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Research Article

Correlating sea lamprey density with environmental DNA detections in the lab

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This study was contributed in relation to the 20th International Conference on Aquatic Invasive Species held in Fort Lauderdale, Florida, USA, October 22–26, 2017 (http://www.icais.org/html/previous20.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Invasive sea lamprey (*Petromyzon marinus* Linnaeus, 1758) are currently managed by the Great Lakes Fishery Commission in an effort to reduce pest populations below levels that cause ecological damage. One technique to improve stream population assessments could be molecular surveillance in the form of environmental DNA (eDNA) monitoring. We developed and validated four probe-based quantitative polymerase chain reaction (qPCR) assays, then used two probes (*cytb, nd1*) to determine whether eDNA concentration was correlated with adult and larval sea lamprey density in the lab. We found a strong positive correlation between adult sea lamprey densities of 2, 20, and 200 individuals/2000L and eDNA concentrations in tanks using both assays (*cytb, nd1*). For larval laboratory tank density trials, eDNA concentrations were generally near our limit of quantification and there was no significant difference in copy numbers detected between larval sea lamprey densities of 1, 5, and 25 individuals/28L. Therefore, we examined detection probability rather than concentration with laboratory tank densities. We observed a trend of increasing detection probabilities with increased larval sea lamprey density that approached significance suggesting that when DNA copy numbers are low, detection rates may be more informative in predicting varying densities of larval sea lamprey. The ability to assess sea lamprey densities from a water sample could be a powerful tool to improve traditional assessment and stream ranking techniques. Further refinement of this method in the field may make eDNA surveillance of sea lamprey a reliable part of stream assessments. Rapid eDNA analysis from many streams may help focus traditional assessment efforts, thereby improving the efficiency of invasive sea lamprey control efforts.

Key words: Great Lakes, eDNA, assessment, invasive species

Introduction

Sea lamprey (SL) *Petromyzon marinus* invaded the Great Lakes upstream of Niagara Falls presumably following the improvements made to the Welland Canal in 1920. First reported in Lake Erie in 1921, SL subsequently spread to the upper Great Lakes and established spawning populations in all of the upper Great Lakes by 1947 (Applegate 1950). Following their introduction, SL devastated the commercial and recreational fisheries of the Great Lakes (Lawrie 1970). Sea lamprey populations are currently managed by

various control techniques employed by the Great Lakes Fishery Commission (GLFC) including chemical control, trapping of adults, as well as physical barriers to prevent upstream migration (Siefkes et al. 2013).

Sea lamprey exhibit adfluvial, semelparous behavior in which they leave the open water of the Great Lakes for tributaries, swim upstream, spawn, and die shortly after spawning. A single female SL lays between 30,000 and 100,000 eggs into a nest while the male simultaneously fertilizes the eggs. The fertilized eggs hatch into ammocoete larvae about 18–21 days post fertilization. The ammocoetes spend approximately 4–6 years burrowed in the sediment of the stream where they filter feed on detritus and algae (Applegate 1950). This sedentary filter-feeding stage makes ammocoetes a prime target for chemical control, which has effectively reduced SL populations (Smith and Tibbles 1980). However, due to limitations in budget, personnel, and overcoming geographic logistics it is not feasible to treat every SL-infested stream of the Great Lakes. Therefore, the GLFC must prioritize streams according to estimated SL abundance, known treatment costs, available personnel, and budget (Siefkes et al. 2013).

Individual stream treatment priorities are currently determined using a variety of assessment techniques which include semi-quantitative surveys of suitable ammocoete habitat as well as SL ammocoete abundance through electrofishing efforts (Christie et al. 2003; Slade et al. 2003). These surveys are combined with expert judgment of streams gained by managers through years of SL control. This information is then used by managers to prioritize streams for treatment. The aim of the assessments is not to obtain actual population densities, but rather to prioritize streams for treatment to achieve the maximum return on investment of SL killed per dollar spent. Occasionally, assessing streams and ranking them for treatment can be difficult because stream conditions including high water, few access points, and other local factors make it dangerous for the assessment crew to gain access. Also, traditional assessment techniques can have low precision based on the population size and distribution of the organism of interest (MacKenzie et al. 2005; Darling and Mahon 2011). Providing a quantitative assessment using environmental DNA (eDNA) could assist management with stream prioritization by supplementing traditional assessment techniques.

Molecular surveillance techniques are increasing in use to detect aquatic invasive species with resource agencies beginning to use eDNA as a surveillance technique (Darling and Mahon 2011; Jerde et al. 2013). One example of a molecular surveillance technique is the analysis of water samples for eDNA using polymerase chain reaction (PCR) (Rees et al. 2014). Some species of carp are easily detected using eDNA due to their life history, distribution, and DNA shedding characteristics (Jerde et al. 2011, 2013). The first use of eDNA to survey for invasive species was in detecting the American bullfrog (*Lithobates catesbeianus* Shaw, 1802) in various ponds in France (Ficetola et al. 2008). Detection assays for eDNA are now developed for numerous terrestrial and aquatic species of native or exotic origin (Ficetola et al. 2008; Goldberg et al. 2011; Takahara et al. 2012; Thomsen et al. 2012b; Dejean et al. 2012; Foote et al. 2012; Thomsen et al. 2012a; Takahara et al. 2013; Pilliod et al. 2013; Goldberg et al. 2013; Egan et al. 2013; Piaggio et al. 2014; Carim et al. 2017).

A previous study has shown that SL eDNA can be detected in streams and distinguished from DNA of native lamprey (Gingera et al. 2016). Although the conventional PCR (cPCR) assay was effective at routinely detecting spawning SL in a stream, eDNA of larvae was detectable in only two of the four streams with medium to high larval density, and not in the two streams with low larval density (Gingera et al. 2016). Detection frequency of eDNA, even at higher densities, decreased with higher stream flow rates. Therefore, cPCR analysis of eDNA samples is not sufficiently sensitive in high flow systems or when larval densities are relatively low which could lead to a false negative error. In addition to the challenge of low sensitivity, cPCR analysis requires the additional step of sequence confirmation to rule out non-specific amplification, which increases cost and time for analysis and adds contamination risk to the eDNA laboratory. While it is still likely advisable to sequence a few quantitative PCR (qPCR) amplicons periodically to confirm positive results, this is still far less than sequencing all positives as is required by cPCR.

Development of eDNA methods to quantify SL abundance at multiple life stages may enable rapid and cost-effective stream assessments of SL abundance (Darling and Mahon 2011; Jerde et al. 2013). These rapid assessments could assist traditional assessment techniques in looking for infestation of new streams, checking barrier effectiveness, quantifying adult spawning runs in streams presently not trapped, and helping to prioritize lampricide applications among Great Lakes tributaries.

Probe-based qPCR can provide additional sequence-specificity over cPCR, because both the probe and the primer are required to be complementary, whereas in cPCR only the primer is required to complement the template DNA. Additionally, qPCR assays are more sensitive for eDNA detection than cPCR (Wilcox

Table 1. Oligonucleotide sequences that were used for sea lamprey specific primers and probes. The *coi* primers are those used by Gingera et al. (2016), all other primers and probes were developed for the present study. The *cytb* and *coi* probes were tagged with the FAM fluorophore. The *nd1* and *nd4* probes were tagged with the HEX fluorophore. All used the ZEN-3 Iowa Black FQ double quenched probes. All oligos were manufactured by Integrated DNA Technologies; Coralville, IA (IDT) and purified by standard desalting.

Oligonucleotide	Sequence	Size (bp)
P.mar-*cytb*-F	GGTTTTGTTATTCTACTGGGCAT	154
P.mar-*cytb*-probe	FAM/TTCCCTTTT/ZEN/AGCCCCTAATGCACT/3IABkFQ	
P.mar-*cytb*-R	GTAGAATGGCATAGGCAAATAGA	
P.mar-*nd1*-F	CTTACTCTCAAGTTGGCCTT	119
P.mar-*nd1*-probe	HEX/TCGAACTCC/ZEN/ATTTGATTTAACTGAAGGA/3IABkFQ	
P.mar-*nd1*-R	CTACGTTAAAGCCAGAAACTAG	
P.mar-*coi*-F	GGCAACTGACTTGTACCMCTAATACTTGGT (Gingera et al. 2016)	225
P.mar-*coi*-probe	FAM/TCCCTTAGC/ZEN/CGGAAACCTAGCCCACA/3IABkFQ	
P.mar-*coi*-R	GGCTAAGTGTAAGGAAAAGATTGTTAGGTCGAC (Gingera et al. 2016)	
P.mar-*nd4*-F	AACACACCTTGATCTGAAACCT	122
P.mar-*nd4*-probe	HEX/AATCGCCTG/ZEN/TTTCCTGGCCTTTT/3IABkFQ	
P.mar-*nd4*-R	AGCCTGCGATGGGAGCCT	
P.mar-gBlock	GGTTTTGTTATTCTACTGGGCATTCTTTTCATAATTTCCCTTTTAGCCCCTAATGCACTAGG TGAACCAGACAACTTTATTTATGCTAATCCTCTTAGTACCCCTCCCCATATTAAACCAGAA TGATACTTTCTATTTGCCTATGCCATTCTACATGCATCTTACTCTCAAGTTGGCCTTTAGCA GCAATATGATTTGTTTCTACTTTAGCAGAAACAAATCGAACTCCATTTGATTTAACTGAAG GAGAGTCAGAACTAGTTTCTGGCTTTAACGTAGATGCATGGCAACTGACTTGTACCCCTA ATACTTGGTGCTCCTGATATGGCCTTCCCTCGTATAAACAACATAAGTTTTTGACTACTTC CGCCCTCTTTACTTTTACTCTTAGCCTCTGCAGGAGTTGAAGCTGGGGCAGGAACAGGAT GAACTGTATATCCTCCCTTAGCCGGAAACCTAGCCCACACCGGGGCCTCTGTCGACCTAA CAATCTTTTCCTTACACTTAGCCATGCATAACACACCTTGATCTGAAACCTTATGATGAAT CGCCTGTTTCCTGGCCTTTTTAATCAAAATACCCCTATATATCTTTCACTTATGATTACCAA AAGCTCACGTAGAGGCTCCCATCGCAGGCT	

et al. 2013; Piggott 2016). Furthermore, some studies suggest that qPCR can correlate species density and distribution with eDNA quantity (Takahara et al. 2012, 2013; Lacoursière-Roussel et al. 2016). Therefore, we developed a SL-specific qPCR probe that could be coupled with the previously described SL cPCR primers designed by Gingera et al. (2016). We also developed additional SL-specific qPCR assays for comparison or to be used in concert with the cytochrome *c* oxidase subunit 1 (*coi*) marker. We then used a duplex reaction to evaluate how SL eDNA levels (detection probability and eDNA copy number) correlate with larval and adult SL abundance under controlled laboratory settings.

Methods

Marker development

A Taqman hydrolysis probe was designed to target the 225-bp region of the SL (*coi*) gene amplified using the SL-specific primers developed by Gingera et al. (2016) (Table 1). We designed additional SL-specific primers and Taqman probes targeting three other mitochondrial genes: cytochrome *b* (*cytb*), NADH dehydrogenase subunit 1 (*nd1*), and NADH dehydrogenase subunit 4 (*nd4*). The new markers were developed by aligning sequences of mitochondrial DNA of SL and closely related lamprey species using BioEdit Sequence Alignment Editor and by selecting areas for primers and probes that were identical across all targets for SL and maximized base-pair mismatches with non-target species (Table 2). Accession numbers for the sequences used are located in Table 2. American brook lamprey, chestnut lamprey, silver lamprey, and Northern brook lamprey are all found in waters where invasive SL are managed. All oligonucleotide components designed each have at least two mismatches to all non-target species sequences compared, and most have at least four. Mismatches to non-target species were abundant and occurred throughout the entire length of the oligonucleotides including 1 or more near the 5' end of each primer.

Candidate primer sequences were tested *in-silico* using Primer-BLAST software (Ye et al. 2012). All three primer pairs aligned with SL DNA as expected in the Primer-BLAST output. One of the three markers tested (*cytb*) also aligned with the far Eastern brook lamprey (*L. reissneri* Dybowski, 1869). However, because far Eastern brook lamprey are not expected to inhabit the waters used in our experiments, we proceeded with the *cytb* marker along with the rest. See Table 1 for primer and probe sequences developed in this study. We tested a variety of fish species (Table 3) *in-vitro* with all four qPCR assays as

Table 2. Accession numbers for DNA sequences used for comparison when designing oligos.

Species	Sequence Locus	Number of Sequences Compared	Accession Numbers
sea lamprey (*Petromyzon marinus*)	Mitochondrion	1	U11880
	cytb	3	EU404058,EU404057, GQ206148
	coi	5	KF930255, KC015795, KC015794, EU524271, KJ128573
Arctic lamprey (*Lethenteron camtschaticum*)	Mitochondrion	3	KJ866209, KJ866208, KF701113
American brook lamprey (*Lethenteron appendix*)	Mitochondrion	2	NC_025583, KM267719
	cytb	1	GQ206179
Northern brook lamprey (*Ichthyomyzon fossor*)	Mitochondrion	2	KM267716, NC_025552
	nd4	3	DQ889824, DQ889823, DQ889822
	nd1	3	DQ889759, DQ889758, DQ889757
silver lamprey (*Ichthyomyzon unicuspis*)	Mitochondrion	2	NC_025553, KM267717
	cytb	1	GQ206171
	nd4	3	DQ889818, DQ889817, DQ889816
	nd1	3	DQ889755, DQ889754, DQ889753
chestnut lamprey (*Ichthyomyzon castaneus*)	*cytb*	1	GQ206168
	nd4	1	DQ889829
	nd1	1	DQ889760
	coi	3	EU524089, EU524088, EU524087

Table 3. Fish species for which tissue-derived DNA was tested for specificity using the four newly developed sea lamprey qPCR assays. Native lamprey species are in bold. Amp (Y/N): Y = positive amplification, N = negative amplification.

Species	Amp (Y/N)	Species	Amp (Y/N)
American brook lamprey (*Lethenteron appendix*)	N	lake trout (*Salvelinus namaycush* Walbaum in Artedi, 1792)	N
bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845)	N	largemouth bass (*Micropterus salmoides* Lacepède, 1802)	N
bigmouth buffalo (*Ictiobus cyprinellus* Valenciennes in Cuvier and Valenciennes, 1844)	N	mosquitofish (*Gambusia affinis* Baird and Girard, 1853)	N
bluegill (*Lepomis macrochirus* Rafinesque, 1819)	N	**Northern brook lamprey (***Ichthyomyzon fossor*)	N
bluehead sucker (*Catostomus discobolus* Cope, 1871)	N	paddlefish (*Polyodon spathula* Walbaum, 1792)	N
brook trout (*Salvelinus fontinalis* Mitchill, 1814)	N	pallid sturgeon (*Scaphirhynchus albus* Forbes and Richardson, 1905)	N
brown trout (*Salmo trutta* Linnaeus, 1758)	N	rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792)	N
channel catfish (*Ictalurus punctatus* Rafinesque, 1818)	N	sea lamprey (*Petromyzon marinus*)	Y
chestnut lamprey (*Ichthyomyzon castaneus*)	N	silver carp (*Hypophthalmichthys molitrix* Valenciennes in Cuvier and Valenciennes, 1844)	N
common carp (*Cyprinus carpio*)	N	speckled dace (*Rhinichthys osculus* Girard, 1856)	N
fathead minnow (*Pimephales promelas* Rafinesque, 1820)	N	spotfin shiner (*Cyprinella spiloptera* Cope, 1867)	N
gizzard shad (*Dorosoma cepedianum* Lesueur, 1818)	N	tilapia (*Tilapia nilotica x aurea* hybrid)	N
golden shiner (*Notemigonus crysoleucas* Mitchill, 1814)	N	walleye (*Sander vitreus* Mitchill, 1818)	N
grass carp (*Ctenopharyngodon idella* Valenciennes in Cuvier and Valenciennes, 1844)	N	yellow perch (*Perca flavescens* Mitchill, 1814)	N

described below using 1uL of 1ng/uL genomic DNA as the template. We also viewed completed reactions on 2% agarose gels so we could be aware of any non-specific amplification that is not detected by the probe which may influence post-reaction sequencing analysis. Genomic DNA used for specificity testing was extracted from fin clips similarly to our eDNA samples as described below. The SL used for *in-vitro* testing were captured from Great Lakes' streams in Michigan, U.S.

Limit of detection and limit of quantification

We defined our limit of quantification (LOQ) as the lowest concentration of the linear range covered by our standard curves (10 copies per reaction). We calculated the limit of detection (LOD) for all four assays independently using methods similar to Agersnap et al. 2017 and Ellison et al. 2006. We defined LOD as the number of DNA copies that can

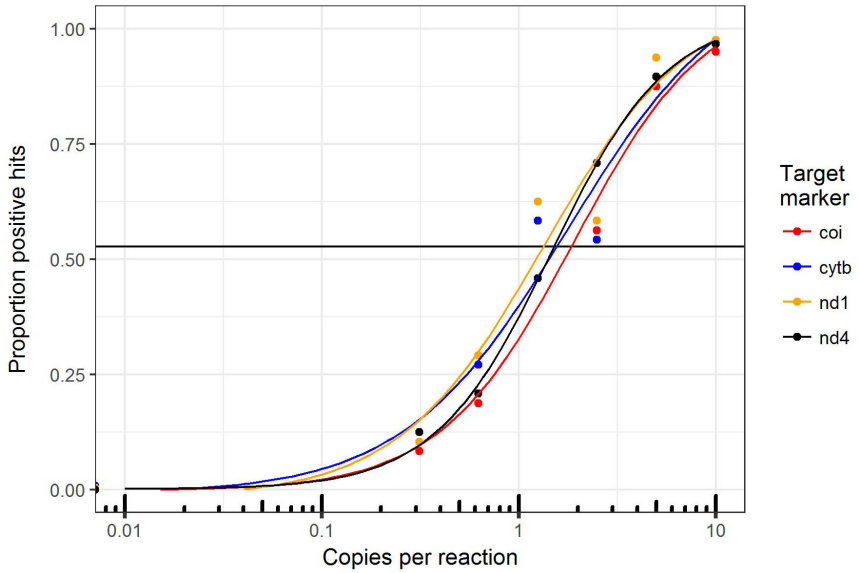

Figure 1. Dose response curve showing proportion of positive hits detected out of 48 replicate reactions across a series of 5 dilutions for each assay. Solid line represents the proportion of positive hits required to have 95% confidence that one in four reactions had a positive detection. This is defined as our Limit of Detection (LOD).

be detected within at least one of four replicate reactions with 95% confidence. Serial dilutions (5, 2.5, 1.25, 0.625, and 0.3125 copies per reaction) of gBlock template DNA were assayed in 48 replicates. We fit a dose-response curve to this data, and we set our LOD as the concentration expected to result in at least one positive detection per four replicates. While we analyzed the data for each assay independently, the qPCRs were carried out in duplex as described below. The dose-response curve was fitted to the data for each assay using DRC package (Ritz 2005) in R (R Core Team 2016) (Figure 1, Table 4).

Table 4. Limit of detection (LOD), standard error and 95% confidence intervals for 4 qPCR assays. LOD is defined as the number of DNA copies that can be detected within at least one of four replicate reactions with 95% confidence.

	LOD (copies/reaction)	Std. Error	95% CI upper and lower	
coi	2.0765	0.8772	0.1652	3.988
cytb	1.9619	1.1904	-0.6318	4.556
nd1	1.4623	0.6504	0.0452	2.879
nd4	1.5798	0.4656	0.5653	2.594

Laboratory density trials

Experimental animals

Laboratory density trials utilized larval and adult SL that were housed at Hammond Bay Biological Station (HBBS) in Millersburg, MI. Sea lamprey were housed and cared for in accordance with HBBS SOP No. 424 (Standard Operating Procedures for Sea Lamprey Housing and Care). Additionally, this protocol was reviewed by the Upper Midwest Environmental Sciences Center (UMESC) Animal Care and Use Committee and approved as AEH-15-GLFC-eDNA-01. All larval SL (n = 93 used in the test) were collected by U.S. Fish and Wildlife Service (USFWS) assessment personnel via backpack electroshockers from the Pentwater River, Oceana County, MI according to methods described in Weisser and Klar (1990). Approximately 1000 adult SL were collected in traps on the Manistique River, Alger County, MI. Adult SL were transported to the HBBS by truck approximately 1 month prior to the start of the test and were held in holding tanks prior to being distributed to the test tanks. Of these, n = 666 were initially introduced into tanks, and additional individuals were used to replace moribund individuals throughout the course of the experiment. All water used in the density trials was sourced from Lake Huron. Aeration was provided to larval and adult SL during testing, but they were not fed. All dead and moribund test animals were removed daily from the test chambers and surviving fish were euthanized by tricaine methanesulfonate (MS-222) overdose at the termination of the study and disposed of in accordance with HBBS SOP No. 424.

Sample filtration

Filtration was performed at a suitable clean location in the lab where the samples were collected. A Masterflex peristaltic pump-head (Cole Parmer 07015-21) was used with Masterflex #15 silicon tubing (Cole Parmer 96410-15). Upstream of the pump-head,

a Pall 47-mm filter holder (VWR 28144-257) was loaded with a glass microfiber filter (1.5 µm pore size, Whatman 934-AH, CAT No. 1827-047). One 47-mm filter was placed in the filter holder grid-to-grid and the filter holder was assembled, ensuring the O-ring was properly seated. The 30.5-cm tubing was attached to the other side of the filter holder. One end of the 61-cm tubing was placed into the sample bottle. The other end of the 61-cm pump-head tubing was placed into the filtrate bottle. Operating the drill in forward (clockwise) motion pulled water out of the sample container, through the filter, and finally through the pump-head tubing and into the filtrate container. Once a sample was filtered, the filter holder was dis-assembled and the filter carefully rolled using clean forceps and transferred to a labeled 15-mL centrifuge tube pre-filled with approximately 10 mL absolute ethanol. The tube was capped and placed in a tube rack which was then placed in a zip-top bag and stored on ice in a cooler. When switching to a different tank density, the filter tubing that was in contact with the pre-filtered sample was replaced with clean tubing, and the filter holder assembly was rinsed with DI water. Once filtration was complete, total volume filtered was recorded, and the filtrate discarded. Most samples consisted of exactly 1 L of filtered water; however, if more than 1 L was filtered, the recorded volume allowed for a standardized calculation of DNA copies per L of water. All ethanol-preserved filters were kept on wet-ice in a cooler for approximately 24 hours until return to UMESC, where they were stored at −80 °C until further processing.

Adult SL laboratory density trials

Adult male SL were stocked in 2000-L tanks in triplicate treatments with densities of 0, 2, 20, and 200 per tank. The inflow water was supplied from Lake Huron at a rate of 16.67 L/min, a turnover rate of once every 2 hours.

Previous research on common carp (*Cyprinus carpio* Linnaeus, 1758) has shown that eDNA levels were relatively high in the water on the first 1–2 days after transfer to the tank and reached equilibrium on about day six (Takahara et al. 2012). Maruyama et al. (2014) found a similar trend in bluegills (*Lepomis machrochirus*), but that eDNA concentrations stabilized around days 3–4. Although we cannot assume that SL have the same shedding rates, eDNA in the water likely persists similarly so we collected samples 8 days after tanks were stocked with the SL. Temperature and mortality were recorded daily in each tank and moribund animals were replaced daily as needed to maintain the desired number of lamprey in each tank. Of the 666 adult SL initially stocked in the tanks, 142 animals died over the 8-day holding period. This relatively high mortality was expected because the semelparous SL were captured on their way upstream to spawn where they would have died shortly after spawning. We collected triplicate 1 L samples from each tank along with one field blank (1 L of deionized water from UMESC). We also utilized a cooler blank to catch possible cross-contamination during storage and transport of the samples in the cooler. The cooler blank consisted of a 15-mL centrifuge tube containing ethanol and a filter used to filter 1 L of deionized water prior to any sampling. To minimize our risk of contamination, we collected samples in order of increasing SL density and conducted filtering in the same order. Only 0.5 L of water from the tanks with 200 animals could be passed through a single filter before the filter began to clog, 1 L of water could easily pass through a single filter from all other tanks. Each sample filter was extracted for DNA and assayed in four qPCR replicates (n = 36 for each density). The data were modeled for each target (*cytb* and *nd1*) separately. We ran linear mixed models of log-transformed copy numbers using tank as the random effect and density as the fixed effect (log10(copies) ~ log10(density) + (1|Tank)) (Bates et al. 2015). The slopes and the p-values were obtained from the model estimates. We did not include control tank data in the model because most reactions (21 *cytb*, 22 *nd1*) did not detect any SL DNA, and the ones that did could not be accurately quantified as they were below our LOQ.

Larval SL laboratory density trials

Aquaria (28 L) were filled with 6.3 L of sand from Lake Huron to an approximate depth of 5 cm and were set up with a flow-through design using water from Lake Huron with a turnover rate of once every two hours (0.23 L/min). Larval lamprey were stocked in triplicate treatment densities of 0, 1, 5, and 25 per aquarium. Temperature of the source water was recorded daily and water samples were collected after 6 days of holding. Because larval lamprey were burrowed in the sediment it was impossible to look for mortality among the test animals, but mortality was checked at the end of the test.

Water samples were filtered as they were in the adult study using an in-line filter attached to Masterflex #15 silicon tubing. This tubing was attached via suction cup to the tank with the intake halfway down into the water column. Samples (1 L) were filtered from the aquaria in order of increasing density (larval SL per tank), and included a field blank.

Each sample was analyzed for SL eDNA in quadruplicate qPCR reactions and results were recorded as mean copy number and were analyzed with a linear

model to look for trends between copy number and SL densities. Results were also analyzed as presence/absence data with a generalized linear mixed model with binomial distribution family with logit link function using the glmer function in the lme4 package (Bates et al. 2015) in R (R Core Team 2016) to look for any trends between detection probabilities with SL densities. We had detections predicted by SL density and target gene as fixed effects and tank as a random effect using the formula: detection probability ~SL density + gene target + (1|tank).

DNA extraction

In the laboratory, each filter was removed from its conical tube and folded in half and the unused outer ring of the filter ripped off by hand, wearing fresh gloves for each sample. Most of the ethanol was blotted off the folded filter using clean paper towels before the filter was folded twice more and placed into an Investigator Lyse & Spin basket (Qiagen; Valencia, CA) for DNA extraction. DNA was extracted from the filters using the gMax mini genomic DNA extraction kit following the manufacturer's guidelines (IBI Scientific; Peosta, IA) with a final elution volume of 100 µL. Each extraction batch included a negative control which consisted of a clean filter wetted with 500 µL of molecular grade nuclease/nucleotide free water treated the same as all other samples.

Quantitative PCR

Quantitative PCR was carried out for all DNA samples collected in each of the studies. Samples were run in 96-well skirted twin.tec PCR plates (EppendorfNA; Hauppage, NY) on Bio-Rad CFX96 Touch™ thermocyclers (Bio-Rad Laboratories; Hercules, CA) in quadruplicate reactions, and this level of replication is comparable to what others have done (Carim et al. 2017; Ostberg et al. 2018; Rice et al. 2018; Eiler et al. 2018). We analyzed amplification plots with CFX Manager software version 3.1. Cycle of quantification (C_q) values were determined using the single threshold method. Plates were set up manually or using an epMotion5075 robot (EppendorfNA; Hauppage, NY) with 20 µL reactions containing 1 µL of template DNA, 500 nM each forward and reverse primers, 125 nM each probe, and 1x environmental master mix (Life Technologies; Carlsbad, CA). All four assays performed equally well in sensitivity and specificity to SL (Tables 2, 4). We found that the markers were able to be duplexed together in the same reaction, because 1) there was no loss of efficiency when duplexed, 2) *cytb* and *nd1* had similar annealing temperatures (60 °C) and *coi* and *nd4* had similar annealing temperatures (63 °C), and 3) the probes had compatible fluorophores (Table 1). While all four assays performed equally well, we felt it was adequate to continue analysis only using two markers and therefore we used *cytb* and *nd1* markers (Table 1) for analyzing our tank samples.

All probes used the ZEN-3 Iowa Black FQ double-quenched probes from Integrated DNA Technologies; Coralville, IA (IDT). Reactions were subjected to an initial denaturation step at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s, 70 °C for 20 s, followed by a final extension at 70 °C for 5 min for the *cytb/nd1* duplex reaction. The *coi/nd4* duplex reaction was run with the same conditions but with an annealing temperature of 63 °C. Annealing and extension temperatures were chosen based on temperature gradient optimizations shown in Supplementary material Figure S1. Briefly, we assayed the same concentration of SL genomic DNA in four replicates at each gradient temperature and compared the C_q values obtained. We determined optimal annealing and extension temperatures to be as stringent as we could without loss of efficiency (evidenced by obtaining higher C_q values with the same starting concentration of target DNA). Approximately 100 copies of a synthetic gBlock gene fragment (IDT) containing the targeted SL sequences (Table 1) was spiked into each of three additional reactions as an internal positive control to detect PCR inhibition. Samples were determined to be inhibited if any of the three spiked replicates failed to amplify or if they resulted in a higher C_q value than a threshold set for each plate. The inhibition C_q threshold was set as the mean C_q value for four replicate 100 copy standards plus 1. We determined that there were no inhibited samples in either the adult or larval SL density trials. Each qPCR plate had duplicate 4-point standard curves for quantification of the SL targets using the same synthetic gBlock gene fragments at 10, 100, 1,000, and 10,000 copies. Plates were considered as acceptable if the standard curves had efficiencies between 80–120% (Shanks et al. 2012) and R^2 values above 0.93. All plates had a range of efficiencies between 111.5%–93.5% and a range of R^2 values between 0.984–0.998.

Results

Validation of assays and limit of detection

All four qPCR assays successfully detected SL and did not detect any non-target species tested. Additionally, the gels indicated that no non-target species tested produced any amplification that was not detected by probe hydrolysis. The LOD values for the four individual assays showed similar sensitivity

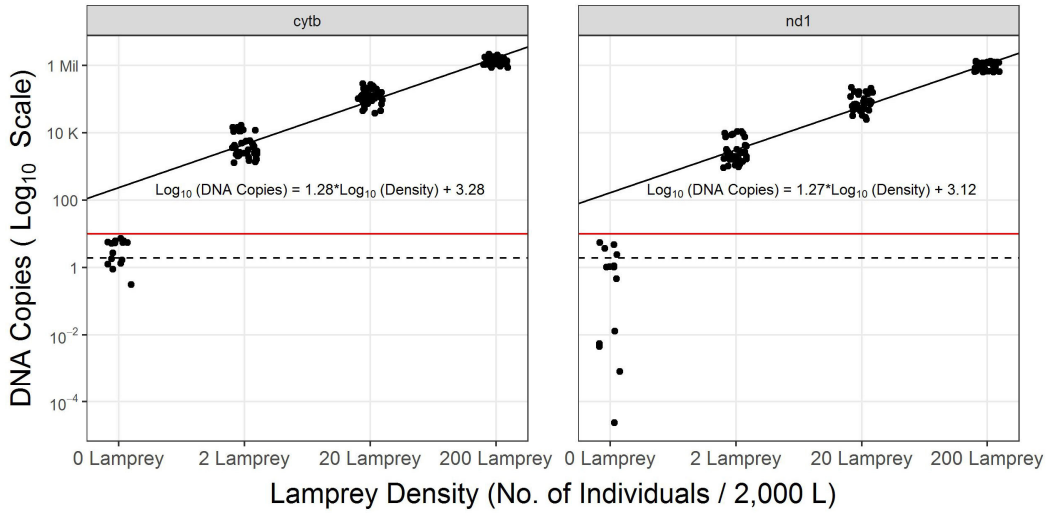

Figure 2. Adult sea lamprey DNA copies vs sea lamprey densities (Both axes are log scale, except log[+0.2] for the 0 lamprey tanks). Slope for the *cytb* assay is 1.28, the slope for the *nd1* assay is 1.27. Jittering was used to reduce over-plotting. There were 43 qPCR reps (21 *cytb*, 22 *nd1*) that were negative among the control tanks which aren't included on the plot. Control tanks were included on the plot to show base-line DNA copies. However, they were all below our limit of quantification (LOQ) and were not used to calculate the regression lines. Red solid line represents the limit of quantification (10 copies); black dashed line represents the limit of detection (1.9619 copies).

among all four assays with a limit of detection of 2.0765 copies/reaction for the *coi* assay, 1.9619 copies/reaction for the *cytb* assay, 1.4623 copies/reaction for the *nd1* assay, and 1.5798 copies/reaction for the *nd4* assay. We report the higher of *cytb/nd1* LOD of 1.9618 copies per µL template for adult and larval density (Figure 1, Table 4).

Adult laboratory density trials

Data indicate that DNA concentration increased significantly with increasing SL density (*cytb* and *nd1* $P < 0.01$, Figure 2). All qPCR replicates from tanks containing SL were positive except one replicate for one of the 20 SL tank samples (i.e., SL eDNA was detected in 11 of the 12 qPCR replicates). In the 0 SL tanks, 21/36 of the *cytb* reactions had no detection and 22/36 of the *nd1* reactions had no detection. The remaining positive reactions in the 0 SL tanks all were below our LOQ of 10 copies. The slope of the line for the *cytb* assay was 1.28 and the slope of the line for the *nd1* assay was 1.27. The 2 SL tanks had mean copy numbers of 5,260 copies/L (*cytb*) and 3,700 copies/L (*nd1*), the 20 SL tanks had 138,000 copies/L (*cytb*) and 95,000 copies/L (*nd1*), and the 200 SL tanks had 1,480,000 copies/L (*cytb*) and 984,000 copies/L (*nd1*) (Figure 2).

Larval laboratory density trials

There was no significant correlation between log copies of SL eDNA copy numbers and SL density (*cytb* R^2: 0.01091 p-value = 0.60, *nd1* R^2: 0.1126 p-value = 0.065, Figure 3). In addition, most of the values are close to our limit of quantification (10 copies). The 0 lamprey tank on the graph was included on the graph to show the base eDNA concentrations observed in the source water, but it was not used to create the line. There were no mortalities in the larval SL trials.

Detection probability increased as SL density increased. However, the trend was not significant ($p = 0.11$). Mean detection probabilities for each density were: 0 lamprey – 20.8%, 1 lamprey – 66.7%, 5 lamprey – 83.3%, 25 lamprey – 91.7% (Figure 4). The assay used (*cytb* or *nd1*) had no significance in detection probability ($p = 0.22$).

Discussion

Like all fisheries techniques to assess population density, limitations exist with eDNA. The DNA that is sloughed into the environment can be diluted, degraded, or might settle out of the water column into the sediment (Strickler et al. 2015; Turner et al. 2015). However, it has been shown that eDNA sampling can have higher detection rates than traditional sampling techniques (Pilliod et al. 2013). Our study suggests that DNA copy numbers provide little value to quantifying population density of SL when eDNA concentrations are low. However, if the sampling protocol can be refined by methods such as increasing the sample size or increasing the amount

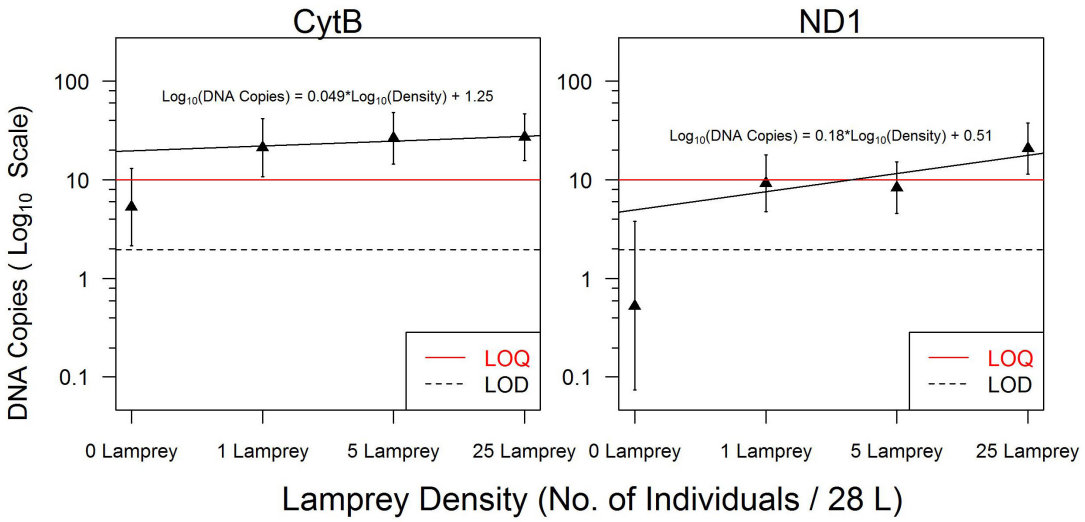

Figure 3. Larval sea lamprey DNA copies vs larval sea lamprey per tank (0, 1, 5, and 25 lamprey per 28 Liters). Both axes are log scale, except log[+0.2] for the 0 lamprey tanks. The 0 lamprey tank on the graph was included on the graph to show the base eDNA concentrations observed in the source water, but it was not used to create the line. Error bars represent 95% confidence intervals of the mean copy numbers, *cytb* R2 = 0.01091 *p*-value = 0.60, *nd1* R2 = 0.1126 *p*-value = 0.065; LOQ = limit of quantification (10 copies); LOD = limit of detection (1.9619 copies).

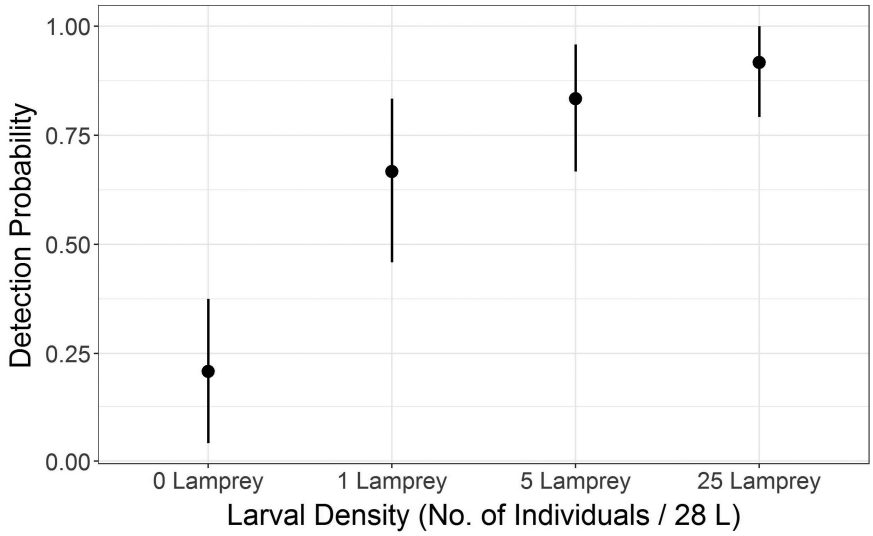

Figure 4. Larval sea lamprey (0, 1, 5, and 25 per tank) and detection probability (detection probability ~ SL density + gene target + (1|tank)) (P = 0.11). Error bars represent 95% credible interval.

of replicate samples taken, the use of detection probability may provide a qualitative measure (i.e., low, medium, and high) for these populations. Analyzing larger volumes of water or extracted DNA may also be optimized to increase sensitivity, although more is not always better as increasing these volumes can also increase PCR inhibitor concentrations and modify reaction buffer concentrations to suboptimal conditions. Reliable qualitative population measures could provide valuable information to resource managers to consider along with the expert judgments and traditional assessment data to maximize the efficiency and success of the SL control program. Although these metrics of low, medium, and high would have to be defined by the management of the Sea Lamprey Control Program.

Our analysis determined that our LOD is 1.9619 copies per µL. We do not expect amplification of partial DNA copies, because on a partial strand 1 or more of the oligonucleotide recognition sequences would be absent. At least 1 full copy must be present in a reaction to result in positive detection.

We would expect that when aliquotting template at such a low concentration, many reactions would get 2 copies of target DNA but some would get 3 or more and others would get 1 or 0. Our ability to detect is likely more dependent on the likelihood of transferring at least 1 copy of SL DNA into one of the replicate reactions rather than our assay's ability to amplify the DNA within the reaction if present. If the target DNA concentration is less than 1 copy per unit volume, then it should be expected that multiple unit volumes must be tested to achieve successful detection. This likely is one reason why not all replicates of a qPCR analysis detect when there is at least 1 positive detection among them, and it is also how one could detect concentrations lower than 1 copy per µL by testing multiple 1 µL subsamples. The LOD of 1.9619 copies per µL also does not indicate that this is the lowest DNA concentration we can detect. Quantitative PCR is a very sensitive technique and will most likely result in positive detection most times when at least 1 target copy of template DNA is in the reaction regardless of starting concentration. Instead, this number indicates that given our level of effort (testing 4 reactions with 1 µL of template each), we could reasonably expect (with 95% confidence) that we would achieve successful detections on any sample with higher than 1.9619 copies per µL of target DNA. Lower concentrations could still be detectable if at least 1 copy of target DNA was transferred into the reaction well, although this would occur at a lower rate.

In our study, we used a combination of copy numbers and detection probability to analyze our results. Our findings indicate that at low concentrations (near our LOQ), DNA copy numbers are not useful for analyzing the data but rather detection rates might be. When DNA copy numbers are high, we observed 100% detection rates and the variability between copy numbers was useful. There is presumably a threshold of DNA copy numbers in a sample that might determine if eDNA data should be analyzed using copy numbers or detection probability. This constitutes an area for further research.

The strong positive correlation between SL density and copy numbers provided by the adult trials shows a proof of concept for our sampling technique as well as our qPCR assays. The slopes for both assays (*cytb* = 1.28, *nd1* = 1.27) show a similar increase in DNA copies with increasing SL density between the two assays. A very strong linear relationship exists between numbers of SL and eDNA concentrations across two orders of magnitude range in SL density.

Adult SL were captured during the spring spawning run and were in poor condition, as would be expected. During their spawning migration, SL stop feeding and induce autophagy. This results in blindness and sloughing of epidermis as their bodies break down (Applegate 1950). This high amount of sloughed skin and mucus may explain the high levels of DNA found in the water samples from the tanks. Our procedure of replacing dead and moribund animals could also increase the amount of DNA being shed into the tanks as additional stress was put on the SL by handling them, however we suggest that this likely had an insignificant impact. No SL were replaced in the 2 SL tanks, only 4 SL were replaced in the 20 SL tanks (with no replacements for 2 days prior to sampling), and less than 10% of SL were replaced in the 200 SL tanks on any given day with less than 5% being replaced on most days. There was so much eDNA in the 200 SL tanks that eDNA introduced by the replacement SL would likely be unnoticed. A detailed summary of mortality is available in Table S1. Similarly increased skin sloughing and mucus production for SL likely occurs in animals that have already spawned within streams; thus, making them easier to detect because only a few animals will likely produce large quantities of DNA. Additionally, actively spawning adults release gametes that could be collected in a water sample possibly increasing the probability of detection. It's possible that the SL used were releasing gametes into the water as they were sexually mature males that had already spermiated, yet had been trapped prior to their ability to spawn.

No statistical difference (*cytb* p-value: 0.6, *nd1* p-value: 0.065) in the copies of SL DNA between tanks containing 1, 5, and 25 ammocoetes was found, but much less DNA was present in water containing ammocoetes than water holding adult SL. For example, the adult SL tanks that contained 200 SL per 2000 L had mean concentrations of approximately 1 million copies of DNA per Liter, whereas the larval SL tanks that contained 25 SL per 28 L had mean concentrations of approximately 20–30 copies per Liter. The larger body size, increased surface area, possible release of gametes, and decaying bodies of adult SL would likely result in greater amounts of cells and increased amounts of SL DNA in the water (Merkes et al. 2014). Also, ammocoetes are primarily burrowed and defecate in the sand. Feces may be the primary source of DNA detected in the water (Klymus et al. 2015; Barnes and Turner 2016). This burrowing behavior might significantly limit the amount of DNA present in a water sample taken near burrowed ammocoetes. Much of the DNA shed in the burrow of the ammocoetes is likely bound to the sediment as it has been shown to have a strong sorptive affinity to sediment (Lorenz et al. 1981; Turner et al. 2015).

When looking at biomass, the mean mass of a single larval SL used in the tank trials was 0.59 g.

This gives us a biomass concentration of 0.021 g/L for the 1 lamprey tanks, 0.105 g/L for the 5 lamprey tanks, and 0.52 g/L for the 25 lamprey tanks. While we didn't collect biomass information for the adult SL used in our study we calculated biomass estimates using 203 g as the mean adult spawning SL mass (Jorgensen and Kitchell 2005). This gives us a biomass concentration of 0.203 g/L for the 1 lamprey tanks, 2.03 g/L for the 20 lamprey tanks, and 20.3 g/L for the 200 lamprey tanks. There is an order of magnitude increase in the biomass estimates of the adult SL tanks when compared with the larval SL tanks. This could be another explanation for the increased copy numbers found in the adult SL tanks.

We observed higher eDNA detection rates in tanks with greater numbers of larval SL. However, because the credible intervals on our detection probabilities were so large, we did not have enough statistical power to achieve significance. We would expect that if more samples had been collected, our credible intervals would be tighter and therefore might observe a significant relationship. Previous research on common carp has shown that eDNA detection is more closely correlated with an increase in individuals rather than an increase in biomass (Doi et al. 2015). This would be interesting to investigate further with SL. For example, do twenty SL with a combined mass of 10 grams shed a similar amount of DNA as ten SL of the same mass? Biomass might be an important variable in the release of eDNA and would presumably vary between species. Another thought to consider is expanding the density intervals of the larval SL in an effort to refine the resolution of our eDNA and detection rate relationship. Further research into how SL density affects eDNA detection rates would help to refine this relationship and lead to more meaningful interpretation of eDNA data when concentrations are low.

The detection of SL DNA in the control tanks was likely caused by SL DNA contained in the source water or sand used during the study. Water was sourced from Lake Huron which is known to harbor a large SL population. For the adult SL laboratory density trials, this did not appear to be a problem, because the background contribution of SL eDNA in the system was less than $1/10{,}000^{th}$ of the total SL eDNA contributed by only 2 individuals in our lowest density experimental tanks. Sand was from a common tank at HBBS that was used for other SL-related testing. Because eDNA persists longer through binding to sediment (Lacoursière-Roussel et al. 2016; Buxton et al. 2017), the sand is a likely additional source of the SL DNA detected in these tanks. For the larval SL laboratory density trials, the background eDNA is slightly more problematic, because the background contribution of SL eDNA in the system was closer to $1/10^{th}$ of the total SL eDNA contributed by having SL present. However, the eDNA concentrations we detected were so low (some below our LoQ) that no inference could be made based on copy numbers in this situation regardless. The background SL eDNA detections could have contributed to the lack of statistical significance in our analysis of detection rates for the larval SL laboratory density trials. The background SL eDNA detections we observed demonstrate the sensitivity of eDNA and highlights the importance of clean sampling procedures and proper study design when conducting eDNA studies. Another potential source of variation would be if eDNA washed off of the filters into the ethanol preservative. We did not observe any material coming off the filters into the tubes we kept them in, and given the decreased solubility of DNA in ethanol compared to water we would expect eDNA to adhere to the filter better in the ethanol preservative. However, some eDNA-containing cells could have physically washed off the filters during sample handling that could have contributed to greater variability and decreased our likelihood of observing statistical significance. This should also be a consideration for future studies.

Testing for inhibition is important for eDNA analysis (Wilson 1997; Green and Field 2012). Most commonly, an internal positive control is used to assay for inhibition. Deciding on a concentration that is appropriate for use in inhibition testing can be a delicate balancing act, because you want to accurately determine inhibition but also not influence the thermodynamics of the actual eDNA analysis of interest. We spiked our samples at 100 copies and this is a low enough concentration that cross-contamination as well as thermodynamic influences are minimal concerns. This is also a good concentration for testing inhibition on low copy number samples as most environmental samples are expected to be. However, when eDNA samples have very high target concentrations this can overwhelm the inhibition test and invalidate it for assaying partial inhibition which can affect quantification results but not detection results. Because we used the same source water for all tanks and zero lamprey tanks were determined to not be inhibited we considered all tanks to not be inhibited despite the very high eDNA concentrations in the adult lamprey tanks.

The use of eDNA for SL would most likely be utilized as a supplement to the traditional assessment techniques that are currently used. The SL are already assumed to be present in the streams and any assessment that is done looks at populations of SL relative to each other between rivers. When used in the field, researchers should include known SL

positive sites and known SL negative sites along with unknown sites that they are interested in monitoring. This will help provide confidence that the assays are performing as expected. Though rare, there may be a few occurrences of using eDNA technology to investigate rivers that are thought to be "new producers" of SL. These "new producer" rivers might be historically negative SL producing rivers due to poor water quality but could eventually attract the attention of spawning SL as the water quality of the river is improved through clean-up efforts. In this situation, the use of eDNA as an early detector of the presence of SL might be suitable and could lead to a more rigorous assessment.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Annealing and extension temperature graphs for all four assays (*coi, cytb, nd1,* and *nd4*).

Table S1. Detailed mortality summary of adult sea lamprey during adult SL density trials.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2018/Supplements/MBI_2018_Schloesser_etal_Figure_S1.pdf
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