Statolith microchemistry as a technique for discriminating among Great Lakes sea lamprey (*Petromyzon marinus*) spawning tributaries

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Abstract: Laurentian Great Lakes fishery management agencies are seeking ways to identify natal origins of parasitic- and spawning-phase sea lamprey (*Petromyzon marinus*) so that efforts to control this invasive species can be prioritized. We developed laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) as a technique to quantify elemental concentrations in larval sea lamprey statoliths and explored the use of statolith microchemistry as a tool to discriminate among larval sea lamprey production streams. Our analyses demonstrate that (*i*) traversing across the statolith with the laser is preferable to drilling down through its apex, (*ii*) preserving specimens in 95% ethanol versus freezing them has minimal effects on elemental concentrations, (*iii*) a minimum of 15 individuals per stream should accurately depict stream-specific statolith elemental signatures, and (*iv*) LA-ICP-MS is preferable to particle-induced X-ray emission (PIXE) for statolith analysis, based on higher precision, lower cost, reduced sampling-time requirements, and wider availability. Using LA-ICP-MS, we could discriminate among larvae from 13 streams located in Lakes Michigan, Huron, and Superior with 82% classification accuracy, indicating that this tool holds promise for determining natal origins of sea lamprey in the Great Lakes.

Résumé : Les agences de gestion des pêches des Grands Lacs laurentiens sont à la recherche de méthodes pour identifier les lieux de naissance des phases parasites et reproductrices de la grande lamproie marine, *Petromyzon marinus*, de manière à établir des priorités dans les efforts pour contrôler cette espèce envahissante. Nous utilisons une technique de spectrométrie de masse à plasma induit couplée à l'ablation laser (LA-ICP-MS) pour mesurer les concentrations d'éléments dans les statolithes des grandes lamproies marines et nous explorons l'utilisation de la microchimie des statolithes comme outil pour discriminer entre les cours d'eau producteurs de larves de grandes lamproies. Nos analyses démontrent que (*i*) il est préférable de traverser le statolithe avec le laser que de le forer à travers l'apex, (*ii*) la préservation des spécimens dans l'éthanol à 95 % plutôt que par la congélation a des effets minimaux sur les concentrations d'éléments, (*iii*) un minimum de 15 individus par cours d'eau devrait permettre de décrire avec exactitude la signature des éléments spécifique à chaque cours d'eau et (*iv*) la LA-ICP-MS est préférable à l'émission de rayons X induite par particules chargées (PIXE) pour l'analyse des statolithes à cause de sa précision plus élevée, son coût moindre, le temps moins long requis pour l'échantillonnage et sa disponibilité plus grande. À l'aide de la LA-ICP-MS, nous avons pu discriminer les larves provenant de 13 cours d'eau situés aux lacs Michigan, Huron et Supérieur avec des classifications réussies dans 82 % des cas; c'est donc un outil prometteur pour la détermination de l'origine à la naissance des grandes lamproies de mer des Grands Lacs.

[Traduit par la Rédaction]

Introduction

Sea lamprey (*Petromyzon marinus*) invaded the upper Laurentian Great Lakes during the early 20th century, following construction of the Welland Canal (Lawrie 1970). Because of their devastating impact on economically and ecologically important species such as lake trout (*Salvelinus namaycush*) and lake whitefish (*Coregonus clupeaformis*) throughout the Great Lakes (Weise and Pajos 1998), fishery management agencies (led by the Great Lakes Fishery Commission, Ann Arbor, Michigan) have implemented strategies to control sea lamprey populations throughout the lakes. These strategies include use of lampricides, physical barriers to block spawning adults, sterile-male release programs, and trapping of spawners. Currently, the application of lampricides (3-trifluoromethyl-4-nitrophenol (TFM), granular Bayluscide) to larval production streams is the most commonly used control method; streams are targeted for treatment

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based on larval densities, growth parameters, and predicted populations of metamorphosed larvae (Smith and Tibbles 1980; Christie et al. 2003). One potential limitation of using larval abundance to prioritize streams for lampricide treatment is that larval mortality rates before and after metamorphosis are unknown and therefore may not be constant among streams. Therefore, larval abundance may not be the best predictor of stream-specific contributions of parasites to open-lake populations (Slade et al. 2003). Because sea lamprey predation remains an ongoing problem despite 40 years of population control efforts, as well as uncertainty concerning the assumption that streams contribute parasites to the open lake in proportion to their larval abundances, fishery management agencies are still seeking a definitive way to quantify the relative contributions of parasites and spawners from spawning tributaries.

Tagging of larvae offers one possible method of identifying the natal stream origins of parasites and spawning adults. Use of artificial tags, however, is problematic because sea lamprey do not home to their natal streams (Bergstedt et al. 1993; Bergstedt and Seelye 1995). A sustained effort over a wide geographic area also is required to acquire sufficient tag recoveries for a robust analysis. Indeed, although Bergstedt and Seelye (1995) achieved 8% tag returns from Lake Huron and a similar study in Lake Champlain yielded 1% returns (Howe et al. 2006), neither of these multiyear studies yielded sufficient data to detect differences in larval survival among streams.

An alternative to artificial tags is the use of natural tags, which include both genetics and otolith microchemistry. Use of genetics as a tool to identify natal origins of sea lamprey, however, is impractical as populations in the upper Great Lakes have been present for less than a century and adult lamprey do not home to their natal streams (Bergstedt et al. 1993; Bergstedt and Seelye 1995). Consequently, it is unlikely that sufficient genetic differentiation is present among sea lamprey subpopulations to delineate among production streams (Bergstedt et al. 1993).

A natural-tag alternative is the trace-metal composition of otoliths. Otoliths are the calcareous structures located in the inner ear of teleost fish used for hearing and balance (Pannella 1971). Otolith microchemistry data have been used to address many fisheries-related problems, including stock delineation, tracking migration pathways, and reconstructing habitat-use patterns (Campana 1999; Thresher 1999). Three unique properties of otoliths allow them to be used for this type of research: (*i*) they are metabolically inert (exhibit no reworking once layers are set down); (*ii*) they continue to grow even when somatic growth is nonexistent; and (*iii*) their elemental composition reflects the physical and chemical environment in which the fish has resided in the past (Campana 1983; Campana and Thorrold 2001).

Herein, we explore the potential extension of otolith microchemistry to statoliths, a calcified inner-ear concretion found in sea lamprey that also exhibits annual banding patterns (Volk 1986; Barker et al. 1997). Statoliths are analogous to the otoliths found in teleost fish (Carlstrom 1963). However, whereas otoliths are composed of calcium carbonate (usually aragonite) and typically continue growing in proportion to body size throughout the life of the fish, statoliths are (*i*) made of calcium phosphate (apatite), (*ii*) small

relative to otoliths from teleost fish (statoliths average ~50 μm across, even in adults, which is equivalent in size to larval otoliths from teleost fish), and (*iii*) the only calcified structures found in an otherwise cartilaginous body. Statoliths also differ from otoliths of most teleost fishes in that the majority of the statolith material reflects the larval environment (Volk 1986). This disparity occurs because of the long time (3–7 years) that sea lamprey spend burrowing as larvae in stream sediments and the short time (<2 years) they spend as parasites and adults, during which little postmetamorphosis statolith growth occurs (Volk 1986). Additionally, the properties of otoliths listed above (e.g., remaining metabolically inert, incorporating elements in proportion to their abundance in the environment), which make them useful for otolith microelemental analysis, have not yet been established for statoliths.

Recently, Brothers and Thresher (2004) used particle-induced X-ray emission (PIXE) analysis to demonstrate that statolith microchemistry can discriminate among larvae collected from four Lake Huron tributaries. We build upon this previous work by analyzing Lake Huron sea lamprey statoliths with laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), a method that allows examination of trace elements at very low levels (parts per billion), even in larval fish otoliths (Ludsin et al. 2006). In determining whether LA-ICP-MS can be reliably used for quantifying the microchemical composition of sea lamprey statoliths and for discriminating among spawning tributaries from three of the five Laurentian Great Lakes (Huron, Michigan, and Superior), we explore factors that might influence the data, including laser-ablation technique, larval preservation method, number of individuals needed to accurately characterize a stream, and instrumental method (e.g., PIXE vs. LA-ICP-MS). We also use our findings to discuss the potential application of statolith microchemistry as a tool for identifying natal origins of sea lamprey in the Laurentian Great Lakes.

Materials and methods

Field collections

Sea lamprey larvae were collected from 17 streams within the watersheds of Lakes Huron, Michigan, and Superior (Fig. 1) by the Canadian Department of Fisheries and Oceans (DFO; Sault St. Marie, Ontario) and the US Fish and Wildlife Service (USFWS; Marquette and Ludington, Michigan, field stations), as well as in one stream (Lewis Creek) from Lake Champlain (New York – Vermont). All collections were made using electrofishing during the summers of 2004 and 2005 as part of assessment surveys in streams recently treated with lampricide. All larvae collected were stored in Nalgene® bottles containing 95% ethanol, except for a subset of larvae used in the comparison of preservation methods (see below), which were stored frozen.

Statolith preparation

Statolith processing occurred in a class-100 cleanroom. Prior to statolith removal, the total length (TL; nearest 1 mm) of each individual was measured, after which the head was removed. Bilaterally dissected heads were soaked in ultrapure milli-Q water (MQW), and then both left and

Fig. 1. Sample-site locations in Lakes Huron, Michigan, and Superior (designated as H, M, and S, respectively). Streams: (1) Sauble River (H), (2) Lauzon Creek (H), (3) Big Manistee River (M), (4) Silver Creek (H), (5) Loeb Creek (M), (6) St. Mary's River (S/H), (7) Black Mallard River (H), (8) Rifle River (H), (9) Bad River (S), (10) Brule River (S), (11) Boardman River (M), (12) Saginaw River (H), (13) Nottawasaga River (H), (14) Musquash River (H), (15) Ford River (M), and (16) White River (M). See text for explanation of symbols used.
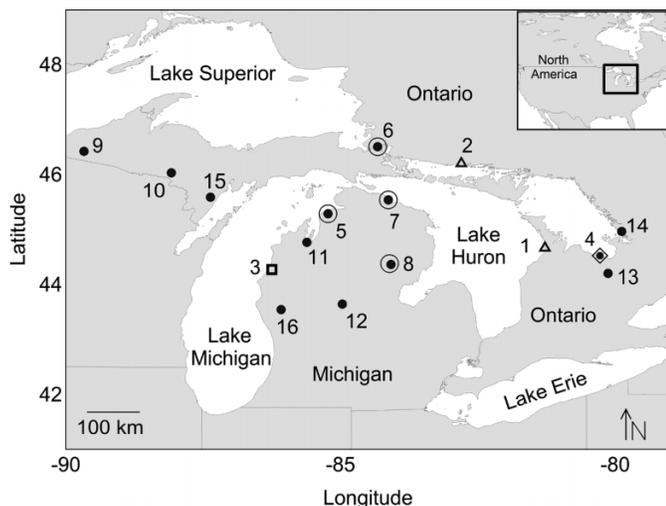
right statoliths were removed onto a clean glass slide using clean glass probes. Removed statoliths were transferred to a drop of MQW in a covered Petri dish using another clean glass probe. After six sets of statoliths had been removed, the covered Petri dishes were cleaned for 5 min using a sonicator (model ULTRAsonik 57X; Ney Dental Inc., Bloomfield, Connecticut) filled with MQW. Sonicated statoliths were then cleaned and rinsed three times with MQW. Statoliths then were mounted dorsal side up on a slide with Scotch™ double-sided tape for LA-ICP-MS analysis (Ludsin et al. 2006). No statolith surface removal was necessary as larvae were captured in their natal streams, and therefore the desired signature is present throughout the entire statolith.

All glass- and plastic-ware were acid-washed before use. Acid washing consisted of preliminary cleaning using Nitrox® soap, followed by a 24-h immersion in 13% nitric acid solution, a 24-h immersion in ultrapure MQW water, and three final rinses with MQW. Finally, everything was dried for 24 h under a class-100 laminar-flow fume hood.

LA-ICP-MS analysis

Statoliths were analyzed using an ICP-MS (Thermo Elemental X7; Thermo Fisher Scientific Inc., Waltham, Mass.) coupled with a Continuum® Surelite® solid-state Nd:YAG laser (wavelength = 266 nm, maximum power = 40 mJ, pulse rate = 20 Hz, primary beam width = 6 mm; Continuum Inc., Santa Clara, Calif.). With exception of the subset of 10 statoliths that were ablated through the central apex, elemental concentrations were determined by traversing the entire width of the statolith. Given the small size of statoliths, the laser power was reduced to <1 mJ and the beam diameter was reduced to 2 mm, which resulted in an abla-

tion spot size of ~10 μm when focused onto the sample through a 10× lens. By beginning and ending each laser transect on the double-sided tape, we could determine where the statolith began and ended in our analysis because mass 120, representative of a carbon molecular ion (instrumentally measured as ^{120}Sn), would spike noticeably when the laser ablated the tape (Ludsin et al. 2006).

A glass reference standard (NIST 610) was analyzed before and after every 16 samples ($n = 2$ replicates, both before and after), which allowed for quantification and correction of instrument drift. In addition, NIST 610 replicates were used to calculate a coefficient of variation (CV = standard deviation/mean × 100) for each element in each run, which served as a proxy for measurement precision (elements were discarded if CV was >10.5%). The argon carrier gas (i.e., background) was analyzed for 60 s before every sample, allowing limits of detection (LODs) to be calculated for individual samples (Ludsin et al. 2006).

In addition to calcium, we quantified 11 elements (Table 1); however, only seven met our criteria for inclusion in analysis: magnesium (Mg), manganese (Mn), zinc (Zn), rubidium (Rb), strontium (Sr), barium (Ba), and lead (Pb). These criteria included a CV that was <10.5% for individual isotopes (per above) and no more than 20% of the samples with concentrations below LOD for a stream. If a sample concentration was below its own LOD, the missing value was replaced with a randomly generated value between zero and the LOD for that specific sample. To correct for ablation-yield differences that resulted from varying laser spot size, we used calcium (measured as ^{43}Ca) as the internal standard, given that this element comprises a relatively large, constant (39.9% by weight) proportion of the statolith apatite ($Ca_5(PO_4)_3(OH)$). For all statistical analyses, we used ^{88}Sr and ^{138}Ba to estimate concentrations of Sr and Ba, respectively.

Experimental setup and data analysis

Toward further developing microelemental analysis of statoliths as a tool for discriminating among sea lamprey production streams, we compared (*i*) two laser-ablation approaches (drilling down through the central apex vs. ablating across the entire statolith), (*ii*) the effects of ethanol vs. freezer storage as a preservation technique, (*iii*) the potential minimum sample sizes needed to accurately represent a stream population, and (*iv*) differences between our LA-ICP-MS results and the PIXE results of Brothers and Thresher (2004). We also determined whether stream-specific statolith elemental signatures differed among Lakes Huron, Michigan, and Superior, as well as within them.

Comparison of laser-ablation approaches

Statoliths grow in a conical shape, with the tip being the oldest material and the base being the most recently deposited (Brothers and Thresher 2004). In an attempt to determine whether the same elemental signature is found throughout the entire statolith, we compared elemental concentrations in statolith pairs wherein one statolith was ablated by traversing across its entire width and the second was ablated by drilling down (vertically) through its apex. Left and right statolith pairs from larvae collected in the Sauble River and Lauzon Creek ($n = 5$ individuals per

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Table 1. Isotopes (not including calcium) quantified using LA-ICP-MS.

	^{7}Li	^{25}Mg	^{55}Mn	^{57}Fe	^{66}Zn	^{85}Rb	^{86}Sr	^{88}Sr	^{137}Ba	^{138}Ba	^{140}Ce	^{208}Pb	^{238}U
Element LOD (ppm)	0.75	13.6	0.47	77.9	0.67	0.291	2.09	0.23	0.52	0.14	0.58	0.53	0.02
CV (%)	6.03	3.5	3.18	12.9	6.32	5.25	2.50	2.22	3.33	3.52	3.67	6.16	5.39
% > LOD	3	100	100	73	96	99	100	100	100	100	32	82	4

Note: Mean limits of detection (LOD) were calculated based on all sample runs. The coefficient of variation (CV), as determined from NIST 610 standards, is the average for all runs. The percentage of samples greater than detection limits for a stream (% > LOD) also is provided. Isotopes in bold type met our criteria for inclusion in our analyses.

stream; triangular symbols in Fig. 1) were compared using paired t tests. To determine the best approach for ablating statoliths with LA-ICP-MS, we compared differences in average elemental concentrations, average LODs, and average statolith dwell times (i.e., analysis durations). No data transformations of the elemental data were necessary (Kolmogorov–Smirnov test for normality, all $p \geq 0.20$). Because seven individual t tests were conducted to test differences in both average concentrations and average limits of detection for each element, we applied a Bonferroni correction to reduce the likelihood of finding a significant result due to chance ($\alpha = 0.05/7 = 0.007$).

Comparison of statolith preservation methods

Because of logistical difficulties in freezing larvae while in the field, as well as difficulties (and cost) associated with preserving parasites and adults in ethanol, it is not practical for the agencies that collect sea lamprey (DFO and USFWS) to use a single preservation technique for all life stages. Thus, we tested whether preservation in 95% ethanol vs. freezing has a significant effect on the trace-elemental concentrations in statoliths. Although preservation in ethanol does not appear to affect trace-elemental concentrations in juvenile and adult teleost fish (Milton and Chenery 1998; Proctor and Thresher 1998; Hedges et al. 2004), we wanted to ensure that ethanol preservation vs. freezing also has no effect on statoliths. To do so, we used fish from both Big Manistee River (Lake Michigan; square symbol in Fig. 1) and Lewis Creek (Lake Champlain). In both streams, 60 larvae were collected, with half being frozen and the other half being preserved in 95% ethanol. Two-sample t tests ($\alpha = 0.05$) were used to determine if elemental concentrations differed as a result of the preservation technique. All elemental data were log-transformed to achieve normal distributions (Kolmogorov–Smirnov test for normality, all $p \geq 0.20$). Mean total length (±1 standard error, SE) was not significantly different between preservation methods (t test: frozen, 75 ± 3 mm; ethanol, 79 ± 2 mm; $p = 0.23$).

Comparison of stream sample sizes

We used three approaches to assess the likely minimum sample size required to adequately characterize stream-specific signatures. First, we used a bootstrapping approach (with replacement) to generate 500 random subsamples consisting of 10, 15, 20, 25, 30, and 40 individuals per stream (i.e., in total, 3000 data sets were generated) from a sample ($n = 47$) of larvae from Silver Creek, Lake Huron (open diamond symbol in Fig. 1). We compared individual element concentrations using a Friedman analysis of variance (ANOVA), which is a nonpara-

metric test for dependent samples. Because seven separate analyses were conducted, we adjusted the alpha level accordingly (i.e., $\alpha = 0.05/7 = 0.007$). Before analysis, we log-transformed Mn, Zn, Rb, and Sr and took the reciprocal of Mg, Ba, and Pb to normalize the data (Kolmogorov–Smirnov test for normality, all $p \geq 0.20$). In addition, we used Pearson's correlation coefficient to determine how variation in the data, as measured by the coefficient of variation (standard deviation/mean × 100; arcsin square-root transformed to achieve normality), was affected by sample size for each element ($\alpha = 0.007$).

Second, using the only five streams in which 25 or more larvae were collected (i.e., Musquash River, Nottawasaga River, Saginaw River, Silver Creek, and St. Mary's River, all draining into Lake Huron; Table 2; Fig. 1), we used a bootstrapping approach (with replacement) to generate 500 random subsamples consisting of 5, 10, 15, 20, and 25 individuals per stream (i.e., in total, 2500 five-stream data sets were generated). We then used linear discriminant function analysis (LDFA) to quantify how well we could discriminate the five streams in each data set (i.e., 2500 LDFAs were conducted). The original (nonbootstrapped) larval sea lamprey elemental signatures from the five streams were used as a cross-validation data set in each LDFA (see second column of Table 2 for sample sizes) to test the ability of LDFA classification functions to correctly classify individuals to their stream of origin.

We used Levene's test to determine whether treatment variances differed among data sets of varying larval subsample size. Because all stream-specific data were highly non-normal (Kolmogorov–Smirnov test for normality, all $p < 0.0001$) and we couldn't normalize data for all treatment groups, even after attempting numerous transformations, we relied on box plots to explore how means differed among treatments of variable subsample size.

Finally, to further assess how variation in stream-specific subsample size could influence the ability to discriminate among streams, we generated a single random subsample of 5, 10, 15, 20, 25, 30, and 40 individuals from our original Silver Creek subsample ($n = 47$). We then conducted multiple LDFAs ($n = 7$; prior classification probabilities set equal for all streams) wherein the data for each analysis only varied in the size of the Silver Creek subsample. For each of the seven analyses, all larvae from any non-Silver-Creek streams were used (see second column of Table 2 for sample sizes) rather than only the five streams with 25 or more sampled larvae (per above). Afterwards, we used jackknifed classification matrices produced as part of each LDFA to compare our ability to discriminate Silver Creek sea lamprey from those collected in other streams.

Table 2. Classification matrix for 13 streams in the watershed of Lakes Huron (H), Michigan (M), and Superior (S).

Stream	N	LD	a	b	c	d	e	f	g	h	i	j	k	l	m	% Correct
Bad River (S)	15	a	**12**		1					1				1		80
Black Mallard River (H)	12	b		**12**												100
Boardman River (M)	10	c			**6**	1		1		1					1	60
Brule River (S)	15	d				**15**										100
Ford River (M)	11	e		1			**9**					1				82
Loeb Creek (M)	10	f	1		1		1	**6**			1					60
Musquash River (H)	25	g							**25**							100
Nottawasaga River (H)	30	h	1							**28**					1	93
Rifle River (H)	13	i					1			1	**4**	4			3	31
Saginaw River (H)	28	j				3					4	**17**			4	61
Silver Creek (H)	15	k											**15**			100
St. Marys River (S/H)	30	l		1		1	1							**27**		90
White River (M)	10	m					1				1	1			**7**	70
Average % correct																82

Note: Letter designations (LD) on the left of the table correspond to those along the top of the table. Letters following stream names (H, M, S) denote the lake into which each tributary drains. The number of correctly classified individuals is shown in bold along the diagonal for each stream. Sample size (N) and the percentage of correct classifications (% Correct) also are reported for each stream. Numbers of misclassified individuals are identified in off-diagonal cells. Streams b, f, i, and l also were used in the comparison of PIXE and LA-ICP-MS.

Comparison of PIXE with LA-ICP-MS

Brothers and Thresher (2004) used PIXE to discriminate among sea lamprey larvae collected in four Lake Huron tributaries ($n = 4$–18 individuals per site): St. Mary's River (two sites), Pigeon River, Rifle River, and Black Mallard River. We collected larvae from the same rivers (but different sites), except Loeb Creek was substituted for the nearby Pigeon River (open circle symbols in Fig. 1). We evaluated similarities in our results using untransformed values for both methods. We then performed two LDFAs (priors set equal in each analysis) to compare results between the two methods.

Among- and within-lake comparisons

We used LDFA to explore whether sufficient elemental variation exists in statoliths to differentiate among individuals spawned in different streams in the Great Lakes. For this analysis, we analyzed sea lamprey larvae from 13 tributaries spanning three Great Lakes (solid circles in Fig. 1). Collection sites included two tributaries from Lake Superior (Bad and Brule rivers), four tributaries from Lake Michigan (Boardman River, Ford River, Loeb Creek, White River), and seven tributaries from Lake Huron (Black Mallard Creek, Musquash River, Nottawasaga River, Rifle River, Saginaw River, Silver Creek, St. Mary's River), with 10–25 individuals per stream (15 individuals were used from Silver Creek based on findings from the Comparison of stream sample sizes; see below). Prior classification probabilities were set equal for all streams. The ability to discriminate among streams was tested using a forward stepwise LDFA. For an element to be included in the final model, its F value had to exceed 1 and its tolerance had to be >0.01. Classification accuracies for individual streams were determined by a jackknifing procedure, conducted as part of the LDFA. All data were log-transformed to ensure normality (Kolmogorov–Smirnov test for normality, all $p \geq 0.20$). Mean total length did significantly differ among streams (range 68–130 mm; $p < 0.001$); however, total length was unrelated to individual statolith elemental concentrations ($r < 0.28$ for all seven elements; C. Hand, unpublished data).

Results

Comparison of laser-ablation approaches

For the seven elements analyzed (Mg, Mn, Zn, Rb, Sr, Ba, and Pb), no significant differences ($\alpha = 0.07$) were found between ablation methods for any element (Table 3), although Mg (<500 ppm difference) and Sr (<20 ppm difference) would have been marginally significant had a Bonferroni correction not been applied. For both Mg and Sr, concentrations were higher in statoliths that were ablated across vs. down through the apex. The LODs did not differ between methods for any element (Table 3). By contrast, we found that the average (±1 SE) ablation analysis time was significantly longer for statoliths that were traversed across (22.9 ± 8.3 s, ranging from 12.7 to 39.8 s) vs. those that were ablated down through the apex (9.4 ± 3.1 s, ranging from 4.2 to 14.9 s; $p < 0.0002$).

Comparison of statolith preservation methods

For both Lewis Creek and the Big Manistee River, no differences were found for Mg, Mn, Zn, Sr, Ba, and Pb in statoliths stored in 95% ethanol vs. those frozen (univariate t tests, all $p > 0.05$). However, for both streams, we found a small but significant difference in Rb, which was higher for larvae preserved in 95% ethanol than for those that were frozen (univariate t tests, both $p \leq 0.005$); the average difference was 0.55 ppm for the Big Manistee River and 0.12 ppm for Lewis Creek.

Comparison of stream sample sizes

Analysis of average elemental signatures generated from bootstrapped Silver Creek samples of varying size ($n = 10$, 15, 20, 24, 30, and 40 individuals) demonstrated no differences for Mg, Mn, Sr, Rb, and Ba (Friedman's ANOVA, all $p \geq 0.20$). Average Zn ($p = 0.04$) and Pb ($p = 0.02$) values,

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Table 3. Comparison of average elemental concentrations (ppm ± SE) and average limits of detection (LOD ± SE) of paired statoliths ablated across the statolith versus down through the statolith's apex (N = 10 pairs) using LA-ICP-MS.

Element	Method	Concentration	p	LOD	p
Mg	Across	3792±148	0.02	9.7±0.8	0.41
	Down	3324±157		10.6±1.2	
Mn	Across	23.8±1.5	0.99	0.41±0.02	0.06
	Down	23.8±2.6		0.45±0.03	
Zn	Across	20.7±6.2	0.56	0.58±0.06	0.10
	Down	29.2±12.7		0.83±0.13	
Rb	Across	3.06±0.69	0.45	0.15±0.01	0.16
	Down	3.21±0.74		0.16±0.01	
Sr	Across	367±51	0.03	0.30±0.04	0.50
	Down	348±49		0.37±0.10	
Ba	Across	19.7±7.0	0.52	0.08±0.03	0.50
	Down	22.5±10.7		0.05±0.01	
Pb	Across	0.20±0.05	0.94	0.024±0.004	0.37
	Down	0.21±0.09		0.020±0.002	

Fig. 2. Box plots of classification accuracies for linear discriminant function analyses (LDFAs) conducted on a five-stream data set containing bootstrapped samples of varying sample size (n = 5, 10, 15, 20, or 25 individuals per stream; n = 500 LDFAs per sample size). Bottom whiskers identify 10th percentiles; top whiskers identify 90th percentiles; the bottoms and tops of the shaded boxes identify 25th and 75th percentiles; horizontal lines inside shaded boxes identify medians; and solid circles identify outliers.

although not significantly different among sample size treatments because of the Bonferroni adjustment of the α level, were both consistently higher in samples of 10 larvae than in subsamples with 15 or more individuals. Further analysis of these bootstrapped means also demonstrated an effect of subsample size on variability in the data. For all elements, the CV decreased significantly with each increasing sample size (correlation: all $r \geq 0.94$, all $p \leq 0.004$).
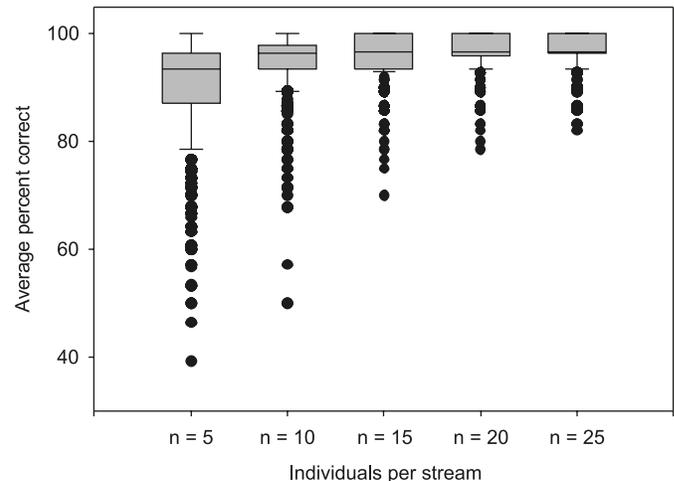
Analysis of bootstrapped samples of varying size (n = 5 to 25 individuals per stream) from our five streams with 25 or more collected larvae revealed that sample size had an effect on our ability to discriminate among streams. Variances in classification success differed (Levene's test: $F = 88$, $p < 0.0001$), with the variance in average classification accuracies being strongly negatively related to sample size (correlation: $r = 0.93$, $p < 0.02$, df = 5; see Fig. 2). Increasing subsample sizes improved classification success. The lowest classification success was 91% for data sets with five individuals per stream, and classification success was ≥95% for data sets with ≥10 individuals per stream (Fig. 2).

Finally, the size of the Silver Creek subsample used in our analyses did not affect our ability to discriminate this stream from all other streams. In all cases, the average classification success of LDFAs ranged from 90% to 100%, regardless of sample size (Silver Creek subsample size, average percent correct: n = 5, 100%; n = 10, 90%; n = 15, 93%; n = 20, 100%; n = 25, 100%; n = 30, 93%; n = 40, 93%; n = 47, 94%).

Comparison of PIXE with LA-ICP-MS

Brothers and Thresher (2004) used PIXE to quantify 10 elements (not including Ca), finding all elements (i.e., Rb, Zn, Pb, Mn, Sr, Fe, Hg, Cu, and Ni) except Ba to be reliable for inclusion in their analyses (i.e., above their detection limits). (Note that measurement precision was seemingly not used as a criterion to determine reliability, which differs from our study.) We quantified 11 elements (Table 1), finding only seven of them (i.e., Mg, Mn, Rb, Sr, Zn, Ba, and Pb) useable, based on our criteria described above. A comparison of those elements that were analyzed by both PIXE

and LA-ICP-MS from the same streams (n = 4) revealed that LA-ICP-MS estimates of Rb, Sr, Pb, and Ba were in the same general range as those of Brothers and Thresher (2004); however, for each of these elements, the measured concentrations were lower with LA-ICP-MS than with PIXE and the measured concentration data were tighter with LA-ICP-MS (Fig. 3). Manganese differed from other elements in that LA-ICP-MS produced lower concentrations and smaller ranges than PIXE for two streams (St. Mary's River and Loeb Creek), whereas the opposite was true for the other two streams (Black Mallard River and Rifle River; Fig. 3). Zinc concentrations also were comparable between methods, though LA-ICP-MS demonstrated a potential for contamination, as indicated by a couple of extreme, high concentrations in the Black Mallard and St. Mary's rivers (Fig. 3).

Similar to Brothers and Thresher (2004), we ran two LDFAs to learn (i) whether the St. Mary's River, an important larval sea lamprey production stream, could be discriminated from the other three streams (all located in lower Michigan) and (ii) whether we could successfully discriminate St. Mary's River fish from those of the lower peninsula of Michigan (the other three streams were pooled). In the first LDFA, our average classification accuracy for the four streams was 88%. We also found that our first two discriminant functions, which explained 95% of the variation in our data, were dominated primarily by Rb, with both Sr and Mn being important (Table 4). Our second LDFA demonstrated that St. Mary's River fish could be distinguished from those from other streams (pooled) in the lower peninsula of Michigan with 89% accuracy, with only 3 of 35 lower peninsula Michigan fish classified as St. Mary's River individuals and only 4 of 30 St. Mary's River fish misclassified.

Among- and within-lake comparisons

A LDFA conducted using 13 streams from Lakes Supe-

Fig. 3. Concentrations (parts per million) of magnesium (Mg), zinc (Zn), rubidium (Rb), strontium (Sr × 100), lead (Pb), and barium (Ba), as measured by PIXE (from Brothers and Thresher 2004) and LA-ICP-MS, from four Lake Huron and Lake Michigan streams: (*a–f*) St. Mary's River, (*g–l*) Black Mallard River, (*m–r*) Rifle River, and (*s–x*) Pigeon River (PIXE) / Loeb Creek (LA-ICP-MS).
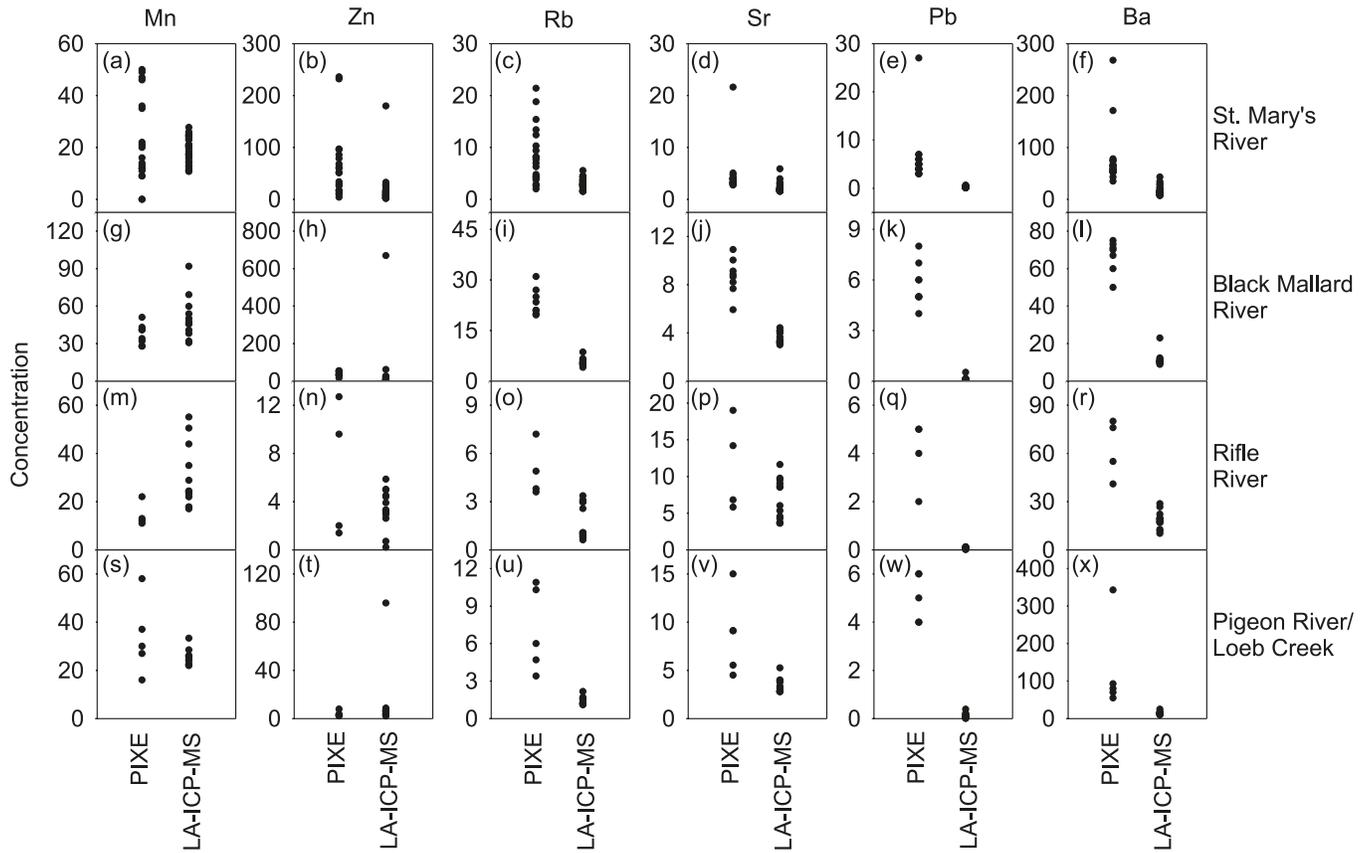

Table 4. Standardized canonical scores for discriminant functions (DF) 1 and 2, from analysis of St. Mary's River versus three other streams in the lower peninsula of Michigan, using LA-ICP-MS.

Element	DF 1	DF 2
Rb	**–0.891**	0.094
Sr	**1.223**	–0.273
Mn	–0.349	**–0.797**
Ba	–0.695	0.119
Zn	–0.336	–0.578
Pb	0.333	0.634
Mg	0.164	–0.167
Cumulative proportion	0.641	0.955

Note: The cumulative proportion of variation explained also is provided. Bolded values indicate values most strongly associated with each discriminant function (DF).

rior, Huron, and Michigan allowed us to classify larvae back to their correct stream with an average accuracy of 82%, with individual stream accuracies ranging from 31% to 100% (Table 2). Eight of the 13 streams had ≥80% correct reassignment, including two streams from Lake Superior, one from Lake Michigan, and five from Lake Huron (Table 2). We illustrate the difference between two streams with good separation (Brule River and Black Mallard River)

and two streams that consistently were misclassified as one another (Rifle River and Saginaw River) (Fig. 4). Inclusion of all seven elements (Mg, Mn, Zn, Rb, Sr, Ba, and Pb) in our all-streams LDFA suggests that each was important in explaining variation among streams; however, we found that Rb, Sr, and Ba were the most important elements, dominating discriminant functions 1 through 3, which explained 83% of the variation in the data (Table 5). Magnesium, Pb, and Zn were largely unimportant for discriminating among sites, being most related to axes that explained relatively little variation (<7%), and Mn was somewhat more important (Table 5).

Discussion

Our investigation indicates promise for statolith microchemistry as a technique to discriminate among sea lamprey produced in different streams, similar to the results of Brothers and Thresher (2004). Further, as we explain below, this technique seems robust in that there appears to be considerable flexibility in sample-size requirements and methods for statolith preservation, ablation, and analysis (LA-ICP-MS vs. PIXE).

Comparison of laser-ablation approaches

We found no statistically significant difference between concentrations of Mg, Mn, Zn, Rb, Sr, Ba, and Pb in statoliths ablated across (horizontal traverse) versus those ablated

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Fig. 4. Linear discriminant function (DF) 1 versus DF 2, which explained 70% of the variation among three Lake Huron (H) and one Lake Superior (S) streams. The rivers shown are the Black Mallard (H, solid triangles), Saginaw (H, open squares), Rifle (H, solid circles), and Brule (S, open circles).
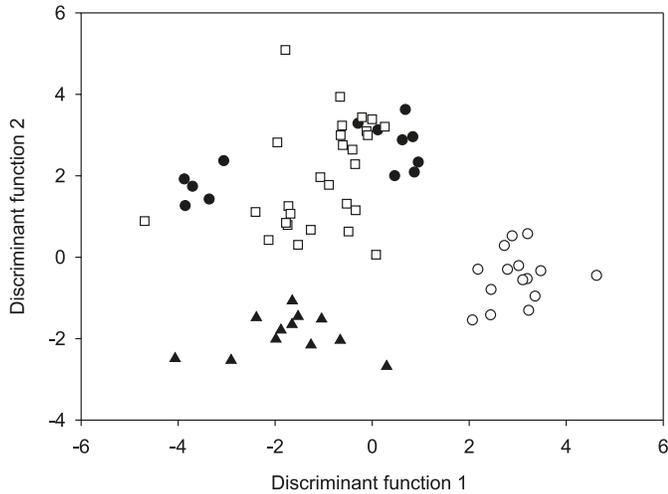
through the apex (vertically). This result was expected given that larval sea lamprey burrow into stream sediments for the entire larval period (Volk 1986). Thus, based on this criterion, it appears that either approach could be used to measure elemental concentrations.

Visual inspection of LODs between ablation approaches suggests that vertical ablation produces higher LODs than those obtained by traversing the width of the statolith. This result is intuitive given that average dwell times on statoliths were approximately threefold longer when traversing across (i.e., more material is ablated) vs. drilling down through the apex and our sample LODs are, in part, based on the amount of material ablated (i.e., LODs decrease as ablated material increases; Ludsin et al. 2006). Importantly, however, the differences in LODs were not statistically significant. These findings ultimately suggest that both techniques could be used to ablate statoliths. Even so, we recommend using a horizontal traverse (which we used for all other analyses) because it may lead to lower LODs for less abundant elements and it allows more of the larval statolith (both base and apex material) to be analyzed, likely enabling more of an individual sea lamprey's life to be integrated.

Comparison of statolith preservation methods

The issue of preservation is of particular concern for the use of statolith microchemistry as a tool, as it is logistically difficult and cost prohibitive for regulatory agencies to preserve adults and larvae using the same method. For collections of larvae, it is difficult to carry a cooler to remote field locations because of space and energy limitations, whereas for adult collections, it is not cost effective to store bodies in 95% ethanol because of their large size. Fortunately, analysis of statoliths from the two storage techniques demonstrated only minor differences between freezing and 95% ethanol storage. Specifically, we found no differences in concentrations of Mg, Mn, Zn, Sr, Ba, and Pb, which is consistent with findings from previous otolith–statolith investigations (Milton and Chenery 1998; Brothers and

Thresher 2004; Hedges et al. 2004), with the exception of Rb, which was slightly higher in ethanol-preserved fish than in those that were frozen. Although this difference is statistically significant, the biological significance is unclear. We believe, based on limited testing of sea lamprey from two streams, that the observed difference in average Rb concentrations is not large enough in magnitude to degrade the discriminatory power of this element in linear discriminant function analyses. Our two test streams support this idea, given that the mean difference in Rb concentration between freezing and ethanol storage within our two study streams was <0.53 ppm, whereas the mean difference in Rb between our two test streams was >1.4 ppm. Additionally, Hand (2006) found that the average Rb concentrations from >40 streams across Lake Huron ranged from 0.63 ppm to 8.36 ppm, indicating that differences of <0.53 ppm resulting from storage technique are unlikely to play a major role in discriminating among streams. Although we still would recommend that all specimens, both adults and larvae, be preserved in the same manner (if possible), our results suggest that the current practice of preserving larvae in ethanol and adults in a freezer is acceptable.

Comparison of stream sample sizes

The Great Lakes Fishery Commission is currently seeking a cost-effective method for identifying which natal streams are producing the most parasitic- and spawning-phase sea lamprey. Owing to the large size of the Laurentian Great Lakes, applying statolith microchemistry will require comparison of a large number of streams (e.g., Lake Huron has >60 tributaries draining into it). For this reason, identifying the smallest possible sample size that will still accurately represent individual streams would be of great benefit by reducing processing time and costs.

Our suite of analyses suggests that researchers will have a lot of flexibility in choosing how many larvae should be processed per stream. For example, our combined bootstrapping–LDFA analysis indicated that sample sizes as low as five individuals per stream could result in an average discrimination success of 91%, whereas analyses with 10 or more individuals per stream would provide discrimination success of ≥95%. Further, the sample sizes used to represent Silver Creek in a suite of seven LDFAs (5–40 individuals per stream) had no effect on our ability to discriminate this stream from all others. This result likely arose because elemental signatures of Silver Creek larvae did not differ among samples of varying size (10–40 individuals per stream). However, our ability to discriminate fish might have been significantly reduced had elements such as Zn and Pb been more important for discriminating among sites in this analysis. Both elements had marginally higher concentrations in subsamples of 10 individuals than in subsamples generated with 15 or more individuals; no differences were found for means generated from sample sizes of 15–40 individuals for any element.

Given these findings, we recommend using a minimum sample size of 15 larvae per stream. Average classification accuracies only improved by <2% with sample sizes of 20–25. Also, sample sizes required will likely increase as the number of sites to be discriminated increases or differences among sites decreases. Thus, sample sizes ultimately should

Table 5. Factor correlations from canonical analysis of 13 streams within the watersheds of Lakes Huron ($n = 7$), Michigan ($n = 4$), and Superior ($n = 2$).

	Discriminant functions						
Element	1	2	3	4	5	6	7
Rb	–0.331	**–0.760**	0.271	0.522	0.078	0.187	–0.057
Sr	**–0.926**	**0.771**	0.213	0.247	0.054	–0.201	0.192
Ba	0.617	–0.462	**–0.918**	0.443	0.165	0.184	0.177
Mn	–0.476	–0.159	–0.351	**–0.828**	–0.254	0.113	0.063
Zn	0.237	–0.058	0.449	–0.233	**0.551**	0.214	**0.788**
Mg	–0.182	0.162	–0.087	–0.157	0.771	–0.039	**–0.619**
Pb	–0.053	–0.225	–0.116	–0.007	–0.266	**–1.047**	–0.109
Cumulative proportion	0.423	0.696	0.830	0.936	0.974	0.995	1.000

Note: The cumulative proportion of variation explained by each axis is presented. The use of bold type indicates the elements most strongly correlated with that discriminant function.

be determined for each study by considering the number of spawning areas and the degree of signature overlap among them.

Our analyses also demonstrate that subsample size can influence variance structure in the data. Specifically, we found that no single transformation could normalize data for any element across our suite of analyses, primarily because of the subsample sizes being used. For example, in our analysis of the effect of laser-ablation technique (e.g., horizontal traverse vs. drilling down through the apex), no data transformations were necessary to normalize data for any element ($n = 5$ larvae per stream), whereas $\log_{10}$ transformations were required to normalize microelemental data for our preservation-method comparison ($n = 30$ individuals per stream). Similar, different transformations were required to normalize data in our bootstrapped samples from Silver Creek, whereas no single transformation could normalize data for our five streams for any element across all subsample-size groupings. Thus, although we feel that the results of our subsample-size comparison are robust, because of the large number of bootstrapped samples used, our analysis is likely imperfect. Given these findings, researchers need to recognize that variable distributions are highly dependent on subsample size (e.g., we found CVs to decrease with increasing sample size) and that the choices made with regard to transformations used to normalize microelemental data need to be considered with great care.

Comparison of PIXE with LA-ICP-MS

Overall, comparison of our results with those of Brothers and Thresher (2004) indicate that both PIXE and LA-ICP-MS are suitable for trace-elemental analysis of statoliths, but also that neither is perfect. For both methods, concentrations of elements were comparable, though LA-ICP-MS consistently exhibited a tighter range of concentrations (i.e., was more precise) for several elements, including Rb, Sr, Pb, and Ba. In addition, LA-ICP-MS consistently produced lower concentrations of Rb, Sr, Pb, and Ba than PIXE, which may indicate sample contamination stemming from the apparently less rigorous cleaning protocol used by Brothers and Thresher (2004) than we used, rather than differences arising from analytical technique. By the same token, occasional high Zn values (>600 ppm) arising from LA-ICP-MS also may be due to sample contamination (Brophy et al. 2003; Ludsin et al. 2006), not analytical error,

even after rigorous cleaning. Aside from possible contamination, differences in elemental concentrations between methods may be due to differences in the exact collection sites within a stream, as well as the year in which larvae were collected (i.e., interannual variation in chemical signatures). Because larvae burrow in the sediment for 3–7 years and the local geology is consistent over a short-term (i.e., annual) scale, we assumed that any annual differences would not greatly influence this comparison.

Brothers and Thresher (2004) also ran a LDFA to learn whether the St. Mary's River, an important larval sea lamprey production stream, could be discriminated from the other three streams (all located in lower Michigan). They were able to accurately classify 89% of the fish to their natal stream. In their LDFA, the first two discriminant functions were dominated by Rb, with additional discriminant functions showing Fe, Mn, Zn, and Sr as being important for discrimination. In our similar analysis, our average classification accuracy for the four streams was 88%. We also found that our first two discriminant functions, which explained 95% of the variation in our data, were dominated primarily by Rb, with both Sr and Mn being important. Thus, both studies concur that Rb is the most important discriminator, regardless of method, and that Mn and Sr also are important.

Brothers and Thresher (2004) also successfully discriminated the St. Mary's River from the lower peninsula of Michigan (the other three streams were pooled). In fact, they were able to discriminate the St. Mary's River with 94% accuracy, with only 1 of 18 St. Mary's River larvae misclassified as being from the lower peninsula. Likewise, only 1 of 17 samples from the lower Michigan peninsula was misclassified as a St. Mary's River fish. We found similar (89%) discrimination success with LA-ICP-MS, with only 3 of 35 lower peninsula Michigan fish classified as St. Mary's River individuals and only 4 of 30 St. Mary's River fish misclassified.

A number of previous studies have demonstrated that both PIXE and LA-ICP-MS are capable of analyzing a large number of elements at low concentrations. Likewise, both techniques can be limited by individual element LODs. For example, although PIXE was capable of estimating Fe concentrations in statoliths (Brothers and Thresher 2004), LA-ICP-MS was not, owing to its low abundance in statoliths and high LOD. Similar difficulties in estimating Fe concen-

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trations in teleost otoliths have been demonstrated (Campana et al. 1997). Conversely, LA-ICP-MS was able to precisely and accurately estimate Ba, an element that PIXE is unable to analyze at low levels (Campana et al. 1997; Brothers and Thresher 2004). Given the strengths and weaknesses of both approaches, we recommend that researchers take care in deciding which technique to use and glean information from previous studies. For example, previous otolith microchemical research in the Great Lakes has demonstrated that Ba is an important element for discriminating among yellow perch (*Perca flavescens*) spawning locations in Lakes Superior and Erie (Brazner et al. 2004; Ludsin et al. 2006). As such, future research investigations involving yellow perch (on these systems) may want to use LA-ICP-MS to ensure that Ba is reliably quantified. By contrast, investigations in which elements such as Hg, Ni, and Fe may be important might want to lean toward using PIXE.

Three other major differences between analyzing statoliths using PIXE versus LA-ICP-MS are notable: (*i*) time required for analysis, (*ii*) invasiveness of the technique, and (*iii*) cost and availability. Excluding sample preparation time, PIXE requires between 10 and 15 min for each sample, whereas LA-ICP-MS requires <3 min. This difference in time may prove crucial, particularly when taking into account the number of replicates required per location to apply the tool to fisheries management problems. PIXE is a relatively nondestructive technique, whereas LA-ICP-MS destroys the sample as it ablates it. Thus, PIXE can allow for statoliths–otoliths to be examined for other purposes (e.g., aging, growth determination) after analysis, whereas LA-ICP-MS requires these determinations to be made prior to analysis (or use of the second statolith–otolith). In addition to these technical differences between methods, a major advantage of LA-ICP-MS is the wider availability of machines and lower cost for analysis than PIXE, logistics that will greatly influence the implementation of statolith microchemistry as a technique by fisheries management agencies. Ultimately, although both techniques are suitable for this type of analysis, we recommend the use of LA-ICP-MS when analyzing trace elements in sea lamprey statoliths because of the savings of time and money and because of the likely importance of Ba for discrimination in freshwater systems.

Among- and within-lake comparisons

Our analysis of sea lamprey producing streams from Lakes Huron, Superior, and Michigan demonstrates promise for statolith microchemistry as a tool for discriminating among spawning tributaries within the Great Lakes. Overall, individual streams from these three lake basins could be discriminated with about 80% classification accuracy. Importantly, however, our ability to discriminate among lakes, when all streams within a lake were grouped into a single category (i.e., Huron vs. Superior vs. Michigan), was only about 60% (Hand 2006), indicating that stream elemental signatures within a lake basin also vary substantially. Thus, no single stream could represent an entire Great Lake. The fact that we could discriminate among individual streams within Lakes Huron, Michigan, and Superior with almost 20% higher accuracy demonstrates that stream-specific signatures indeed differ within each of these systems and that local geology, watershed runoff, and pollutant sources may

overwhelm regional, basin-wide geology. This finding indicates that sufficient variation may exist to discriminate among natal streams of parasitic- and spawning-phase sea lamprey in any single Great Lake, as supported by previous Great Lakes otolith microchemistry studies that have successfully discriminated among local spawning locations. For example, Brazner et al. (2004) found that age-0 yellow perch from different Lake Superior wetlands could be discriminated with an average classification accuracy of 76%, and Ludsin et al. (2006) could discriminate larval yellow perch from different spawning areas in Lake Erie with 69%–100% accuracy.

Our ability to discriminate among streams was imperfect; several streams share a common signature. This commonality may be dictated by regional environmental factors that influence a stream, such as local geology or location in a watershed, as the larval period is spent burrowing in sediment. For example, we found that 4 of 13 Great Lakes tributaries (Loeb Creek, Rifle River, Saginaw River, and White River) located in close proximity to one another in the lower peninsula of Michigan were consistently misclassified as one another, most likely because they shared a common geological source. By contrast, two streams (Nottawasaga and Musquash rivers) located in relatively close proximity around Georgian Bay, but in different watersheds and geology, were not misclassified as one another and were almost perfectly differentiated. From these results, we can see that relative geographical location holds little bearing over our ability to discriminate, but that local geology and watershed influences can be important. One possible approach that could effectively address the local environmental commonalities, when combined with otolith microelemental analysis, is statolith Sr isotope analysis, a technique that has proven effective for differentiating among juvenile Atlantic salmon (*Salmo salar*; Kennedy et al. 2000).

Though we have reached many conclusions regarding statolith microchemistry as a technique, several issues still need to be addressed prior to its implementation into control efforts. All tests performed herein were carried out using larval sea lamprey, working under the assumption that the analogy between otoliths and statoliths extends to all properties. That acknowledged, the question of whether physiological changes experienced by sea lamprey during metamorphosis significantly impact the statolith still remains. The results presented here are conclusive for larvae and invite further research regarding parasitic- and spawning-phase adults.

Overall, our results indicate that statolith microchemistry shows strong potential as a technique for discriminating among individual sea lamprey produced in different spawning tributaries, especially those of the Great Lakes. Although PIXE is a valid method of quantifying statolith trace-elemental composition and should be used in investigations where elements such as Hg, Ni, and Fe are likely to be important, we advocate using LA-ICP-MS over PIXE for freshwater investigations (including statolith microchemistry) because it (*i*) more precisely measures concentrations of elements often times important for discrimination (e.g., Rb, Sr, Pb, and Ba), (*ii*) is more widely available, (*iii*) is faster, and (*iv*) has lower analytical costs. In using LA-ICP-MS, we recommend a minimum sample size of 15 individu-

als per stream and suggest that both freezing and 95% ethanol can be used to preserve fish (and still be comparable). Finally, despite its promise as a tool for discriminating among local sea lamprey producing tributaries, our findings also suggest that statolith microchemistry may not provide perfect discrimination for all streams owing to the likely influence of local geology and watershed effects. We therefore recommend further research to fully explore the impact of these factors on discrimination abilities, other potential limitations of the application of the technique (e.g., interannual variability, physiological effects), and whether other approaches (e.g., statolith Sr isotope analysis; Kennedy et al. 2000) could be used in combination with statolith elemental concentrations to improve our ability to discriminate among individual streams.

Acknowledgments

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