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

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2 **hatchery propagated Lake Trout *Salvelinus namaycush* in Michigan**
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42 Abstract

43 Over the past century, populations of Lake Trout *Salvelinus namaycush* have declined
44 throughout the Great Lakes basin due to over-fishing, habitat destruction, introduction of
45 invasive species and associated recruitment issues from high thiaminase, as well as emerging
46 infectious diseases. To combat these declines, state and federal fishery management agencies
47 undertook substantial stock enhancement efforts, including more stringent regulation of sport and
48 commercial catch limits and increasing hatchery propagation of Lake Trout stocked into Great
49 Lakes basin waterways. One state fish hatchery involved in these rehabilitation efforts
50 experienced a mass mortality event in 2012 and a milder mortality episode in 2017. In 2012,
51 following a period of abnormally heavy rain, hatchery staff observed abnormal behavior
52 followed by increased mortalities in two strains of Lake Trout fingerlings reaching upwards of
53 20% and totaling a loss of approximately 100,000 fish. In 2017, following another heavy rain
54 season, 6-8% of two-year old Lake Trout experienced morbidity and mortality similar to that
55 observed in 2012. During the 2012 event, Brook Trout (*Salvelinus fontinalis*) and splake (Lake
56 Trout x Brook Trout hybrid) reared in flow-through systems receiving water from diseased Lake
57 Trout remained clinically unaffected. Molecular analyses revealed all lots of affected Lake Trout
58 were infected with the Salmonid Herpesvirus-3 (Epizootic Epitheliotropic Disease Virus,
59 EEDV), a disease that caused complete depopulation of this hatchery in the late 1980s and since
60 has never been detected in this hatchery or in Michigan. Further sampling detected EEDV in
61 apparently healthy 5-year old Lake Trout, and in wild Mottled Sculpin (*Cottus bairdii*) collected
62 in the hatchery source water. Rivers' postulates were fulfilled by exposing naïve Lake Trout to
63 the infectious material, resulting in similar disease signs. Despite going undetected for many

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64 years, these two EEDV episodes clearly demonstrate the continued presence of this deadly
65 herpesvirus in the Great Lakes basin.
66
67 Introduction
68 The Lake Trout *Salvelinus namaycush* is an invaluable native constituent of the fish
69 fauna residing within the Laurentian Great Lakes Basin of North America (Bronte et al. 2008). In
70 addition to its intrinsic ecological value as a keystone species, Lake Trout are also a valuable
71 commercial and sports fishery commodity (Redick 1967). Through the 1970s, Great Lakes Lake
72 Trout fisheries had steadily declined due to overfishing, habitat degradation, predation by the Sea
73 Lamprey *Petromyzon marinus*, and the invasion of Alewife *Alosa pseudoharengus* that carried
74 high levels of thiaminase which disrupted Lake Trout recruitment processes (Hile et al. 1951;
75 Eschmeyer 1957; Redick 1967; Wells and McLain 1973; Holey et al. 1995; Eshenroder and
76 Amatnagelo 2002; Cline et al. 2013). As a result, state and federal fisheries management
77 agencies developed programs to regulate Lake Trout harvest and create self-sustaining wild
78 populations (Redick 1967), which included bolstering depleted wild populations by the stocking
79 of hatchery-raised Lake Trout as well as supplementing wild populations in water bodies with
80 low natural egg survival rates (Redick 1967).
81 During their tenure in hatcheries, Lake Trout are susceptible to a number of infectious
82 diseases (Faisal et al. 2010, 2013), including one particularly devastating disease caused by
83 Epizootic Epitheliotropic Disease Virus (EEDV; *Herpesvirales*, *Alloherpesviridae*). This
84 herpesvirus, also known as Salmonid Herpesvirus-3, led to the loss of approximately 15 million
85 juvenile, hatchery-reared Lake Trout in the 1980s. These mortalities occurred in seven state and
86 federal hatcheries across three states in the Great Lakes region following very heavy summer

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87 rainfall events, leading to the first description of EEDV (Bradley et al. 1988, 1989; McAllister
88 and Herman 1989). In an attempt to control this virus and limit its spread, fisheries managers
89 opted for depopulation and disinfection of affected hatcheries, along with the implementation of
90 movement restrictions for Great Lakes basin Lake Trout (Kurobe et al. 2009). It appeared these
91 control efforts were largely successful because reports of EEDV outbreaks, characterized by a
92 rapid onset of mortality in young (< 2 years of age) Lake Trout, hyperplastic lesions of the skin
93 and gill epithelia, ocular hemorrhage (Bradley et al. 1988), and secondary infections (Kurobe et
94 al. 2009) ceased. However, in 2003, 2006 and 2007, the virus was detected in Wisconsin Lake
95 Trout. In 2003 and in 2006, EEDV was detected in the reproductive fluids of wild, apparently
96 healthy spawning adult Lake Trout in Lake Superior, and in the skin of hatchery-raised juvenile
97 Lake Trout experiencing mortalities, although the severity of mortalities was unreported (Kurobe
98 et al. 2009). In 2007, it was detected in the skin of apparently healthy juvenile Lake Trout in the
99 same Wisconsin hatchery (Kurobe et al. 2009).
100 Herein, we report the resurgence of EEDV and describe two mortality events associated
101 with this deadly virus that occurred in Lake Trout at a state fish hatchery in Michigan's Upper
102 Peninsula during the fall of 2012 and 2017. This apparent resurgence of EEDV following
103 decades of covertness highlights the need to better understand the biological properties of this
104 virus, along with the intricacies of the host-virus interactions.
105
106 [A]Methods
107 [B]Lake Trout mortality events
108 In September of 2012, Michigan's Upper Peninsula experienced several days of heavy,
109 warm rain, resulting in the flooding of many smaller streams and creeks including Cherry Creek,

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110 which is a surface water source for Marquette State Fish Hatchery (MSFH; Marquette County,
111 Lake Superior watershed). At the time, MSFH was raising two strains of Lake Trout (i.e., Lake
112 Superior (LS) and Seneca Lake (SE) strains), Brook Trout *Salvelinus fontinalis*, and splake
113 (Lake Trout x Brook Trout hybrid), all of which were housed in covered, outdoor raceways
114 (12,786-14,793 gallons) with an average of 90,000 fish/raceway. The original Lake Superior
115 (LS) strain Lake Trout were collected from Lake Superior in the early 2000s (2001, 2003 and
116 2004) in order to establish a lean Lake Trout strain. Seneca Lake (SE) strain Lake Trout came
117 from the U.S. Fish and Wildlife Service hatchery system as fertilized eggs. As a whole, the
118 hatchery receives both well water and surface water from nearby Cherry Creek, a very high
119 quality, cold, and stable water source; production aged fish receive Cherry Creek water at an
120 approximate rate of 1,200 gallons/minute. As an additional precaution, all water supplying
121 broodstock fish is passed through an ultraviolet sterilization unit before entering the raceways.
122 Among the production fish, the Brook Trout, LS strain Lake Trout, and a portion of the SE strain
123 Lake Trout were receiving first pass water, whereas the splake and the remainder of the SE strain
124 Lake Trout were receiving second pass water and were housed immediately downstream of the
125 first pass raceways. Shortly after the period of abnormally heavy and warm rains, mortalities
126 began to climb in both strains of juvenile (approximately 8 months post-hatch) Lake Trout
127 (Figure 1). The signs of disease were first expressed in the LS strain Lake Trout and within
128 several weeks were followed by signs in the SE strain Lake Trout. Between October 2nd and
129 November 8th, 2012, moribund 2011 year class Lake Trout from both strains were collected alive
130 for clinical examination at the Michigan State University – Aquatic Animal Health Laboratory
131 (MSU-AAHL). Over the five-week period, diagnostic examinations were performed on a total of
132 60 LS and SE strain Lake Trout (30/strain).

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133 In September of 2017, elevated mortality was once again reported at MSFH, this time in
134 one lot of 2-year old future broodstock LS strain Lake Trout. This mortality episode also
135 occurred following a heavy, warm rain event during a period of unusually warm late season
136 ambient air temperatures. Moribund fish (n = 10) were collected alive and sent to the MSU-
137 AAHL for clinical examination.
138
139 [B]Clinical examination
140 Upon receipt at the MSU-AAHL, fish behavior was observed and noted, followed by
141 euthanasia with an overdose of tricaine methanesulfonate (MS-222; Western Chemical, Ferndale,
142 Washington, USA; 0.25 mg/mL) and fish were immediately subjected to gross external clinical
143 examination. Wet mounts of gill tissues and skin lesions were prepared and examined for the
144 presence of parasites, fungi and bacteria via light microscopy. Tissues were collected as detailed
145 below. Next, fish were surface disinfected with 70% ethanol and gross internal clinical
146 examination and aseptic tissue collections performed following guidelines of the American
147 Fisheries Society Fish Health Section (AFS-FHS) Blue Book (2016) for the diagnostic assays
148 detailed below.
149
150 [B]Bacteriology
151 For primary bacterial isolation, 10 µL sterile disposable loops were used to streak kidney
152 tissues directly onto trypticase soy agar (TSA; Remel, San Diego, California, USA), Hsu Shotts
153 medium (HSM) (Bullock et al. 1986), and cytophaga agar (CA) (Anacker and Ordal 1959),
154 which were incubated aerobically at 22°C (TSA and HSM) or 15°C (CA) for up to seven days.
155 Additionally, representative brain and gill tissues, as well as tissues from skin/muscle lesions,

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156 were streaked onto HSM and/or CA due to suspicion of flavobacterial involvement. Resultant
157 bacterial growth was recorded, sub-cultured, and identified as recommended in the AFS-FHS
158 Blue Book (2016). Specifically for *Flavobacterium psychrophilum,* molecular confirmation was
159 performed as previously described (Van Vliet et al. 2015).
160
161 [B]Virus isolation
162 Kidney, spleen and heart tissue samples were aseptically collected, diluted 1:4 (w/v) with
163 Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific,
164 Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St
165 Louis, Missouri, USA), penicillin (100 IU/mL; Invitrogen), streptomycin (100 µg/mL;
166 Invitrogen), and amphotericin B (250 µg/mL; Invitrogen). Tissues and diluent were then
167 homogenized and centrifuged at 4,700 x *g* for 30 minutes, and the supernatant clarified by a
168 second centrifugation at 2,700 x *g* for 20 minutes. The final supernatant was used to inoculate
169 cell cultures of *Epithelioma papulosum cyprini* (EPC) (Fijan et al. 1983) and Chinook Salmon
170 embryo (CHSE-214) (Fryer et al. 1965) cell lines and examined for cytopathic effects as per the
171 guidelines of the AFS-FHS Blue Book (2016). Skin, fin, gill (2012 and 2017), and eye (2017
172 only) tissues were collected and stored at -20°C for further molecular diagnostics (see below).
173
174 [B]Histopathology
175 Skin, muscle, fin, gill, eye, kidney, spleen, heart, and liver tissues, as well as transverse
176 and sagittal whole body sections, were collected from representative fish and preserved in
177 phosphate-buffered 10% solution of formalin for histopathological assessment. After embedding

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178 within paraffin, tissues were sectioned at 5 µm, stained with hematoxylin and eosin (H&E)
179 (Prophet 1992) and examined under a light microscope.
180
181 [B]Molecular analysis
182 In the years following the 2012 mortality episode and as EEDV molecular assays were
183 being designed and improved, two novel salmonid herpesviruses (i.e., Salmonid Herpesvirus-4;
184 Atlantic Salmon papillomatosis virus (Doszpoly et al. 2013), and Salmonid Herpesvirus-5;
185 Namaycush Herpesvirus (Glenney et al. 2016a)) were identified and found to possess some
186 terminase gene sequence similarity with EEDV (Glenney et al. 2016b). However, glycoprotein
187 gene sequence analysis allowed for development of highly sensitive qPCR assays specific to
188 each of Salmonid Herpesviruses-3, -4, and -5 (Glenney et al. 2016b). Therefore, in the present
189 study, the following three separate PCR assays were employed (Glenney et al. 2016b):
190
191 1. End-point PCR (terminase gene; amplifies DNA from Salmonid Herpesvirus-3, -4, and -5)
192 (Glenney et al. 2016b)
193 2. TaqMan qPCR (terminase gene; amplifies DNA from Salmonid Herpesvirus-3, -4, and -5)
194 (Glenney et al. 2016b)
195 3. SYBR Green qPCR (glycoprotein gene; amplifies DNA from Salmonid Herpesvirus-3 only)
196 (Glenney et al. 2016b)
197
198 End-point PCR was used in the 2012 and 2017 EEDV outbreaks for gene sequencing and
199 phylogenetic analyses (see below) whereas the TaqMan qPCR was used for screening purposes

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200 from 2012 through the summer of 2016 until the SYBR Green qPCR assay was developed and
201 optimized to definitively identify EEDV infected fish (Glenney et al. 2016b).
202 Available molecular assays changed between 2012 and 2017, so too did the knowledge
203 of EEDV tissue tropism and optimal diagnostic samples (data not shown). As a result, early
204 EEDV testing following the 2012 mortality episode was performed on pools of kidney, spleen
205 and gill tissues lethally collected from juvenile fish, while mucous was non-lethally collected
206 from adult broodstock in order to allow for screening of a larger number of fish without
207 significantly influencing broodstock availability for future fish production. Historical samples
208 from 2007-2012 consisted of kidney, spleen and heart pools previously collected for virological
209 screening and stored at -20°C.
210 For viral DNA extraction, one of two extraction methods was used. For the TaqMan
211 qPCR, the MagMax™ 96 Viral RNA isolation kit (Life Technologies, Grand Island, New York,
212 USA) was used manually, following manufacturer's instructions. Samples were lysed using
213 Proteinase K and Lysis buffer (Qiagen, Germantown, MD, USA), and incubated in a water bath
214 at 55°C for 1 hour. Following lysis, samples were centrifuged at 21,000 x *g* for 10 minutes and
215 the supernatant used in the extraction process. Following the development of the SYBR Green
216 qPCR assay, viral DNA extractions were performed manually using the Mag Bind® Blood and
217 Tissue DNA Kit (OMEGA Bio-tek, Inc., Norcross, Georgia, USA), following the manufacturer's
218 instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate
219 (OMEGA Bio-tek, Inc.) after tissue digestion (Glenney et al. 2016b). Following all nucleic acid
220 extractions, DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer
221 (Life Technologies) and diluted to a standard concentration using nuclease free water.

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222 All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR
223 machine (Eppendorf, Hauppauge, New York, USA). Both the TaqMan and SYBR Green assays
224 were performed as described previously (Glenney et al. 2016b) with the exception that the total
225 reaction volume of the SYBR Green assay was 20 µL; 30-60 ng total DNA was added to each
226 qPCR reaction. Using the Mastercycler ep *realplex*^{2} S accompanying software at the
227 manufacturer's default settings, samples were considered positive based on a threshold setting of
228 the computer default noiseband for the TaqMan assay and 10% maximum florescence for the
229 SYBR Green assay with a limit of 35 cycles for all samples. Positive extraction controls
230 consisted of EEDV-positive tissue samples from diseased Lake Trout collected during a natural
231 EEDV outbreak. Positive amplification controls and standards were produced for both the
232 TaqMan and SYBR Green assays as previously described (Glenney et al. 2016b). Negative
233 controls consisted of water as well as negative tissue extraction controls from disease-free Lake
234 Trout.
235
236 [B]Gene sequencing and phylogenetic analysis
237 Representative samples (*n* = 4/episode) that were positive via the TaqMan qPCR (in
238 2012) or SYBR Green qPCR (in 2017) were selected for endpoint PCR and subsequent gene
239 sequencing and phylogenetic analysis. Amplicons for sequence analysis were produced using
240 primers 194F (5' - TAG TCT GAT CCC CCT CAT GC - 3') and 249R (5' - GTC GAG TCC
241 GAC ACC AGA TT - 3'), which amplify a 324 bp fragment of the terminase gene (Glenney et
242 al. 2016b). Each 50 µL reaction mixture was comprised of 25 µL 2x Go-Taq Green Master Mix
243 (Promega, Madison, Wisconsin, USA), 250 mM of each primer, 50 ng of DNA template, and
244 DNase-free water. Cycling parameters consisted of an initial denaturation step at 95°C for 15

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245 minutes, followed by 35 cycles of 94°C for 30 seconds, 59.5°C for 30 seconds and 72°C for 1
246 minute with a final step of 72°C for 10 minutes, and were carried out in a Mastercycler Pro
247 Thermal Cycler (Eppendorf). Amplicons and a 1 kb molecular ladder (Roche Applied Science,
248 Penzberg, Germany) were combined with SYBR Green (Cambrex Bio-Science, Lonza Group,
249 Basel, Switzerland), electrophoresed through a 1.5% agarose gel at 50V for 45 minutes, and
250 visualized under ultraviolet light. Amplicons were then purified using a QIAquick PCR
251 Purification Kit (Qiagen) and Sanger sequenced at the Michigan State University Research
252 Technology Support Facility using both the forward and reverse primers.

253 For phylogenetic analyses, contigs were assembled using the contig assembly program in
254 Bioedit Sequence Alignment Editor (Hall 1999). Multiple sequence alignment was done using
255 ClustalW in the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0)
256 (Tamura et al. 2013), whereby reference terminase gene sequences for Salmonid Herpesvirus-1
257 through -5 were downloaded from GenBank (NCBI) and included in the alignment (a total of
258 303 bases were included in the final data alignment set). The optimal model for phylogenetic
259 reconstruction was assessed in MEGA 6.0 and the model with the lowest Bayesian Information
260 Criterion (Kimura Two Parameter model with gamma distribution, K2+G) was selected.
261 Neighbor-joining analysis was carried out in MEGA 6.0 with 1,000 resamplings. Bayesian
262 analysis was conducted in MRBAYES version 3.1.2 (K2+G model) (Ronquist et al. 2012). The
263 Markov chains ($n = 4$) were run for up to one million generations, with a stopping rule in place
264 once the analysis reached an average standard deviation of split frequencies of <0.01. Two
265 independent analyses were conducted, with the initial 25% of Markov chain Monte Carlo
266 samples being discarded as burnin and sampling occurring every 500 generations. Results from
267 Bayesian analyses were visualized in FigTree v1.3.1 (Rambaut 2009).

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268

269 [B]Experimental challenges and fulfillment of Rivers' Postulates

270 To confirm the virulence of the EEDV strain associated with the 2012 hatchery disease
271 outbreak and fulfill Rivers' Postulates, experimental challenges were performed. Apparently
272 healthy juvenile LS strain Lake Trout (approximately 6 months post hatch) were obtained from
273 MSFH while maintained on a closed (i.e., well) water system and transported live to the
274 Michigan State University - University Research Containment Facility (MSU-URCF, East
275 Lansing, Michigan, USA). The originating lot of fish used for experimental challenges was not
276 present in the hatchery system during either mortality episode. Upon receipt, fish were held in a
277 680 L fiberglass tank supplied with continuous flow-through oxygenated well water (12.0 ±
278 1.0°C). Fish were fed 1.0 mm sinking feed (Bio-Oregon, Westbrook, Maine, USA) daily and
279 allowed to acclimate to laboratory conditions for at least one month prior to use in experimental
280 challenges. Sixty fish were randomly collected and clinically examined, as per the guidelines of
281 the AFS-FHS Blue Book (2016), and determined to be free from fish pathogens of concern as
282 per the Great Lakes Model Program (Phillips et al. 2014). Likewise, EEDV qPCR confirmed an
283 absence of EEDV. Experimental challenges were performed in accordance with MSU's
284 Institutional Animal Care and Use Committee.

285 All experimental challenges were performed in 42 L continuous, flow-through tanks
286 receiving oxygenated, chilled, well water (9.0 ± 0.5°C), and fish were allowed to acclimate for a
287 minimum of 48 hours to experimental conditions prior to start of challenges. A stock of
288 infectious EEDV was produced from the skin of MSFH-naturally infected Lake Trout following
289 mortality. Skin was homogenized in a sterile phosphate buffered saline solution (PBS; pH 7.5 ±

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290 0.5; Sigma-Aldrich) at a 1:3 (w/v) ratio, and clarified via low speed centrifugation (1,400 x *g*) for
291 20 minutes at 4°C.
292 Next, LS strain Lake Trout (*n* = 5 challenge group and *n* = 5 control group) were
293 anesthetized using MS-222 (0.1 mg/mL) then IP injected with either EEDV stock (1.22×10^7 viral
294 copies/fish) or sterile PBS. Following recovery from anesthesia, fish were transferred back to
295 experimental tanks for the duration of the studies.
296 All fish were fed *ad lib* and monitored daily for development of clinical signs of disease,
297 morbidity or mortality for up to 2 months following injection. Any moribund fish displaying a
298 combination of severe clinical signs such as altered behavior, inability to maintain balance,
299 difficulty respiring, significantly pale gills, or severe external lesions was euthanized with MS-
300 222 (0.25 mg/mL). At the end of the two-month period, surviving fish were euthanized. Skin
301 tissues were collected from all fish immediately following death and tested for the presence of
302 EEDV using the TaqMan qPCR protocol described above.
303 *Intra- and extra-hatchery EEDV surveillance*
304 After the 2012 mortality event, all lots of fish at the hatchery were screened for the
305 presence of EEDV. Between the fall of 2012 and the spring of 2013, pools of kidney, spleen and
306 gill were collected from a total of 120 juvenile LS strain Lake Trout, 240 juvenile SE strain Lake
307 Trout, 480 juvenile Brook Trout, and 240 juvenile splake while mucous was collected non-
308 lethally from 60 adult Brook Trout and 270 adult LS strain Lake Trout. Routine EEDV
309 surveillance screening continued among MSFH Lake Trout and splake through 2017 for
310 production fish (*n* = 60/lot) and broodstock fish (*n* = 10/lot) with the testing of kidney, spleen,
311 gill, fin, skin, and eye tissues (Table 2.1).

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312 Additionally, wild fish were collected by standard electrofishing from Cherry Creek,
313 upstream of MSFH, and tested for the presence of EEDV (Table 2.2). In 2012, 70 each of Brook
314 Trout, Brown Trout (*Salmo trutta*), and Mottled Sculpin (*Cottus bairdii*) were collected from
315 Cherry Creek, and in 2013, 60 of each of these three species were collected and tested for EEDV.
316 From 2014-2017, 60 Mottled Sculpin/year were collected from Cherry Creek for EEDV
317 screening.
318
319 [A]Results
320 [B]Description of MSFH mortality events
321 In 2012, approximately 10 days after the heavy rains, juvenile LS strain Lake Trout
322 began exhibiting decreased appetites, evidenced by a lack of interest in food. Within three days,
323 the LS strain Lake Trout in raceways 5a and 5b began riding high in the water column,
324 displaying flashing behavior, and were generally lethargic. Similar changes were noted 10 days
325 later in the SE strain Lake Trout in raceway 6, followed one month later by the SE strain Lake
326 Trout in raceways 4a and 4b. Affected fish developed multifocal skin pallor that in some
327 instances became overgrown with whitish fungal masses. The mortality episode spanned over
328 200 days, with the cumulative mortality by raceway ranging from >15% to >25% (Figure 1) and
329 total losses exceeding 100,000 Lake Trout. Based upon initial clinical examinations, disease
330 signs, and the detection of *Flavobacterium psychrophilum* in external lesions (see below),
331 affected Lake Trout initially received an immersion treatment with Chloramine-T (Halamid®,
332 Syndel USA, Ferndale, Washington, USA; 10 mg/mL for 1 hour for 3 consecutive days, repeated
333 a second time after two days of no treatment). After no discernible improvements, this was
334 followed by two treatment courses with Florfenicol medicated feed (Aquaflor; Merck Animal

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335 Health, Madison, New Jersey, USA; 10 mg/kg body weight/day for 10 days), which resulted in
336 only a slight and brief decrease in mortality.
337 Likewise, in late September 2017, 2-year old LS strain Lake Trout (20-27 centimeters in
338 length) at MSFH began developing disease signs similar to those seen in 2012. Initial mortalities
339 were approximately 5 fish/day with an additional 12-15 showing signs of morbidity by day.
340 These levels of mortalities continued through the following month.
341

342 [B]Clinical examination

343 During the 2012 mortality event, both strains of moribund Lake Trout showed a number
344 of external disease signs, including ocular hemorrhage with or without corneal opacity (Figure
345 2A, 2B), gill pallor, multifocal to diffuse skin " blotchiness" (Figure 2C) that was sometimes
346 accompanied by erythema (Figure 2D), ulcerations that penetrated through the epidermis and
347 dermis of the skin into the underlying muscle (Figures 2E, 2F), as well as ulcers that were
348 overgrown by water mold hyphae (Figure 2G). In some instances, ulceration progressed to the
349 point where the caudal fin had eroded completely (Figure 2H) and was also accompanied by a
350 yellowish discoloration (Figure 2I), which is commonly associated with flavobacteria. Other
351 notable disease signs included erythema of the fins (Figure 2D), oral cavity, isthmus and
352 ventrum, and excess mucus production of the skin. Internally, occasional hemorrhage within the
353 adipose tissue, hemorrhagic enteritis, splenomegaly, and renal congestion were observed.
354 Clinical findings were similar between both strains of Lake Trout; however, those seen in the LS
355 strain tended to be more severe than those in the SE strain.
356 During the 2017 outbreak, disease signs similar to the 2012 outbreak were observed and
357 were once again predominated by ocular hemorrhage (Figure 3A), varying degrees of skin

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358 ulceration (Figure 3B, 3C), and overgrowth by water mold (Figure 3D). In both the 2012 and
359 2017 outbreaks, microscopic examination of skin and gill preparations revealed the presence of
360 occasional monogeneans (*Gyrodactylus* spp.), gliding filamentous bacterial rods consistent with
361 flavobacteria, and aseptated hyphae consistent with the oomycete *Saprolegnia*.
362
363 [B]Bacterial and viral isolation
364 *Flavobacterium psychrophilum* was recovered from a portion of the external lesions, and
365 motile *Aeromonas* spp. were occasionally recovered in kidney cultures, albeit in relatively low
366 quantities. No signs of viral replication were observed in cell lines following inoculation with
367 tissue homogenate supernatant.
368 *Histopathology*
369 Histopathological findings in EEDV-infected Lake Trout collected during the 2012
370 outbreak included corneal epithelial necrosis and/or ulceration (Figure 4A), epithelial necrosis
371 and ulceration of the skin (Figure 4B), lamellar edema (Figure 4C), gill epithelial
372 swelling/hypertrophy (Figure 4D), proteinaceous exudate within both Bowman's space and the
373 renal tubules (Figure 4E), renal tubular epithelial necrosis (Figure 4F), and multifocal necrosis of
374 the renal interstitium (Figure 4F). Similar microscopic changes were observed in EEDV-infected
375 2 year old LS strain Lake Trout in 2017; however, individual necrosis of the gill lamellar
376 epithelium and a moderate dermatitis in the skin were also observed, as was hemosiderosis
377 within the spleen.
378
379 [B]Molecular identification

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380 PCR-based molecular assays clearly demonstrated the presence of a salmonid herpesvirus
381 in affected MSFH Lake Trout tissues from 2012. Skin, gill and kidney/spleen tissues from 16
382 fish (8 LS and 8 SE strain Lake Trout) tested positive via endpoint and TaqMan qPCR, although
383 sequencing was required for confirmation of EEDV identity (see below). EEDV was detected in
384 the LS strain Lake Trout from 7 of 8 gill samples, 7 of 8 kidney/spleen sample pools, and 8 of 8
385 skin samples, and in the SE strain Lake Trout from all 24 tissues tested. Viral gene copy number
386 per reaction in the LS strain Lake Trout ranged from $3.0x10^1$ to $2.3x10^3$ (median = $2.01x10^2$) in
387 the gills, to $1.4x10^1$ to $6.56x10^2$ (median = $1.34x10^2$) in the kidney/spleen, and $1.98x10^4$ to
388 $9.80x10^5$ (median = $9.22x10^4$) in the skin. Viral gene copy number per reaction in the SE strain
389 Lake Trout ranged from $1.92x10^3$ to $9.63x10^4$ (median = $1.26x10^4$) in the gills, to $1.0x10^1$ to
390 $5.45x10^4$ (median = $4.06x10^1$) in the kidney/spleen, and $2.70x10^2$ to $1.44x10^4$ (median =
391 $2.56x10^3$) in the skin.
392 The presence of EEDV in the 2017 mortality event was confirmed using the SYBR green
393 qPCR assay, as well as with endpoint PCR and gene sequencing (see below). All tissues
394 collected from moribund fish were positive for EEDV: 10 of 10 skin lesions ($2.57x10^4$ to
395 $1.42x10^6$; median = $2.04x10^5$ copies) and 10 of 10 eye tissues ($8.68x10^3$ to $2.38x10^7$; median =
396 $1.38x10^6$ copies).
397
398 [B]Sequencing and phylogenetics
399 Amplification and sequencing of a portion of the EEDV terminase gene from four
400 naturally infected Lake Trout from each of the 2012 and 2017 EEDV outbreaks led to the
401 generation of gene fragments totaling 311-322 bps in length. Percent similarity analysis revealed
402 that three out of four MSFH EEDV isolates from 2012 and four out of four 2017 isolates were

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403 100% similar to the Salmonid Herpesvirus-3 isolate from Wisconsin (accession # EU349284) at
404 this locus, whereas one 2012 isolate was 99.7% similar (310/311 bp) to the Wisconsin reference
405 isolate. Phylogenetic analyses placed the eight MSFH EEDV isolates into a robustly supported
406 clade (i.e., posterior probability and bootstrap values >70) that also contained the Wisconsin
407 reference isolate, which shared a most recent common ancestor with Salmonid Herpesvirus-4
408 and -5 (Figure 5).
409
410 [B]Pilot experimental challenges
411 In the EEDV experimentally challenged Lake Trout, 80% mortality was reached at 29
412 days post-infection with previous mortalities occurring at days 6, 13, and 20 post-infection.
413 EEDV was detected in multiple tissues from infected fish that died on days 13, 20, and 29 post-
414 infection. The virus was found in the skin/fin pools (3 of 5 fish; 9.40×10^4 to 2.50×10^6; median =
415 5.85×10^5 copies/reaction), gill (2 of 5 fish; 4.61×10^3 to 5.26×10^3; median = 4.93×10^3
416 copies/reaction), and kidney/spleen pools (3 of 5 fish; 3.60×10^2 to 2.72×10^3; median = 8.68×10^2
417 copies/reaction) of experimentally challenged fish. Clinical signs were consistent with those seen
418 in the natural epizootics and included ocular hemorrhage (Figure 6A), skin pallor, erosions and
419 ulcerations (Figure 6B, 6C), with congestion and erosion of the fins. The control group
420 experienced only a single mortality, and no evidence of EEDV infection was detected in any
421 control fish.
422
423 [B]Intra- and extra-hatchery EEDV surveillance
424 Using qPCR as detailed above, EEDV was not detected in any of the adult Brook Trout
425 or Lake Trout broodstock tested in 2012. Of the 120 juvenile LS strain Lake Trout and 240

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426 juvenile SE strain Lake Trout tested in 2012 following the mortality episode, EEDV was
427 detected in 24 fish (21 to 113 virus copies/reaction; median = 36) and 21 fish (20 to 1,828 virus
428 copies/reaction; median = 69), respectively. It is interesting to note that, while beyond the
429 established cut off of 35 cycles, amplification was observed between 35 and 40 cycles from a
430 total of 132 additional juvenile Lake Trout tested in 2012. Likewise, while no juvenile splake or
431 Brook Trout had detectible levels of EEDV, amplification was observed between 35 and 40
432 cycles in two Brook Trout samples and four splake samples (kidney, spleen, and gill tissues).
433 Following the 2012 mortality event, EEDV screening at MSFH continued through 2017, as
434 detailed in Table 2.1, including the testing of archived historical samples from 2007 and 2011.
435 During routine surveillance in 2017, EEDV was detected in 8 adult (5 years post-hatch)
436 and 5 juvenile (2 years post-hatch) LS strain Lake Trout in fin and eye tissues ranging from
437 1.27×10^2 to 1.1×10^7 viral copies in the fin (median = 3.89×10^3) and 6.9×10^3 to 2.5×10^5 viral
438 copies in the eye (median = 1.28×10^5). Of note, these samples were collected just prior to the
439 appearance of disease signs in the affected lot.
440 EEDV screening in fish collected from Cherry Creek (i.e., the surface water system
441 feeding MSFH) is detailed in Table 2.2. All Brook Trout and Brown Trout collected were
442 EEDV-negative (*n* = 150 and *n* = 185, respectively). All Mottled Sculpin were EEDV-negative
443 except for five pools (*n* = 25 fish) in 2013.
444
445 [A]Discussion
446 The Lake Trout-lethal herpesvirus, EEDV, has re-emerged in one hatchery within the
447 Great Lakes basin, where it is again associated with substantial mortality, severe disease signs,
448 and high viral loads in multiple strains of fingerling Lake Trout, with disease signs in 2 year old

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449 Lake Trout and virus detection in 5 year old Lake Trout as well. Moreover, laboratory
450 experiments aimed at fulfilling Rivers' postulates confirmed the ability of the causative virus
451 strain to produce clinical signs and mortality consistent with those seen in natural EEDV
452 outbreaks, despite the absence of secondary invaders (e.g., oomycetes, *F. psychrophilum*, etc.)
453 that were present in the natural outbreaks. The resurgence of EEDV after decades of an apparent
454 absence is both surprising and perplexing. On one hand, it is known that some human and animal
455 herpesviruses can run a covert, low-level infection in which the host survives and becomes a
456 viral reservoir within a system, particularly with a long lived fish species with a low mortality
457 rate in the hatchery (Bonsall et al. 2005). On the other hand, sub-lethal infections are uncommon
458 among the other fish-pathogenic alloherpesviruses, although shedding of infectious virus has
459 been detected following survival from Cyprinid Herpesvirus-3 infection (an OIE reportable
460 pathogen) (Yuasa et al. 2008; Hanson et al. 2011; Baumer et al. 2013). In the case of EEDV, the
461 1980s reports demonstrated its high pathogenicity to Lake Trout and suggested that survival of
462 infected fish was unlikely. This concept prevailed until Kurobe et al. (2009) developed a novel
463 PCR assay based on the terminase gene sequence and reported the presence of EEDV in
464 apparently healthy Lake Trout collected from Wisconsin waters, signifying that EEDV may be
465 capable of causing sub-lethal infections within Lake Trout. Indeed, such infections have
466 subsequently been reported for EEDV (i.e., Salmonid Herpesvirus-3) and Salmonid Herpesvirus-
467 5 in wild, clinically normal, adult Lake Trout throughout the northeastern United States (Glenney
468 et al. 2016a, 2016b). In this context, it is possible that MSFH Lake Trout harbored a sub-clinical
469 EEDV infection prior to the fall of 2012 and that stressors, such as the heavy, warm rain events
470 that preceded each of the outbreaks and resulted in an influx of sediment-laden water into
471 hatchery rearing units, led to clinical outbreaks of EEDV. This is a particularly key point when

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472 discussing species such as Lake Trout whose natural habitats rarely see such decreases in water
473 quality. In combination with high hatchery densities (also not seen naturally), this demonstrates a
474 clear stress trigger for disease outbreaks caused by pathogens such as EEDV.

475 Alternatively, it is possible the virus found its way into the hatchery via the source water,
476 as, in 2013, EEDV DNA was detected in Mottled Sculpin residing upstream of MSFH in Cherry
477 Creek, the stream supplying water to the affected fish. Indeed, it was a surprise to detect EEDV
478 DNA in non-salmonids, as alloherpesviruses are known to be highly species-specific. Detection
479 of viral DNA on external tissues alone would be questionable as to whether these fish were truly
480 infected, or if EEDV genetic material was present in the water only. However, the detection of
481 EEDV DNA within pools of kidney, spleen and heart tissues raises the possibility that these fish
482 were truly infected, although conclusive determination of whether the virus was active or not
483 was unfortunately not possible. Nevertheless, experiments examining the susceptibility of
484 Sculpin to EEDV are currently underway. It is noteworthy that the UV system installed to treat
485 the incoming water serving the juvenile Lake Trout malfunctioned in the summer and fall of
486 2017. It is not known how long the system was nonfunctional but the timing of fall rains and
487 resulting water turbidity and the loss of UV treatment could have synergistically contributed to
488 the 2017 outbreak of EEDV in the 2012 year class of juvenile LS strain Lake Trout and to the
489 2017 detection of the virus in the 2015 year class of juvenile LS strain Lake Trout.

490 Finally, it is also possible that the LS strain Lake Trout broodstock collected as eggs from
491 the waters of western Lake Superior in 2001, 2003, and 2004 harbored a native sub-clinical
492 EEDV infection and acted as a source of infection for the progeny in 2012 and 2015 year classes.
493 Parental brood lots for the 2012 year class were the 2001, 2003, and 2004 Lake Trout. The
494 parental brood lots for the 2015 year class were the 2003, 2004, and F2 generation 2009 (2001

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495 and 2003) Lake Trout. Although there are no reports of EEDV vertical transmission, Kurobe et
496 al. (2009) detected EEDV DNA in the ovarian fluids of spawning Lake Trout from Lake
497 Superior, and both Salmonid Herpesvirus-3 and -5 have been detected in the ovarian fluid of
498 clinically normal Lake Trout from Lake Champlain, Vermont (Glenney et al. 2016b). Likewise,
499 it was suggested that broodstock may be a source of infectious virus after multiple EEDV
500 outbreaks in the 1980s occurred in juvenile Lake Trout reared on well water (McAllister and
501 Herman 1989; Kurobe et al. 2009), indicating a source of infection other than the water supply.
502 Additionally, EEDV outbreaks occurred in first year progeny from wild source broodstock in the
503 1980s (Kurobe et al. 2009), and while infection source tracking wasn't possible at the time, the
504 more recent detection of EEDV genomic material in apparently clinically normal fish (Kurobe et
505 al. 2009; Glenney et al. 2016b) allows for the supposition that the MSFH broodstock were
506 harboring an undetected EEDV infection despite a two year period of juvenile isolation which
507 included stress and fish health testing prior to the fish arriving at MSFH.

508 The EEDV outbreak in 2017 was equally surprising given that: a) the virus had not been
509 detected in MSFH since 2012 despite regular surveillance of broodstock and production fish with
510 the highly sensitive TaqMan and SYBR green qPCR assays; b) it led to mortality in 2-year old
511 Lake Trout as opposed to fingerlings or yearlings, which, to our knowledge, has not been
512 reported previously; and c) it was associated with severe signs of disease and high virus loads in
513 older fish. Importantly, during each mortality event, detection of EEDV was limited to a single
514 cluster of raceways that shared common attributes such as age or proximity, further supporting
515 the importance of biosecurity within the hatchery. As was the case in the 2012 outbreak, heavy
516 rains preceded the 2017 mortality episode, during which raceway water temperatures were also
517 within what is believed to be the optimal temperature range (i.e., 9.0 ± 1.0°C) for EEDV

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518 outbreaks. Thus, this study suggests that EEDV is not only capable of causing mortality and
519 disease in fingerling and yearling Lake Trout, but under certain conditions in older fish as well,
520 further demonstrating the effect this virus can have on Lake Trout rehabilitation efforts.
521 Typically, only two year classes of production Lake Trout are housed on hatchery grounds at any
522 given time, the youngest of which are held in indoor "nursery" raceways on well water until the
523 older fish in the production building are stocked into the wild. Because of this process, a
524 mortality event in production aged fish, while problematic to that year's stocking goals, can
525 potentially be compensated for in following years with alterations in the number of spawning
526 family pairs and in stocking management plans. Significant mortalities in captive broodstock,
527 however, have the potential to be catastrophic to Lake Trout rehabilitation and population
528 management, as these fish are frequently used to produce many consecutive years of production
529 fish. Additionally, Lake Trout are slow-maturing fish, taking typically 6-7 years to reach sexual
530 maturity, meaning that if a hatchery loses a younger lot of broodstock to EEDV, it could take
531 years to rebuild a new line of reproductive stock.

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

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541 event revealed comparable levels of EEDV between the two strains in the kidney, spleen and
542 skin while the SE strain Lake Trout had higher viral loads in the gills. However, in future
543 screening, the virus was detectible either at comparable levels between strains (2012), or only in
544 the LS strain Lake Trout (2017). Knowledge of strain variation in disease resistance and research
545 into specific genetic markers provides resource managers with the option to focus or tailor
546 management strategies toward producing more resistant strains of fish or protecting more
547 susceptible ones. Such a strain variation in susceptibility of Rainbow Trout (*Oncorhynchus*
548 *mykiss*) to the Infectious Pancreatic Necrosis Virus (IPNV) has been well documented (Ozaki et
549 al. 2001). Armed with the ability to experimentally induce clinical EED, future experiments can
550 focus on dissecting these potential strain variations as well as more closely examining the non-
551 Lake Trout species susceptibility to EEDV.
552 In addition, the observed temporal pattern of disease also suggests that a water borne
553 route of transmission (i.e., from LS strain fish in raceways 5a and 5b to SE strain fish in raceway
554 6), without the need for direct fish to fish contact, may be important in EEDV contagion.
555 Similarly, the initiation of disease signs and mortality in SE strain Lake Trout maintained next
556 to, not up- or down-stream from, the LS strain Lake Trout that first showed disease signs,
557 highlights the importance of biosecurity and the potential for virus spread without rearing unit
558 interconnectivity. Lastly, Brook Trout, which were reared in parallel raceways and next to the
559 affected SE strain Lake Trout, and splake, which were receiving 2nd pass water from the EEDV-
560 infected SE and LS strain Lake Trout rearing units, never developed EED. These findings are in
561 line with the reports of Bradley et al. (Bradley et al. 1988) and McAllister & Herman (McAllister
562 and Herman 1989) as no other salmonids on hatchery grounds during the 1980s EEDV outbreaks
563 experienced mortalities, and experimental challenge of Brook Trout did not result in clinical

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564 disease or mortalities (Bradley et al. 1989; McAllister and Herman 1989). Interestingly, although
565 there is no peer-reviewed data, it has been reported that Lake Trout hybrids can be
566 experimentally infected with EEDV (McAllister 1991).
567 Gross and histopathologic findings in these cases were consistent with those seen in the
568 initial description of EEDV in the 1980s. As the name of this virus implies, the most significant
569 microscopic lesions noted in this study were in the skin and gill epithelia, which may contribute
570 to death of the host through osmoregulatory impairment and/or respiratory dysfunction.
571 Additionally, the outer layers of the skin and gills serve as an important line of defense against
572 fish pathogens, and as a result, any insult to this layer can predispose the affected host to a suite
573 of opportunistic microbial pathogens, as was observed in this study in the form of *F.*
574 *psychrophilum, Aeromonas* spp., and water mold infections in the more progressed EEDV-
575 associated skin lesions.
576 Recent advancements in phylogenetics have allowed for improvements in our knowledge
577 of the relationships among fish herpesviruses (Waltzek et al. 2009). Gene sequencing and
578 phylogenetic analyses performed in this study confirmed the identity of the etiological agent as
579 EEDV and also showed that seven of the eight isolates from the 2012 and 2017 outbreaks were
580 identical to the Salmonid Herpesvirus-3 reference isolate (Waltzek et al. 2009) over the
581 sequenced portion of the terminase gene. One isolate displayed a single nucleotide
582 polymorphism (SNP) when compared to the other seven MSFH isolates and the reference isolate
583 (Figure 5). Of note, this SNP led to an amino acid shift from a glutamine to a leucine (data not
584 shown), but its effects on the functionality of the terminase gene product, which involve
585 packaging viral DNA into the virus capsid (Sigamani et al. 2013), are currently unknown.
586 Nevertheless, this study confirms the continued presence of highly similar EEDV strains in

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587 multiple Great Lakes states, a matter of concern in the context of Lake Trout rehabilitation and
588 conservation efforts in the Laurentian Great Lakes.

589 These recent outbreaks of EEDV within the Great Lakes basin have highlighted the
590 magnitude of work remaining to be completed in order to fully understand this disease. As
591 molecular diagnostic assays continue to improve, other testing strategies must improve to match.
592 This includes identifying and screening all at-risk populations, particularly gametes and live fish
593 slated to enter a hatchery system, as well as focusing diagnostic efforts on sample collections
594 most likely to highlight an EEDV infection by identifying viral target tissues. Increasing our
595 working knowledge of Lake Trout immunology will allow for the identification of previously
596 exposed fish and potential susceptibility differentiation between strains of Lake Trout, leading to
597 potential alterations in management strategies to produce larger numbers of more resistant fish,
598 assuming this process does not select against other important survival characteristics.

599 Efforts must be made to culture this virus, as the current lack of an EEDV-susceptible
600 cell line hampers both diagnostic options as well as research opportunities. Without the ability to
601 culture and produce an infectious stock of EEDV, frozen tissues and epizootic-surviving fish,
602 both potentially containing active EED virus, are of vital importance. Possession of these unique
603 materials will allow this much-needed research to be conducted. Moreover, further research into
604 the pathogenesis and biological properties of this deadly virus in hatcheries will provide fishery
605 management agencies with the tools and information necessary to not only prevent future
606 outbreaks of EEDV, but also continue the successful rehabilitation of Lake Trout populations
607 across North America. As a result of the noted issues with detection in the past, we also suspect
608 EEDV has a much wider distribution throughout the Great Lakes than currently known, and is

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609 likely present in many other hatchery systems, further highlighting the importance of continued
610 work on this virus.
611
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617
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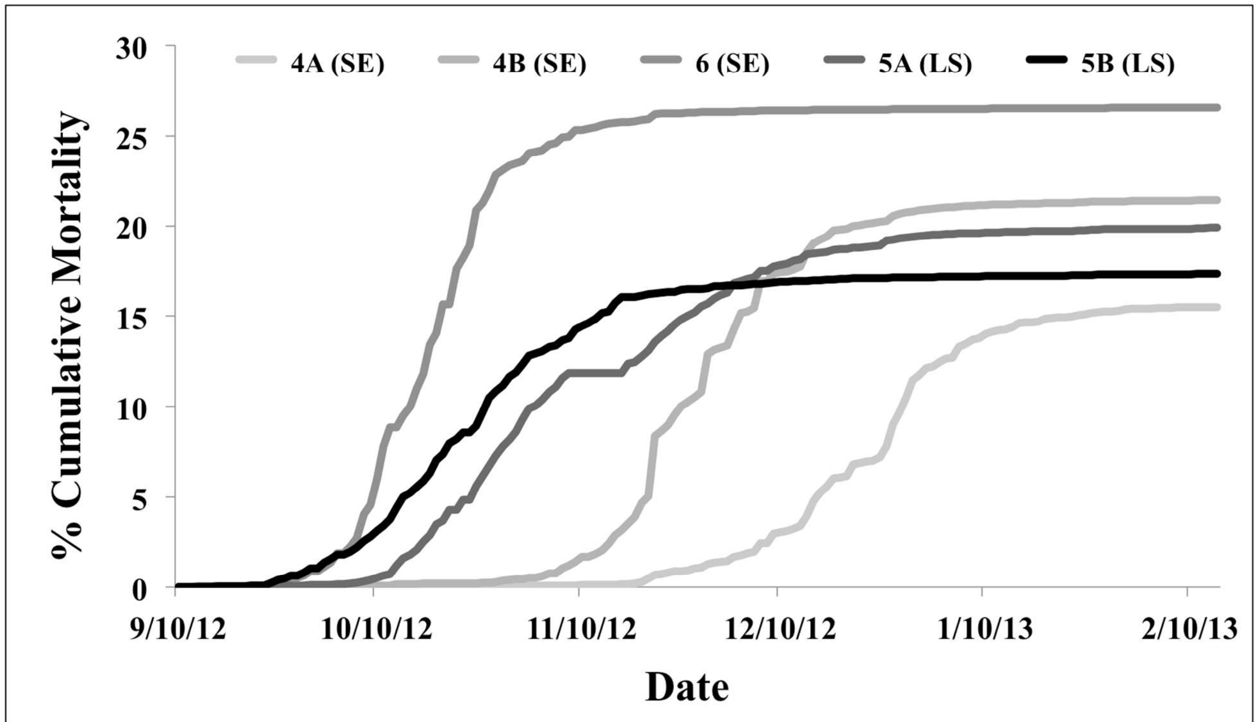

Figure 1. Faisal.

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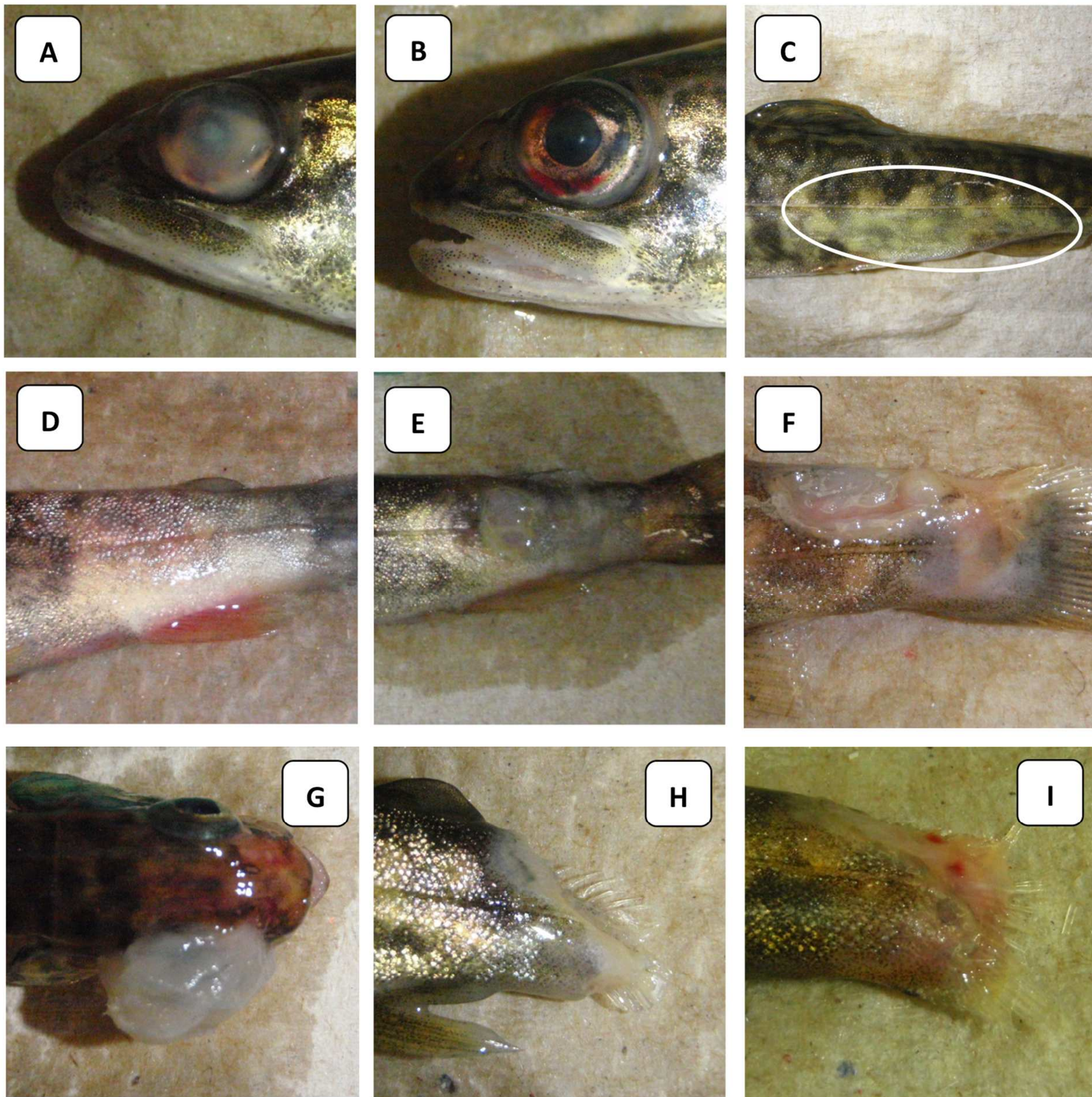

Figure 2. Faisal.

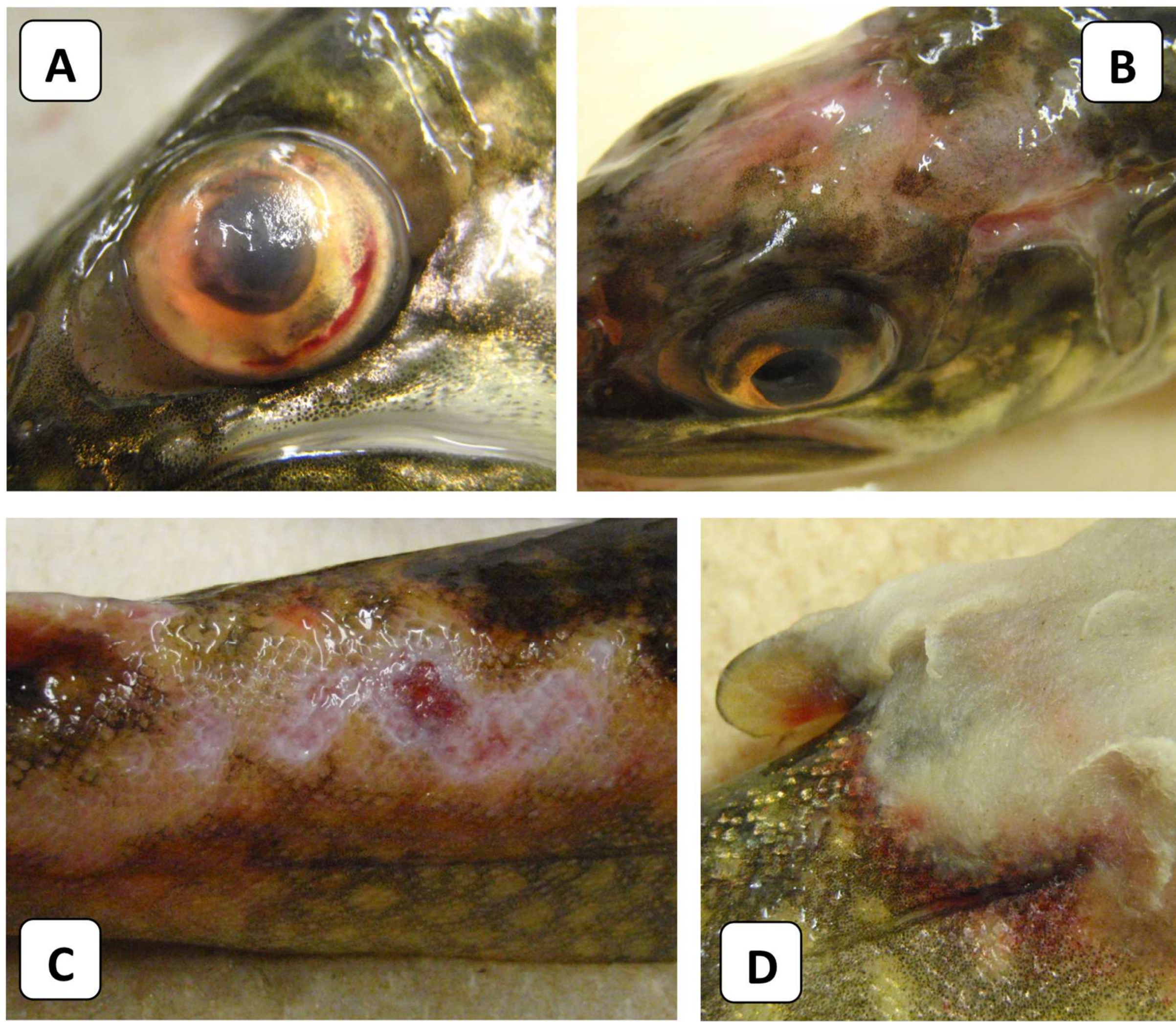

Figure 3. Faisal.

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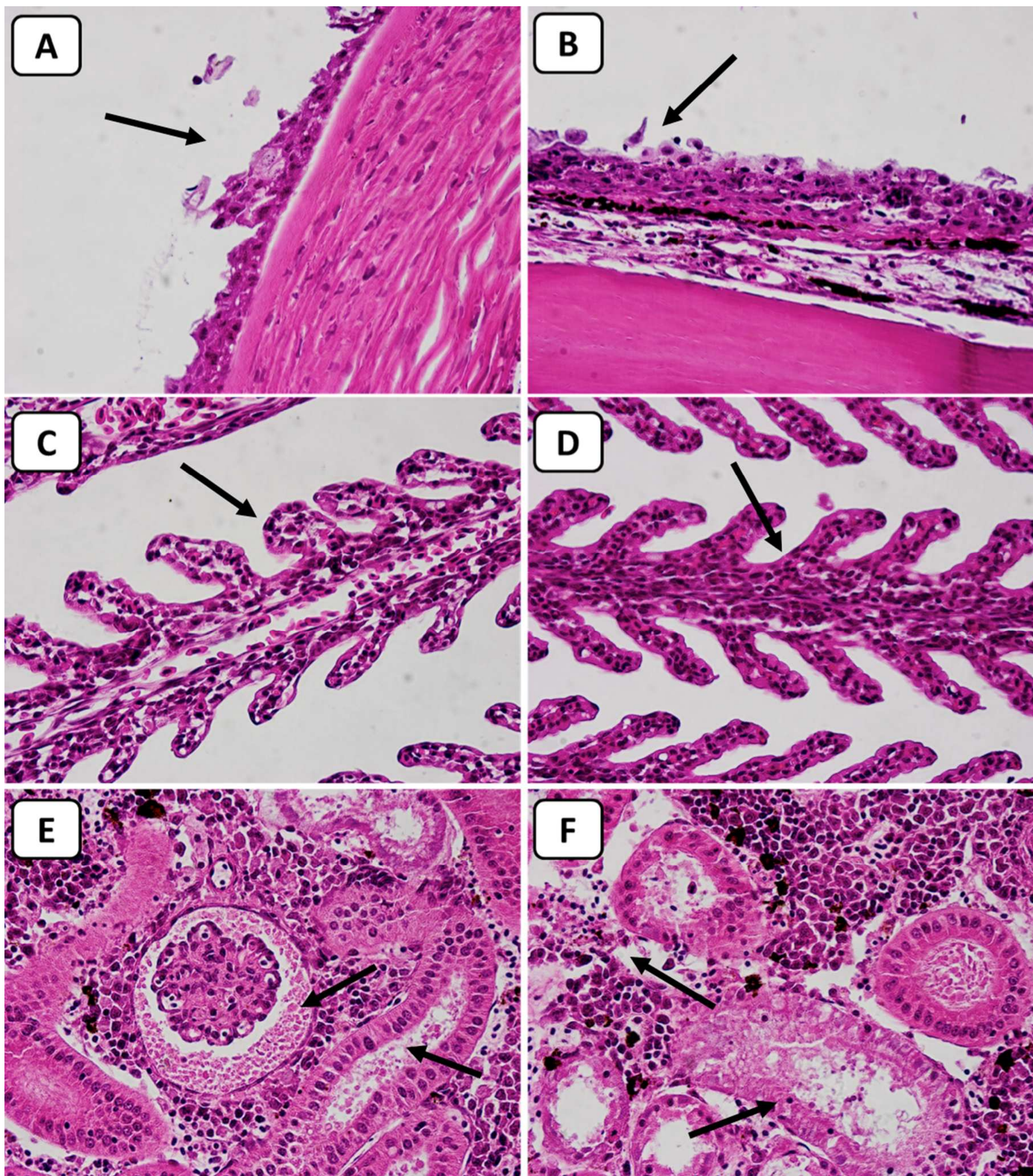

Figure 4. Faisal.

Figure 5. Faisal.

Review Only

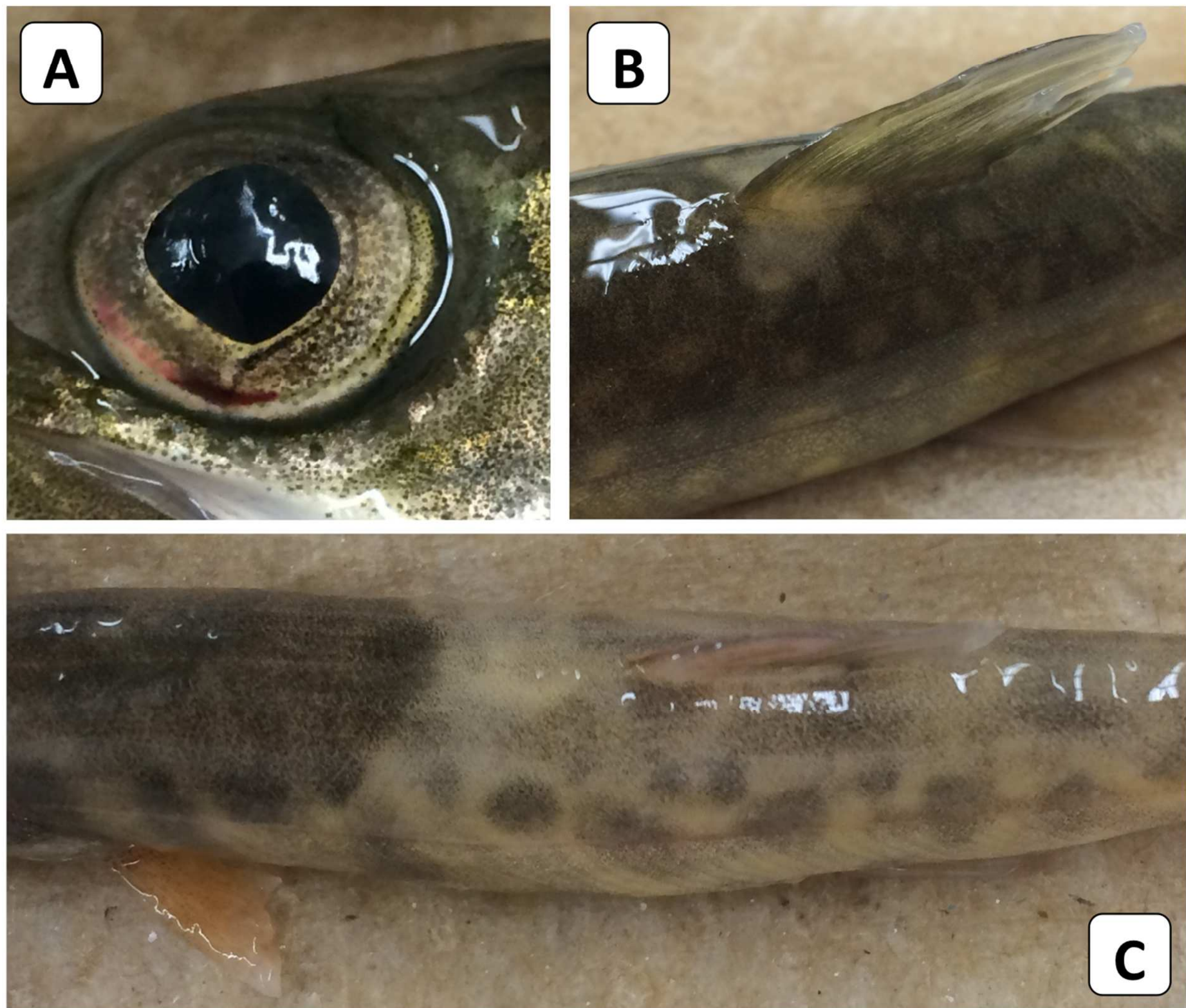

Figure 6. Faisal.

Only

Figure Captions:

Figure 1. Cumulative percent mortalities during the 2012 mortality event at Marquette State Fish Hatchery (MSFH), Michigan, among juvenile production Lake Trout (*Salvelinus namaycush*) by rearing unit raceway. Prior to mortality event, production fish numbers were approximately as follows: Raceway 4A - 125,105 fish; Raceway 4B - 124,782 fish; Raceway 5A - 75,031 fish; Raceway 5B - 76,085 fish; and Raceway 6 - 56,167 fish.

Figure 2. Gross clinical signs exhibited by Lake Trout (*Salvelinus namaycush*) naturally infected with Epizootic Epitheliotropic Disease Virus (EEDV) at Marquette State Fish Hatchery (MSFH), Michigan, in 2012; **(A, D, E, G)** Lake Superior strain Lake Trout and **(B, C, F, H)** Seneca Lake strain Lake Trout. (A) advanced stage ocular degeneration with hemorrhage and corneal opacity; (B) ocular hemorrhage; (C) diffuse skin "blotchiness," dermal erosion and excess mucous production; (D) dermal erosion, "blotchiness" and erythema, anal fin congestion; (E) dermal erosion, ulceration of trunk and caudal peduncle; (F) caudal peduncle ulceration, necrosis and dermal erosion; (G) ocular degeneration with substantial water mold overgrowth; (H) caudal fin ulceration with exposed vertebrae; (I) caudal fin ulceration, necrosis, petechial hemorrhage and yellow discoloration, exposed fin rays.

Figure 3. Gross clinical signs exhibited by Lake Superior strain Lake Trout (*Salvelinus namaycush*) naturally infected with Epizootic Epitheliotropic Disease Virus (EEDV) at Marquette State Fish Hatchery (MSFH), Michigan, in 2017. **(A)** ocular hemorrhage and ulceration; **(B)** cranial epithelial erosion, ulceration and hemorrhage; **(C)** skin erosion, ulceration and hemorrhage of trunk and dorsum; **(D)** skin ulceration with secondary overgrowth and hemorrhagic margins.

Figure 4. Hematoxylin and eosin (H&E) stained tissue sections from hatchery-reared Lake Trout (*Salvelinus namaycush*) that were naturally infected with Epizootic Epitheliotropic Disease Virus (EEDV). **(A)** corneal epithelial ulceration and necrosis (400x magnification); **(B)** epithelial ulceration and necrosis of the skin (400x magnification); **(C)** gill lamellar edema (400x magnification); **(D)** gill epithelial swelling/hypertrophy (400x magnification); **(E)** proteinaceous exudate within both Bowman's space and the renal tubular epithelium (400x magnification); and **(F)** renal tubular epithelial necrosis and multifocal necrosis of the renal interstitium (400x magnification).

Figure 5. Dendrogram depicting the relationships of eight Epizootic Epitheliotropic Disease Virus (EEDV) isolates (denoted in bold) from the 2012 and 2017 outbreaks at Marquette State Fish Hatchery (MSFH), Michigan, with isolates representing the five currently described salmonid herpesviruses. The dendrogram was generated in MRBAYES 3.1.2 (*120*) using the Kimura Two Parameter model with gamma distribution based upon the lowest Bayesian Information Criterion. The Markov chains ($n = 4$) were run until an average standard deviation of split frequencies of < 0.01 was attained. Two independent analyses were conducted, with the initial 25% of Markov chain Monte Carlo samples being discarded as burnin. Posterior probabilities ≥ 70 are displayed at the nodes, where an * denotes that the same node was supported in Neighbor-Joining analysis (i.e., boot strap value ≥ 70). The final data set contained 303 bp of the terminase gene.

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Figure 6. Gross pathology associated with Epizootic Epitheliotropic Disease Virus (EEDV) experimentally challenged Lake Trout (*Salvelinus namaycush*). **(A)** ocular hemorrhage; **(B)** multifocal pallor, skin and fin erosion; **(C)** generalized pallor with skin and fin erosion.

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

Table 1. Number of positive fish detected by EEDV screening at Marquette State Fish Hatchery, Michigan, 2007-2017 (number positive/number tested). Sampling during mortality episodes denoted by a (*). Tissues tested included kidney (K), spleen (Sp), heart (H), mucous (M), gill (G), skin (Sk), fin (F), and eye (E) and were either pooled (e.g., KSpH) or individual (e.g., K, Sp, H). All samples tested using EEDV TaqMan qPCR as described, except where indicated: (❖) tested with EEDV SYBR green qPCR, (§) tested with both qPCR assays.

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

Table 2. EEDV screening in Cherry Creek, Michigan, 2011-2017. No sampling was associated with a mortality episode. All testing was performed in pools of five fish per pool. All samples tested using EEDV TaqMan qPCR as described, except where indicated: (❖) tested with EEDV SYBR green qPCR.

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

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1. Abstract

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

challenged splake (lake trout x brook trout hybrid) that died 10 days post-infection at titers substantially higher than the initial inoculum ($3.8x10^7$ viral copies/mg skin), indicating EEDV replication had occurred. EEDV DNA was also detected in one mottled sculpin (*Cottus bairdii*), although the role this species plays in supporting EEDV replication and serving as EEDV reservoir appears unlikely. Collectively, study results show that lake trout strains vary in their susceptibility to EEDV, that in some cases, splake may serve as a short term EEDV reservoir, and overall affirm the complexities of EEDV disease ecology.

2. Introduction

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

Herpesviruses are thought to have emerged approximately 400 million years ago, and have since been associated with infections across an array of animal phyla. The fish herpesviruses make up a monophyletic family, the Alloherpesviridae (Waltzek et al., 2009). Several alloherpesviruses have been associated with severe economic losses in a variety of cultured fish species (lake trout (Bradley et al., 1988), catfish (Alborali et al., 1996) and common carp (*Cyprinus carpio*, Garver et al., 2010)). As is the case with terrestrial herpesviruses, those affecting fish are known to have a very narrow host range, typically causing disease in only one fish species (Hanson et al., 2011). Following this trend, to date, EEDV epizootics have only been reported in lake trout (Bradley et al., 1989), but the ability of other fish species to harbor EEDV in the absence of clinical disease signs is not well understood.

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

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

others have suggested that specificity problems may be at the core of these positive results. As a result, Glenney et al. (2016b) developed a new sensitive and specific quantitative PCR (qPCR) assay, which has since been employed to investigate EEDV infection status in Great Lakes fishes. In 2017, Shavalier (2017) found that mottled sculpin (*Cottus bairdii*) harbored EEDV DNA.

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

3. Materials and methods

3.1. Fish maintenance

Eleven fish species currently residing within the Great Lakes basin, representing four families (i.e., Salmonidae, Cottidae, Centrachidae, and Esocidae) were selected for species susceptibility experiments (Table 1). This included two strains of lake trout (Lake Superior and Seneca Lake strains) that are a primary focus of hatchery-based lake trout rehabilitation efforts in the Great Lakes basin, as well as splake, a lake trout x brook trout hybrid. Fish were either obtained from the Michigan Department of Natural Resources State Fish Hatcheries, the Little Traverse Bay Bands of Odawa Indians Hatchery, or were collected from the wild (Table 1). All experimental animals were maintained at the Michigan State University Research Containment Facility (MSU-URCF). At the time of experimental challenge, fish ranged from 4 to 14 months old and from 11.5 to 16.5 cm length in Seneca (SE) strain lake trout, 9.5 – 16.9 cm in Lake Superior (LS) strain lake trout, 6.4 – 12.5 cm in splake, 10.5 – 14.8 cm in brook trout, 6.5 – 10.2 cm in rainbow trout, 10.2 – 13.0 cm in brown trout, 10.4 – 13.0 in Atlantic salmon, 8.0 – 13.0 in coho salmon, 5.4 – 7.1 cm in lake herring, 5.7 – 9.1 cm in mottled sculpin, 7.0 – 12.3 cm in muskellunge, and 5.4 – 9.4 in largemouth bass (Table 1). Fish were housed in 680 L flow-through fiber glass tanks supplied with oxygenated, dechlorinated water (9 to 14 °C), and fed commercial pellets twice per day. All fish handling and maintenance was performed in accord with the Michigan State University Institutional Animal Care and Use Committee (IACUC) committee approval.

3.2. Infectious inoculum preparation

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

3.3.Infection challenges

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

After injection, fish were returned to their respective tanks, monitored for recovery, and then maintained for 30 – 100 days (challenge period modified based upon the observation of disease signs; Table 2). Daily fish care was done to include feeding and monitoring water flow, air flow, water temperature, fish behavior and clinical signs. Any severely moribund fish were euthanized using a lethal dose of MS-222 (0.25 mg/mL, buffered with sodium bicarbonate at dose of 0.5 mg/mL). Complete external and internal examinations were performed on all dead or moribund fish. During the necropsy, clinical signs were noted and skin samples collected from the caudal peduncle area and stored at -20 °C for molecular analysis. At the end of the study period, surviving fish were euthanized and analyzed as described above.

3.4.DNA extraction

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

3.5.Molecular detection of EEDV

All qPCR reactions were carried out in a Mastercycler ep realplex^2 real-time PCR machine (Eppendorf, Hauppauge, New York) and were performed as described by Glenney et al.

(2016a) using the primers 5' – TGG GAG TCC GTC GTC GAA – 3' (SalHV3_23F) and 5' – TCC ACA CAG GAG CTC ACG AA – 3' (SalHV3_23F). The 20 µl reaction contained 10 µl of SYBR® Select Master Mix, 2 µl of nuclease-free water (Promega), 2 µl of forward primer, 2 µl of reverse primer, and 4 µl of template containing 50 nmol total DNA. The qPCR cycling parameters consisted of step at 50 °C for 2 minutes; 95 °C followed for 10 minutes; and 40 cycles of 95 °C for 15 seconds, 60 °C for 60 seconds and were carried out in a Mastercycler Pro Thermal Cycler (Eppendorf, Hamburg, Germany). Samples were considered positive if the cycle threshold (Ct) value was < 35 (Shavalier, 2017). Utilized controls included previously confirmed EEDV-positive tissue homogenate (positive extraction control, PEC), sample diluent (negative extraction control, NEC), EEDV-positive DNA extract positive reaction control, PRC), and nuclease-free water (negative reaction control, NRC). The quantification of EEDV copies present in a sample was determined based upon comparison to the standard curve generated by 8 serial 10-fold dilutions of EEDV PRC (Shavalier, 2017) that were run with each 96 plate.

3.6. Data analysis

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

4. Results

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

Throughout the course of this study, no Seneca strain lake trout, brook trout, brown trout, rainbow trout, Atlantic salmon, coho salmon, lake herring, or largemouth bass died in any of the challenge groups, nor was EEDV detected in any of these species (Table 2). Three muskellunge died in the LD group and one muskellunge died in the HD group; in all cases, however, these were due to aggression (LD group) or cannibalism (HD group) and EEDV was never detected in any muskellunge (Table 2). The number of mortalities and/or gross disease signs for the remaining fish species/strains are presented in Table 2 and discussed in sections 4.1.1-4.1.4.

2.1.1. Lake Superior strain lake trout

In the HD LS strain lake trout group, disease signs consistent with EED were observed in all fish and included mild to severe ocular hemorrhage, mild to severe corneal opacity, mild to severe exophthalmia, mild to severe skin erosion of the caudal peduncle, congestion at the base of the fins, mild petechial hemorrhage on the caudal peduncle, and gill pallor with concurrent mucous accumulation. Mortality in the HD group began 21 days post-infection (pi) and continued to day 66 (Table 3). The two surviving fish were euthanized on day 66. All 10 fish in the HD group were EEDV-positive according to qPCR (Table 2), whereby EEDV loads ranged from $1.3 \times 10^4 – 2.0 \times 10^9$ virus copies/mg of skin tissue taken from the caudal peduncle area in each individual fish (Table 3).

No disease signs consistent with EED were observed in the LD group and all fish survived until the end of the study period (Table 2). Likewise, EEDV was not detected in any LD fish (Table 3). Four fish died in the negative control group and were attributed to aggression; however, a low EEDV load (i.e., 8.9×10^3 virus copies/mg of skin tissue) was detected in one negative control fish (Table 2).

2.1.2. SE strain lake trout

No mortality occurred in the HD SE lake trout group; however, exophthalmia, ocular hemorrhage, congestion at the base of the fins, mild hemorrhage within the caudal peduncle, and multifocal skin pallor were observed, albeit less severe when compared to the LAT-LS HD group. EEDV was detected in three of ten fish in this group via qPCR at the end of the experiment (Table 2), with loads ranging from $1.6x10^7$ – $7.2x10^7$ virus copies/mg of skin tissue (Table 3). No mortality occurred in the LD or NC groups, nor was EEDV detected via qPCR in either group (Table 2).

2.1.3. Splake

Three splake died in the HD group on days 5, 7 and 10 p.i. (Table 2), whereby severe lethargy, erythema along the lateral line, gill pallor, and congestion/hemorrhage at the base of the fins were observed. However, EEDV was only detected in one of these three fish, but at a load of $3.8x10^7$ viral copies/mg skin (Table 3). In the 7 HD fish surviving until the end of the experiment, no EEDV was detected. No disease signs consistent with EED were observed in the LD or NC groups, nor was EEDV detected in either group. However, three fish died in the LD group, and one fish died in the NC group (Table 2).

2.1.4. Mottled Sculpin

Six of ten mottled sculpin in the HD group died (Table 2) during days 4 – 40 pi. Observed disease signs varied and included combinations of moderate exophthalmia, congestion at the base of the fins, and/or hemorrhage on the isthmus. Despite this, EEDV was not detected in the six fish succumbing to death, nor in any of the four survivors. Five fish died in the LD group (Table 2); EEDV was detected in one of the five mortalities and at a low load (i.e., $1.1x10^2$ viral copies/mg skin; Table 3). Four fish died in the NC group, but EEDV was not detected in these fish, nor in the survivors (Table 2).

3. Discussion

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

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

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

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

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

When EEDV re-emerged in a state fish hatchery in Michigan in 2012, Shavalier (2017) observed differences in cumulative percent mortality within the rearing units housing the SE and LS lake trout strains. However, differences in rearing unit densities, first vs. second pass water, and other epidemiological variables made any conclusive determination of differential strain susceptibility to EEDV all but impossible at that time. In this study, lake trout from both strains were challenged with identical EEDV doses and maintained under the same rearing conditions so that EEDV susceptibility within two lake trout strains could be assessed, a matter of importance for Great Lakes lake trout conservation and stock enhancement efforts. Surprisingly, no mortality occurred in the SE lake trout strain (HD group) compared to 80% mortality in the comparable LS lake trout strain. Likewise, EEDV loads were orders of magnitude higher in most LS strain lake trout compared to the SE strain (Table 3), but interestingly were higher in the SE strain lake trout that survived until the end of the study compared to the two surviving LS lake trout (Table 3). This is even more interesting considering that the SE lake trout were maintained for 100 days compared to 66 for the LS lake trout, perhaps indicating some SE lake

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

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

Table 1: Origin and median length/weight of the fish species that were assessed for Epizootic epitheliotropic disease virus (EEDV) susceptibility in this study. (*) (LS) Lake Superior strain lake trout; (SE) Seneca strain lake trout; (MSFH) Marquette State Fish Hatchery; (OSFH) Oden State Fish Hatchery; (PRSFH) Platte River State Fish Hatchery; (WLSFH) Wolf Lake State Fish Hatchery; (LTBBFH) Little Traverse Bay Bands of Odawa Indians Fish Hatchery.

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

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

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

Table 3: Days to death post- epizootic epitheliotropic disease virus (EEDV) infection, whether EED disease signs were present, and the estimated EEDV loads that were detected in the skin of fish that were EEDV positive throughout the course of this study. (*) Euthanized; (+) present; (-) absent; (PI) post-infection; (LS) Lake Superior strain lake trout; (SE) Seneca strain lake trout. Lake trout (*Salvelinus namaycush*), Mottled sculpin (*Cottus bairdii*), Splake (*Salvelinus namaycush* x *Salvelinus fontinalis*).

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

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