Research Article

In vitro Culture of Lake Trout (*Salvelinus namaycush*) Cells and Trials to Isolate Salmonid Herpesvirus-3 (syn. Epizootic Epitheliotropic Disease Virus)

Megan Shavalier[1,2*] and Mohamed Faisal[1,2,3]

[1]Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI, USA; [2]Comparative Medicine and Integrative Biology Program, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA; [3]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA.

Abstract | The lake trout (*Salvelinus namaycush*) is an ecologically and economically important fish species in the Great Lakes basin of North America. Primary cultures of lake trout cells were produced following manual and enzymatic digestion of tissue and incubation at 15°C with Earle's salt-based minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS). Primary cultures of both yearling gonad tissue and sac fry body cell types were readily established and subculturing occurred within 2-4 weeks of initial seeding. Repeated passaging of cells resulted in gonad cells (designated LYR) reaching subculture number 35 and fry cells (designated LTF) reaching 52. Additional primary cell cultures were produced from yearling fin and broodstock liver tissues. Morphologically, both LTF and LYR cells started out as mixed populations with a substantial percentage of fibroblast-like cells, however as passages went on, cell populations became increasingly epithelial-like. Species origin was confirmed using DNA barcoding. Infection of novel lake trout cell lines with pathogenic aquatic viruses VHSV, IPNV and EEDV resulted in development of cytopathic effect.

Received | December 23, 2024; **Accepted** | February 05, 2025; **Published** | July 22, 2025
***Correspondence** | Dr. Megan Shavalier, Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, 1129 Farm Lane, Room 343, East Lansing, MI 48824, USA; **Email:** shavali1@msu.edu
Citation | Shavalier, M. and M. Faisal. 2025. *In vitro* culture of lake trout (*Salvelinus namaycush*) cells and trials to isolate salmonid herpesvirus-3 (syn. epizootic epitheliotropic disease virus). Journal of Virological Sciences, 13: 01-10.
DOI | https://dx.doi.org/10.17582/journal.jvs/2025/13.01.10
Keywords | Lake trout, Cell culture, *Salvelinus namaycush*, Virus susceptibility

Copyright: 2025 by the authors. Licensee ResearchersLinks Ltd, England, UK.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Introduction

The lake trout (*Salvelinus namaycush*) is an important native fish species in the Laurentian Great Lakes Basin of North America (GLB) (Bronte *et al.*, 2008). This cold water, apex predator is well adapted to life in the Great Lakes, has a steadying effect on local ecosystems, and is prized by the sports and commercial fishing industries alike (Redick, 1967; Bronte *et al.*, 2008). Tragically, fishery managers and scientists have been confronted with significant population threats and declining numbers over the past 60 years due to a conglomerate of ecological and anthropogenic factors including invasion by non-endogenous species (i.e., sea lamprey (*Petromyzon marinus*) and alewife (*Alosa pseudoharengus*)), habitat

destruction, over fishing, and emerging infectious diseases (Hile *et al*., 1951; Eschmeyer, 1957; Wells and McLain, 1973; Hansen *et al*., 1995; Holey *et al*., 1995; Eshenroder and Amatnagelo, 2002; Bronte *et al*., 2008; Cline *et al*., 2013). Rehabilitation programs focused on the recovery of this important fish rely heavily on the use of captive breeding programs. Unfortunately, the intensive nature of salmonid aquaculture serves as a ripe location for the eruption of infectious diseases, particularly those caused by viruses (Redick, 1967; Bronte *et al*., 2008).

One key aspect of aquatic animal health programs is the diagnosis, prevention, and study of pathogens in such environments, tasks that often utilize cell culture techniques and assays. Unfortunately, while American Type Culture Collection (ATCC) carries over 3,400 commercially available distinct cell lines, less than 20 of those are derived from fish tissues, leaving many researchers and diagnosticians no choice but to develop their own cell lines for projects. Attempts were made several decades ago to produce a cell line of lake trout origin, unfortunately, the cell cultures are no longer preserved (Cheng *et al*., 1993; personal communications). To date, there remain no established long-term cultures of immortal cell lines originating from lake trout tissues. Such a cell line is badly needed due to the reemergence of a deadly virus infection caused by salmonid herpesvirus-3 (*Salmovirus salmonidallo3*), the causative agent of Epizootic Epitheliotropic Disease, that decimated millions of hatchery fish and keeps reemerging despite strict biosecurity measures (Faisal *et al*., 2019). These continued outbreaks underscore the urgency to develop continuous cell cultures from the only susceptible host, the lake trout. It is noteworthy that isolation of EEDV from available cold water fish cell lines has been unsuccessful (Faisal *et al*., 2019). While some aquatic herpesviruses can be isolated in cell culture, many are either difficult to isolate, slow to replicate and develop cytopathic effect, or do not have a susceptible cell line at this time (Hanson *et al*., 2024). To this end, the present study describes our attempts to establish and characterize two novel cell lines from lake trout tissues, and to test their susceptibility to support EEDV among other viruses known to infect lake trout.

Materials and Methods

Fish and tissue collection

For this study, three different groups of lake trout were obtained as tissue donors for primary cell cultures: (1) a single sexually mature fish (>12 years of age), which had been spawned and housed its entire life at the University Research Containment Facility (Michigan State University, East Lansing, Michigan); (2) yearling fish collected from the Marquette State Fish Hatchery (MSFH, Marquette, Michigan); and (3) sac fry collected from the Marquette State Fish Hatchery (MSFH, Marquette, Michigan).

Tissues were collected in a similar manner from the adult and yearling lake trout. Fish were euthanized with an overdose of tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.25 mg/mL) prior to tissue collection. All tissues were placed in sterile cell culture media (MEM-0) that consisted of Earle's salt-based minimal essential medium (MEM; Invitrogen, Carlsbad, California) and supplemented with 10% tryptose phosphate broth (BD Biosciences, San Jose, California), 29.2 mg/mL L-glutamine (Invitrogen), penicillin (100 IU/mL; Invitrogen), streptomycin (0.1 mg/mL; Invitrogen), amphotericin B (2.50 µg/mL; Invitrogen) and sodium bicarbonate (7.5% w/v; Sigma) for temporary holding and to prevent drying during transport between facilities. External tissues (i.e., skin and fin) were briefly flame sterilized to remove external pathogens and then dissected and placed into a solution of MEM-3x, which contained triple antibiotic/antifungal (penicillin (300 IU/mL), streptomycin (0.3 mg/mL) amphotericin B (5.50 µg/mL)), for 30 minutes, after which time tissue were transferred to sterile cell culture media (MEM-0) while remaining tissues were collected. Blood (adult fish only) was drawn directly from the ventricle following a cut down dissection of the heart and diluted 1:2 with MEM-0 containing heparin at a minimum of 30 IU/mL blood. Internal tissues were aseptically collected and placed directly into MEM-0. Blood, skin, fin, gill, liver, testes, anterior kidney, and posterior kidney were collected from the adult fish. The same tissues were collected from the juvenile fish with the exception of blood, and gonads rather than testes as the fish were immature.

For lake trout sac fry, fish were dissected in order to remove the yolk sac and the head (cranial to opercular margin), with the remaining body processed as a singular tissue sample. Approximately 20 individual fry were processed together until an adequate amount of tissue was collected.

Isolation and in vitro culture of primary cells

Individual tissues collected from all three groups of fish were transferred to sterile petri dishes where they were manually trimmed using scissors until they reached a size of approximately 1-2 mm diameter. Next, enzymatic digestion was performed at room temperature using 0.25% Trypsin-EDTA (Gibco, Life Technologies, Carlsbad, California) until tissues were visually determined to have reached complete digestion. The tissue-trypsin suspension was then filtered through sterile gauze to remove any remaining tissue pieces, after which, MEM-10 (10% fetal bovine serum; Hyclone Laboratories Inc.) was added to the resulting filtrate at a ratio of 2:1 (medium/trypsin) in order to deactivate the trypsin. The single cell suspension was then centrifuged at 190 x *g* for 5 minutes at 15˚C and resuspended in 10 mL of MEM-10 three times. The final cell suspension was seeded into 25cm^2 cell culture flasks (Corning) and incubated at 15˚C (closed system, subambient temperature). This process was repeated for all tissue types with the exception of the blood. Heparinized blood diluted with MEM-0 was centrifuged at 4,700 x *g* for 10 minutes at 4˚C. Next, buffy coats were collected, mixed with sterile water 1:1 to hemolyze red blood cells, then washed with MEM-10 and seeded into culture flasks as above.

Culture maintenance and subculture

Primary cell flasks were monitored daily for evidence of attachment and replication. Unattached, nonviable cells (as later determined using Trypan Blue (Sigma-Aldrich, St. Louis, Missouri) exclusion staining) and spent media were replaced every 2-3 day (75%) by fresh MEM-10.

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

Optimization of culture conditions

The effect of incubation temperature, serum type/concentration and growth medium base on cell growth was evaluated for the two most promising cell lines: yearling gonad tissue (LYR; subculture 4) and fry tissue (LTF; subculture 11). LYR and LTF cells were seeded into 12.5 cm^2 culture flasks (CELLTREAT Scientific Products, Pepperell, Massachusetts) at a density of 4x10^5 and 1.5x10^5 cells per flask respectively. For temperature trials, flasks were incubated at 15, 21, and 25˚C while all other culture conditions remained consistent as described above. For serum trials, cells were grown at 15˚C with growth medium supplemented with either 10% fetal bovine serum (FBS), 15% FBS, or 15% FBS plus 1% heat inactivated lake trout serum. For growth medium trials, cells were seeded in growth medium containing 15% FBS and incubated at 15˚C. Growth medium was produced using either Earle's salt-based minimal essential medium as described above, or Leibovitz's L-15 medium. All other culture conditions remained identical. Every 2-5 days, cells were detached using trypsin, from n = 2 flasks per day, and counted using a hemacytometer in order to assess relative cell growth and density. The total number of viable cells was counted in the 4 large squares of the hemacytometer (1 mm^2 each). Cell density (cells/mL) was calculated by multiplying the average viable cells by the dilution factor and dividing by the volume of one square (see below).

$$Cell\ Density = \frac{Average\ Number\ Viable\ Cells \times Dilution\ Factor}{Counted\ Square\ Volume\ (mL)}$$

This number was then multiplied by the total volume of cell suspension and divided by 12.5 (area in cm of one flask) to get total number of viable cells per cm^2 within one flask. After being removed from the flask and counted, cells were disposed; no flask was counted more than once. A total of 84 LTF flasks (14 sampling events) and 78 LYR flasks (13 sampling events) were counted for the temperature trials, 30 (5 sampling events) and 24 (4 sampling events) respectively for the serum concentration, and 28 (7 sampling events) and 20 (5 sampling events) for the growth media base.

Confirmation of species of origin

In order to establish that these novel cell lines were indeed of lake trout origin, a DNA barcoding technique was employed to amplify and sequence the cytochrome *c* oxidase 1 (*COI*) gene (Cooper *et al*., 2007; Ivanova *et al*., 2007). An early and late passage cell sample from both the LTF and LYR cell lines were used, with lake trout skin tissue serving as a positive control and *Epitheliosum papulosum cyprini* (EPC; ATCC) cells as a negative control (Winton *et al*., 2010). DNA extractions were performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions for extractions from cell cultures. All PCR reactions were carried out in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The COI-3 primer cocktail designed by Ivanova *et al*. (2007) was used to amplify a 631 bp fragment of the *COI* gene. Each 25 µL reaction mixture was comprised of 12.5 µL 2x Go-Taq Green Master Mix (Promega, Madison, Wisconsin, USA), 0.8 µM of each primer, and 4.5 µL DNA template (56.25 ng). Cycling parameters were as described by Ivanova *et al*. (2007) for the COI-3 primer cocktail. Amplicons and a 1 kb Plus molecular ladder (Roche Applied Science, Penzberg, Germany) were electrophoresed through a 2% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) at 100V for 30 minutes, and visualized under ultraviolet light.

Amplicons were prepared for sequencing using ExoSAP-IT (Thermo Fisher Scientific). 1 µL of each PCR product was combined with 3 µL 1x $MgCl_2$ Buffer and 0.25 µL ExoSAP-IT reagent. The ExoSAP-IT mixture was placed in the thermocycler with a program of 37°C for 20 minutes followed by 95°C for 10 minutes. After clean up, amplicons were Sanger sequenced at the Michigan State University Research Technology Support Facility using M13 forward and reverse primers (Ivanova *et al*., 2007). Sequences and chromatograms provided by the Michigan State University Research Technology Support Facility were visually examined using 4Peaks software (http://nucleobytes.com/4peaks/; Version 1.8) and contigs were assembled and aligned using ClustalW in the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0) (Tamura *et al*., 2013). Resulting contigs were then entered into the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007) search function where each sequence was compared against the ID System to identify nearest neighbors using a global alignment of more than 3,000,000 barcode sequences from 180,000 animal species.

Viral susceptibility

Flasks of LTF cell cultures were exposed to isolates of viral hemorrhagic septicemia virus (VHSV) genotype IVb (family Rhabdoviridae, genus *Novirhabdovirus*), and infectious pancreatic necrosis virus (IPNV; family Birnaviridae, genus *Aquabirnavirus*), while both LTF and LYR cells were inoculated with filtered tissue homogenates obtained from epizootic epitheliotropic disease virus (EEDV; family Alloherpesviridae, genus *Salmovirus*, species *Salmovirus salmonidallo3*) infected fish showing the typical disease signs as detailed below. All three virus isolates originated from natural disease outbreaks in the State of Michigan with stocks produced by the Aquatic Animal Health Laboratory at Michigan State University (Faisal et al., 2012, 2013, 2019, unpublished data). Viral stocks of VHSV-IVb and IPNV were inoculated into 25cm^2 flasks of LTF cells (containing MEM with 2% FBS) and incubated at 15°C. After inoculation, cells were monitored via light microscopy for development of cytopathic effect (CPE) at 48 and 72 hours post infection.

As a current *in vitro* model of replication does not exist for EEDV, our lake trout cells were exposed to virus-positive tissue homogenate supernatant (first passage on cells), followed by a second passage on cells of either first pass supernatant or first pass cells. The EEDV-positive tissue homogenate was produced by homogenizing skin from experimentally infected lake trout in sample diluent (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 (2.5 µg/mL; Invitrogen)). As optimal incubation temperature for EEDV *in vitro* is unknown, infected cells were incubated at a range of temperatures (i.e., 4, 9, and 15°C). The presence of EEDV in fish tissues was verified by performing quantitative PCR as described below.

Quantification of EED viral DNA

A TaqMan quantitative PCR (qPCR) described by Glenney *et al*. (2016) was used to compare

viral titers in tissue samples to those in cells and supernatant following inoculation of lake trout cells. In this manner, a relative increase in viral loads would suggest replication by active virus rather than merely the presence of viral genetic material. For DNA extractions, the MagMax™ 96 Viral RNA isolation kit (Life Technologies, Grand Island, New York, USA) was used manually, following manufacturer's instructions, after which, extracted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with qPCR protocols as previously described (Glenney *et al*., 2016). The EEDV glycoprotein gene was targeted in the qPCR reaction (as outlined by Glenney *et al*., 2016). Positive control standards for quantification were produced using known positive skin samples in the method as described by Glenney *et al*. (2016). A total of 50ng DNA was used in each qPCR reaction.

Results and Discussion

Primary culture and routine subculture

Out of the eight tissues collected from the lake trout broodstock, no or poor cell attachment was observed in the gills, skin, testes, anterior kidney, posterior kidney, fin or blood cultures. A small number of liver cells (~5% confluence) were attached within two days of seeding. Following a media change on day 5, small clusters of cells had begun to develop (Figure 1A). The number and size of these small clusters increased through Day 10, however by Day 15 the cells were beginning to detach, and the flask was subcultured. Following subculture, the flask was monitored, with media changes performed once weekly until the flask reached 50% confluence around Day 85 at which time a second subculture was performed. A total of 9 subcultures were performed before cell growth began to significantly decrease, with the flask reaching 100% confluence (Figure 1B) within an average of 3-4 weeks after 3-8 subcultures.

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

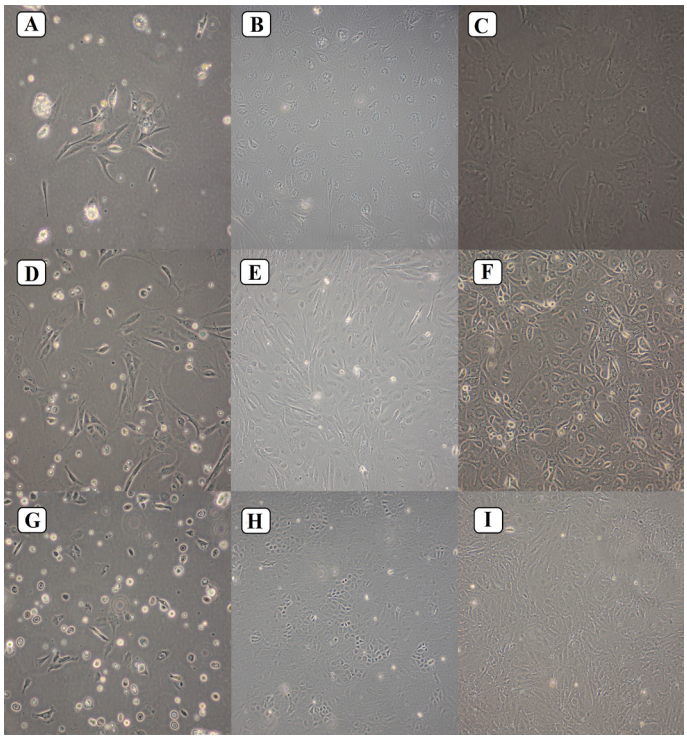

Figure 1: A, B: *Liver cells cultured from an adult lake trout (Salvelinus namaycush). A) primary culture, 6 days after seeding (original magnification 200x); B) 6th subculture, confluent (original magnification 100x).* **C:** *Fin cells cultured from yearling lake trout (Salvelinus namaycush), primary culture (original magnification 200x).* **D, E, F:** *LYR cells cultured from yearling lake trout (Salvelinus namaycush). A) primary culture, 3 days after seeding (original magnification 200x); B) 6th subculture (original magnification 100x); C) 32nd subculture (original magnification 200x).* **G, H, I:** *Cell cultures established from body tissue of lake trout (Salvelinus namaycush) sac fry. A) primary culture, 2 days after seeding (original magnification 200x); B) 14th subculture (original magnification 100x); C) 47th subculture (original magnification 100x).*

Primary cultures established from fry tissues (Figure 1G) reached 50% confluence within the first 3 days after seeding (albeit with patchy growth), and 75-100% confluence by Day 14 at which time they were subcultured. The next 3 subcultures occurred 3-4 weeks apart (flasks reaching 75-80% confluence), and after the 4th subculture the flasks were reaching 100% confluence within 2 weeks (Figure 1H). In later passages (e.g., >20), the LTF cells could be subcultured weekly. LTF cells have been successfully cultured up to 52 subcultures (Figure 1I).

Morphologic characteristics

Primary cultures of the LYR cells (Figure 1C) displayed fibroblast-like morphologic characteristics. Cells appeared to be bipolar with a length > 2x cell width. However, by the 6th subculture, a more mixed population of fibroblast-like and epithelioid cell were observed, with the epithelial-like cells appearing more polygonal and in discrete patches between the other cells. This trend toward an epithelial-like cell morphology continued through the later subcultures as pictured in Figure 1F.

Morphologically, the LTF cells appeared to be a mixture of fibroblast-like and epithelial-like cells in the primary cultures (Figure 1G). However, through passages, they became consistently more epithelioid with regular dimensions growing in discrete patches.

Optimization of culture conditions

Both LTF and LYR cells grew extremely poorly at 25°C (Figure 2A, B, light grey lines). While some level of growth was achieved in both cell types at 21 and 15°C the trend was for best growth at the coldest temperature (although statistical strength is low due to the size). In fact, in the LTF cells, the number of cells per cm^2 was higher at all time points beyond 3 days post seeding. As such, all further growth of lake trout cells was performed at 15°C. When comparing the three different serum supplement concentrations, it was clear that a 15% FBS concentration was preferred over 10% FBS (Figure 2C, D). The addition of the lake trout serum did not appear to have a positive effect on cell growth. As such, 15% FBS was used in all future medium preparations. A comparison of the two main growth medium bases (i.e., MEM vs. L-15) showed mixed results. While 100% confluence was achieved in the LTF cells with both media types (Figure 2E, F), relatively poor growth was observed in the LYR cells grown in MEM (Figure 2F), which was uncharacteristic compared to all previous LYR cell growth. In spite of the inconsistencies in this single trial, all cells continued to be grown in MEM rather than changing to L-15.

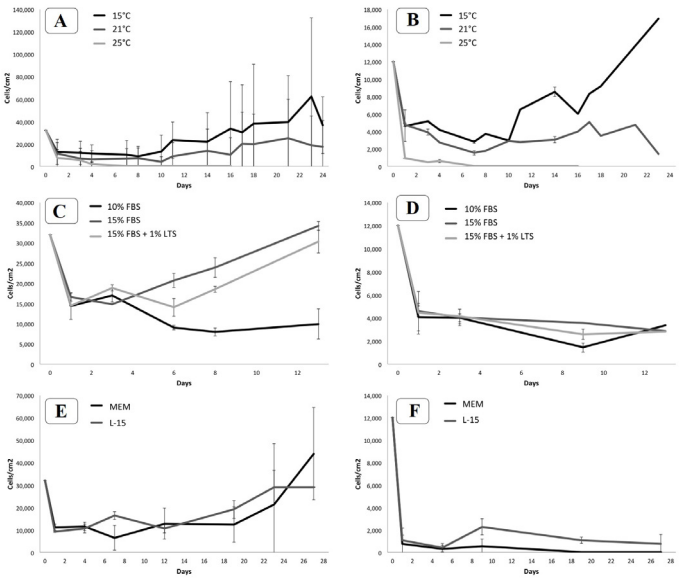

Figure 2: A, B: *Influence of incubation temperature on growth of novel lake trout cell cultures. A) LTF cells (11th subculture); B) LYR cells (4th subculture). Solid lines indicate cell density (cells per cm^2). 2 flasks examined for cell density per sampling day.* **C, D:** *Influence of serum supplement concentration and type on growth of novel lake trout cell cultures. A) LTF cells (11th subculture); B) LYR cells (4th subculture). Solid lines indicate cell density (cells per cm^2). 2 flasks examined for cell density per sampling day.* **E, F:** *Influence of growth medium base on growth of novel lake trout cell cultures. A) LTF cells (11th subculture); B) LYR cells (4th subculture). Solid lines indicate cell density (cells per cm^2). 2 flasks examined for cell density per sampling day.*

Confirmation of species of origin

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

Virus susceptibility

Clear changes were observed in the LTF cells infected with both VHSV and IPNV within 48 hours post infection (Figure 3A, B, C). LTF cells began to round,

Links Researchers

shrink and detach from the culture surface, disrupting the monolayer. This lysis and rounding of cells were comparable with the typical CPE associated with these viruses.

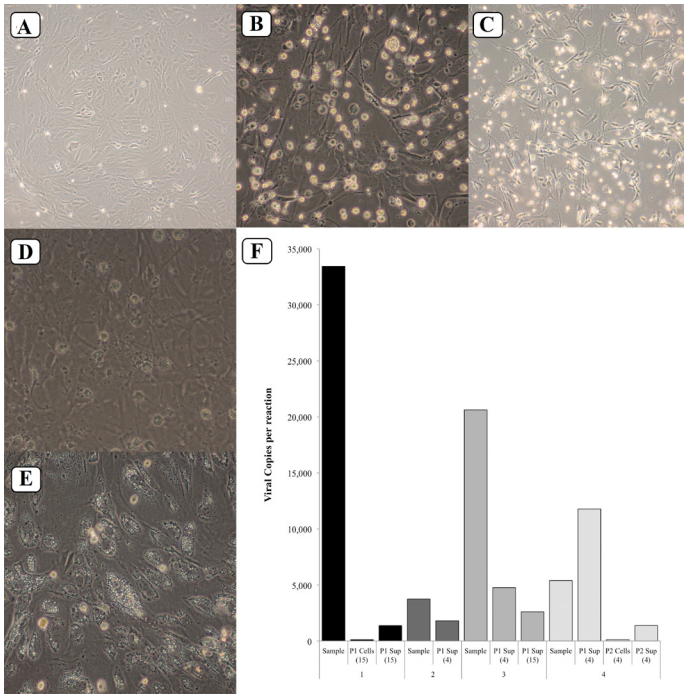

Figure 3: A, B, C: *Lake trout (Salvelinus namaycush) cell cultures (14th subculture) infected with VHSV or IPNV. A) LTF cell negative control (original magnification 100x); B) LTF cells infected with VHSV, cell lysis (original magnification 200x); C); LTF cells infected with IPNV, cell lysis (original magnification 100x).* **C, D:** *Lake trout cells inoculated with EEDV-positive tissue homogenate (9°C). A) LTF cells (47th subculture), cell rounding, some lysis (original magnification 200x); B); LYR cells (32nd subculture), vacuolation, some rounding (original magnification 200x).* **E:** *Viral loads following inoculation of lake trout LTF cells with EEDV positive tissue homogenate; represented as number of viral copies per qPCR reaction. Sample = original sample inoculated onto cells, P1 = 1st pass infection, P2 = 2nd pass infection, Cells = cell pellet tested for presence of EEDV, Sup = flask supernatant tested for presence of EEDV. Number in parentheses indicates temperature of incubation. Numbers at bottom indicate separate infection trials.*

Both LYR and LTF cells were infected with various EEDV-positive samples as confirmed by qPCR. While cytological changes were observed following initial inoculation, including cell rounding (Figure 3D, E), piling of cells, vacuolation (Figure 3E), and mild areas of cell lysis, upon subsequent passages such changes were no longer observed. In order to account for the potential necessity of cell to cell contact for *in vitro* infection as is seen with Marek's Disease Virus (Schumacher *et al.*, 2000), EEDV infectivity trials *in vitro* were completed using both supernatant and cell suspensions for second passages. However, while mild cytotoxicity was observed from the previous cells being introduced onto new cultures, no overt CPE was observed. Cellular changes were more severe when infected flasks incubated at 9°C than at 4°C or 15°C.

The viral loads from four separate *in vitro* infectivity trials with LTF cells are presented in Figure 3F. This data demonstrates that EEDV continued to be detected in cultured cells as determined by the presence of viral DNA copies as well as by visualization of cellular changes. The virus DNA copy numbers, however, decreased with subculturing. In some cultures, the virus load was relatively high in first pass samples, yet it diminished in the second passage.

The unfortunately small number of well established fish cell lines presents a challenge in the isolation and study of certain aquatic pathogens such as epizootic epitheliotropic disease virus (Bradley *et al.*, 1988; Shavalier, 2017). As demonstrated by Hedrick *et al.* (1991), host species specific cell lines are important for the isolation of certain viruses. In this study, we established primary cultures of multiple different lake trout tissues, and subcultured, expanded and characterized two: One from yearling gonads (LYR) and one from fry (LTF). The development of these cell cultures resulted in a much-needed expansion of the available arsenal of fish cell lines.

The methods described herein resulted in the creation of primary cell cultures from all three age groups of lake trout tested: liver from the broodstock, fin and gonads from the yearling fish and whole sac fry. While neither the yearling fin nor the adult liver cells survived beyond 10 subcultures, the establishment of primary cultures from both of these tissues indicates that they remain potential options for future studies utilizing primary non-transformed cells *in vitro*. Both yearling gonad and fry cells on the other hand readily established monolayers and produced stable subcultures, suggesting that they pose a possibility for use in a myriad of experiments to better understand the pathogenesis of viral infections in an important species in the Great Lakes Basin.

We determined that both gonad (LYR) and fry (LTF) cells were well adapted for growth in either MEM or L-15 growth media, supplemented with 15% fetal bovine serum and incubated at 15˚C. These conditions are comparable to those required by other salmonid cell lines (Fryer and Lannan, 1994), however differ slightly from those used in previous attempts at producing lake trout cell cultures which grew best at a higher temperature (18-21˚C) (Cheng *et al*., 1993).

Morphologically, in early passages, all cultures contained mixed populations of cells, both epithelial- and fibroblast-like. This was particularly clear in the LTF cells, which was not surprising as these cells originated from whole body tissues as opposed to from a single organ from the older fish. However, as passage number increased, proportions of fibroblast-like and epithelioid cells changed with both cell types becoming more epithelial-like. In many individual flasks, it became clear that with mixed cell populations, the fibroblast-like cells were out competing the epithelioid cells, however through regular subculturing and splitting of flasks, certain cultures of epithelial-like fry cells were able to prevail. A similar trend was reported by Swaminathan *et al.* (2010) in cultures of pearlspot fin cells. These cells were originally composed of a mixture of fibroblastic- and epithelial-like cells, however after ten subcultures were primarily epithelial-like.

A crucial component of cell line characterization is definitive identification of species origin. Historically, methods such as karyotyping and isoenzyme analysis have been popular (Freshney, 2011), however recent advancements in molecular diagnostics have helped cement a new protocol for cell line species identification, DNA Barcoding, which has been used successfully to determine the species of origin of a wide range of cell lines from all animal kingdoms (Cooper *et al*., 2007). By sequencing a stretch of the cytochrome *c* oxidase 1 (*COI*) gene in early and late passages from both the LTF and LYR cell lines, and comparing the sequence to the BOLD database, we established that these cells were indeed of lake trout origin.

Following inoculation of LTF cells with VHSV and IPNV we demonstrated the development of cytopathic effect (CPE) including rounding and lysis of cells (Figure 3A, B). This is crucial, as it suggests the susceptibility of lake trout cells to two key aquatic viruses in the Great Lakes Basin. If further investigation reveals that these cultures are truly capable of supporting replication of VHSV and IPNV, they have the potential to serve as a diagnostic tool in the detection and identification of these pathogens. On the contrary, inoculation of both LTF and LYR cells with EEDV resulted in the development of mild, inconsistent CPE that included some lysis and rounding of cells, as well as vacuolation of cells exposed to the virus, indicating a decreased health of the cells. These inconclusive results with EEDV may indicate that these lake trout cells cannot support the replication of EEDV and the search should continue to find other cells or an alternative culture protocol that would support EEDV replication. Alternatively, these cells and culture conditions may support the integration of EEDV DNA into that of the cultured cells, resulting in a dormant infection. Such virus-host DNA integration has been reported for several members of the order Herpesvirales (Morissette and Flamand, 2010). For example, Delecluse and Hammerschmidt (1993) demonstrated that the Marek's Disease Virus DNA gets integrated in a number of cell lines and remains dormant, yet can be reactivated (Delecluse *et al*., 1993). Imajoh *et al.* (2015) also developed an *in vitro* model that resulted in latent versus lytic infection of cyprinid herpesvirus 3 depending on incubation temperature. Indeed, our experience with this disease *in vivo* included an event where the acute form of the disease reemerged in quarantined lake trout, two years after surviving a previous EEDV outbreak (Faisal *et al*., 2019). Whether EEDV also gets integrated into the host DNA requires further investigation. Despite our inability to achieve consistent replication of EEDV in lake trout cells, achieving the establishment long term culture from several organs as well as two cell lines from lake trout will enable scientists to better understand pathophysiological processes and genetics of this poor-studied salmonid.

Acknowledgement

The authors would like to thank the Michigan Department of Natural Resources, specifically Marquette State Fish Hatchery and staff for supplying the fish used in this study.

Novelty Statement

This study produced primary cultures of lake trout cells susceptible to inoculation with multiple key

aquatic viruses in the Great Lakes Basin.

Author's Contribution

MS and MF prepared the manuscript and were involved in the analysis of the data. MS conducted the study.
Megan Shavalier conducted the study. MS and MF were.

Funding information
This work was supported by the Great Lakes Fishery Trust (Award # 2014.1455).

Consent for publication
Not applicable.

Conflicting interests
The authors have declared no conflict of interest.

References

Bradley, T.M., Newcomer, C.E. and Maxwell, K.O., 1988. Epitheliocystis associated with massive mortalities of cultured lake trout *Saivelinus namaycush*. Dis. Aquat. Organ., 4: 9–17. https://doi.org/10.3354/dao004009

Bronte, C., Krueger, C., Holey, M. and Toneys, M., 2008. A guide for the rehabilitation of lake trout in Lake Michigan. Great Lakes Fishery Commission, Miscellaneous Publication 2008-01. Ann Arbor, Michigan

Cheng, L.L., Bowser, P.R. and Spitsbergen, J.M., 1993. Development of cell cultures derived from lake trout liver and kidney in a hormone-supplemented, serum-reduced medium. J. Aquat. Anim. Health, 5: 119–126. https://doi.org/10.1577/1548-8667(1993)005<0119:DOCCDF>2.3.CO;2

Cline, T.J., Bennington, V. and Kitchell, J.F., 2013. Climate change expands the spatial extent and duration of preferred thermal habitat for lake superior fishes. PLoS One, 8: https://doi.org/10.1371/journal.pone.0062279

Cooper, J.K., Sykes, G., King, S., Cottrill, K., Ivanova, N.V., Hanner, R. and Ikonomi, P., 2007. Species identification in cell culture: A two-pronged molecular approach. *In Vitro* Cell Dev. Biol. Anim., 43: 344–351. https://doi.org/10.1007/s11626-007-9060-2

Delecluse, H.J. and Hammerschmidt, W., 1993. Status of Marek's disease virus in established lymphoma cell lines: Herpesvirus integration is common. J. Virol., 67: 82-92. https://doi.org/10.1128/jvi.67.1.82-92.1993

Delecluse, H.J., Schuller, S. and Hammerschmidt, W., 1993. Latent Marek's disease virus can be activated from its chromosomally integrated state in herpesvirus-transformed lymphoma cells. EMBO J., 12: 3277-3286. https://doi.org/10.1002/j.1460-2075.1993.tb05997.x

Eschmeyer, P.H., 1957. The near extinction of lake trout in Lake Michigan. Trans. Am. Fish Soc., 85: 102–119. https://doi.org/10.1577/1548-8659(1955)85[102:TNEOLT]2.0.CO;2

Eshenroder, R.L. and Amatnagelo, K.L., 2002. Reassessment of the lake trout population collaspe in Lake Michigan during the 1940s. Gt Lakes Fish Comm. Tech. Rep., 65: 1–38.

Faisal, M., Loch, T.P., Shavalier, M., VanDeuren, M.G., Standish, I., Winters, A., Glenney, G., Aho, J., Wolgamood, M., VanAmberg, J., Eisch, E. and Whelan, G.E., 2019. Resurgence of salmonid herpesvirus-3 infection (epizootic epitheliotropic disease) in hatchery-propagated lake trout in Michigan. J. Aquat. Anim. Health, 31(1): 31-45. https://doi.org/10.1002/aah.10051

Faisal, M., Schulz, C.A., Loch, T.P., Kim, R.K., Hnath, J. and Whelan, G., 2013. Current status of fish health and disease issues in the Laurentian Great Lakes: 2005 – 2010. In: (eds. Taylor, W.W., Lynch, A.J., Leonard, N.J.,) Great Lakes fisheries policy and management: A binational perspective, 2nd edn. Michigan State University Press, East Lansing, pp. 259–302. https://doi.org/10.14321/j.ctt7ztc19.13

Faisal, M., Shavalier, M., Kim, R.K., Millard, E.V., Gunn, M.R., Winters, A.D., Schulz, C.A., Eissa, A., Thomas, M.V., Wolgamood, M., Whelan, G.E. and Winton, J., 2012. Spread of the emerging viral hemorrhagic septicemia virus strain, genotype IVb, in Michigan, USA. Viruses, 4. https://doi.org/10.3390/v4050734

Freshney, R.I., 2011. Culture of animal cells: A manual of basic technique and specialized applications: Sixth edition. John Wiley & Sons, Inc., Hoboken, NJ, USA. https://doi.org/10.1002/9780470649367

Fryer, J.L. and Lannan, C.N., 1994. Three decades of fish cell culture: A current listing of cell lines derived from fishes. J. Tissue Cult. Methods, 16:

87–94. https://doi.org/10.1007/BF01404816

Glenney, G.W., Barbash, P.A. and Coll, J.A., 2016. A quantitative polymerase chain reaction assay for the detection and quantification of epizootic epitheliotropic disease virus (EEDV; salmonid herpesvirus 3). J. Aquat. Anim. Health, 28: 56–67. https://doi.org/10.1080/08997659.2015.1121935

Hansen, M.J., Peck, J.W., Schorfhaar, R.G., Selgeby, J.H., Schreiner, D.R., Schram, S.T., Swanson, B.L., MacCallum, W.R., Burnham-Curtis, M.K., Curtis, G.L., Heinrich, J.W. and Young, R.J., 1995. Lake trout (*Salvelinus namaychus*) populations in Lake Superior and their restoration in 1959-1993. J. Great Lakes Res. 21(Sup. 1): 152–175. https://doi.org/10.1016/S0380-1330(95)71088-7

Hedrick, R., McDowell, T. and Rosemark, R., 1991. Two cell lines from white sturgeon. Trans. Am. Fish. Soc., 120: 528–534. https://doi.org/10.1577/1548-8659(1991)120<0528:TCLFWS>2.3.CO;2

Hile, R., Eschmeyer, P.H. and Lunger, G.F., 1951. Decline of the lake trout fishery in Lake Michigan. Fish Bull. U. S. Fish Wildl. Serv., 52: 77–95.

Holey, M.E., Rybicki, R.W., Eck, G.W., Brown, Jr. E.H., Marsden, J.E., Lavis, D.S., Toneys, M.L., Trudeau, T.N. and Horrall, R.M., 1995. Progress toward lake trout restoration in Lake Michigan. J. Great Lakes Res., 21: 128–151. https://doi.org/10.1016/S0380-1330(95)71087-5

Imajoh, M., Fujioka, H., Furusawa, K., Tamura, K., Yamasaki, K., Kurihara, S., Yamane, J., Kawai, K. and Oshima, S., 2015. Establishment of a new cell line susceptible to Cyprinid herpesvirus 3 (C y HV-3) and possible latency of C y HV-3 by temperature shift in the cells. J. Fish Dis., 38(6): 507-514. https://doi.org/10.1111/jfd.12252

International Office of Epizootics, Aquatic Animal Health Standards Commission, 2017. Aquatic animal health code, 20th edn. World Organisation for Animal Health, Paris, France.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H. and Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes, 7: 544–548. https://doi.org/10.1111/j.1471-8286.2007.01748.x

Morissette, G. and Flamand, L., 2010. Herpesviruses and chromosomal integration. J. Virol., 84: 12100-12109. https://doi.org/10.1128/JVI.01169-10

Ratnasingham, S. and Hebert, P.D.N., 2007. Barcoding, bold: The barcode of life data system (www.barcodinglife.org). Mol. Ecol. Notes, 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Redick, R.R., 1967. A review of literature on lake trout life history with notes on Alaskan management. State of Alaska, Department of Fish and Game, Homer, Alaska.

Schumacher, D., Tischer, B.K., Fuchs, W. and Osterrieder, N., 2000. Reconstitution of marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-Negative MDV-1 Mutant. J. Virol., 74: 11088–11098. https://doi.org/10.1128/JVI.74.23.11088-11098.2000

Shavalier, M., 2017. Investigations on the intricate interactions between epizootic epitheliotropic disease virus (Salmonid Herpesvirus-3) and its Host, the Lake Trout (*Salvelinus namaycush*). Michigan State University.

Swaminathan, T.R., Lakra, W.S., Gopalakrishnan, A., Basheer, V.S., Khushwaha, B. and Sajeela, K.A., 2010. Development and characterization of a new epithelial cell line PSF from caudal fin of Green chromide, Etroplus suratensis (Bloch, 1790). *In Vitro* Cell Dev. Biol. Anim., 46(8): 647-656. https://doi.org/10.1007/s11626-010-9326-y

Tamura, K., Stecher, G. and Peterson, D., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol., Volume 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Wells, L. and McLain, A.L., 1973. Lake Michigan: Man's effects on native fish stocks and other biota.

Winton, J., Batts, W., DeKinkelin, P., LeBerre, M., Bremont, M. and Fijan, N., 2010. Current lineages of the epithelioma papulosum cyprinid (EPC) cell line are contaminated with fathead minnow, Pimephales promelas, cells. J. Fish Dis., 33(8): 701-704. https://doi.org/10.1111/j.1365-2761.2010.01165.x