similar lesions were found in fish examined at later days (Figure 13).

Also at 28 days post exposure, both examined fish had severe lymphoid necrosis of the spleen (Figures 20, 21) and lymphohistiocytic perivasculitis affecting multiple organs, but being most severe in the omentum (Figures 22, 23), heart (Figure 24) and, as previously discussed, in the liver. Lymphohistiocytic perivasculitis was most severe in the omentum of one fish euthanized on Day 28 and there were rare intranuclear inclusion bodies in monocytes in the lumens of affected blood vessels (Figure 23). While proliferative branchitis with focal lymphohistiocytic inflammatory cell infiltrates (Figure 25) was observed in numerous EEDV infected fish, similar lesions were also found in control fish.

Microscopic lesions in the kidneys (Figures 26-31) were not observed until Day 28 post exposure; however, once these changes began to occur, they were noted in every fish examined from that point through the end of the study. Renal pathology was characterized by depletion and multifocal necrosis of interstitial hematopoietic cells. At higher magnifications, pyknotic and degenerate nuclei were found in hematopoietic cells (Figures 28, 29), while at low magnifications, lesions were characterized by a marked reduction in the number of interstitial cells (Figures 30, 31). While these changes were observed in both anterior and posterior kidney sections, no significant pathology was noted in the renal tubules at any time points.

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

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

## Discussion

In this study, we have demonstrated that infection with Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) leads to development of a lethal, systemic disease in lake trout and have further elucidated the pathogenesis of EEDV in its primary host species. Clinical signs and gross and microscopic lesions induced through experimental immersion challenge were consistent with those observed in natural EEDV outbreaks.[2,13]

Challenges outlined in Table 1 served multiple purposes: to maintain virus infectivity, as some herpesviruses are known to lose their virulence following extensive frozen storage,[14] to serve as a guide for future experimental challenge dose range choices, to increase viral titers in available stocks, and to expand our knowledge about the disease course and associated clinical signs following exposure to EEDV. With these trials, we were able to successfully maintain an active and virulent stock of EEDV over the span of 12 months, increase the relative viral loads within each stock batch, and reproduce clinical EED consistent with natural outbreaks in a controlled laboratory environment.

Based on the results of the dose-dependent challenges, for future *in vivo* experiments with EEDV to produce clinical morbidity and mortality in juvenile lake trout, we would recommend an immersion challenge model as follows: virus dose at or above $4.7x10^4$ copies/mL water, exposure of at least 1 hour, and constant maintenance during

and after viral exposure of water temperature at 9 ± 1°C. The use of these conditions with EEDV has demonstrated the ability to cause juvenile LS strain lake trout to develop clinical disease. Finally, we would recommend a monitoring period of at least 60 days rather than the 30 used here, as this virus appears to be rather slow growing and may take additional time to develop an active infection.

The incubation period was significantly longer for EEDV infected fish compared to other viral diseases of fish and the earliest lesions were observed in the eyes, skin and fins. While exophthalmia and ocular hemorrhage were described grossly, no ocular lesions were reported microscopically. This discrepancy may simply reflect our inability to track individual fish with gross lesions across subsequent sampling days. The grossly observed skin lesions corresponded with the reported degenerative and necrotic epidermal lesions described microscopically and represent the first manifestation of EEDV in lake trout. Early cutaneous lesions were followed by viremia that resulted in severe lesions in internal organs and mortality at Day 28. The severity of gross lesions, histopathologic lesions, and mortalities also coincided with peak viral identification around 28 days p.i. via qPCR (data not shown). These results demonstrate the prolonged and delayed development of clinical disease following exposure to EEDV. This is ecologically and epidemiologically important in terms of identifying potential viral sources following a mortality event. The point of exposure may have been up to a month prior to observation of diseased fish given the extended incubation period in the present study.

Interestingly, while EEDV nucleic acid was not identified in the liver until Day 21 (data not shown), pathological changes were observed among hepatocytes soon after

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

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

salmonids. While not particularly life threatening to the fish, this shortening allows for an increase in gill exposure to environmental conditions. This could have led to increased exposure to waterborne infectious agents, but also increased exposure of the sensitive gill lamellae to non-infectious debris and particles in the environment, leading to thickening and increased cellular infiltrates as described. So while the EED virus may cause degenerative and inflammatory changes within the gills, variation in individual fish anatomy may cause similar changes that could obscure such lesions. Additionally, an oblique angle when sectioning gill arches can have a great affect on the ability to interpret lamellar changes. In order to more definitively identify EEDV-related lamellar changes, multiple gill arches from each fish should be examined.

This study also provides evidence for the potential cause of death of EEDV infected fish. During the last three weeks of the experiment, cutaneous lesions were observed in 80-100% of infected fish. Epithelial necrosis as observed here is known to lead to hypo-osmotic shock and death in fish infected with other viruses such as Koi Herpesvirus (cyprinid herpesvirus-3).[10] Additionally, by Day 28 p.i., infected fish had developed lesions in multiple organs that were primarily centered on vessels. Observation of intranuclear inclusion bodies in blood monocytes indicates transmission of virus from affected surface epithelium through infected monocytic cells causing viremia and secondary infection of internal organs. Such lesions are most likely indicative of an overall poor systemic health of viremic fish. As renal tubular damage was not appreciated throughout the study, renal function was likely unaffected. However, the combination of lymphoid necrosis causing immune suppression, perivasculitis most likely resulting in a systemic cytokine response, and epithelial

damage coupled with the general stress caused by high densities and standard operating procedures within salmonid hatcheries is enough to tip the scales, resulting in the observed mortalities.

Finally, when comparing the relatively minor mortality rates observed in the current study with the high viral titers demonstrated in the previous chapter, a concern arises that lake trout are able to not only survive an EEDV infection, but such fish may in fact be able to act as a viral reservoir and subsequently pose a substantial risk to younger, more susceptible populations of fish. While we have demonstrated that EEDV causes a lethal disease in experimentally challenged lake trout, we have also shown that fish are able to survive despite high viral loads and advanced pathological lesions. This highlights the need for aggressive and persistent screening for EEDV in captive lake trout in order to rapidly identify and quarantine any potentially infected populations before the virus can spread throughout the hatchery. Recent mortality events[13] have highlighted the fact that EEDV remains present and a threat within the Great Lakes basin, and this current study has provided us with key information regarding the host-virus interactions.

Supplementary tables are provided. Additional data used in this study can be made available upon reasonable request.

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## Figure Legends

**Figures 1-3.** Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection, juvenile lake trout (*Salvelinus namaycush*). **Figure 1.** Multifocal skin erosions with erythema and excess mucous accumulation. **Figure 2.** Ocular hemorrhage. **Figure 3.** Anal and caudal fin erosion with congestion, caudal peduncle erosion with erythema, generalized pallor.

**Figure 4.** Dose-dependent percent cumulative mortality of juvenile lake trout (*Salvelinus namaycush*) following immersion bath exposure to Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3). Doses presented as a base-10 log of the number of viral copies per mL immersion bath water.

**Figure 5.** Box plot highlighting calculated lethal immersion doses of Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) based on dose dependent cumulative mortalities and calculated following methods developed by Reed and Muench (1938). Lower hash mark represents $LD_{10}$ (i.e., dose required to cause 10% mortality), lower box margin represents $LD_{25}$, middle box bar represents $LD_{50}$, upper box bar represents $LD_{75}$, and upper hash mark represents $LD_{90}$.

**Figures 6-9.** Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection (bath immersion), juvenile lake trout (*Salvelinus namaycush*). **Figure 6.** Ocular hemorrhage. **Figure 7.** Pectoral fin congestion. **Figure 8.** Engorgement or hyperemia of major hepatic vasculature. **Figure 9.** Caudal and anal fin

congestion, generalized pallor of the caudal peduncle, erosion of caudal peduncle and caudal fin.

**Figures 10-13.** Control and Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection, juvenile lake trout (*Salvelinus namaycush*), liver. HE. **Figure 10.** Negative control, Day 1, severe hepatic lipidosis. **Figure 11.** Negative control, Day 9, moderate hepatic lipidosis. **Figure 12.** Infected, Day 18, focal hepatocellular necrosis (arrow) and lymphohistiocytic perivascular inflammation (arrowhead), severe hepatic lipidosis. **Figure 13.** Infected, Day 42, focal hepatocellular necrosis and lymphohistiocytic inflammation (arrowhead), mild hepatic lipidosis.

**Figures 14-19.** Control and Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection, juvenile lake trout (*Salvelinus namaycush*), skin. HE. **Figure 14.** Negative control, Day 35. **Figure 15.** Infected, Day 21, individual epithelial cell necrosis (arrow) and intracytoplasmic eosinophilic vacuoles in few cells (arrowhead) as well as rare intraepithelial inflammatory cells are the earliest lesions. **Figure 16.** Infected, Day 21, early epithelial erosion with sloughing of degenerated epithelial cells (arrow). **Figure 17.** Infected, Day 28, more severe epithelial erosions with extensive intraepithelial inflammatory infiltrates and cellular degeneration (arrowheads). **Figure 18.** Infected, Day 28, advanced stage with epithelial cells undergoing degeneration and necrosis throughout all layers of the epidermis (arrowheads). **Figure 19.** Infected, Day 35, late stage with massive epithelial ulceration and complete epidermal loss.

**Figures 20-25.** Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection, juvenile lake trout (*Salvelinus namaycush*). HE. **Figure 20.** Spleen, Day 28, diffuse lymphoid depletion with focal areas of necrosis (arrowhead). **Figure 21.** Spleen, Day 28, focal areas of necrosis (arrowhead). **Figure 22.** Omentum, Day 28, severe lymphohistiocytic perivasculitis (arrowhead). **Figure 23.** Omentum, Day 28, lymphohistiocytic perivasculitis (arrowhead) with intranuclear inclusion body in blood monocyte (arrow). **Figure 24.** Heart, Day 28, lymphohistiocytic perivasculitis (arrowhead) and epicarditis. **Figure 25.** Gill, Day 28, proliferative branchitis with focal lymphohistiocytic inflammatory cell infiltrate (arrowhead).

**Figures 26-31.** Control and Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) experimental infection, juvenile lake trout (*Salvelinus namaycush*), kidney. HE. **Figure 26.** Negative control, Day 35. **Figure 27.** Negative control, Day 35. **Figure 28.** Infected, Day 28, hematopoietic cellular necrosis and depletion. **Figure 29.** Infected, Day 28, hematopoietic cellular necrosis. **Figure 30.** Infected, Day 28, hematopoietic cellular depletion. **Figure 31.** Infected, Day 28, hematopoietic cellular depletion.

**Table 1.** Mortality data following *in vivo* passage of Epizootic Epitheliotropic Disease Virus (EEDV) stocks through juvenile lake trout (*Salvelinus namaycush*). Virus stock passage # represents stock batch used to infect specified group of fish. Dose received is presented in viral copies per fish (IP) or viral copies per mL water (Imm). First and last mortalities are presented as the number of days post virus exposure. Viral titers calculated per mg skin tissue collected from infected fish following mortality.

| Virus Passage Number | Method of Infection | Viral Dose Received | Day of First Mortality | Day of Last Mortality | Total Mortalities/ Infected | Virus Titer (per mg tissue) |
|---|---|---|---|---|---|---|
| 1 | IP | $1.22x10^{7}$ | 6 | 29 | 4/5 | $5.39x10^{5}$ |
| 1 | IP | $1.50x10^{7}$ | 22 | 66 | 10/10 | $6.40x10^{4}$ |
| 1 | IP | $1.50x10^{7}$ | 36 | 116 | 10/10 | $2.21x10^{4}$ |
| 2 | IP | $8.85x10^{7}$ | 113 | 134 | 8/10 | $1.07x10^{6}$ |
| 2 | IP | $1.23x10^{4}$ | 26 | 30 | 4/5 | $6.64x10^{4}$ |
| 2 | Imm | $6.18x10^{4}$ | 25 | 45 | 9/10 | $5.49x10^{6}$ |
| 3 | IP | $7.32x10^{8}$ | 24 | 41 | 10/10 | $2.99x10^{5}$ |
| 3 | IP | $7.32x10^{8}$ | 11 | 26 | 2/3 | $5.49x10^{6}$ |
| 4 | IP | $3.55x10^{6}$ | 7 | 13 | 2/3 | $2.66x10^{4}$ |
| 5 | Imm | $7.09x10^{3}$ | 19 | 23 | 4/5 | $2.13x10^{4}$ |
| 6 | Imm | $1.46x10^{4}$ | 11 | 33 | 9/10 | $4.38x10^{7}$ |

Abbreviations: IP, intraperitoneal injection; Imm, immersion bath challenge

**Table 2.** Dose-dependent mortalities and qPCR identification of Epizootic Epitheliotropic Disease Virus (EEDV) following experimental exposure of naïve, juvenile lake trout (*Salvelinus namaycush*).

| Viral Dose (copies/mL water) | Cumulative Mortalities | qPCR Positive Fish |
|---|---|---|
| 0 | 2[a]/10 | 0/10 |
| $2.08 \times 10^1$ | 3/10 | 0/10 |
| $7.95 \times 10^2$ | 1/10 | 0/10 |
| $1.82 \times 10^4$ | 0/10 | 0/10 |
| $1.92 \times 10^5$ | 8/10 | 1/10 |
| $1.01 \times 10^6$ | 10/10 | 10/10 |

[a]Negative control mortalities showed no evidence of EEDV infection.

**Table 3.** Calculated lethal immersion doses of Epizootic Epitheliotropic Disease Virus (EEDV) in juvenile lake trout (*Salvelinus namaycush*) based on dose dependent cumulative mortalities calculated following methods developed by Reed and Muench (1938).

| % Mortality | Viral copies per mL immersion water |
|---|---|
| 10 | $2.30 \times 10^{1}$ |
| 25 | $1.07 \times 10^{3}$ |
| 50 | $4.70 \times 10^{4}$ |
| 75 | $1.21 \times 10^{5}$ |
| 90 | $3.83 \times 10^{5}$ |

**Table 4** Identification of Epizootic Epitheliotropic Disease Virus (EEDV) genomic material in skin or kidney tissues of experimentally challenged lake trout (*Salvelinus namaycush*) collected in parallel on predetermined days using a SYBR Green qPCR assay. "-" indicates no EEDV genetic material detected; "**+**" indicates the presence of EEDV DNA.

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

Abbreviation: P.I., post-infection


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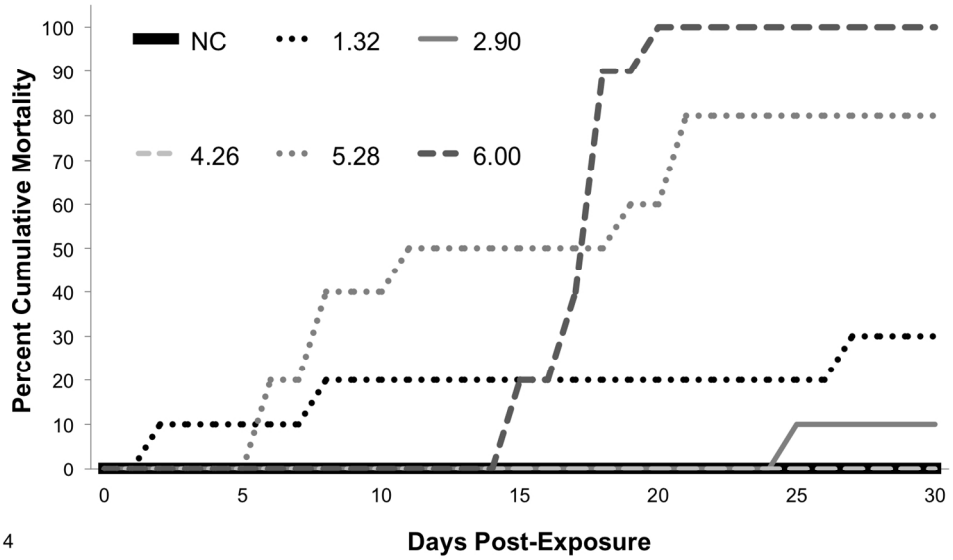


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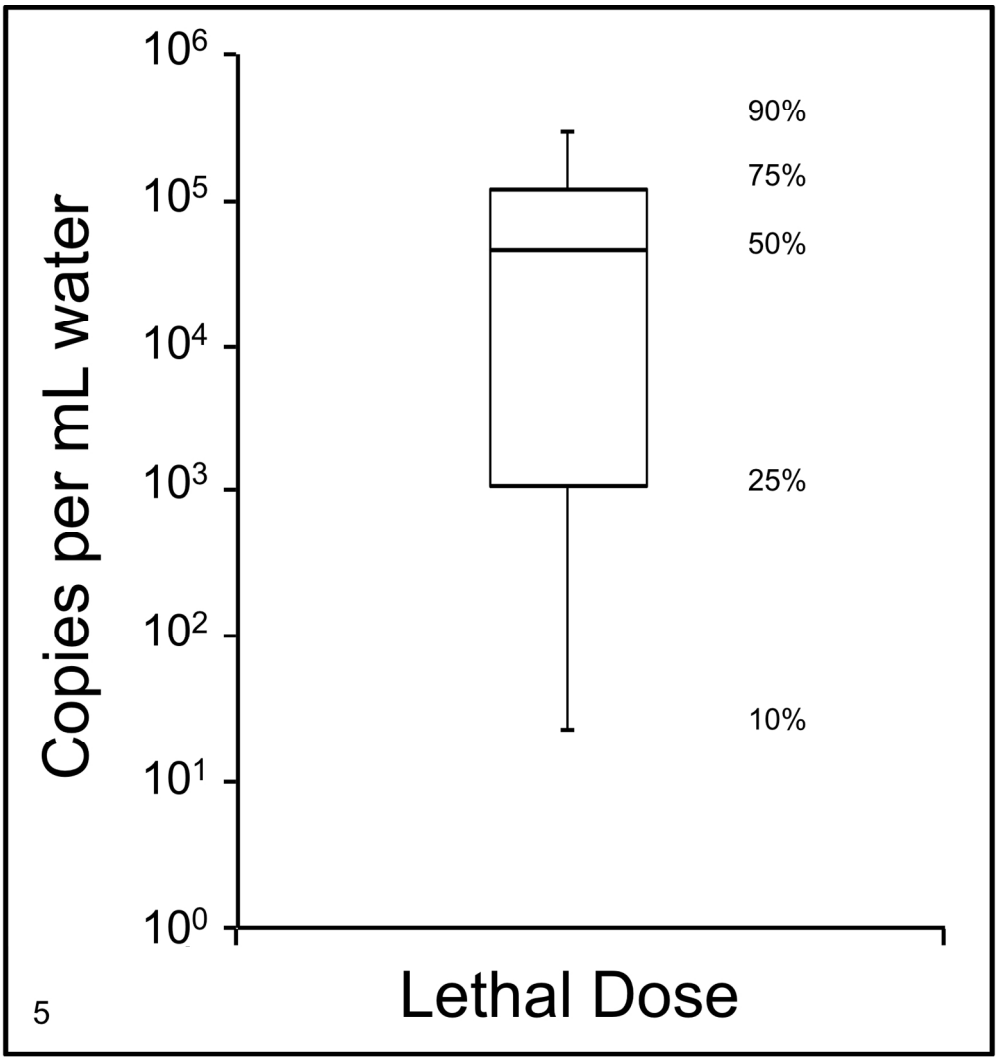


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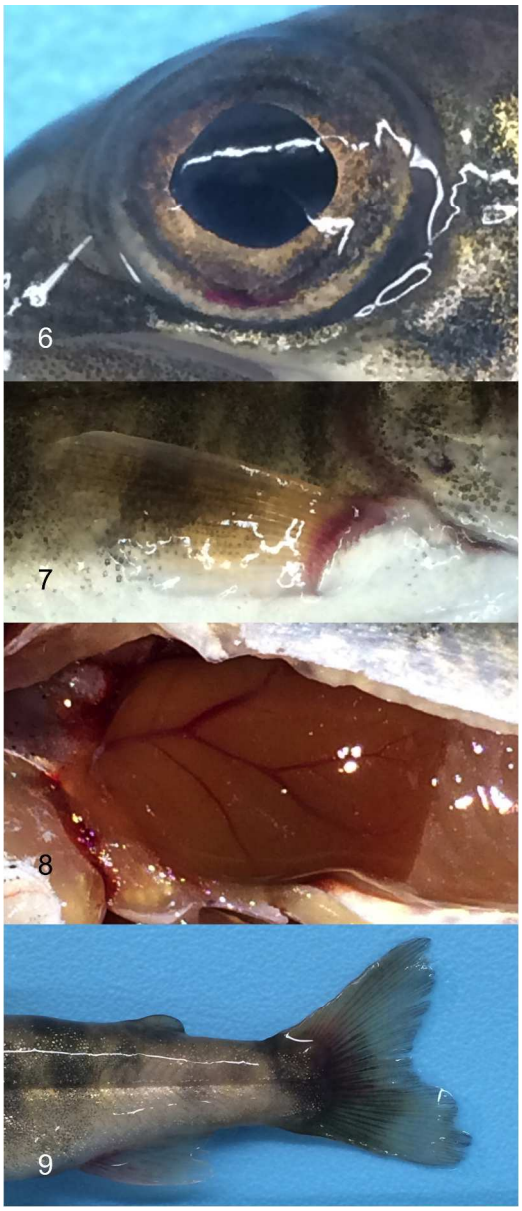


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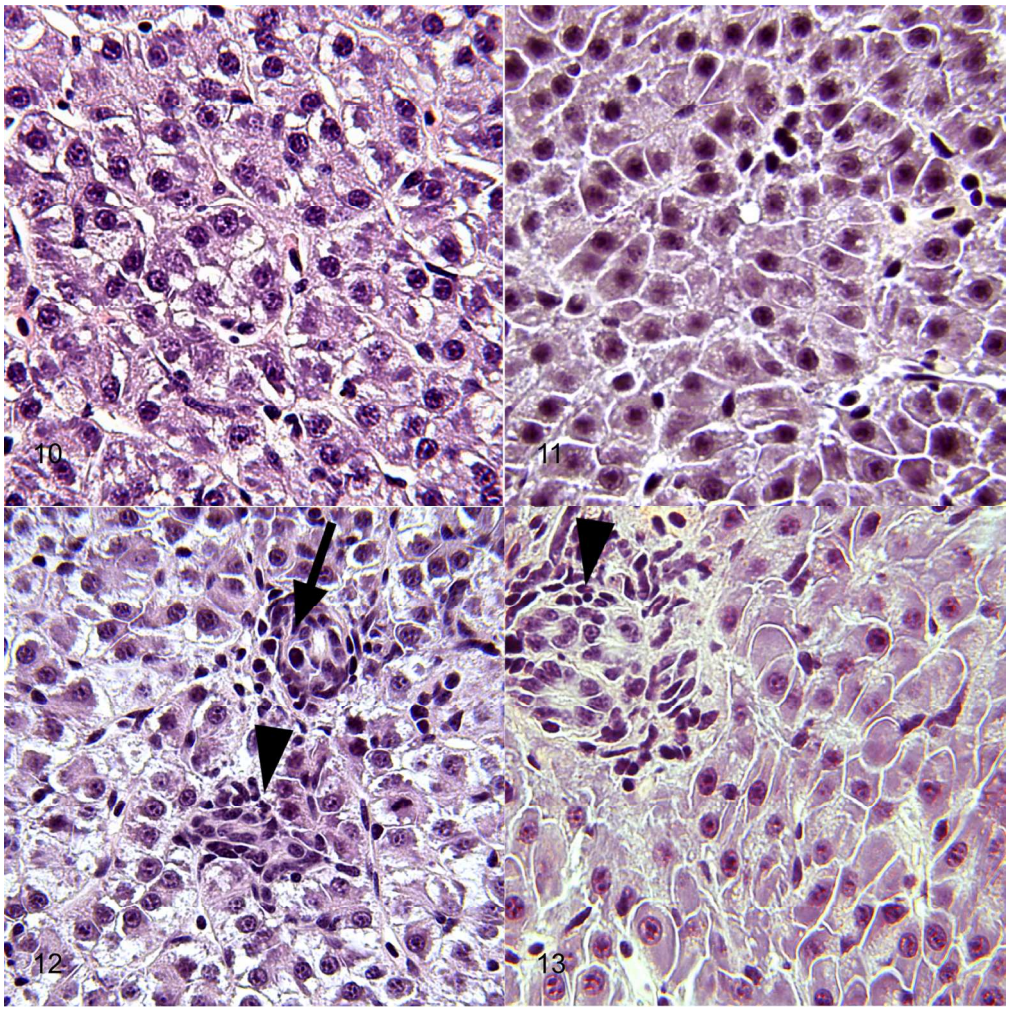


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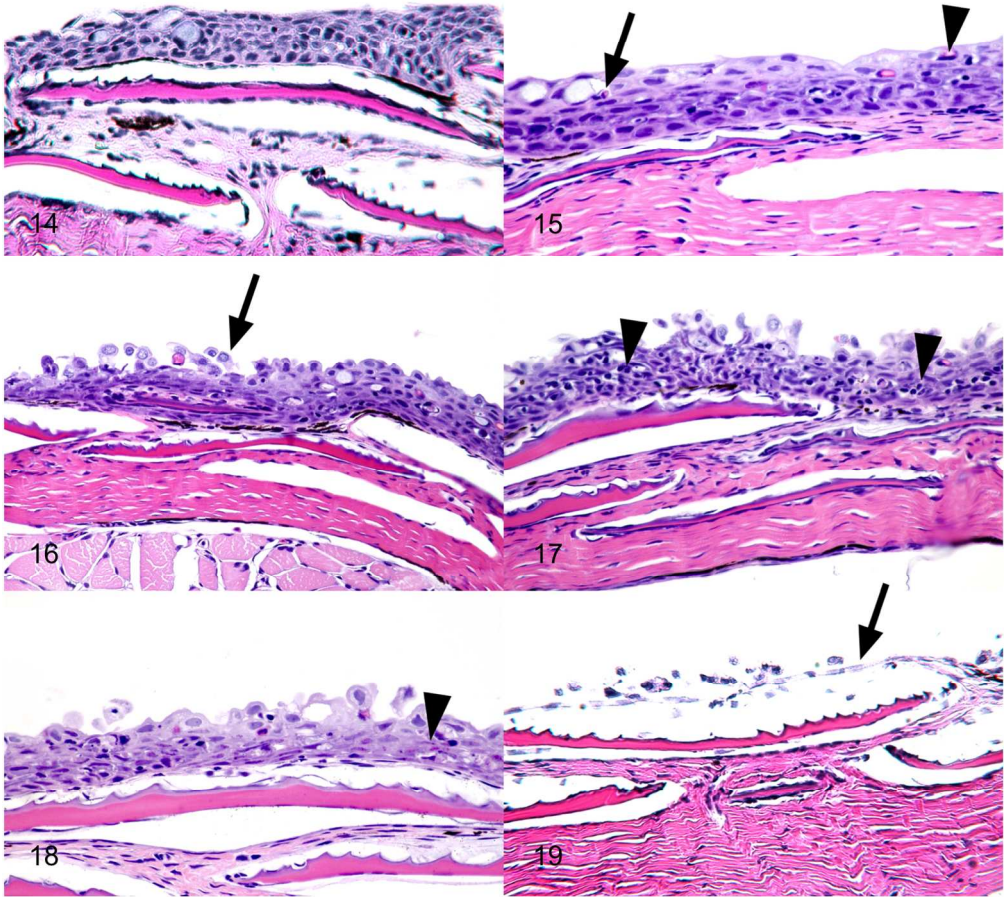


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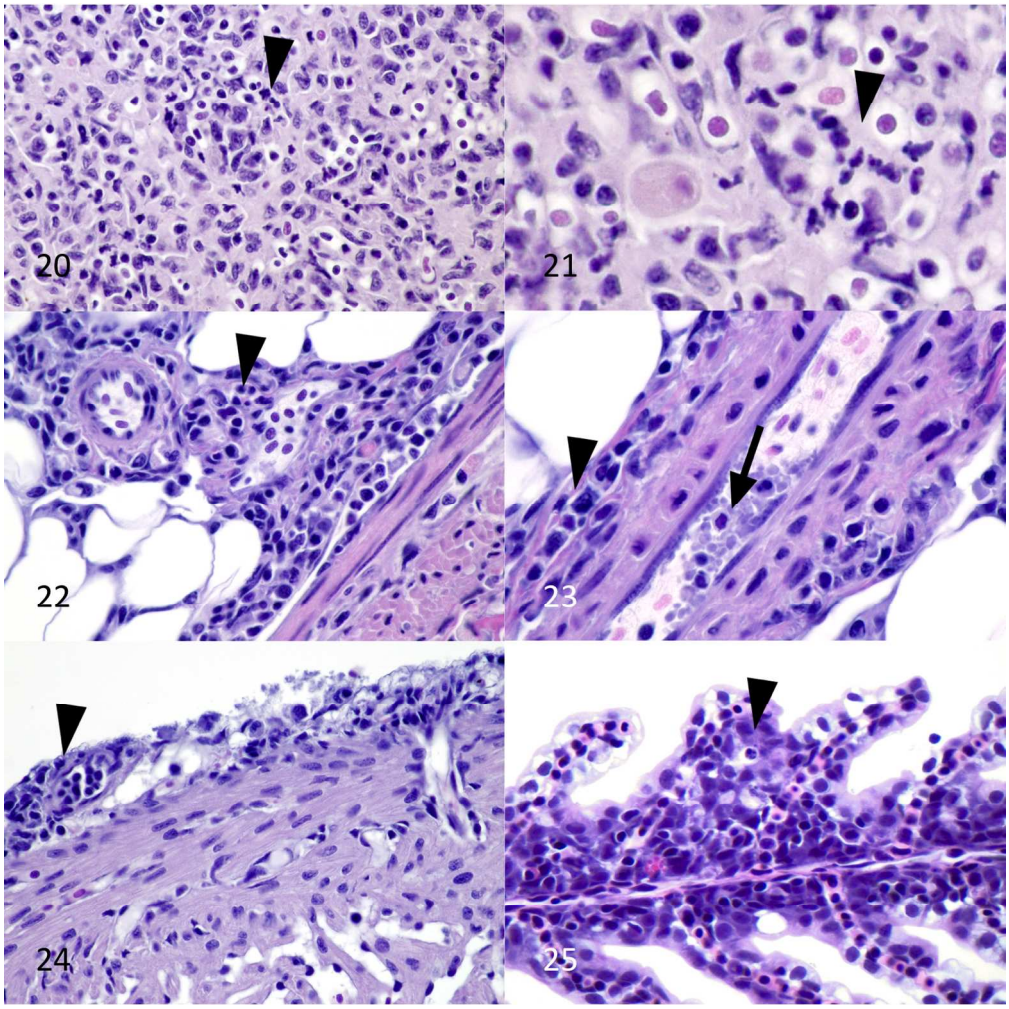


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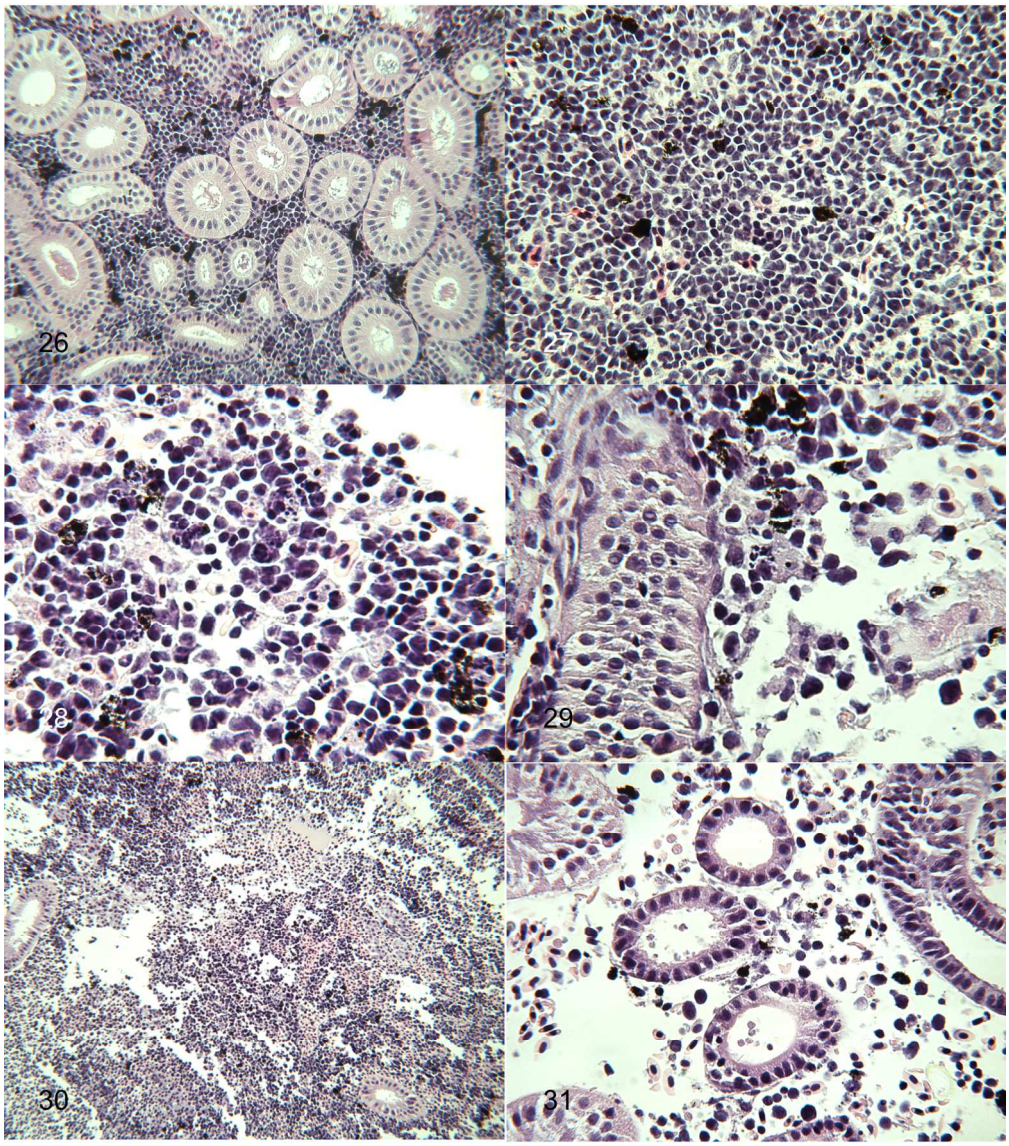


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Supplemental Table 1. Summary of gross pathologic changes observed following immersion challenge of naïve juvenile lake trout (*Salvelinus namaycush*) with Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3).

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

Supplemental Table 2. Number of juvenile lake trout (*Salvelinus namaycush* ) per sampling day with appreciable histopathologic lesions by tissue type associated with Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) infection.

| Tissue | Day 0 | Day 1 | Day 3 | Day 6 | Day 9 | Day 12 | Day 15 | Day 18 | Day 21 | Day 28 | Day 35 | Day 42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fin** | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 1/2 | 1/4 | 4/5 |
| **Gill** | 3/4 | 4/5 | 5/5 | 5/6 | 5/6 | 1/4 | 5/5 | 1/3 | 4/5 | 1/2 | 3/5 | 3/6 |
| **Intestine** | 3/6 | 2/6 | 1/6 | 3/6 | 0/6 | 2/6 | 3/6 | 5/6 | 2/6 | 2/2 | 0/6 | 0/6 |
| **Kidney** | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 2/2 | 5/5 | 6/6 |
| **Liver** | 5/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 2/2 | 5/5 | 6/6 |
| **Skin** | 0/4 | 0/3 | 0/3 | 0/6 | 0/6 | 0/6 | 0/5 | 0/6 | 2/6 | 2/2 | 4/5 | 4/5 |
| **Spleen** | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/5 | 0/6 | 0/6 | 0/5 | 2/2 | 4/5 | 5/6 |
| **Heart** | 0/4 | 0/3 | 0/3 | 0/6 | 0/6 | 0/6 | 0/5 | 0/6 | 0/6 | 2/2 | 0/6 | 0/6 |

Supplemental Table 2. Summary of histopathologic changes seen in juvenile lake trout (*Salvelinus namaycush* ) exposed to Epizootic Epitheliotropic Disease Virus (EEDV; salmonid herpesvirus-3) on all samples days. No appreciable lesions were eobserved in the eyes or brains, on any days.

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

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

Mohamed Faisal[1,2], Mochamad A. Purbayu[1], Megan A. Shavalier[1], Thomas P. Loch[1,2]

[1]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, and [2]Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan 48824, USA

ABSTRACT

Salmonid herpesvirus-3, commonly known as the epizootic epitheliotropic disease virus (EEDV), is 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 lake trout that were intraperitoneally (IP) injected with EEDV. To assess virus shedding, each infected fish was housed individually in a static aerated aquarium for 8 hours, then water was assessed for the presence of EEDV DNA using quantitative PCR assay. Water sampling was conducted every seven days for up to 93 days post-infection (pi). Our results demonstrated that infected lake trout start to shed EEDV into the water as early as 9 days pi and shedding peaked ~three weeks pi and continued for up to ~9 weeks pi. 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.

KEY WORDS: lake trout, EEDV, salmonid herpesvirus-3, shedding, water

## INTRODUCTION

Alloherpesviruses are ubiquitous pathogens that infect a wide range of fish and amphibians (**Waltzek et al., 2009**). Water appears to be the main vehicle for alloherpesviruses transmission, although transmission nature and long-term kinetics have never been assessed (**Kancharla and Hanson, 1996; Yuasa et al., 2008**). This is particularly true for the salmonid herpesvirus-3, commonly known as epizootic epitheliotropic disease virus (EEDV), that causes serious losses in its host; the lake trout (*Salvelinus namaycush*). Despite the dearth of knowledge about EEDV biological characteristics, 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 (**McAllister and Herman, 1987; 1989; Bradley et al., 1988; 1989; Shavalier, 2017**). Most recently, a quantitative PCR assay for the detection of EEDV in infected tissues has been developed. The aim of the present study is to assess the magnitude and duration of EEDV shedding by experimentally infected lake trout.

## MATERIALS AND METHODS

### *Fish maintenance*

Twenty-nine Lake Superior (LS) strain lake trout (*Salvelinus namaycush*), age 25 months post-hatch (> 55 mm in length) 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, to which the fish were allowed to acclimate over a period of 15 days.

***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. 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. Pit tag microchips were placed into the N125 needle (Biomark®) for insertion in the body cavity of fish following the manufacturer's instructions and identification number of each fish was read and recorded using the pit tag reader (Pocket Reader 098494; Destron-Fearing™, Eagen, Minnesota). Following microchips implantation, fish were transferred into their respective tanks and were maintained for additional 15 days before infection challenges.

***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 (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, 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 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.

***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 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. Sterile 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 use. After the collection, all fish in the glass aquariums were transferred back into their respective tanks. All aquaria, air-lines and air-stones were 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.

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. Water sample (250 µL) 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.

***Quantification of EEDV DNA in water samples***

The EEDV-specific SYBR Green qPCR assay of **Glenney et al. (2016)** that targets the glycoprotein gene was utilized throughout this study. 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., 2016**). 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. 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-

**Commented [O1]:** Tom: Did Aji divided the rate by 40 as requested by Kim?

116 fold dilutions of EEDV positive standards (**Shavalier, 2017**). Virus titer among groups and
117 sampling periods was analyzed using repeated measures ANOVA test. Calculation was
118 performed using SPSS statistical software 23.0 version.
119
120 **Results**
121 ***Mortalities and clinical signs***
122 During the course of the experiment, infected fish developed typical signs of EEDV
123 infection in the form of patches of skin pallor, hemorrhage in the lower canthus of the eye
124 (Figure 1 A), and skin erosions in the trunk (Figure 1 B) and around the nares. Mortalities in the
125 EEDV-infected group started by day-40 pi and cumulative mortality rate peaked by Day-68 pi
126 and remained steady at ca 74% (17 of 23 fish) through the end of the observation period (Day-
127 93). There were no further mortalities through the end of the study period (day-93 pi) and 6 fish
128 survived the infection (Figure 2, Table 1). External lesions of surviving fish healed, and the fish
129 resumed feeding and growth by the end of observation period. On the contrary, none of the
130 negative control fish developed clinical signs, with one of them died 11 pi due to causes not
131 related to EEDV.
132
133 ***Levels of EEDV DNA in the water***
134 The shedding of EEDV from infected lake trout into the water, as indicated by the
135 presence of EEDV DNA, started from the first sampling period and continued into the $9^{th}$
136 sampling period (Table 1, Figure 3). Only one fish shed EEDV at day-9 pi and the number of
137 shedding fish increased to four at the second sampling period. Shedding reached its maximal
138 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 $4^{th}$, $5^{th}$, $7^{th}$, $8^{th}$, and $9^{th}$ sampling period. From the $10^{th}$ sampling period through the end of observation period, shedding ceased as the number of alive fish decreased due to EEDV-mortalities (Table 1, Figure 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 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). Repeated measures ANOVA using Mauchly's test statistic was significant ($p < 0.05$) means that there were significant differences in shedding/fish among sampling periods with the third sampling period being the highest $p<0.002$.

Also, of note was the observation of three shedding trends in this experiment: the first trend was characterized by shedding at relatively high titers until death, the second trend was in

the form of intermittent periods shedding followed by no periods of no shedding, and the third trend was that of survivors where shedding ceased and signs of recovery started to appear.

**Discussion**

Findings of this study unravel details of EEDV 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 3 and Table 1 clearly demonstrates that EEDV needs up to three weeks for the shedding to reach its maximal level. We believe that shedding took place through the sloughed skin cells, however, urine, feces, and other fish body fluids may also have contributed to the observed EEDV shedding loads.

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**). The matter is further complicated by the relatively long time of high shedding levels 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.

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.

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 (≤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 virus reservoirs.

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Table 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 shed the virus.

| Group | Fish # | Sampling period | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **1 (Day-7, 8, 9)** | **2 (Day-14, 15, 16)** | **3 (Day-21, 22, 23)** | **4 (Day-28, 29, 30)** | **5 Day-35, 36, 37)** | **6 (Day-42, 43, 44)** | **7 (Day-49, 50, 51)** | **8 (Day-56, 57, 58)** | **9 Day-63, 64, 65)** | **10 (Day-70, 71, 72)** | **11 (Day-77, 78, 79)** | **12 (Day-84, 85, 86)** | **13 (Day-91, 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 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.

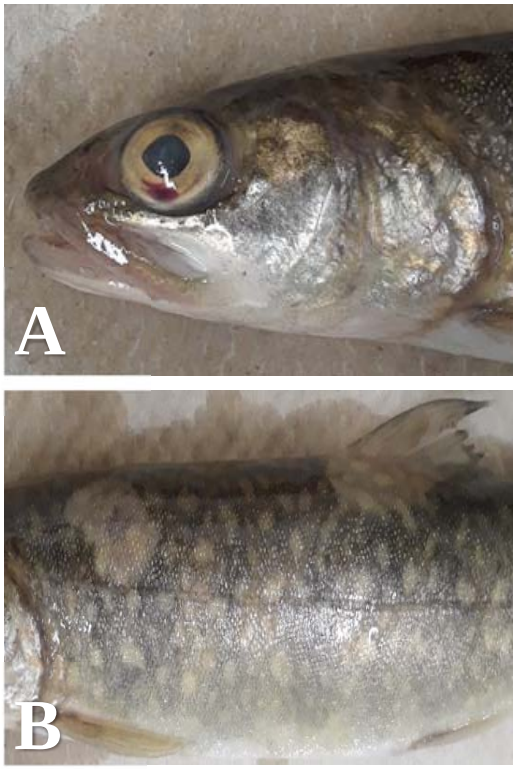

Figure 2: Cumulative mortality of lake trout (*Salvelinus namaycush*) in the EEDV group (n=23) experimentally infected with EEDV.

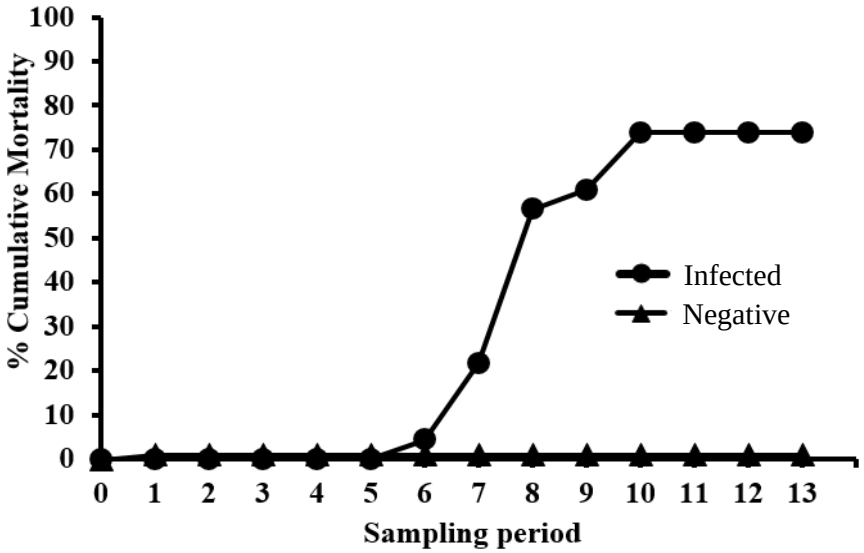

Figure 3: Shedding rate of lake trout in the EEDV group exposed EEDV at a dose of $4.5x10^5$ viral copies/mL (did we inject the whole mL????). The box plots showing minimum, 1st quartile, median, 3rd quartile and maximum viral copies/fish/hour by sampling period in water samples.