NATIVE *Ichthyomyzon* LAMPREYS OF THE GREAT LAKES BASIN: DEVELOPMENT OF GENETIC MARKERS AND A MORPHOLOGICAL KEY TO AMMOCOETES

Final Report submitted to the Great Lakes Fishery Commission

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TABLE OF CONTENTS

EXECUTIVE SUMMARY

The native lampreys of the Great Lakes appear to be declining in parts of the drainage and, as a result, *Ichthyomyzon fossor* and *Ichthyomyzon unicuspis* have been given special conservation status by several jurisdictions. A major barrier to truly understanding current distributions and populations trends in the native lampreys is the difficulty in identifying ammocoetes to species, particularly *Ichthyomyzon* species. Several keys have been proposed in the past and have been primarily based on meristic, morphometric and pigmentation characters (e.g. Hubbs and Trautman 1937, Vladykov and Kott 1980, Lanteigne 1981, Fuiman 1982). However, these keys often contradict one another and are generally not useful in the field. A likely source of error in these keys is the erroneous identification of the ammoecoetes used in the original datasets to create the keys. We proposed to develop a key that can be readily used in the field, and is based on external features measured on ammocoetes identified to species by genetic analysis. The objectives of the completed research were: 1) to develop a key to the ammocoetes of the Great Lakes readily useable in the field; and, 2) to develop genetic markers for the native lampreys of the Great Lakes.

To develop a morphological key and to examine geographic variation, 1461 ammocoetes and 139 adult samples of each native lamprey species were obtained from each of the Great Lakes basins (where present) with the assistance of control agents. The original plan was to develop genetic markers for each species using adult specimens, and then use the markers to identify each ammocoete to species. Although the chestnut lampreys was readily distinguishable using mitochondrial sequence and RFLP analyses, northern brook lamprey and silver lamprey could not be distinguished from one another using mitochondrial sequence data (10,255 bp), mitochondrial RFLP, or nuclear ITS sequence analysis (Appendix 1). This raises the question of

whether or not the north brook lamprey and silver lamprey are different species or simply different ecomorphotypes.

Another means of distinguishing between the ammocoetes of these species was required. Based on previous studies, ooyctes counts may differ substantially between parasitic and non-parasitic paired species. A total of 104 ammocoetes were sectioned to obtain oocyte counts. Although the oocyte count was substantially lower in specimens from streams known to only contain northern brook lamprey adults, there was no clear separation in counts in specimens from streams containing adults of both species (Appendix 2).

Morphological and pigmentation measurements were derived from digital images of 1461 live ammocoetes. Morphometric, geometric (based on truss measurements) and pigmentation measurements were analyzed using univariate and multivariate (principal components analysis (PCA), discriminant function analysis (DFA) and MANOVA) analyses (Appendix 2). The potential effect of size was controlled by performing the analyses on subsets of similar-sized specimens. The DFA was performed on the entire dataset using two groups based on genetics (chestnut lamprey vs. others) and on a subset of the dataset three groups based on specimens from three streams believed to have only one of the three species. Although some significant differences were identified among groups using the univariate analyses, none of the measurements were different enough to act as a diagnostic character in a key. The exception was the presence of pigmented lateral line organs in only chestnut lampreys greater than 105 mm. Similarly, the multivariate analyses failed to identify any strong differences among the three species.

To determine the influence of preservation methods, a subset of the measurements were repeated after 3 weeks, 6 weeks, 3 months and 6 months of preservation in ethanol and formalin.

A significant change in many of the measurements was detected at 3 weeks; however, little additional change was found after 3 weeks (Appendix 3). Therefore, morphological and pigmentation characters differ between live and preserved specimens.

In conclusion, we were unable to identify any genetic, gonadal, morphological, meristic or geometric characters that could unequivocally separate ammocoetes of silver lamprey and northern brook lamprey. The chestnut lamprey can be distinguished from the other two species using mtDNA analyses, or the lateral line pigmentation character in specimens larger than 105 mm. The lack of variation in the morphological, meristic and geometric characters would make it difficult to develop a diagnostic key based on external features even if species identity could be independently verified.

PROBLEM STATEMENT AND OBJECTIVES

The native lampreys of the Great Lakes appear to be declining in parts of the drainage and, as a result, *Ichthyomyzon fossor* and *Ichthyomyzon unicuspis* have been given special conservation status by several jurisdictions. A major barrier to truly understanding current distributions and populations trends in the native lampreys is the difficulty in identifying ammocoetes to species, particularly *Ichthyomyzon* species. Several keys have been proposed in the past and have been primarily based on meristic, morphometric and pigmentation characters (e.g. Hubbs and Trautman 1937, Vladykov and Kott 1980, Lanteigne 1981, Fuiman 1982). However, these keys often contradict one another and are generally not useful in the field. A likely source of error in these keys is the erroneous identification of the ammoecoetes used in the original datasets to create the keys. We propose to develop a key that can be readily used in the field, and is based on external features measured on ammocoetes identified to species by genetic analysis. The objectives of the proposed research are: 1) to develop a key to the ammocoetes of the Great Lakes readily useable in the field; and, 2) to develop genetic markers for the native lampreys of the Great Lakes. This proposal is submitted at the request of the GLFC Research Priorities Working Group.

RATIONALE AND RELEVANCE TO COMMISSION OBJECTIVES

As part of its Strategic Vision, the Great Lakes Fishery Commission (GLFC) is committed to undertake research in support of healthy Great Lakes ecosystems (Taylor 1995). One component of this commitment is to "identify and characterize anthropogenic causes of loss of species ..." (Taylor 1995). As part of its "Integrated Management of Sea Lamprey Vision", the GLFC is also concerned about mortality of nontarget species associated with the use of lampricides (Taylor 1995). The native lampreys are one group of nontarget species that are probably significantly impacted by lampricide use and may be impacted by control barriers. The inability to identify ammocoetes to species is a major impediment to fully understanding the impact of lampricide and barriers on native lampreys. A key to ammocoetes will allow lamprey assessment crews to identify native ammocoetes to species in the field. This will result in a better understanding of the distribution of, and population trends in, native lampreys. This information can then be used to identify the possible impacts of sea lamprey control (e.g. barriers, lampricide) on native lampreys. Therefore, the proposed research addresses and integrates the GLFC vision statements on healthy Great Lakes ecosystems and integrated management of sea lamprey.

DELIVERABLES

Deliverable 1. *A morphological key to native lamprey ammocoetes of the Great Lakes basin.*

This study failed to identify any morphological or pigmentation variables that could be used as diagnostic characters in a morphological key to the native lampreys of the Great Lakes basin. Appendix 2 outlines the analyses undertaken in an attempt to identify such characters.

Deliverable 2. *A set of genetic markers for the native lampreys of the Great Lakes basin.*

Appendix 1 outlines the development of a set of genetic markers for the native lampreys of the Great Lakes basin.

Deliverable 3. *Annual report and final report.*

This document constitutes the final report. The annual report for 2002/2003 is found in Appendix 1.

Deliverable 4. *Graduate student thesis.*

Appendices 2 and 3 constitute the bulk of Fraser Neave's M.Sc. thesis to be submitted to the University of Guelph and defended in Summer 2004.

Deliverable 5. *Corresponding publications.*

Three publications are planned: 1) genetic markers; 2) morphological and pigmentation analysis; and, 3) preservation effects on morphological and pigmentation characters. These manuscripts will be submitted by December 2004.

REFERENCES

Fuiman, L.A. 1982. Family Petromyzontidae, lampreys. Pp. 23-37. In: N.A. Auer (ed.). 1982. Identification of larval fishes of the Great lakes basin with emphasis on the Lake Michigan drainage. Great Lakes Fish. Comm. Spec. Publ. 82-3.

Hubbs, C.L. and M.B. Trautman. 1937. A revision of the lamprey genus Ichthyomyzon. Misc. Pub. Mus. Zool. Univ. Mich. 35.

Lanteigne, J. 1981. The taxonomy and distribution of the North American lamprey genus *Ichthyomyzon*. MSc. Thesis. University of Ottawa.

Taylor, W. W. 1995. Strategic vision of the Great Lakes Fishery Commission for the decade of the 1990s. Great Lakes Fish. Comm. Misc. Rept. 40 p.

Vladykov, V.D. and E. Kott. 1980. Description and key to metamorphosed specimens and ammocoetes of Petromyzontidae found in the Great Lakes region. Can. J. Fish. Aquat. Sci. 37: 1616-1625.

APPENDIX 1

NATIVE *Ichthyomyzon* LAMPREYS OF THE GREAT LAKES BASIN: DEVELOPMENT OF GENETIC MARKERS AND A MORPHOLOGICAL KEY TO AMMOCOETES

Annual Progress Report submitted to the Great Lakes Fishery Commission

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INTRODUCTION AND SUMMARY

This report comprises our summary of progress for year 1 of a project entitled *Native Ichthyomyzon Lampreys Of The Great Lakes Basin: Development Of Genetic Markers And A Morphological Key To Ammocoetes*. Over the past year substantial progress has been made on both the ammocoete key and the genetic markers. For the development of the larval key, ammocoetes were collected from four of the five Great Lakes (Lake Ontario was excluded due to very low numbers of *Ichthyomyzon*), and were digitally imaged for morphometric analysis and sampled for subsequent genetic analysis. The processing of the digital images (meristic and morphometric measurements, and analyzing pigmentation patterns) has commenced, and this analysis of potential diagnostic characteristics will continue throughout the year 2 of the project. Preliminary results lend tentative support to one of the two existing morphological keys, i.e. that chestnut lampreys can be distinguished based on lateral line pigmentation and that caudal fin pigmentation may help differentiate between silver and Northern brook lampreys. The morphological component of this project will form the basis of Fraser B. Neave's M.Sc. thesis at the University of Guelph. In the second component of the study, a genetic marker was developed that easily and unambiguously identified the chestnut lamprey. To develop similar markers to distinguish between silver and Northern brook lampreys (and thus to test the ability of caudal fin pigmentation or other morphometric characters to distinguish between these species), we sequenced 10,255 bp (approximately 60%) of the mitochondrial genome in several juvenile or adult specimens. We found considerable intraspecific variation and no diagnostic differences were present that would permit all populations of the two species to be distinguished. Preliminary results, however, suggest that genetic differences exist between the two species in Lake Michigan tributaries, and genetic work is ongoing in an attempt to develop individual markers for each basin.

PROBLEM STATEMENT AND OBJECTIVES

The native lampreys of the Great Lakes appear to be declining in parts of the drainage and, as a result, *Ichthyomyzon fossor* and *Ichthyomyzon unicuspis* have been given special conservation status by several jurisdictions. A major barrier to truly understanding current distributions and population trends in the native lampreys is the difficulty in identifying ammocoetes to species, particularly *Ichthyomyzon* species. Several keys have been proposed in the past and have been primarily on meristic, morphometric and pigmentation characters (e.g. Hubbs and Trautman 1937, Vladykov and Kott 1980, Lanteigne 1981, Fuiman 1982). However, these keys often contradict one another and are generally not useful in the field. A likely source of error in these keys is the erroneous identification of the ammoecoetes used in the original datasets to create the keys. We propose to develop a key that can be readily used in the field, and is based on external features measured on ammocoetes identified to species by genetic analysis. The objectives of the proposed research are: 1) to develop a key to the ammocoetes of the Great Lakes readily useable in the field; and 2) to develop genetic markers for the native lampreys of the Great Lakes.

RATIONALE AND RELEVANCE TO COMMISSION OBJECTIVES

As part of its Strategic Vision (Taylor 1992), the GLFC is committed to undertake research in support of healthy Great Lakes ecosystems. One component of this commitment is to "identify and characterize anthropogenic causes of loss of species ..." (Taylor 1992). As part of its "Integrated Management of Sea Lamprey Vision", the GLFC is also concerned about mortality of nontarget species associated with the use of lampricides (Taylor 1992). The native lampreys are one group of nontarget species that are likely significantly impacted by lampricide use and may be impacted by control barriers. The inability to identify ammocoetes to species is a major impediment to fully understanding the impact of lampricide on native lampreys. A key to ammocoetes will allow lamprey assessment crews to identify native ammocoetes to species in the field. This will result in a better understanding of the distribution of, and population trends in, native lampreys. This information can then be used to identify the possible impacts of sea lamprey control (e.g., barriers, lampricide) on native lampreys. Therefore, the proposed research

addresses the GLFC vision statements on healthy Great Lakes ecosystems and integrated management of sea lamprey.

PROJECT DELIVERABLES

1. A morphological key to native lamprey ammocoetes of the Great Lakes Basin.
2. A set of genetic markers for the native lampreys of the Great Lakes Basin.
3. One annual report at the end of Year 1 and one final report at the end of Year 2.
4. One graduate student thesis (Fraser B. Neave, Year 2).
5. Corresponding publications (Year 2).

PROGRESS ON DELIVERABLES DURING YEAR 1

Deliverable 1. *A morphological key to native lamprey ammocoetes of the Great Lakes Basin.* The morphological component of this project will form the basis of Fraser B. Neave's M.Sc. thesis at the University of Guelph. Ammocoete collection commenced in May 2002, with the aid of DFO workers at the Sea Lamprey Control Centre in Sault Ste. Marie and United States Fish and Wildlife Service workers from Marquette and Ludington. Spring and summer sampling led to collections of 160, 340, 110, and 180 ammocoetes, respectively, for Lakes Huron, Michigan, Superior, and Erie; Lake Ontario has been excluded due to very low numbers of *Ichthyomyzon*. Each of these 790 specimens was digitally imaged while fresh, and a tissue sample from each individual was preserved in 95% ethanol for future genetic analysis. Half of the ammocoetes were fixed in formalin and transferred to ethanol, and the remainder was left in formalin. In order to examine the effects of preservation over time, a subsample of the preserved ammocoetes was digitally imaged after periods of 3 weeks, 6 weeks, 3 months and 6 months.

The digital images are being analyzed for the existence of diagnostic characteristics (both morphometric and pigmentation measurements) that will be useful in a morphological key. Image analysis has been completed for approximately half of the 790 images of the freshly preserved ammocoetes. Morphometric measurements include eight traditional lengths (total length, head length, branchial length, trunk length, tail length, maximum diameter of trunk,

maximum diameter of branchial region, and height of suprabranchial non-pigmented band). In addition, 11 measurements of pigmentation density are being conducted (on the whole body, head, oral hood, suprabranchial area, sub-branchial area, branchial area, lateral line area, trunk area, dorsal fin, tail area, and caudal fin) on each image.

We have performed preliminary analysis on the pigmentation measurements, having tentatively identified ammocoetes to species based on the historical adult catches from each stream. Identification will be verified when diagnostic genetic markers become available (see Deliverable 2, below). We observed a high degree of variation within each species, but large chestnut lamprey (*Ichthyomyzon castaneus*) ammocoetes could be readily distinguished from silver (*I. unicuspis*) and Northern brook (*I. fossor*) lampreys based on their pigmented lateral lines, a diagnostic characteristic described in other keys (e.g., Lanteigne 1981).

Diagnostic characters described previously to differentiate between silver and Northern brook lampreys, however, were not as straightforward. In particular, the wide (three millimetre) non-pigmented band described by Lanteigne (1981) in the suprabranchial region of the Northern brook lamprey does not appear in ammocoetes in tributaries to Lake Huron and Lake Superior (Figure 1). The widest bands observed so far are approximately one millimetre, and we are reasonably certain that all three species of lampreys have been sampled in this collection. Tributaries to the other Great Lakes have not yet been analyzed. A second characteristic, the degree of caudal fin pigmentation described by Vladykov and Kott (1980), shows more promise. Vladykov and Kott (1980) found that silver lamprey ammocoetes were darker in both the head and caudal fin regions than were Northern brook lamprey ammocoetes, and our preliminary results similarly show potential species-specific differences, at least in the caudal fin. We compared degree of caudal fin pigmentation (mean grey value) in ammocoetes from two streams, Coldwater Creek and Sibley Creek (Figure 2). Based on juvenile and adult capture records (Appendix 1), we know that Coldwater Creek contains both silver and Northern brook lampreys, whereas we are reasonably certain that Sibley Creek is inhabited only by Northern brook lampreys. The greater range of mean grey values in the Coldwater Creek population suggests that the upper values represent the silver lampreys not found in Sibley Creek. If we are able to develop diagnostic genetic markers for ammocoetes from Lake Huron tributaries, we will test

this hypothesis. We will also test the degree of overlap in pigmentation values; at present, there does not appear to be an unambiguous distinction between the two species.

Further image analysis and statistical testing in the second year of the project may point to other potential diagnostic characteristics. Truss measurements have not yet been conducted, but we are now familiar with the software that will allow us to complete these measurements and allow comparison of body forms between the species.

Deliverable 2. A set of genetic markers for the native *Ichthyomyzon* lampreys of the Great Lakes Basin.

To ensure accurate species identification, only juvenile and adult lampreys were used for genetic analysis. A total of 86 Northern brook lampreys (*Ichthyomyzon fossor*) and 47 silver lampreys (*I. unicuspis*), were collected from tributaries to Lakes Huron, Michigan, and Superior, and one Northern brook lamprey and two silver lampreys were collected from Lakes Erie and Ontario, respectively (Appendix 1). Northern brook lampreys from New York state (Allen Brook, St. Lawrence Co.) and Vermont (Lake Champlain) were also included in our genetic analysis. Three chestnut lampreys (*I. castaneus*) were collected from two Lake Michigan streams.

A) Distinguishing Northern Brook and Silver Lampreys

i) *Mitochondrial Sequence Data*: In an attempt to find diagnostic genetic differences between these two species, we sequenced up to 10,255 bp from the mitochondrial genome (Table 1) of seven Northern brook lampreys and five silver lampreys (Table 2). Because we expected significant geographic variation in addition to possible species-specific differences, our strategy involved: i) focusing on a population from Lake Huron (Coldwater Creek) where both species were found (so that any differences between species would not be due to geographic variation); and ii) inclusion of Northern brook and silver lampreys from a large geographic range (Table 2). This allowed us to assess the maximum amount of intraspecific variation expected at a given gene and to test whether potential species-specific differences were observed across both species' ranges.

A total of 17 variable sites were observed in the mitochondrial genome of both species (Table 2). Substitutions at two of these sites were unique to the Northern brook lamprey from Vermont, and

a further eight sites varied between this lamprey and most of the Great Lakes lampreys, with the exception of two silver lampreys from Lake Huron. These eight sites were all correlated, so that the mitochondrial type of these three lampreys (Type B) was quite distinct from others in the Great Lakes Basin (Type A). This divergent mitochondrial type may represent lampreys from a second glacial refugium, and indicates that intraspecific variation in both Northern brook and silver lampreys may be large and as great as interspecific variation.

Of the seven remaining variable sites, six were not diagnostic for species; rather, they showed intraspecific variation within one or both species (Table 2). One site (ND4-807) may be promising, although only two Northern brook lampreys and one silver lamprey have been sequenced to date. More specimens will be sequenced at this gene, and the remainder of the ND5 gene will also be sequenced since this was shown to be the most variable mitochondrial gene; four variable sites were detected in the first 741 bp (of a total of 1797 bp).

ii) *Mitochondrial Restriction Fragment Length Polymorphism (RFLP) Analysis*: Eight of the observed sequence polymorphisms could be differentiated using restriction enzymes (Table 2), thus allowing a large number of specimens to be screened relatively easily (e.g., Figure 3). Five of the sites, those recognizing mitochondrial type B (see above), were correlated so that restriction of any one or two genes (e.g., ND3 and ND6) were sufficient to distinguish between these two divergent mitochondrial lineages. Out of a total of 133 lampreys screened to date, mitochondrial type B was found in a total of five: the Vermont Northern brook lamprey, three silver lampreys from Lake Huron, and one from Lake Ontario (Table 3, Appendix 1).

At the remaining three polymorphic sites that could be analyzed using RFLP analysis (ND5-201, ND5-711, and COI-669), both alleles were found in both species and most populations (Table 3). As the sequencing data indicated, no diagnostic species differences were found at any individual site. A total of nine composite haplotypes were observed when the results of the five RFLP assays were combined (Table 4) and again, there were no diagnostic species differences. A difference in the frequency of each haplotype (Table 5) was observed among populations and species, however, particularly with respect to the ND3/ND6 (i.e. type A vs. B) and ND5 assays (Figure 4). Species-specific differences were significant in Lake Michigan streams ($p = 0.0003$,

exact test for allele frequency distribution differences): only one Northern brook lamprey with possessed mitochondrial type A4 whereas 13 of the 17 (76%) of silver lampreys were observed with this type; likewise, only one silver lampreys possessed the common Northern brook lamprey type. Although these species differences are not diagnostic and remain to be tested in a Lake Michigan stream where both species occur (Appendix 1), they are promising in that they indicate that there is a significant barrier to gene flow between these two species and an increased likelihood that other differences (e.g., in ND4 or the complete ND5 gene or in the nuclear genome) may exist. Given the level of geographic variation observed, however, it is highly unlikely that such differences can be used to distinguish between all populations of the two species, making it necessary to develop markers for each basin.

iii) *Nuclear ITS sequence*:

In order to determine if the internal transcribed spacer region (ITS1) of the nuclear rRNA gene could provide greater resolution than the mitochondrial genome, we sequenced this region in two Northern brook and three silver lampreys (Table 1). We chose lampreys with diverse mitochondrial types (A1b, A2a, and B1a), yet found the ITS1 to be identical in all five specimens. Consequently, this region was not investigated further.

B) Distinguishing Chestnut Lamprey from Northern Brook and Silver Lampreys

i) *Mitochondrial Sequence and RFLP Analysis*: We sequenced 4,363 bp from the mitochondrial genome (Table 1) of one chestnut lamprey from the Manistee River, Lake Michigan, and found a total of 306-316 nucleotide substitutions (7.2%) at these genes relative to Northern brook and silver lampreys. Consequently, dozens of restriction enzymes are available to distinguish chestnut lampreys from the other two species. The most efficient RFLP for our purposes at present is the ND5 *Bst*NI assay (Figure 3) since it is also being used to detect intraspecific variation in Northern brook and silver lampreys. A single assay will recognize the two alleles in Northern brook and silver lampreys, and since a third pattern is characteristic of the chestnut lamprey, will also allow for identification of this species.

Other Deliverables

The remaining deliverables listed above are not expected until Year 2.

REFERENCES

Fuiman, L.A. 1982. Family Petromyzontidae, lampreys. Pp. 23-37. In: N.A. Auer (ed.). 1982. Identification of larval fishes of the Great lakes basin with emphasis on the Lake Michigan drainage. Great Lakes Fish. Comm. Spec. Publ. 82-3.

Hubbs, C.L. and M.B. Trautman. 1937. A revision of the lamprey genus Ichthyomyzon. Misc. Pub. Mus. Zool. Univ. Mich. 35.

Lanteigne, J. 1981. The taxonomy and distribution of the North American lamprey genus *Ichthyomyzon*. MSc. Thesis. University of Ottawa.

Taylor, W. (ed.) 1992. Strategic vision of the Great Lakes Fishery Commission for the decade of the 1990s. Great Lakes Fish. Comm. Misc. Rept. 40 p.

Vladykov, V.D. and E. Kott. 1980. Description and key to metamorphosed specimens and ammocoetes of Petromyzontidae found in the Great Lakes region. Can. J. Fish. Aquat. Sci. 37: 1616-1625.

Table 1. Summary of mitochondrial and nuclear genes sequenced, including total number of base pairs (bp) and specimens sequenced for each of the three species examined (Northern brook lamprey, *Ichthyomyzon fossor*; silver lamprey, *I. unicuspis*; chestnut lamprey, *I. castaneus*).

GENE REGIONS	bp	DETAILS OF REGION SEQUENCED	N		
			fossor	*unicuspis*	*castaneus*
MITOCHONDRIAL					
12S rRNA	1030	tRNA-Pro, tRNA-Phe, 5'-end of 12S rRNA (879/900 bp)	4	2	0
ATPase 6/8	872	Entire ATPase8 (168 bp) and ATPase6 (714 bp)	2	2	0
COI	627	627/1554 bp of COI (positions 61–687)	4	4	0
COII	800	tRNA-Ser, tRNA-Asp, entire COII (690 bp)	3	2	1
COIII	786	Entire COIII (786 bp)	3	2	0
ND1	966	Entire ND1 (966 bp)	4	3	1
ND2	1425	tRNA-Ile, tRNA-Gln, tRNA-Met, entire ND2 (1044 bp), tRNA-Trp, tRNA-Ala	3	3	1
ND3/4L	661	Entire ND3 (351 bp), tRNA-Arg, 5'-end of ND4L (231/291 bp)	3	2	1
ND4	1029	3'-end of ND4 (1029/1377 bp)	2	1	1
ND5	1049	tRNA-His, tRNA-Ser, tRNA-Leu, 5'-end of ND5 (741/1797 bp)	6	3	1
ND6/D-loop	1010	Entire ND6 (519 bp), entire noncoding region II, "D-loop" (491 bp)	5	1	1
Total	10,255				
NUCLEAR					
ITS1	523	3'-end of 18S rRNA (125 bp), entire ITS1 (321 bp), 5'-end of 5.8S rRNA (77 bp)	2	3	0

Table 2. Summary of the 17 variable sites found in the mitochondrial genome of Northern brook and silver lampreys (*I. fossor* and *I. unicuspis*, respectively). Sites are labeled according to gene name and variable nucleotide position, and restriction enzymes that cut differently at the variable sites (producing restriction fragment length polymorphisms, RFLPs) are indicated below each site label. The type of variation detected is noted at the bottom of the table: two sites (VT) detect differences between *I. fossor* from Vermont and all other specimens; eight sites (TypeB) detect a divergent mitochondrial type found in *I. fossor* from Vermot and some silver lampreys from Lakes Huron and Ontario (see text); six sites (Intra) show variation within one or both species; one site (Inter) may show variation between the two species, although this gene has been sequenced in only four specimens to date.

Species	Lake	Stream	12S-419	ATP6-645	COI-669 *Pst*I	COII-327 *Acc*I	COIII-195 *Rsa*I	COIII-507	ND1-273	ND2-984	ND3-180 *Fok*I	ND4-502	ND4-807	tRNA-Leu *Aci*I	ND5-201 *Bst*NI	ND5-271	ND5-573	ND5-711 Bsr*DI*	ND6-72 *Alw*26I
fossor																			
F01	S	Pearl			G				C	A	T			G	G	A	T	A	C
F03	H	Coldwater	A	T	G	A	C	C	C	A	T	G	A	G	A	A	C	A	C
F15	H	Coldwater										A	A						C
F16	H	Coldwater													G	A	T	G	
F35	M	Manistee	A				C	C						G	A	A	T	A	
Ich2	NY		A		A	A			C					G	A	A	T	A	C
F04	VT		G	C	A	G	T	C	T	G	C	A	A	A	A	G	T	A	T
unicuspis																			
U01	O	Cobourg	A		G				C	A	T								
U02	H	Coldwater	A	C	A	G	T	C	T	G	C			A	A	A	T	A	T
U03	H	Coldwater		T	A	A	C	A						G	G	A	T	G	
U04	H	Coldwater							T	G									
U34	H	Coldwater			G							A	G	G	A	A	C	A	
			VT	TypeB	Intra	TypeB	TypeB	Intra	TypeB	TypeB	TypeB	Intra	Inter?	TypeB	Intra	VT	Intra	Intra	TypeB

Table 3. Frequency of indicated nucleotides at variable mitochondrial sites in each species and lake basin (as detected by RFLP). With the exception of one silver lamprey (*I. unicuspis*) from Lake Ontario (where a third COI allele was detected), only two alleles were noted per site (i.e. 0.88 of *I. unicuspis* from Lake Huron possessed TC at the variable sites in ND3 and ND6; the remaining 0.12 possessed CT at these sites).

		N	ND3-180/ND6-72 *Fok*I / *Alw*26I TC	ND5-201 *Bst*NI A	ND5-711 *Bsr*DI A	COI-669 *Pst*I A
Huron	*fossor*	61	1.00	0.31	0.33	0.66
	unicuspis	25	0.88	0.44	0.44	0.68
Michigan	*fossor*	13	1.00	0.31	0.38	0.69
	unicuspis	17	1.00	0.18	0.94	0.82
Superior	*fossor*	12	1.00	0.18	1.00	0.67
Erie	*fossor*	1	1.00	1.00	1.00	0.00
Ontario	*unicuspis*	2	0.50	1.00	1.00	0.00*

* both individuals with G at position 669, but one lacking additional *Pst*I cut site

Table 4. Nine composite mitochondrial (mt) haplotypes detected in Northern brook and silver lampreys by RFLP. Overall mt type is described according to ND3 and ND6 restriction type (which distinguishes two major mitochondrial lineages, A or B), ND5 type (1, 2, 3, or 4), and COI type (a, b, or c).

	ND3-180 / ND6-72 *Fok*I / *Alw*26I	ND5-201 *Bst*NI	ND5-711 *Bsr*DI	COI-669 *Pst*I	mt Type
1	TC	A	A	A	A1a
2	TC	A	A	G	A1b
3	TC	G	G	A	A2a
4	TC	G	G	G	A2b
5	TC	A	G	A	A3a
6	TC	G	A	A	A4a
7	TC	G	A	G	A4b
8	CT	A	A	A	B1a
9	CT	A	A	G*	B1c

*G at COI position 669 but lacking *Pst*I cut site present in types a and b at position 573, thus producing a third COI type

Table 5. Frequency of the nine composite mitochondrial haplotypes detectable by RFLP in each species and lake basin.

		N	A1a	A1b	A2a	A2b	A3a	A4a	A4b	B1a	B1c
Huron	*fossor*	61		0.30	0.65	0.03	0.02				
	unicuspsis	25		0.32	0.56					0.12	
Michigan	*fossor*	13		0.31	0.62			0.08			
	unicuspsis	17	0.18		0.06			0.59	0.18		
Superior	*fossor*	12		0.17				0.75	0.08		
Erie	*fossor*	1		1.00							
Ontario	*unicuspsis*	2		0.50							0.50
NY	*fossor*	1	1.00								
VT	*fossor*	1								1.00	

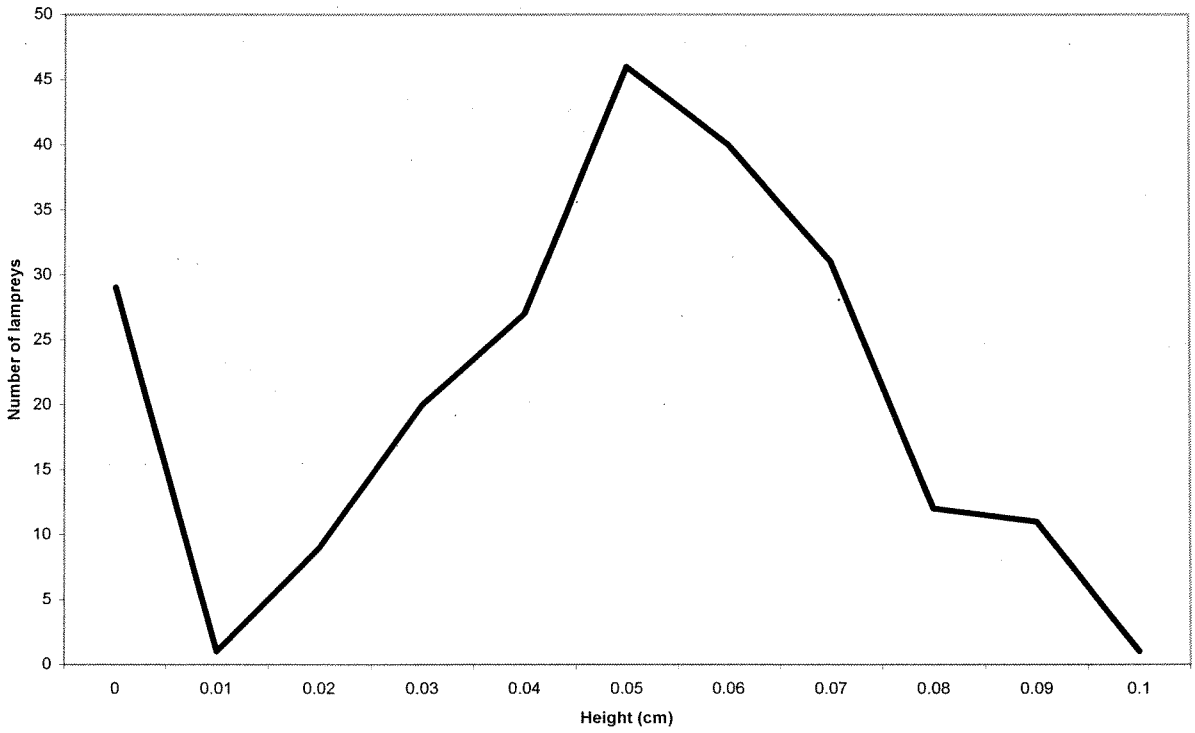


Figure 1. Suprabranchial non-pigmented band in 229 *Ichthyomyzon* ammocoetes of Lake Huron and Lake Superior tributaries.

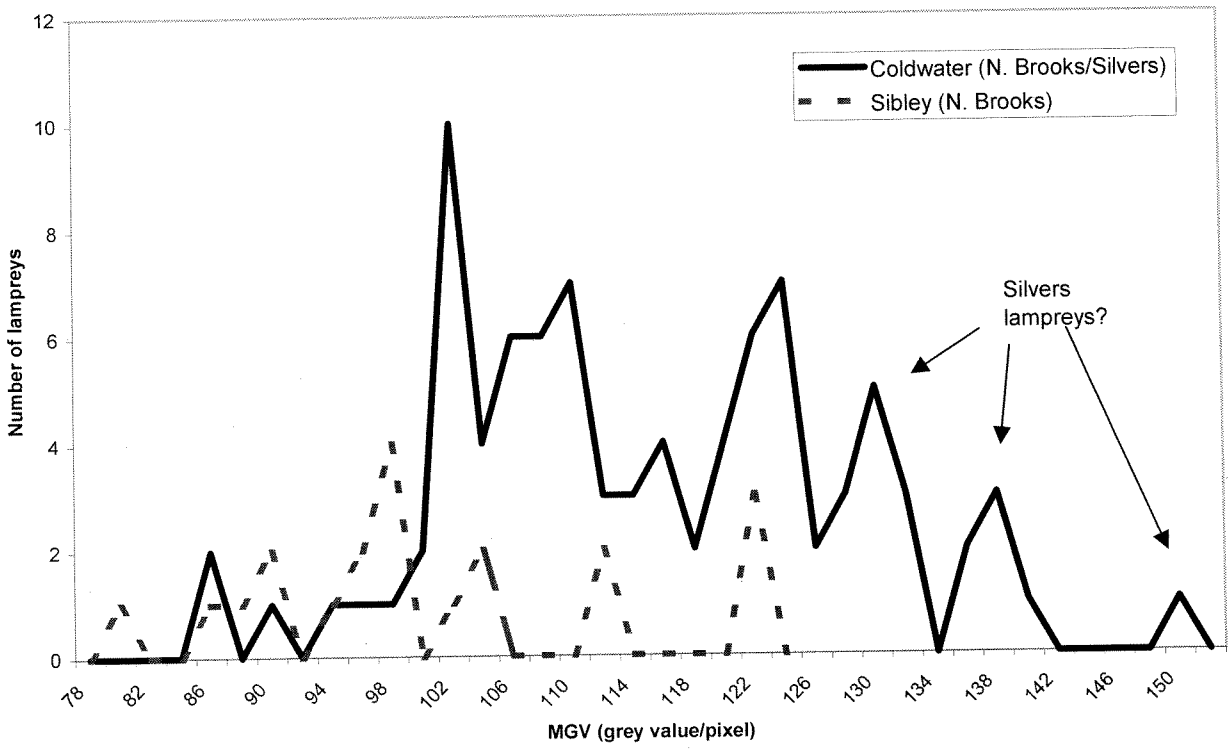


Figure 2. Degree of caudal pigmentation in *Ichthyomyzon* ammocoetes of two streams, Coldwater Creek and Sibley Creek.

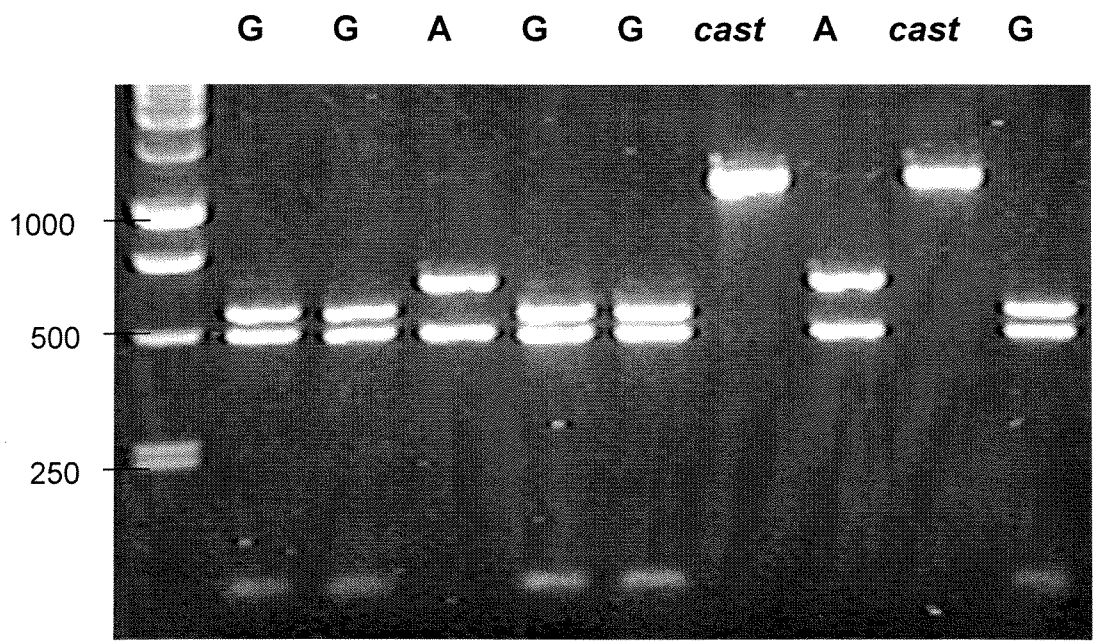


Figure 3. Gel photo showing restriction fragment length polymorphism (RFLP) in ND5 PCR product digested with *Bst*NI. The PCR product is uncut (1119 bp) in the chestnut lamprey (*cast*), cut into three fragments (90, 488, 541 bp) in Northern brook and silver lampreys with a G residue at ND5 position 201, and cut into two fragments (488 and 631 bp) in Northern brook and silver lampreys with an A at position 201. The DNA size standard is in the left lane.

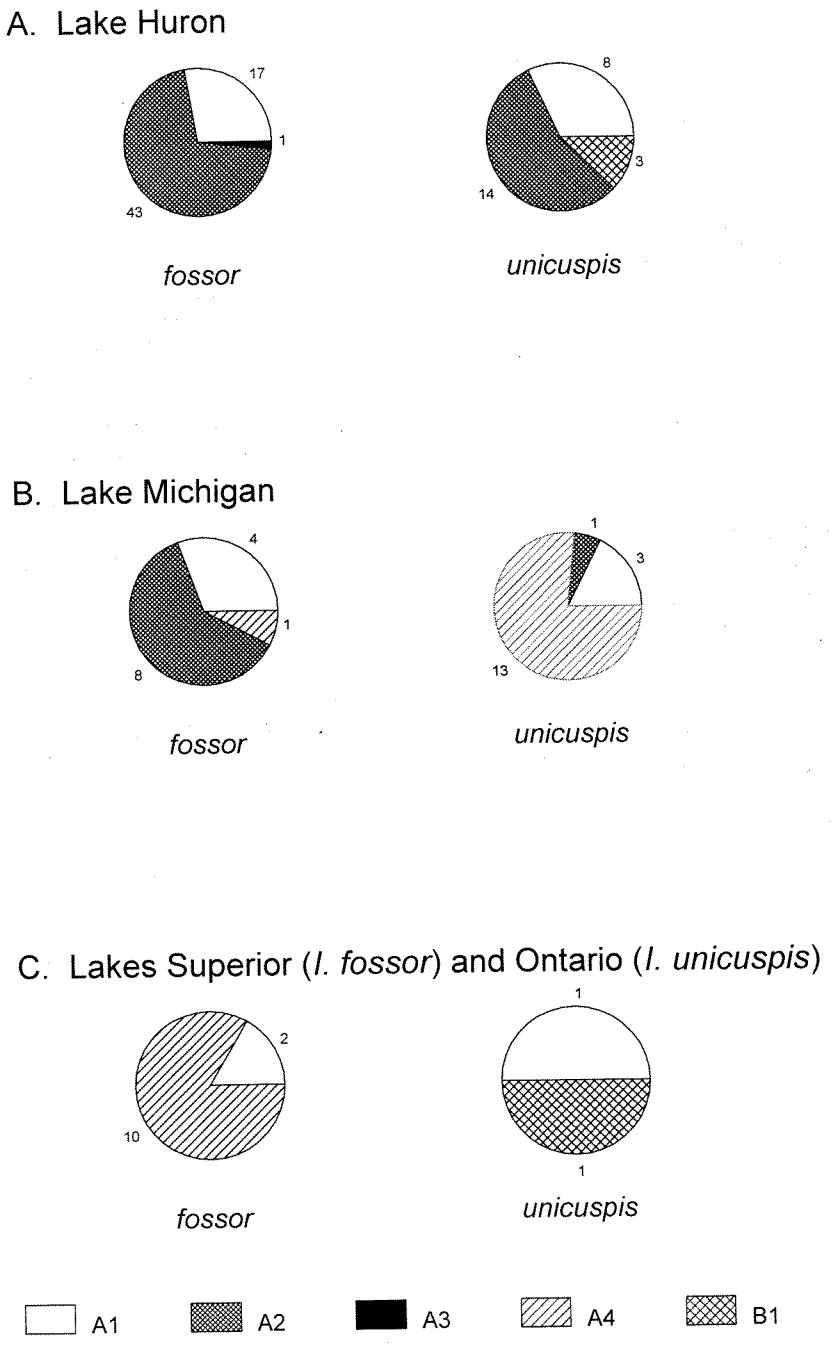


Figure 4. Frequency of the five major mitochondrial haplotypes in Northern brook lamprey (*I. fossor*) and silver lamprey (*I. unicuspis*) in tributaries to Lakes Huron, Michigan, Superior, and Ontario. Northern brook lampreys from Lake Erie, New York, and Vermont (one specimen from each) were types A1, A1, and B1, respectively.

Appendix 1. RFLP data for each Northern brook and silver lamprey (*I. fossor* and *I. unicuspis*, respectively), by individual stream and lake basin (H, M, S, O, and E). Nine composite mitochondrial (mt) haplotypes were detected; mt type descriptions indicate ND3 and ND6 restriction type (which distinguishes two major mitochondrial lineages, A or B), the combined ND5 type (1, 2, 3, or 4), and COI type (a, b, or c). Five silver lampreys were collected from Lake Superior, but DNA extractions have not been successful to date.

Lake	Stream	ND3-180 *Fok*I	ND6-72 *Alw*26I	ND5-201 *Bst*NI	ND5-711 *Bsr*DI	COI-669 *Pst*I	mt Type Number	mt Type Description
GREAT LAKES BASIN								
H	**Coldwater**							
fossor	F-03	T	C	A	A	G	2	A1b
	F-15	T	C	A	A	G	2	A1b
	F-16	T	C	G	G	A	3	A2a
	F-17	T	C	A	A	G	2	A1b
	F-58	T	C	A	G	A	6	A4a
	F-59	T	C	A	A	G	2	A1b
	F-60	T	C	G	G	G	4	A2b
	F-76	T	C	G	G	A	3	A2a
	F-77	T	C	G	G	A	3	A2a
	F-78	T	C	A	A	G	2	A1b
	F-79	T	C	G	G	A	3	A2a
	F-80	T	C	A	A	G	2	A1b
	F-81	T	C	A	A	G	2	A1b
unicuspis	U-02	C	T	A	A	A	8	B1a
	U-07	T	C	G	G	A	3	A2a
	U-08	T	C	G	G	A	3	A2a
	U-09	T	C	G	G	A	3	A2a
	U-10	T	C	G	G	A	3	A2a
	U-34	T	C	A	A	G	2	A1b
	U-35	T	C	A	A	G	2	A1b
	U-36	T	C	A	A	G	2	A1b
	U-37	C	T	A	A	A	8	B1a
	U-40	T	C	G	G	A	3	A2a
	U-41	T	C	A	A	G	2	A1b
	U-42	T	C	G	G	A	3	A2a
	U-43	T	C	G	G	A	3	A2a
	U-44	T	C	A	A	G	2	A1b
	U-45	T	C	G	G	A	3	A2a
	U-46	T	C	A	A	G	2	A1b
	U-47	T	C	G	G	A	3	A2a
H	**Hog Cr**							
fossor	F-13	T	C	G	G	A	3	A2a
	F-14	T	C	G	G	A	3	A2a
	F-19	T	C	G	G	A	3	A2a

Lake	Stream	ND3-180 *Fok*I	ND6-72 *Alw*26I	ND5-201 *Bst*NI	ND5-711 *Bsr*DI	COI-669 *Pst*I	mt Type Number	mt Type Description
	F-44	T	C	A	A	G	2	A1b
	F-55	T	C	G	G	A	3	A2a
	F-56	T	C	G	G	A	3	A2a
	F-57	T	C	G	G	A	3	A2a
	F-61	T	C	G	G	A	3	A2a
	F-62	T	C	G	G	A	3	A2a
	F-63	T	C	A	A	G	2	A1b
	F-64	T	C	G	G	A	3	A2a
	F-65	T	C	G	G	A	3	A2a
	F-66	T	C	G	G	A	3	A2a
	F-67	T	C	G	G	A	3	A2a
	F-68	T	C	G	G	A	3	A2a
	F-69	T	C	A	A	G	2	A1b
	F-70	T	C	G	G			
	F-71	T	C	A	A	G	2	A1b
	F-72	T	C	A	A	G	2	A1b
	F-73	T	C	G	G	A	3	A2a
	F-74	T	C		G	G		
	F-75	T	C	G	G	A	3	A2a
unicuspis	U-03	T	C	G	G	A	3	A2a
	U-04	C	T	A	A	A	8	B1a
	U-05	T	C	G	G	A	3	A2a
	U-06	T	C	G	G	A	3	A2a
	U-11	T	C	G	G	A	3	A2a
H	**Saugeen**							
fossor	F-07	T	C	A	A	G	2	A1b
	F-08	T	C	A	A	G	2	A1b
	F-46	T	C	A	A	G	2	A1b
	F-47	T	C	G	G	A	3	A2a
unicuspis	U-32	T	C	A	A	G	2	A1b
	U-33	T	C	A	A	G	2	A1b
H	**Beaver R**							
fossor	U-52	T	C	G	G	A	3	A2a
H	**Nine Mile**							
fossor	F-02	T	C	G	G	A	3	A2a
	F-05	T	C	G	G	A	3	A2a
	F-06	T	C	G	G	A	3	A2a
H	**Nottawasaga**							
fossor	F-11	T	C	G	G	A	3	A2a
	F-12	T	C	G	G	A	3	A2a

Lake	Stream	ND3-180 *Fok*I	ND6-72 *Alw*26I	ND5-201 *Bst*NI	ND5-711 *Bsr*DI	COI-669 *Pst*I	mt Type Number	mt Type Description
	F-49	T	C	G	G	A	3	A2a
	F-50	T	C	G	G	A	3	A2a
	F-51	T	C	G	G	A	3	A2a
	F-52	T	C	G	G	A	3	A2a
	F-53	T	C	G	G	A	3	A2a
	F-54	T	C	G	G	A	3	A2a
	F-82	T	C	A	A	G	2	A1b
	F-83	T	C	G	G	A	3	A2a
	F-84	T	C	G	G	A	3	A2a
	F-85	T	C	G	G	A	3	A2a
	F-86	T	C	G	G	A	3	A2a
	F-87	T	C	A	A	G	2	A1b
	F-88	T	C	G	G	A	3	A2a
	F-89	T	C	G	G	A	3	A2a
	F-90	T	C	G	G	A	3	A2a
H	**Sauble**							
fossor	F-09	T	C	G	G	G	4	A2b
	F-10	T	C	G	G	A	3	A2a
	F-48	T	C	A	A	G	2	A1b
M	**Big Sauble**							
fossor	F-32	T	C	G	G	A	3	A2a
M	**Cool**							
fossor	F-26	T	C	A	A	G	2	A1b
	F-27	T	C	G	G	A	3	A2a
	F-28	T	C	G	G	A	3	A2a
	F-41	T	C	A	A	G	2	A1b
M	**Manistee**							
fossor	F-33	T	C	G	G	A	3	A2a
	F-34	T	C	G	G	A	3	A2a
	F-35	T	C	A	A	G	2	A1b
	F-36	T	C	G	G	A	3	A2a
	F-37	T	C	G	A	A	6	A4a
	F-39	T	C	G	G	A	3	A2a
	F-40	T	C	G	G	A	3	A2a
M	**Pere Marquette**							
fossor	F-31	T	C	A	A	G	2	A1b
M	**Peshigo and Middle**							
unicuspis	U-12	T	C	G	A	A	6	A4a

Lake	Stream	ND3-180 *FokI*	ND6-72 *Alw26I*	ND5-201 *BstNI*	ND5-711 *BsrDI*	COI-669 *PstI*	mt Type Number	mt Type Description
	U-13	T	C	G	A	G	7	A4b
	U-14	T	C	G	A	A	6	A4a
	U-15	T	C	G	A	A	6	A4a
	U-16	T	C	G	A	A	6	A4a
	U-17	T	C	G	A	G	7	A4b
	U-23	T	C	A	A	A	1	A1a
	U-24	T	C	G	A	A	6	A4a
	U-25	T	C	A	A	A	1	A1a
	U-26	T	C	G	A	A	6	A4a
	U-27	T	C	G	A	A	6	A4a
	U-28	T	C	A	A	A	1	A1a
	U-29	T	C	G	A	A	6	A4a
	U-30	T	C	G	A	G	7	A4b
	U-49	T	C	G	A	A	6	A4a
	U-50	T	C	G	G	A	3	A2a
	U-51	T	C	G	A	A	6	A4a
S	**Pearl R**							
fossor	F-01	T	C	G	A	G	7	A4b
	F-29	T	C	G	A	A	6	A4a
	F-30	T	C	A	A	G	2	A1b
	F-42	T	C	A	A	G	2	A1b
	F-43	T	C	G	A	G	7	A4a
S	**Sibley**							
fossor	F-20	T	C	G	A	A	6	A4a
	F-21	T	C	G	A	A	6	A4a
	F-22	T	C	G	A	A	6	A4a
	F-23	T	C	G	A	A	6	A4a
	F-24	T	C	G	A	A	6	A4a
	F-25	T	C	G	A	A	6	A4a
	F-45	T	C	G	A	A	6	A4a
O	**Cobourg**							
unicuspis	U-01	T	C	A	A	G	2	A1b
O	**Wilmot Cr**							
unicuspis	U-48	C	T	A	A	G*	9	B1c
						*And 573-G; lacking both *PstI* sites		
E	**Little Buffalo R**							
fossor	F-91	T	C	A	A	G	2	A1b
OUTGROUPS (*I. fossor*)								
NY	Ich2	T	C	A	A	A	1	A1a

Lake	Stream	ND3-180 *Fok*I	ND6-72 *Alw*26I	ND5-201 *Bst*NI	ND5-711 *Bsr*DI	COI-669 *Pst*I	mt Type Number	mt Type Description
VT	F-04	C	T	A	A	A	8	B1a

APPENDIX 2

The utility of meristic, morphometric, pigmentation and gonad analysis to differentiate Great Lakes *Ichthyomyzon* lamprey larvae

Introduction

The *Ichthyomyzon* Genus

Three of the five lamprey species found in the Great Lakes basin are in the genus *Ichthyomyzon* (Cudmore-Vokey and Crossman 2000). Both the chestnut lamprey (*I. castaneus*) and the silver lamprey (*I. unicuspis)* reside within streams as filter-feeding larvae (ammocoetes), and then feed on various host fishes after metamorphosing into considerably larger adults. The non-parasitic northern brook lamprey (*I. fossor*) resides in the stream both as an ammocoete and adult, and is substantially smaller in size in the adult form than the silver and chestnut lampreys. The current distribution of the chestnut lamprey is limited to a small number of streams tributary to Lake Michigan (USFWS, unpublished data). The northern brook and silver lampreys are more widely distributed throughout tributaries to the Great Lakes, with very low densities in the Lake Ontario basin (Scott and Crossman 1973).

Distributions of these lampreys have been documented as changing dramatically since sea lamprey (*Petromyzon marinus*) control was initiated in the late 1950's (Schuldt and Goold 1980). Lampricide applications have successfully reduced sea lamprey populations, but have simultaneously impacted native lamprey populations (Davis 1970). Schuldt and Goold (1980) found substantial reductions in *Ichthyomyzon* lamprey populations around Lake Superior, as well as reductions in the American brook lamprey (*Lampetra appendix)*, another native species. Unpublished data from the Canadian Sea

Lamprey Control Centre (SLCC) and the United States Fish and Wildlife Service (USFWS) over a 20 year time period subsequent to the study of Schmidt and Goold (1980) indicate further declines in native lamprey populations all around the Great Lakes, most notably in silver lamprey populations. The northern brook lamprey has been listed as special concern in Canada by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). The COSEWIC status report cited restricted distribution as the primary concern for this species (Lanteigne 1991). The northern brook lamprey is also listed as Threatened or Endangered in five American states (Renaud 1997). The silver lamprey is not currently listed in Canada, but a COSEWIC status review is likely forthcoming. The chestnut lamprey is currently listed in Canada as special concern (Lanteigne 1992).

As filter feeders (Hardisty and Potter 1971), larval lampreys contribute to the cycling of nutrients within a stream and act as a prey source for some fish species (Vladykov 1973). Despite poor public sentiment towards lampreys in North America caused by the invasive sea lamprey, native lampreys do not belong under this umbrella and should be protected (Renaud 1997). The first step in protection is determining their distribution and population trends.

Identification of *Ichthyomyzon*

A requirement for determining the status of a species is a reliable technique to identify the species. Identification of adult *Ichthyomyzon* species is not problematic, as differences in both size and external characteristics, particularly dentition, allow for relatively easy identification (Scott and Crossman 1973). However, due to their sedentary nature and lotic habitat preference, ammocoetes are the life stage usually encountered.

Ammocoetes lack teeth; therefore species are much more difficult to distinguish. Most *Ichthyomyzon* species have been considered indistinguishable as ammocoetes (Hubbs and Trautman 1937) until relatively recently, Three published taxonomic keys currently exist (Vladykov and Kott 1980; Fuiman 1982; Lanteigne 1988) with respect to the classification of ammocoetes of the *Ichthyomyzon* genus. These keys, however, have inconsistent diagnostic traits for identification of silver and northern brook lampreys. Vladykov and Kott (1980) stated that silver lamprey ammocoetes have a more heavily pigmented caudal fin and head than northern brook ammocoetes. In contrast, Lanteigne (1988) advocated the use of supra-branchial pigmentation patterns, finding that northern brook lampreys possessed a narrow unpigmented band immediately dorsal to the branchial pores. A key by Fuiman (1982) reiterated the Vladykov and Kott (1980) key, and presented no new data.

Chestnut lamprey ammocoetes have been identified using their pigmented lateral line organs (Hubbs and Trautman 1937; Vladykov and Kott 1980; Lanteigne 1981, 1988), a character not possessed by other Great Lakes lamprey larvae. However, several authors have found this feature becoming apparent at different lengths: 56 millimetres (Lanteigne 1981); 60 millimetres (Vladykov and Kott 1980); and up to 150 millimetres (Hubbs and Trautman 1937). One objective of the current study is to verify this diagnostic characteristic.

My original intentions were to identify ammocoetes to species using genetic markers before examining external characters, but an inability to find species-specific markers (Mandrak *et al.* 2002) precluded their use in this study. Three other techniques were used to identify diagnostic differences between species (Hughes and Potter

1969;Beamish and Thomas 1983). Some other lamprey species can be separated using: 1) morphometric and meristic contrasts (Potter and Strahan 1968; Potter *et al.* 1968; Potter and Osborne 1975; Bird and Potter 1979); 2) differences in pigmentation patterns (Vladykov 1950; Vladykov 1960); 3) differences in internal organs (Hardisty 1961). This study investigated each of these techniques to try to identify *Ichthyomyzon* lamprey ammocoetes.

Lamprey Genetics

Genetic analyses performed concurrently, and in association with this study were unsuccessful in identifying species-specific markers for northern brook and silver lampreys (Docker, unpubl. data). Over 13,000 base pairs of nine regions of the mitochondrial genome and 523 base pairs from the nuclear genome were sequenced. No consistent diagnostic character was obtained. Other studies have had similar difficulties finding markers for paired lamprey species in other genera (Docker *et al.* 1999), Pedro Raposo de Almeida, University of Evora, Portugal, pers. comm.). Filcek et al. (in press) found promise of microsatellite markers in two Great Lakes populations of northern brook and silver lampreys. Further work is currently investigating possible microsatellite markers for basin-specific applications (Docker, pers comm.).

Genetic markers using restriction fragment length polymorphisms (RFLP) have been successfully obtained to separate chestnut lampreys from silver and northern brook lampreys (Mandrak *et al.* 2002). This study used these existing markers to establish identity of chestnut lamprey ammocoetes. The RFLP made use of the many differences in the ND5 gene between these groups, and of the many enzymes that were able to

differentiate the chestnut lamprey, *Rsa*I and *Bst*NI were selected for this study as reliable markers to obtain and verify identification (Docker, pers. comm.).

Gonad Analysis

Paired species, also termed satellite species, are lamprey species that are closely related, but possess distinctly different life styles: parasitic and non-parasitic (Hardisty and Potter 1971). The parasitic representative of a paired species has been documented as possessing significantly higher numbers of oocytes (the precursors to eggs) even in the larval stage, which has allowed specific determination of individual ammocoetes (Hardisty 1961). This method involves sectioning the ovary of sufficiently sized female ammocoetes (over 80 millimetres) and enumerating oocytes in ovarian cross sections. This technique has been used to differentiate other paired species such as the chestnut and southern brook lamprey (*I. gagei*) (Hall and Moore 1954; Malmquist 1978; Beamish and Thomas 1983); the European brook lamprey (*Lampetra planeri*) and the European river lamprey (*L. fluviatilis*) (Hardisty 1961, 1963, 1964); and the Australian lamprey (*Mordacia mordax)* and the Australian non-parasitic lamprey (*M. praecox*) (Hughes and Potter 1969).

The two paired species in this study have considerably different adult fecundities: the northern brook lamprey has been documented as having an average of between 1,200 (Leach 1940) and 1,524 oocytes (Vladykov 1951). This is in contrast to the silver lamprey, the fecundity of which has been estimated to be between 10,800 and 29,412 (Vladykov 1951). If these differences are reflected in the ammocoetes of the silver and

northern brook lampreys, this technique may provide a method of differentiation for large female ammocoetes of these two species.

External Characteristics

The techniques discussed above (genetic and gonad analysis) were investigated in the present study as a means of differentiating species. However, the principle intent of the current study was to find species-specific external differences such that an identification key could be created and used in the field, using meristics, pigmentation and/or morphometrics. The study of morphometrics, defined as the comparison of body forms, has been used extensively with fishes, and has been used successfully to enable species and morphotype differentiation (Neira *et al.* 1988; Parsons *et al.* 2003). Morphometrics have also been considered a useful tool in comparing groups of lampreys (Potter *et al.* 1968; Kott 1974; Lyons *et al.* 1997). This study utilized both traditional morphological methods (using linear distances) and geometric morphometrics (using relationships between homologous landmarks). Geometric techniques were included because they have been found, in some cases, to be superior to traditional methods in describing shape differences (Winans 1984; Parsons *et al.* 2003). Geometric morphometric analyses result in partial warp scores for each individual, and describe the relative positioning of the landmarks. These partial warp scores contain information about shape from each individual that can be used in multivariate analysis.

Pigmentation patterns have also been used to differentiate larval lampreys. Historically, lamprey pigmentation measures have been subjective, using categorical

rating (Lanteigne 1988) or non-quantitative measures (Vladykov 1950; Manion 1972; Kott 1974; Potter and Osborne 1975; Richards *et al.* 1982). The current study used a free computer program, Scion Image for Windows (Rasband 2000) to quantitatively assess the level of pigmentation from digital images of the lampreys.

A meristic character of ammocoetes often used to separate both genera and species of lampreys is the number of myomeres (Scott and Crossman 1973; Vladykov and Kott 1980). Myomeres are longitudinal muscle bands found along the external surface in both ammocoetes and adults. The enumeration of trunk myomeres has warranted consideration in this study, given that Lanteigne (1981) found small but significant differences in this character in her analysis of the *Ichthyomyzon* genus, as did Potter and Osborne (1975) when comparing the paired European river lamprey and the European brook lamprey.

The presence of mixed species samples in this study (i.e., the silver/northern brook samples) was fitting for principal component analysis, as this statistical method condensed multiple variables down to a smaller set of new dimensions. This allowed for the determination of the variables which contributed most to variation within the data set. Polarity, represented by grouping of individuals in the projection of the principle components, allowed for the investigation of variation within previously designated species groups. Therefore, dilution of pure silver lamprey populations by northern brook lampreys was not problematic in the multivariate analyses, as any ammocoetes demonstrating common differences would appear as an outgroup, regardless of their initial species designation.

The objective of this study was the development of a taxonomic key that can be used on live individuals. Identifying species is an essential first step before trying to find differences among species; therefore gonad analysis was investigated as a technique to differentiate species. RFLP analysis allowed for the identification of chestnut lampreys, but silver and northern brook lampreys are for all intents and purposes indistinguishable. External characters were the primary focus of this study, as differences in body proportions, trunk myomere counts, body form, or level of pigmentation in different regions of the different species of ammocoete may allow for interspecific differentiation. In the event that this was not possible (i.e. no diagnostic differences between species are observed in live specimens), a secondary objective was to be able to differentiate species using preserved specimens. Although this is obviously detrimental to individuals, this key would still be beneficial in determining the status of Great Lakes *Ichthyomyzon* species using preserved specimens. Knowledge of the distribution and abundance of these species is fundamental in determining their conservation status, and when obtained, will subsequently enhance any necessary conservation and rehabilitation efforts.

Methods

Specimen Identification

To develop a taxonomic key, the identity of the specimens used to construct the key must be known. Identifying specimens using features not used as diagnostic characteristics in the key to avoid lack of independence bias. The objective of this study is to develop a taxonomic key to identify both live and preserved specimens. Therefore, external features, such as pigmentation and morphology need to be incorporated into the key. Genetic analysis is an independent method of determining species identity; however

recent analyses have developed markers for chestnut lampreys, but not for silver and northern brook lampreys (Docker et al. unpubl. data). Gonad analysis was investigated as a method of determining *Ichthyomyzon* species identity, but sample locations (and hence species identity) were designated using historic catch records of identified adult and metamorphosed silver and northern brook lampreys, as outlined below.

Specimen collection

Lampreys are most susceptible to sampling while in the larval phase, as they often burrow into shallow water substrate where they remain until they metamorphose into juveniles. Many native ammocoetes are collected incidentally with larval sea lamprey sampling efforts conducted by Sea Lamprey Control and United States Fish and Wildlife Service personnel. This represents a valuable source of information, as efforts to monitor sea lamprey ammocoetes have resulted in an extensive set of all Great Lakes lamprey species distribution data. All collected *Ichthyomyzon* ammocoetes however, were identified only to genus due to the absence of a reliable identification key. Metamorphosed and adult *Ichthyomyzon* lampreys have been successfully identified to species in this extensive SLCC and USFWS dataset (unpublished data), and this was used to for species identification of the ammocoetes from collection streams in the current study (as performed by Potter and Osborne 1975; Beamish 1993). One subset of streams was sampled where only northern brook lamprey adults or juveniles were captured. Another subset was sampled where catch records also indicated the presence of silver lampreys; however, no streams were documented to contain only silver lampreys. Hence silver lamprey samples were designated 'silver/northern brook lamprey' streams, and

sample sizes from these rivers were increased due to the assumption that both species of lampreys would be included in the sample. Three northern brook lamprey streams and three silver/northern brook lamprey streams were sampled from each of Lake Superior, Huron, Michigan and Erie to achieve basin-wide representation. Another subset of three streams in Lake Michigan was sampled, targeting chestnut lamprey ammocoetes. This was again based on historic catch records of adults, and again sample sizes were increased because of the assumed presence of other species of lampreys.

Ammocoetes were collected from 25 Great Lakes tributaries (Fig. 1) by the USFWS and the Department of Fisheries and Ocean (DFO) agencies using ABP-2 backpack electrofishers in the summers of 2002 and 2003. In total, 303 ammocoetes were collected from Superior, 360 from Lake Huron, 618 from Lake Michigan, and 180 from Lake Erie. Table 1 lists the number of individuals collected from each stream and the species that were targeted. The presence of a single dorsal fin and a low myomere count enabled identification to genus (Vladykov 1950).

After collection, the specimens were shipped in coolers containing stream water and a shallow bottom layer of sand and silt, maintained live in flow-through tanks, and then digitally imaged within two weeks. After imaging, specimens were preserved in one of two ways. All ammocoetes (1461) were first fixed in 10% formalin for approximately 48 hours, and then approximately half of the ammocoetes from a subset of 15 streams (309 ammocoetes) were transferred to 95% ethanol as part of a separate study investigating the effects of different preservative techniques. The other 1152 ammocoetes remained in 10% formalin. All ammocoetes were stored in 200 millimetre screw-top glass tubes to maintain their longitudinal shape, after Hardisty and Huggins (1970).

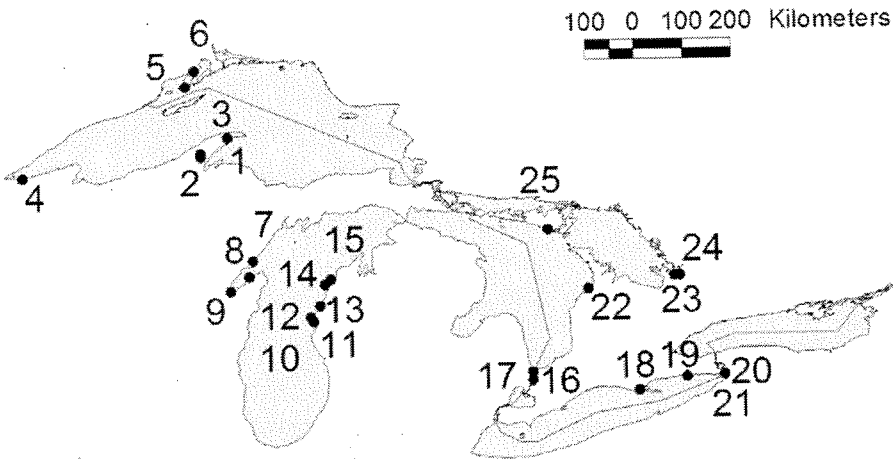


Figure 1. Locations of sample sites in the Great Lakes. Stream names corresponding to numbers are displayed in Table 1.

Table 1. Stream names, map number (corresponding to Fig. 1), target species and number of ammocoetes collected.

Lake	Stream	Map Label	Number of Ammocoetes	Targeted Species
Superior	Otter R. (Sturgeon R.)	1	30	N. brook
	Pilgrim R.	2	43	Silver/N. brook
	L. Gratiot R.	3	90	Silver/N. brook
	Upper Middle R.	4	90	Silver/N. brook
	Sibley Cr.	5	20	N. brook
	Pearl R.	6	30	N. brook
Michigan	Menominee R.	7	90	Silver/N. brook
	Peshtigo R.	8	90	Silver/N. brook
	Fox R.	9	90	Silver/N. brook
	Big Sable R.	10	50	Chestnut
	Bray R. (Pere Marquette R.)	11	30	N. brook
	Cool Cr. (Manistee R.)	12	30	N. brook
	Manistee R.	13	120	Chestnut
	Betsie R.	14	88	Chestnut
	Platte R.	15	30	N. brook
Huron	Black R.	16	90	Silver/N. brook
	Pine R.	17	58	Silver/N. brook
	Saugeen R.	22	20	N. brook
	Hog R.	23	90	Silver/N. brook
	Coldwater R.	24	90	Silver/N. brook
	Manitou R.	25	12	N. brook.
Erie	Big Cr.	18	30	N. brook
	Grand R.	19	30	N. brook
	Little Buffalo R.	20	30	N. brook
	Cayuga Cr.	21	90	Silver/N. brook
Total:			1461	

Genetic Analysis

Chestnut lamprey ammocoetes were differentiated from northern brook and silver lamprey ammocoetes using genetic analyses on 205 individuals from streams where adult chestnut lampreys have been collected (Manistee, Big Sable and Betsie Rivers) as determined from USFWS records (USFWS, unpubl. data). After being anaesthetized with clove oil, a small piece of tissue was removed from the left side of the trunk of each individual using a scalpel, and stored in 95% ethanol. DNA was extracted using the Wizard ® Genomic DNA Kit. This involved digesting a rice-sized piece of tissue in 5 µl of 20mg/ml Proteinase K and 250 µl of EDTA/Nuclei Lysis solution. The samples were incubated for one hour at 50°C with rocking. Three µl of RNase Solution were added; the sample was incubated at 37°C for 15-30 minutes; 100µl of Protein Precipitation Solution was added; the pellet was washed with 70% ethanol; and subsequently re-suspended in TE, with a pH of 8.0.

Extracted DNA was amplified on a thermal cycler with a denaturing temperature of 94°C, variable annealing temperatures, and extension temperature of 72°C was used in the PCR cycle for intervals of one minute, one minute and one and a half minutes respectively. Three annealing temperatures were used, with extension temperatures of 56, 54 and 52°C for 10, 10 and 20 cycles respectively. This was followed by a 2 minute denaturation at 94°C, and concluded with a five minute extension period at 72°C, as performed by Docker (unpubl. data).

The ND5 gene was then amplified using a Polymerase Chain Reaction (PCR). Each 25µl reaction was composed of 2.5µl of 10X Invitrogen buffer, 2.5 µl of $MgCl_2$, 0.5µl of dNTP, 17.4µl of ddH_2O, 0.5µl of forward and reverse primers (Docker, unpubl.

data), 0.1µl of Platinum Taq® and 1 µl of DNA. PCR products were visualized on a 1.8% agarose gel to ensure successful amplification, and then a RFLP assay was conducted on the samples using the *Bst*NI and *Rsa*I enzymes (New England Biolabs), using the following: 0.9µl of 10X buffer, 0.25µl enzyme (10U/µl), and 3.35 µl of double distilled water per sample. *Bst*NI required addition of bovine serum albumin (BSA) (1% of total volume) to augment the reaction. Digestion times were two hours at 60°C for *BstN*I and two hours at 37°C for *Rsa*I. RFLP products were then visualized on a 1.8% agarose gel.

Gonad Analysis

To distinguish silver lamprey from northern brook lamprey, gonad analysis was performed on a subset of 104 preserved individuals over 80 millimetres in length from northern brook lamprey streams and mixed silver/northern brook lamprey streams (52 lampreys from each source). Lamprey sections were obtained by removing a five millimetre cross-section with a razor blade, commencing from and then anterior to the measured midpoint of the preserved specimen. This targeted the largest region of the gonad (Docker 1992). Sections were prepared using a series of dehydration steps as outlined by Docker (unpubl. data - See Appendix I for specific dehydration steps used) and processed in an automated Histomatic tissue preparation instrument (Fisher Histomatic Tissue Processor Model 166 MP) before being mounted in paraffin wax on histology cassettes. The mounted tissues were then sectioned at either six or eight µm using a microtome, floated in warm water, mounted on slides, dried, and then stained with haematoxylin and eosin (Appendix II outlines the staining procedure utilized for approximately half the samples; the remainder were prepared using a standard

haematoxylin and eosin procedure (Willey 1971) by the Histology Department at the University of Guelph).

The sex of the ammocoetes was determined under a microscope by the presence or absence of oocytes, and images of the cross sections of females were obtained using a camera-mounted dissecting microscope. Oocyte counts per section for each individual were visually obtained from the images using the counting feature of Image Pro (Image Pro Plus software ver. 4.5) to prevent double counting. A regression analysis was used to explore the relationship between total ammocoete size and oocyte count separately for northern brook lamprey and mixed silver/northern brook lamprey populations. Frequency distributions were plotted for each species source type.

Ammocoete Imaging

All ammocoetes were anaesthetized using a low concentration of clove oil (20 ppm) which was added to stream water in which the specimens had been previously maintained. The right side of each fish was photographed in a 20 centimetre by 30 centimetre wax tray, containing 600 mL of stream water. A background of Rite in the Rain All-Weather Copier Paper® provided a white background to minimize background colour influences on transparent fins. An Olympus C-3020 digital camera was used, with the lens placed 20.5 centimetres above the subject on a tripod with the macro setting enabled. Halogen lights (200 watts) were angled at approximately 45° onto the specimen at a distance of 30 centimetres across from, and above, the ammocoete. A ruler was included in the photograph to provide scale. Where required, the animals were straightened and stabilized with insect mounting pins, and the centre of the cloaca was

indicated by the insertion of an insect pin before taking the photo, as it is rarely visible from a lateral perspective.

Camera settings were an F-stop of F4 with a shutter speed of 1/100 second. No flash was used. The whiteness was increased from standard by one setting to maximize capture of pigmentation levels. The macro setting was enabled, and auto-focus was used, with the ammocoete in the middle of the field of view. The timer function of the camera was used to minimize movement of the camera during the imaging to maximize image clarity. The resolution setting on the camera was 1200 x 1600 pixels. After being preserved for three weeks, the lampreys were retrieved from their individual test-tubes and photographed again in the manner outlined above to determine if the effects of preservation might alter the detectability of differences between the species.

Morphometric and Pigmentation Analysis

All digital images were converted from jpeg files to black and white bitmap files for individual image analysis in Scion Image for Windows (Rasband 2000), an image analysis software program. The scale was standardized on each image before any measurements were made using the ruler that had been placed in each image. Measurements consisted of seven length measurements and 10 pigmentation measurements taken while fresh, and five length measurements and 10 pigmentation measurements three weeks after initial preservation (Table 2, Figure 2). The maximum height of trunk and branchial areas were not measured on preserved specimens, nor was the pigmentation of the caudal fin, as measurement effort was scaled down in the secondary analysis due to very large sample sizes. Using image analysis software, pigmentation measurements for different regions were measured as mean grey values,

which is a measure of the intensity of colouration (ranging from 1 to 255) divided by the total number of pixels in the selected area. The precision of the mean grey values and morphometric measurements using the image processing software was tested using a series of 10 repeated measures of each variable on one individual. Presence or absence of pigmented lateral line organs was noted for each individual.

Statistical Analysis

To compare species, the following groups were used: 292 northern brook lampreys from 11 streams, 1010 silver/northern brook lampreys from 11 streams, and 64 chestnut lampreys from three streams. The chestnut lampreys were identified by RFLP analysis (the remaining lampreys from three chestnut streams were excluded from analysis, as they were from the three streams containing chestnut lampreys but were not genetically tested). The identification of the northern brook and silver/northern brook lamprey groups was based on historical catch data. To correct for variation due to size, traditional morphological measurements were regressed against total length and the residuals were used for all analyses (Reist 1985). Due to unequal variances in the variables, species groups' means of both pigmentation and regressed morphometric variables were compared using Welch's statistic, and was followed by Tamhane's *post hoc* t-tests (Tabachnick and Fidell 1996).

Before comparing different characteristics, the morphometric data were transformed by subtracting the mean and dividing by the standard deviation (standardized). The data were then analyzed in a principal components analysis (PCA) using a correlation matrix. Pigmentation variables were similarly transformed and used in a separate PCA based on a correlation matrix analysis. To remove potential

confounding effects of allometric growth (Vladykov and Kott 1980), the lampreys were divided into three 30 millimetre size classes (50 to 80 millimetres, greater than 80 and up to 110 millimetres, and greater than 110 and up to 140 millimetres), and the principal components analyses were separately performed on these subsets for pigmentation and morphometric data. To determine if the combination of variable types enhanced species detectability, the two measurement types were combined, using both standardized pigmentation and morphometric data in a separate PCA using a correlation matrix on all data.

To detect the maximum amount of multivariate variation between the chestnut lampreys and the other two species, morphometric and pigmentation characters were transformed and compared by discriminant function analyses (DFA). The northern brook and silver/northern brook lamprey groups were combined due to an inability to confidently assign species identity (as required by DFA) and compared to the chestnut lamprey data using pigmentation and traditional morphometric data for all specimens. To eliminate potential size confounding factors, the same data were analyzed again in three 30 millimetre size increments as outlined above.

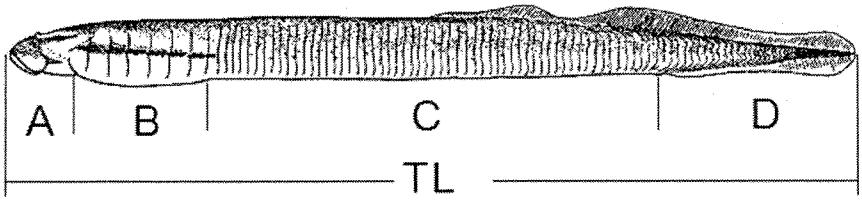
Because of a high transformation rate of Black River ammocoetes into silver lampreys, it was singled out for further analysis. A DFA was performed with these data to test for differences between the chestnut and northern brook lamprey ammocoetes.

To test for geographic variation, another DFA was conducted using data from a small subset of streams. Black River ammocoetes were again assumed to be silver lampreys; Big and Grand River ammocoetes, being geographically proximate were grouped as northern brook lampreys, and chestnut lampreys (60) from the neighbouring

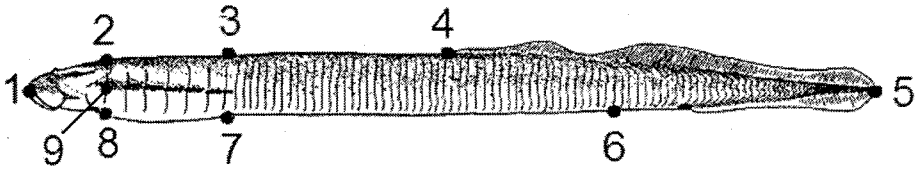
Betsie River, Manistee River, and Big Sauble River were grouped and used in the last analysis.

All of the preceding statistics were performed on measurements of live lampreys. To determine if the effects of preservation might enhance the ability to find species differences, the same statistical procedures were run on the measurements obtained from the specimens which had been preserved for three weeks.

A. Traditional Morphometric Measurements



B. Landmarks for Truss Measurements



C. Areas for Pigmentation Measurements

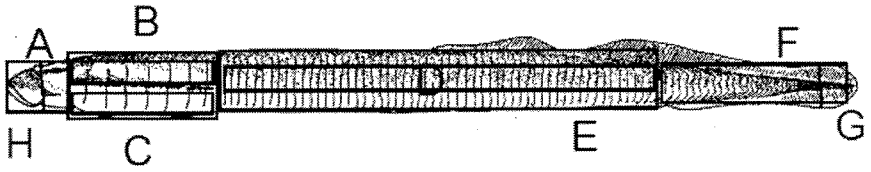


Figure 2. Location of measurements conducted on digital images, including A) traditional morphometric distances B) landmark locations, and C) areas of pigmentation measurements.

Table 2. Ammocoete measurement details for A) traditional morphometrics, B) geometric morphometric landmark locations, and C) pigmentation values. ND means not displayed on Figure 2.

A. Traditional Morphometric Measurements	
A	Head length: from anterior edge of snout to anterior edge of first brachial pore
B	Branchial length: from anterior edge of first branchial pore to anterior edge of last branchial pore
C	Trunk length: from anterior edge of last branchial pore to centre of cloaca
D	Tail length: from centre of cloaca to posterior edge of caudal fin
ND	Total length: anterior edge of snout to posterior edge of tail
ND	Supra-branchial non-pigmented band height: height of white band anterior to the third and fourth branchial pores
ND	Maximum diameter of trunk
ND	Maximum diameter of branchial region
B. Landmarks for Truss Measurements	
1	Tip of snout
2	Dorsal origin of branchial area
3	Dorsal origin of trunk area
4	Origin of dorsal fin
5	Tip of caudal fin
6	Cloaca
7	Ventral origin of trunk area
8	Ventral origin of branchial area
9	Medial origin of branchial area
C. Areas for Pigmentation Measurements	
A	Head
B	Supra-branchial area
C	Sub-branchial area
B+C	Branchial area
D	Lateral line area on trunk
E	Trunk area
F	Caudal area
G	Caudal fin edge
H	Oral hood
ND	Whole body
ND	Caudal fin

Visual Pigmentation Analysis

To augment the new pigmentation analysis software technique, four areas of pigmentation areas were assessed visually, using methods similar to those of Lanteigne (1981). Pigmentation levels of the upper lip, caudal fin, sub-orbital region and pre-branchial regions were scored as outlined in Table 3, using a system adapted from Lanteigne (1981). A Kruskal-Wallis test was performed to determine if differences in pigmentation levels could be detected among species.

Table 3. Pigmentation scoring system used in visual evaluation of pigmentation levels in four different areas of all ammocoetes.

Area Assessed	Score	Description
Upper Lip	1	pigment does not reach margin of the lip, leaving a narrow white band
	2	pigment reaches margin of the lower part of the upper lip
Suborbital Region	1	irregularly spotter appearance
	2	melanophores widely distributed, leaving a white blotch on the cheek
	3	melanophores covering the whole area
Pre-branchial Region	1	non-pigmented
	2	pre-branchial pigmented blotch
	3	dark pre-branchial pigmented blotch
Caudal Fin	1	pigmented not present in region next to dorsal and ventral surface
	2	pigment covers most of caudal fin
	3	pigment follows the fin ray outward to the margin of the caudal fin

Geometric Morphometric Analysis

Size-free analysis of shape was conducted using computer software to analyze the interrelationships of homologous landmarks on each of the fish. Images of 1248 live ammocoetes were landmarked as illustrated in Figure 2B using the following software applications: TpsUtil (Rohlf 2002); TpsDig (Rohlf 1996); TpsRelw (2003), and TpsRegr (Rohlf 1997). The remaining images could not be confidently landmarked due to absence

of unequivocal landmark locations or ammocoetes which were not completely straight. TpsUtil was used to build files containing the images; TpsDig served as a format to digitize the landmarks on each of the images; TpsRelw was used in estimating partial warp scores; and TpsRegr was used to estimate the deformation grids. These deformation grids show the change in shape from the average form of all individuals (the consensus form) by regressing measurements against a dummy variable assigned to each species group. A PCA was performed on the unstandardized partial warp scores using a covariance matrix, and a MANOVA was conducted on the partial warp scores to test for differences between the species groups, i.e. northern brook, silver/northern brook, and chestnut lamprey groups.

Meristic Analysis

Trunk myomeres were counted on 1338 individuals after preservation using a dissecting microscope and an adjustable side illumination source. The most anterior myomere counted had its anterior septum located distinctly posterior to the seventh branchial pore. The last myomere counted was the muscle band which was partly or entirely above the cloacal slit when looking at the specimen from a ventral view (Hubbs and Trautman 1937). A Kruskal-Wallace test was used to compare species groups, and a Mann-Whitney test was conducted to find differences between pairs of species groups.

Results:

Genetics

The RFLP revealed a total of 62 chestnut lampreys from of a total of 205 ammocoetes analyzed. Figure 3 illustrates the differences between the chestnut lamprey and the silver/northern brook lampreys after digestion by *Rsa*I and *Bst*NI enzymes, which

provided easy and reliable identification of the chestnut lamprey when compared to the other two species. The two enzymes concurred in all but one case, and this individual was excluded from subsequent analyses.

Twenty-seven per cent of the 45 chestnut lampreys that were greater than 80 millimetres possessed visibly pigmented lateral line organs, characterized by dark spots along the dorsal and lateral area of the trunk and head (Fig. 4). This characteristic was not observed in any of the ammocoetes below 80 millimetres in length.

Gonad Analysis

Of the 104 ammocoetes analyzed, sex could not be determined in 11 due to poor section quality. Females were identified by the presence of clusters of oocytes (Fig. 5). Forty-five individuals were female, three of which were poor cross-sections which precluded reliable oocyte counts. Results of the analyses of oocyte numbers showed a substantial difference in range between the known northern brook populations and the mixed populations (13-42 oocytes versus 15 – 93 oocytes) (Figure 6). The regression of oocyte count on total length revealed a weak negative relationship between the two variables for the northern brook group (r^2=0.421), and no relationship in the mixed population (r^2=0.088) Fig. 7). This lack of relationship in the mixed silver/northern brook population suggested that there was two species present in the putative mixed population populations, confounding the oocyte to length relationship.

Figure 8 shows the marked contrasts in oocyte numbers in cross sections of two similarly sized (129 and 132 millimetre) lampreys from one mixed silver/northern brook lamprey stream (Coldwater Creek).

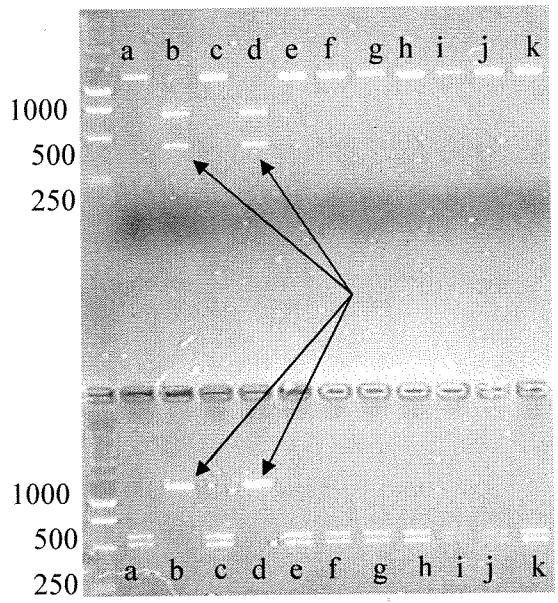


Figure 3. Electrophoretic gel showing RFLP results of 11 ammocoetes. ND5A gene was digested by *RsaI* (top row) and *Bst*NI (bottom row) enzymes. A size standard is in the left lane. Arrows indicate the two chestnut lampreys (b and d); the remainder are silver or northern brook lampreys.

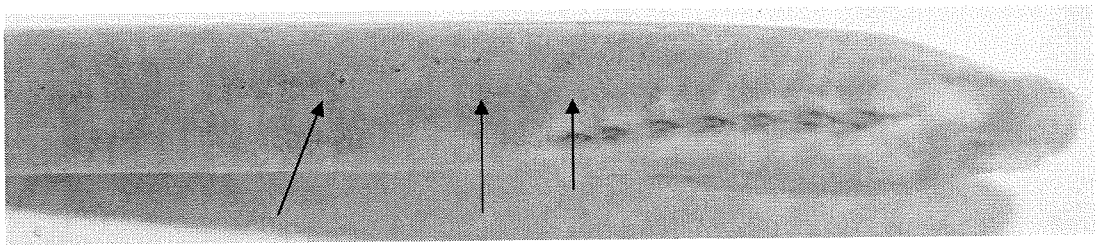
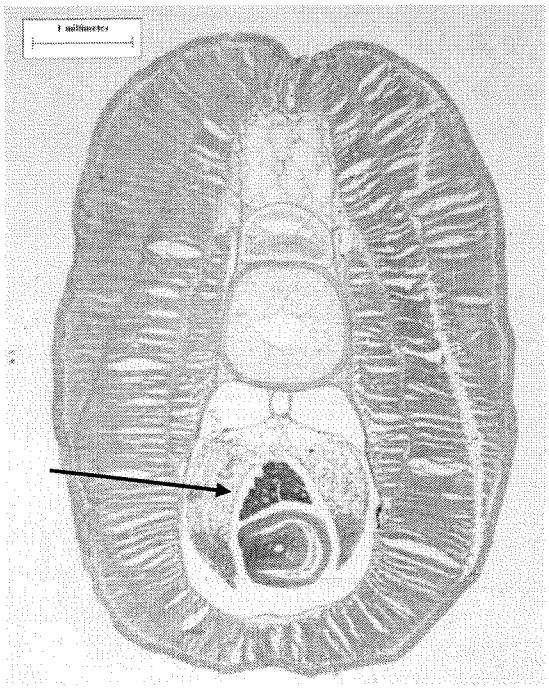


Figure 4. Chestnut lamprey ammocoete identified by RFLP analysis, displaying pigmented lateral line organs (indicated by arrows).

a)



b)

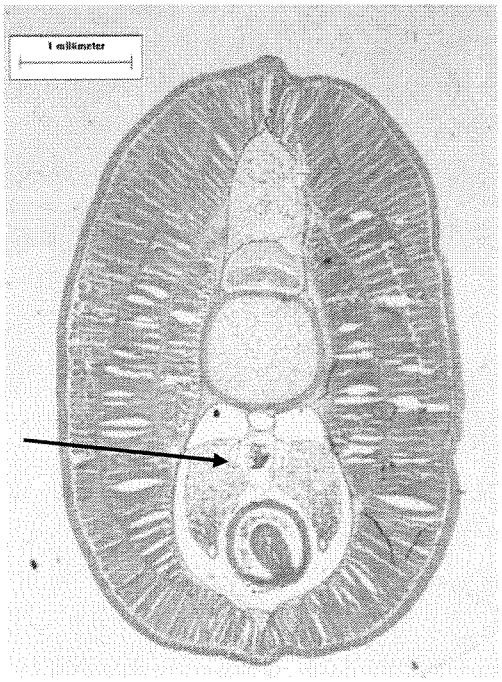


Figure 5. Cross section of a) a 105mm female ammocoete and b) 98mm male ammocoete. Arrows point to the gonadal region.

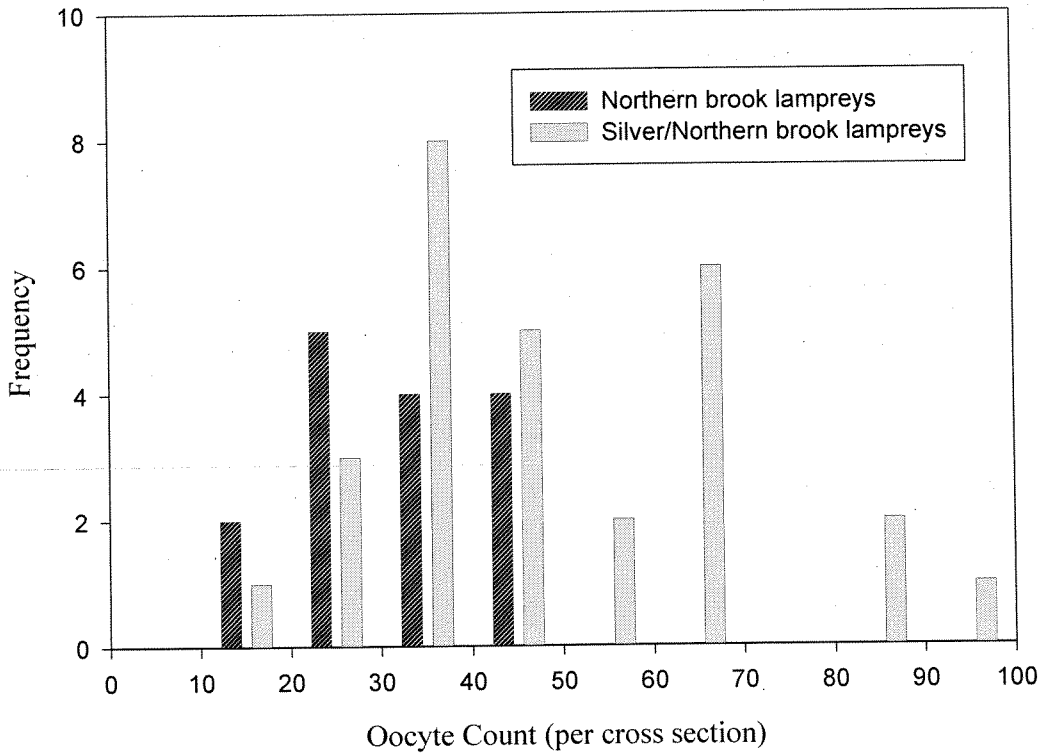


Figure 6. Oocyte counts per cross section in the mixed silver/northern brook population (N= 28), and northern brook population (N=15).

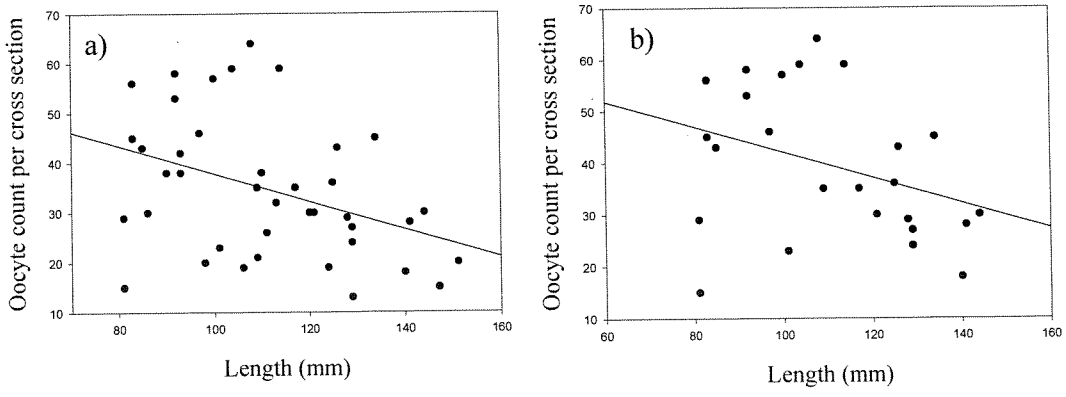
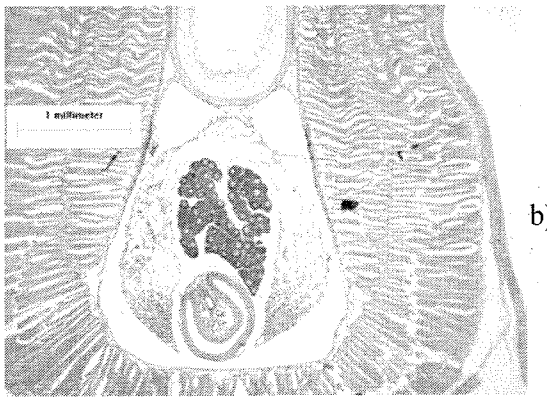


Figure 7. Regression of oocyte count on length of a) northern brook lamprey ammocoetes, (r^2=0.421) and b) mixed silver/northern brook lamprey ammocoetes (r^2=0.088).

a)



b)

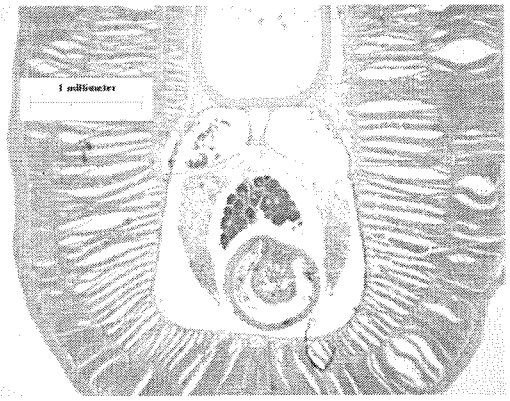


Figure 8. Ovarian cross section of two ammocoetes of similar size, displaying variation in formation and number of oocytes. Specimen a) has a total length of 132mm, and b) is 129 mm in length.

Table 4. Counts of trunk myomeres from northern brook, mixed silver/northern brook, and chestnut lamprey populations with 95% confidence intervals.

Lamprey Species	*N*		Myomere Count 47	48	49	50	51	52	53	54	55	56	57	Mean ± 95% C.I.
Northern brook	279	Count	1	6	25	62	72	48	39	23	3	-	-	51.26 ± 0.18
		Percent	0.4	2.2	9.0	22.2	25.8	17.2	14.0	8.2	1.1			
Northern brook/silver	912	Count	13	30	62	129	179	226	161	88	18	5	1	51.57 ± 0.11
		Percent	1.4	3.3	6.8	14.1	19.6	24.8	17.7	9.6	2.0	0.5	0.1	
Chestnut	60	Count	2	2	1	7	8	12	16	7	4	1	-	52.05 ± 0.50
		Percent	3.3	3.3	1.7	11.7	13.3	20.0	26.7	11.7	6.7	1.7		

Table 5. Morphometric measurements of northern brook, silver/northern brook, and chestnut lamprey populations including mean, standard deviation and range. All measures are residuals of values regressed against total length.

Species		Head Length (mm)	Branchial Length (mm)	Trunk Length (mm)	Tail Length (mm)	Trunk Height (mm)	Branchial Height (mm)	Supra-branchial Band Height (mm)
N.Brook	Mean	0.02	-0.113	0.07	-0.008	0.16	0.05	0.47
	Std. Dev.	0.05	0.77	0.32	0.46	0.70	0.63	1.97
	Range	-0.13 to 0.19	-2.15 to 2.64	-.72 to 1.58	-1.96 to 1.67	-2.14 to 2.49	-1.64 to 1.64	-4.60 to 6.68
Silver/N. Brook	Mean	-0.010	0.07	-0.027	0.01	-0.043	-0.010	0.06
	Std. Dev.	0.05	0.78	0.29	0.50	0.78	0.75	2.19
	Range	-0.19 to 0.17	-2.46 to 3.51	-1.15 to 1.00	-1.57 to 1.77	-2.62 to 2.19	-2.65 to 2.40	-6.26 to 6.99
Chestnut	Mean	0.01	-0.052	-0.091	0.04	-0.271	-0.3782	-1.139
	Std. Dev.	0.03	0.49	0.26	0.39	0.57	0.46	1.76
	Range	-0.06 to .09	-0.83 to 2.19	-0.64 to .64	-1.07 to 0.91	-1.69 to 1.08	-1.18 to .54	-5.45 to 2.17

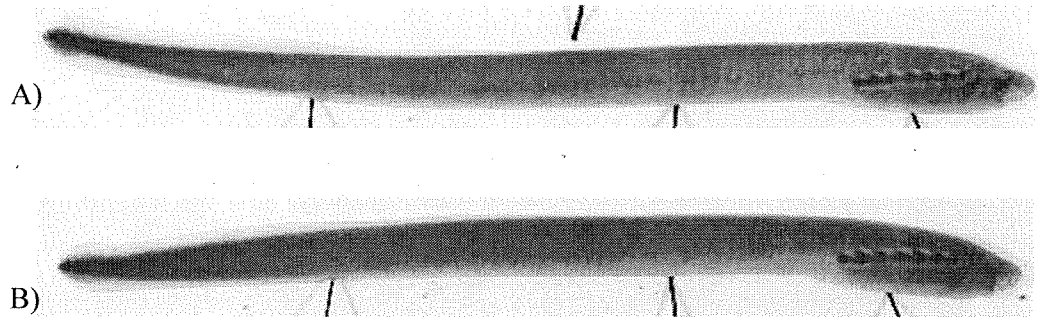


Figure 9. Two similarly sized lampreys possessing widely different oocyte counts. Specimen A is 104 mm long and possessed 90 oocytes/cross section, and B is 109 mm long with 35 oocytes/cross section.

Meristics

Significant differences in myomere counts were found between the species groupings (Kruskal-Wallace test, $p<0.001$), and a Mann-Whitney test found significant differences ($p<0.022$) among all pairings. Means were very similar despite being significantly different, differing by less than one myomere count (Table 4). Intraspecific variability was high for all species groups.

Morphometrics and Pigmentation

Traditional Morphometrics

Results from a series of 10 repeated measures on one individual showed a high rate of precision for the morphometric measurements (standard deviation < 0.03cm). The pigmentation values varied more, but were still low (standard deviation < 2.26% for all), with the exception of the dorsal fin measurement (6.28%), which was subsequently removed from the study.

Welch's tests showed significant differences in many of the characters. Six of the seven morphometric variables were significant ($p<0.001$), with tail length the only insignificant measure ($p=0.66$). Pigmentation analyses showed significant differences among all 11 measurements ($p<0.026$), however differences in the means of both

measurement types were extremely small, and ranges overlapped such that they cannot be used to unequivocally differentiate species (Table 5 and Table 6).

Principal components analysis of the size-corrected morphometric data revealed no distinct groups of individuals (Figures 10, 11), even when broken into 30 millimetre size classes (not shown). Similar results were produced by the principal component analysis of the standardized pigmentation data (Figures 12 and 13). Variation was substantial both within and among species.

Table 6. Ranges, means and standard deviation of mean grey values of eleven body regions of three populations of lampreys.

Species	Pigmentation Character	Mean	Range	Minimum	Maximum	Standard Deviation
N. brook	Head	130.12	86.6	80.45	167.05	14.88
	Supra-branchial	153.16	88.27	107.16	195.43	16.61
	Sub-branchial	131.36	82.54	83.08	165.62	14.45
	Branchial Total	147.65	86.89	94.39	181.28	14.88
	Lateral Line Area	149.18	109.14	88.92	198.06	18.43
	Trunk	139.11	94.32	84.65	178.97	15.95
	Tail	121.68	86.81	78.49	165.3	16.41
	Whole Body	132.70	84.41	87.99	172.4	14.37
	Caudal Edge	85.36	110.54	40.54	151.08	22.17
	Caudal Fin	61.87	56	34.98	90.98	9.64
	Oral Hood	114.38	85.76	68.94	154.7	16.29
Silver/N. brook	Head	130.17	99.66	77.14	176.8	15.30
	Supra-branchial	148.63	95.31	101.38	196.69	15.77
	Sub-branchial	125.85	87.38	78.83	166.21	14.74
	Branchial Total	141.71	83.29	93.92	177.21	14.04
	Lateral Line Area	145.38	105.74	90.75	196.49	15.58
	Trunk	135.76	89.76	89.92	179.68	14.12
	Tail	120.89	94.73	75.71	170.44	15.14
	Whole Body	130.56	84.76	88.1	172.86	13.36
	Caudal Edge	96.70	126.02	45.59	171.61	20.85
	Caudal Fin	56.39	74.41	28.49	102.9	11.57
	Oral Hood	118.81	117.95	65.01	182.96	17.38
Chestnut	Head	125.67	58.32	95.85	154.17	12.11
	Supra-branchial	149.12	70.85	117.58	188.43	13.09
	Sub-branchial	131.81	53.6	104.4	158	9.62
	Branchial Total	144.26	44.66	123.07	167.73	9.42
	Lateral Line Area	151.66	50.12	129.81	179.93	11.60
	Trunk	141.72	47.65	123.09	170.74	10.30
	Tail	125.53	62.4	102.46	164.86	13.21
	Whole Body	135.48	46.04	117.03	163.07	9.53
	Caudal Edge	101.67	103.79	50.48	154.27	22.04
	Caudal Fin	62.79	60.58	29.55	90.13	9.69
	Oral Hood	110.43	66.73	80.91	147.64	14.48

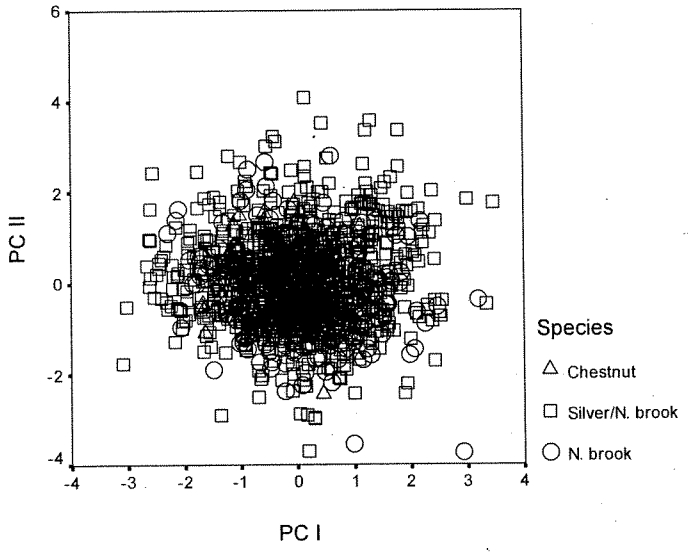


Figure 10. Projection of the first two principal components representing variation in shape within, and among, all chestnut, northern brook, and silver/northern brook lamprey ammocoetes (N=1461).

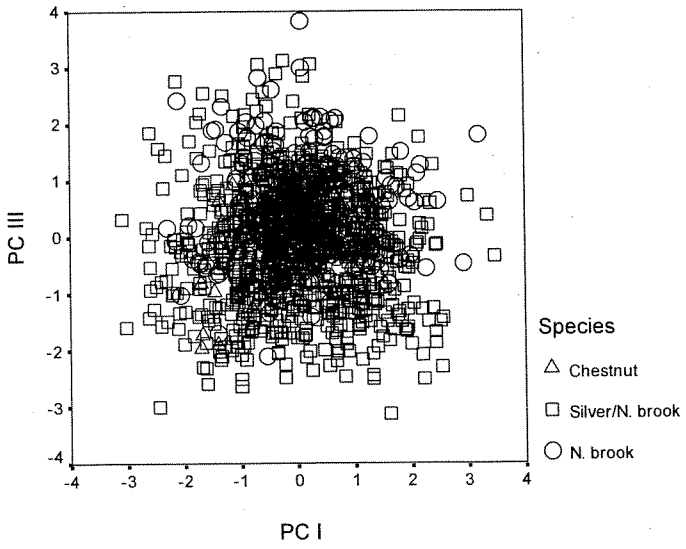


Figure 11. Projection of the first and third principal components representing variation in shape within, and among, all chestnut, northern brook, and silver/northern brook lamprey ammocoetes (N=1461).

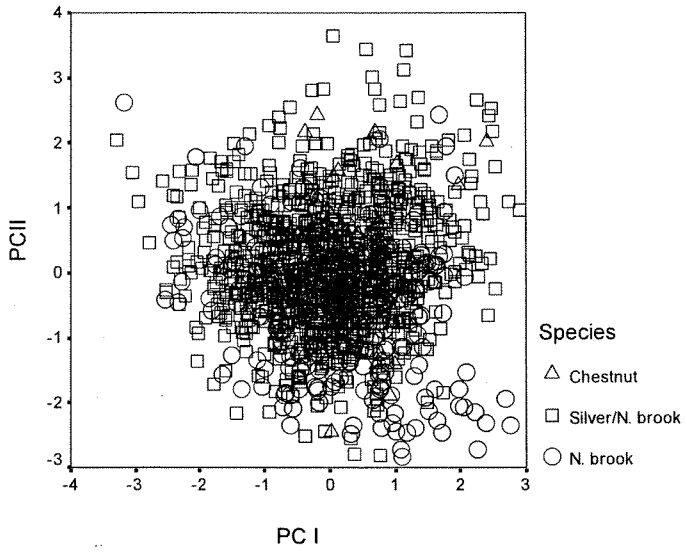


Figure 12. Projection of the first two principal components representing variation in pigmentation within, and among, all chestnut, northern brook, and silver/northern brook lamprey ammocoetes (N=1461).

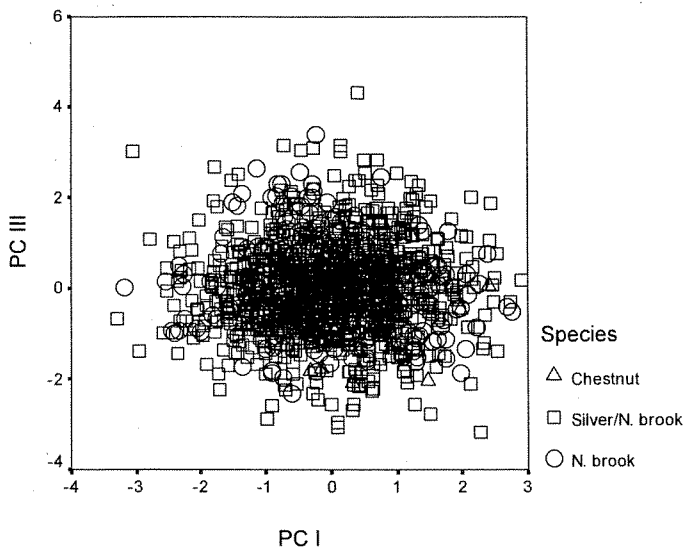


Figure 13. Projection of the first and third principal components representing variation in pigmentation within, and among, all chestnut, northern brook, and silver/northern brook lamprey ammocoetes. (N=1461).

When traditional morphometric and pigmentation data were combined in a PCA, results were not different from the separate analyses, and revealed high variation within and among species groups (not shown).

When data from northern brook lamprey and silver/northern brook lamprey groups were combined and contrasted to chestnut lamprey ammocoetes with discriminant function analysis, the classification rate was high (95.3 %). This however, was misleading, because the classification success rate of the chestnut lampreys was extremely poor at 14.7%. The high overall classification success rate was influenced strongly by the much larger silver/northern brook lamprey group (99.4% success rate), so the overall success acts as a weighted average, and the overall high success rate is deceiving. The differentiation power of this set of data was minimal, as a chestnut lamprey would be classified as a silver/northern brook lamprey 85 times out of 100.

When smaller size classes were considered in similar analyses, the chestnut lamprey classification success rate increased, but was still low. Size classes of 50-80, >80 to 110, and >110 to 140 millimetres provided classification rates of 15, 44 and 50% respectively (Table 7). There was divergence from a common form with increasing size based on loadings on tail pigmentation and head length. The high Wilk's Lambda values showed that the differences between the groups were small, despite their significance in three of the four analyses.

The analysis of three species groups (northern brook, chestnut, and silver/northern brook lampreys) using ammocoetes from Black River to represent silver lampreys showed better classification success rates (77% using pigmentation data, 74% using morphometric data, Table 8), but again these overall success rates were skewed by the

large sample size of northern brook lampreys. The silver and chestnut lampreys had much lower classification success.

Discriminant function analyses investigating the effects of geographic variation within the basin of provided considerably different results (Figure 14). Visualization of different species groups was possible in the data projection, and the classification success was much higher, over 90% (Table 9). This was accompanied by low Wilks Lambda values (Table 9), meaning differences between groups are substantial. However, considerable overlap still occurred between species groups.

Morphometric variables on the first root (head and branchial length) explained 54% of the variation. The second root, which served to separate northern brook and chestnut lampreys, explained 46% of the variation, and the key variable was the degree of pigmentation in the edge of the caudal fin. The characters contributing most to this variation were morphometric: head length, branchial length and tail length had the highest loadings in the DFA (Table 10).

Table 7. Significance level, Wilks lambda values and classification success rates from discriminant function analyses of size classes of two species groups of ammocoete using morphometric and pigmentation data.

Size class (mm)	N	Wilks lambda	p-value	Classification Success % (chestnut)	Classification Success % (silver and n. brook)	Classification Success % (overall)
50-80	161	0.823	0.049	15.4	100	93.1
80-110	568	0.759	<0.001	44.4	97.7	93.4
110-140	462	0.945	0.115	50	99.8	99.6
All	1366	0.882	<.001	14.1	99.4	95.3

Table 8. Significance level, Wilks lambda values and classification success rate from discriminant function analyses of all chestnut lamprey, all northern brook lamprey, and a subset of silver/northern brook (Black R. ammocoetes) using morphometric and pigmentation data.

	N	Wilks lambda	p-value	Class. Success % chestnut	Class. Success % Black R. (silver)	Class. Success % northern brook	Class. Success % overall
Morphometric data	446	0.479	<.001	18.8	77.8	90.0	77.3
Pigmentation data	446	0.365	<.001	42.2	57.8	87.2	74.7

Table 9. Significance level, Wilks lambda values and classification success rate from discriminant function analyses of chestnut, a silver/northern brook, and northern brook lamprey groups using morphometric and pigmentation data. Species groups were selected from widely different geographic areas.

N	Wilks lambda	p-value	Class. Success % chestnut	Class. Success % Black R. (silver)	Class. Success % northern brook	Class. Success % overall
180	.117	<0.001	93.9	93.3	88.3	91.7

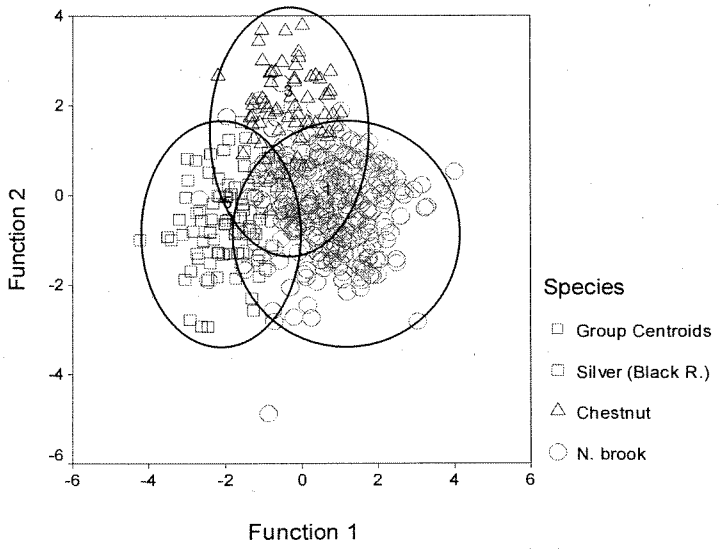


Figure 14. Roots 1 and 2 from discriminant function analysis of standardized morphometric and pigmentation data from a subsample of 90 silver/northern brook lampreys from Black R., 60 northern brook lamprey from Grand R. and Big Cr., and 64 chestnut lamprey ammocoetes from Manistee R., Big Sauble R. and Betsie R.

Table 10. Character coefficients of morphometric and pigmentation (mgv) variables for roots one and two from discriminant analysis of 90 silver/northern brook lampreys from Black River, 60 northern brook lamprey from Grand River and Big Creek, and 64 chestnut lamprey ammocoetes from Manistee, Big Sauble and Betsie Rivers. All measurements are from live ammocoetes.

	Function	
Character	1	2
Head Length	0.579	0.181
Branchial Length	-0.480	-0.187
Tail Length	0.429	0.242
Total Branchial mgv	0.404	0.073
Head mgv	0.403	0.041
Suprabranchial mgv	0.383	0.056
Caudal Fin mgv	0.368	0.213
Oral Hood mgv	0.312	0.027
Subbranchial mgv	0.308	0.155
Trunk Area mgv	0.298	0.219
Total Body mgv	0.267	0.220
Lateral Line Area mgv	0.263	0.189
Maximum Branchial Height	-0.171	-0.400
Suprabranchial Band Height	0.082	-0.347
Maximum Trunk height	0.012	-0.286
Dorsal Fin mgv	-0.222	0.257
Trunk Length	0.009	-0.244
Tail Area mgv	0.172	0.195

Preserved Specimen Analyses

Statistical analysis of pigmentation and morphometric data from preserved specimens did not always concur with live specimen results. *Post hoc* comparisons of each of the variables revealed that 57% of the pairings of variables in both the live and preserved specimens had significant differences, (31 of 54 and 26 of 45 respectively, not shown). However, the differences were not always consistent, suggesting that preservation does have differential effects on ammocoetes (see Chapter 1). Most revealing was that the suprabranchial non-pigmented band height, significant in all three pairings of live silver/northern brook, chestnut and northern brook lamprey groups, was not significant in any of them after preservation (p=0.058). The head length still remained significantly different between the silver and northern brook ammocoetes (p<0.001). Multivariate analyses of the preserved specimens revealed similar results to those discussed above. Distinct non-overlapping groups were not observed in principal component analyses, and discriminant function analyses showed lower classification success rates, but similar characteristics contributed to each function (not shown).

Visual Pigmentation Analysis

One character analyzed, the degree of upper lip pigmentation was found to be uniform, and hence was excluded from analysis. The other three characters varied among individuals, and the Kruskal-Wallis test revealed significant differences among species (p<0.002 for all three characters). However, the mean values once again revealed very small differences (Table 11), and were accompanied by high intraspecific variation.

Geometric morphometrics

A graphical representation of the landmark data is shown as Cartesian deformation grids in Figure 20. The only substantial difference between species groups was a slightly more posteriorly located origin of the dorsal fin in both the northern brook and silver/northern brook lamprey groups when compared to the chestnut lamprey ammocoetes (Figure 16). The differences were small, and were magnified to make then discernable. A MANOVA shows the differences to be significant ($p<0.001$) but the degree of separation of the means was very low (Wilks' Lambda = 0.793). A principal components analysis showed no distinct groups (Fig. 17). The DFA comparing silver/northern brook and northern brook lampreys with chestnut lampreys revealed an overall classification rate of 95.4%, but only 8.8% for chestnut lampreys.

Table 11. Mean and standard deviations of visual pigmentation scores of northern brook, silver/northern brook and chestnut lamprey ammocoetes. Scoring system is displayed in Table 3.

Species	Pigmentation Measure	Mean	Standard Deviation
N. brook	Caudal	2.18	0.51
	Sub-orbital	0.34	0.58
	Pre-branchial	1.23	0.44
Silver/N. brook	Caudal	2.29	0.53
	Sub-orbital	0.02	0.71
	Pre-branchial	1.13	0.35
Chestnut	Caudal	2.78	0.55
	Sub-orbital	0.09	0.74
	Pre-branchial	1.16	0.52

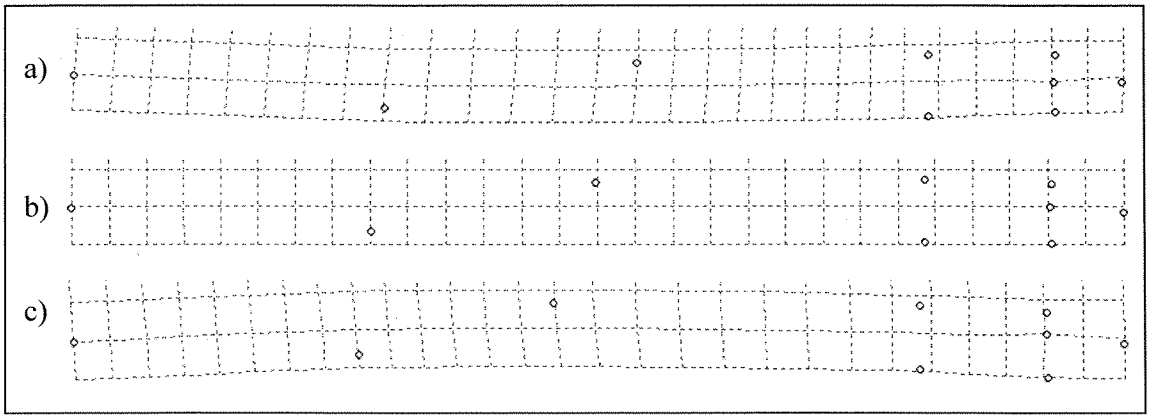


Figure 16. Deformation grids of a) northern brook lampreys, b) silver/northern brook lampreys, c) chestnut lampreys. Differences are magnified by three for visualization purposes.

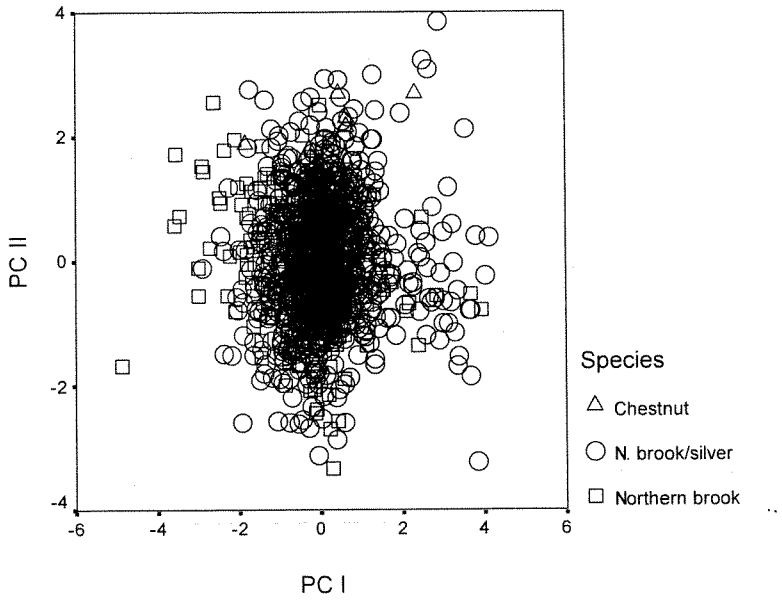


Figure 17. Scatter plot representing variation in shape within, and among, chestnut, northern brook, and silver/northern brook lamprey groups from principal component analysis of geometric morphometric data.

Discussion

The results of this study indicate that there are no clear methods or diagnostic characteristics to consistently differentiate the northern brook and the silver lamprey on a basin-wide scale. The chestnut lamprey could be identified if it had developed pigmented lateral line organs, but small chestnut lamprey ammocoetes (<105mm) failed to display this character, and were very similar to the other two species. Comparisons of pigmentation and morphometric data among all species groups exhibit significant differences. However, these differences were very small and have high intraspecific variation, are hence they are insufficient for use in a taxonomic key. This variation was reflected in the multivariate analyses, as both principal component and discriminant function analyses show no clear differences between the three groups of species when all

ammocoetes were considered. Breaking the samples into size classes still revealed high intraspecific variation.

An analysis of a smaller sub-set of the data from different geographic areas revealed different results. Head lengths and branchial lengths were two of the main factors that had some utility as indicators of specific origin (as indicated by grouping in the principal components analysis and high classification success rate in the discriminant function analysis). There are two possible explanations for these observations. The differences found in this subset, but not in the whole basin, could be attributed not to variation among species, but to geographic variation. This hypothesis is supported by the life history of northern brook lampreys, as they are largely limited to within-stream movement (Scott and Crossman 1973), and inter-stream mixing is likely minimal. (This hypothesis assumes that gene flow between silver and northern brook lampreys does not occur, if it does, silver lamprey could mediate inter-stream mixing of northern brook lamprey populations.) This relative isolation from other streams could have facilitated divergence from other populations.

Another possibility is that the silver lampreys (from silver/northern brook lamprey populations) in the Black River used in this sub-sample analysis (selected because of the high metamorphosis rate of silver lamprey juveniles) could be one of the few streams that had a high relative abundance of silver lampreys. For example, the remaining mixed silver/northern brook lamprey streams may be composed of a greater proportion of northern brook lampreys than silver lamprey ammocoetes, which would explain the high degree of overlapping characteristics found in the overall analysis.

The key by Vladykov and Kott (1980) was not supported at all by the results of the current study. The pigmentation characters that they deemed species-specific had no consistent patterns among species. The supra-branchial band height, which Lanteigne (1981) used as a diagnostic character, was found to be highly variable as well.

Genetics

Ammocoetes displaying pigmented lateral line organs were all verified by RFLP analysis to be chestnut lampreys, but all chestnut lampreys did not display pigmented lateral lines. The RFLP results indicate further that chestnut lampreys rarely possess this character at lengths if less than 105 millimetres in length. This disagreed with the keys published by Lanteigne (1981) and Vladykov and Kott (1980). Identification of small ammocoetes (less than 105 millimetres) cannot reliably use this characteristic, as it is variable in its rate of development. Larger ammocoetes consistently display this character, and it becomes more prominent (i.e. darker pigmentation) with increasing ammocoete body length.

Oocyte analysis

The oocyte counts of northern brook and mixed silver/northern brook lamprey populations overlapped substantially, but had considerably different ranges (13-42 compared to 15-93, respectively). No other research on ammocoete gonads of these species has been conducted, but other paired species reveal similar results. Hardisty (1961) found a range of 25 to 50 oocytes for the non-parasitic European brook lamprey, and 65 to 110 for the European river lamprey, the parasitic representative of that paired species. This reflects the difference in adult fecundity of this pair, ranging from 5,000 to 10,000 for the brook lamprey and 14,000 to 26,000 for the parasitic river lamprey

(Hardisty 1963), which represents a narrower range than the paired species in this study (1500 and 29,000 for northern brook and silver lamprey, respectively) (Vladykov 1951). Hughes and Potter (1968) reported much smaller differences in the post-metamorphic Australian lampreys they examined, finding means of 16 to 20 oocytes in the non-parasitic species and 20 to 23.5 in the parasitic species.

The three lampreys at the high end of the oocyte distribution counts in the mixed population (80, 83, and 93 oocytes) suggested that these may be the only silver lampreys present in the histological analysis as all the other lampreys (39) possessed counts of less than 59. This would indicate very low numbers of silver lampreys, which contradicts catch records of metamorphosed individuals. Post-metamorphic catch data indicate an equal composition of silver lampreys (13 silver lampreys of 26 collected).

Oocyte numbers can be a function of size and age. Hardisty (1964) found that atresia (a form of degeneration) of oocytes was responsible for a reduction of 60-90% of oocytes in the European brook lamprey. If similar processes occurred in northern brook lamprey, the contrast in fecundities of the adults may not be reflected in the ammocoetes, therefore simple comparisons of oocyte numbers per cross section must be considered carefully. However, Hardisty (1971) later speculated that atresia is not necessarily a common feature of non-parasitic lamprey. Hence, the results of the oocyte analysis displayed some potential to determine species identity of individuals on either extreme of an oocyte count distribution, but the majority of ammocoetes which fall in the middle of the distribution cannot be confidently identified. Thus, without extensive additional sampling and some type of verification, this technique is not reliable for species differentiation.

The three individuals with considerably higher oocyte counts were paired with similarly sized ammocoetes with low oocyte counts, and external characters were compared in an effort to detect any obvious differences that might be associated with differences in ovaries. These visual comparisons revealed no obvious differences, suggesting that differences in gonad properties may not be reflected in external characteristics.

Meristics

The myomere counts, while significantly different, varied by less than one myomere among species groups. The myomere counts obtained here largely concur with other studies. The northern brook lamprey mean of 51.2 myomeres was similar to 50.6 and 50.9 as reported by Lanteigne (1981) and Hubbs and Trautman (1937) respectively. Results from the silver lampreys also were similar, with my result of 51.6 compared to 51.7 from Lanteigne (1981), and a mean value of 50.5 in Hubbs and Trautman (1937). The mean obtained here for chestnut lamprey ammocoetes was 52.0, similar to previous findings of 52.6 (Hubbs and Trautman 1937) but considerably different from 54.2 myomeres (Lanteigne 1981). However, when I re-calculated the mean from Lanteigne's (1981) raw data, a corrected new mean of 52.4 corresponds with the other values. The means obtained in the current study are statistically different among all species groups as Lanteigne (1981) found. However, due to the overlap of ranges and high intraspecific variability, is not a practical diagnostic character.

Morphometrics and Pigmentation

The pigmentation and traditional morphometric contrasts also show significant, but not diagnostic, differences among the three species. Principal component analysis

consistently showed extensive overlap between the three species with regards to both pigmentation and body shape. Grouping silver and northern brook lampreys and then comparing them to chestnut lampreys revealed very low classification success rates in discriminant function analysis. Vladykov and Kott's (1980) designation of pigmentation in the head and tail to differentiate northern brook and silver lampreys is not supported. However, they speculated on the variability of this characteristic and questioned its reliability. The key by Lanteigne (1981), which promoted the use of a band of non-pigmentation above the branchial pores, was also not supported in this analysis. This characteristic was also found to be highly variable both within, and among, species groups.

The variance of the pigmentation measurements found in Chapter One resulted in a secondary independent pigmentation assessment. This was conducted visually, and this analysis supported the results of the image analysis software: differences were found among species groups, but these differences were very small, and intraspecific variation was extremely high.

The geometric morphometric results showed small, but not diagnostic, differences between species groups. However, these results did not correspond to the differences produced by traditional morphometric analyses. The shifting of the dorsal fin origin was the main source of the geometric differences, but this was very minor and could not be used for taxonomic purposes. It is unexpected that the geometric analysis did not entirely concur with the traditional morphometrics. Both types of analyses indicated an inability to differentiate species using body form.

Lamprey identification difficulties are not novel, as illustrated by an inability to differentiate other sets of paired lampreys in their larval form. Examples of this include the European river lamprey and the European brook lamprey (Potter et al. 1968), and the paired *Mordacia* species in Australia (Potter and Osborne 1975). Potter and Osborne (1975) investigated differences in two paired species of British larval lampreys, and found after analysis of trunk myomeres, pigmentation patterns and morphometrics, that despite some significant differences (myomeres and rate of change of body proportions) there was no clear method of distinguishing them. They concluded that an existing key published by MacDonald (1959) was flawed, and discredited characters previously viewed as diagnostic for these paired species. Obvious parallels exist with the current study. Published keys for northern brook, silver and chestnut lamprey ammocoetes appear inadequate. It is possible that the existing conflicting keys can be explained by geographic variation within each species. Previous keys were based on a relatively small number of populations (three in Lanteigne (1981), Vladykov and Kott (1980) did not specify the numbers of populations used). Furthermore, the validation of identification of ammocoetes used in their keys was not documented.

This lack of consistent differences between species across the Great Lakes basin might be explained by hybridization. Although crosses between silver and northern brook lampreys have successfully produced larvae which survived into early life stages (Piavis et al. 1970), full development of offspring has never been confirmed. Furthermore, the size discrepancy between the adults of these two species is believed to inhibit hybridization, as size is an important element during the act of spawning (Beamish and Neville 1992). However, accidental hybridization may occur, as other paired lamprey

species have been observed spawning on the same redd (Hardisty and Huggins 1970) Hubbs and Trautman (1937) have given consideration to the possibility that there may be hybridization between different species of the *Ichthyomyzon* genus. They suggested that intermediate forms may be possible between other paired species within the genus, but speculated that the possibility of hybridization between the silver and northern brook lamprey was very small.

Another potential explanation of the difficulty in identifying northern brook and silver lamprey ammocoetes is the relative recent occurrence of their speciation event. Paired *Lampetra* species diverged less than 70 000 years ago (Docker *et al.* 1999), and based on the lack of differences in almost 13,000 base pairs of the mitochondrial genome, Docker (pers. comm.) estimates that northern brook and silver lampreys may have separated less than 5000 years ago. This illustrates that there may have been very little time for species to develop differences in terms of both genetics and external larval characteristics. However, chestnut lamprey probably diverged from other lamprey species at least two to three million years ago (Docker pers. comm.) and small chestnut ammocoetes are indistinguishable from northern brook and silver lamprey ammocoetes. This suggests that had the northern brook and silver lamprey diverged less recently, differences in ammocoetes of these two species may still not have evolved.

These similarities between lamprey species in their larval phase are not unexpected. The ammocoetes of the three species undergo identical selection pressures, as they live in very similar, and often identical, habitat. As adults, the parasitic and non-parasitic life histories are influenced by different selective advantages, and this is

reflected in their considerably different morphological characteristics and pigmentation patterns, in contrast to the ammocoete phase.

This cumulative lack of differences between the northern brook and the silver lamprey ammocoetes raises the question of the validity of the original species designation of these two groups by Hubbs and Trautman (1937), especially given the inconclusive results of the concurrent genetic investigation. Is it possible that one population produces two life history types? That is, could the northern brook and silver lampreys be one species with plastic life histories? Genetic analysis up to this point supports the fact that there is, or has recently been, gene flow between the species. The probability of reproductive isolation as previously discussed implies otherwise. Moreover, the presence of small, but significant, differences of many of the morphometric and pigmentation characters analyzed in this study, in combination with an apparent bimodality in oocyte counts, supports the current species designations.

The high intraspecific variation found in morphometric and pigmentation analyses in this study may be accounted for by examining the history and evolutionary trends of these species. Bell and Andrews (1997) speculated that there may have been multiple formations of non-parasitic lamprey species from one parasitic species, even within the same geographic area. Furthermore, the both the silver and northern brook lamprey colonized the Great Lakes through two different dispersal routes following the most recent glaciation (Mandrak and Crossman 1992), and this may have provided different sources of populations, and could explain the potential evolution of several different forms of each species. These factors could confound analyses looking for common

differences between the species, as multiple colonization times and places of a species could result in populations possessing widely divergent characteristics.

A considerable setback of this study was the inability to develop diagnostic genetic markers for northern brook and silver lampreys. This was compounded by the absence of lamprey populations where silver lampreys were known to be the only species present. This necessitated sampling streams where both species co-existed. As a result, direct comparisons of ammocoetes of pure populations of each of the three species were impossible, and any differences between species were weakened due to the assumed presence of the second species, even if in small numbers. Contrasting northern brook lampreys with a mix of silver and northern brook lampreys had limitations due to potential contamination of silver lamprey samples. However the multivariate analyses used here minimized these effects. Principal component analysis as used in this study is conducive to potentially mixed samples, as it is an exploratory procedure where individuals, although they may be grouped *a priori,* are not necessarily considered together. This allowed investigation of differences both within, and among, species groups, and the dilution factor is not critical, as characters delineating any group or sub-group of individuals would be revealed.

Despite using historical catch records to determine species presence in streams, one of the species may have been under-represented, or absent from the samples (i.e. the mixed silver/northern brook lamprey populations could be composed largely of northern brook lampreys, as mentioned previously). However, a small number of the large ammocoetes collected in conjunction with this study (26 individuals from five mixed

silver/northern brook streams, Appendix III) were held in aquaria throughout their metamorphosis period to juveniles, at which point they were identified using dentition and disc size. Silver lampreys dominated, representing 65% of the juveniles. This supports using historic stream catch data as an indication of species' presence.

This study would have benefited from the establishment of genetic markers distinguishing the silver and northern brook ammocoetes, as the presence or absence of sufficient representation of each species would not be in question. An answer to the dilemma of reliable species identification is the rearing of ammocoetes from known species of parents, as performed with other species by Richards (1980). This would eliminate any uncertainty of species origin, and would facilitate future comparisons of species.

In summary, the original intent of this study, to establish a key for larval *Ichthyomyzon* species, was not met with success due to an inability to find consistent external differences between species. Chestnut lamprey ammocoetes can be differentiated from the silver and northern brook lamprey ammocoetes at all sizes using genetic analysis, or in larger ammocoetes by the presence of pigmented lateral line organs. Chestnut lamprey ammocoetes smaller than 105 millimetres do not always display this pigmentation, therefore the pigmentation characteristic is not always applicable. A smaller scale comparison of ammocoetes from several streams showed differences in head length and one area of pigmentation between the three species, but this was not applicable across the Great Lakes basin, and may be due to geographic, rather than interspecific, differences. Silver, northern brook, and small chestnut lamprey ammocoetes are exceedingly similar when considered across their distribution considered

here. Therefore, previously published keys must be considered with the caveat that they may be applicable to only specific areas, or may be inaccurate, particularly those using pigmentation patterns of chestnut lamprey ammocoetes. Using differences in oocyte counts did show some promise as a method of differentiating species, but a substantially larger sample size is required to understand the applicability of this procedure. Rearing of ammocoetes to metamorphosis or the identification of genetic markers would enhance confidence in determining specific origin of ammocoetes. This in turn may allow for establishment of differences between species across the basin, or facilitate development of taxonomic keys applicable to smaller geographic areas.

References

1. Beamish, F.W. 1993. Environmental sex determination in southern brook lamprey, *Ichthyomyzon gagei*. Canadian Journal of Fisheries and Aquatic Sciences **50**: 1299-1307.

2. Beamish, F.W.H. and Thomas, E.J. 1983. Potential and actual fecundity of the "paired" lampreys, *Ichthyomyzon gagei* and *I. castaneus*. Copeia **2**: 367-374.

3. Beamish, R.J. and Neville, C.M. 1992. The importance of size as an isolating mechanism in lampreys. Copeia **1**: 191-196.

4. Bell, M.A. and Andrews, C.A. 1997. Evolutionary consequences of postglacial colonization of freshwater by primitively anadromous fishes. Evolutionary Ecology of Freshwater Animals **82**: 323-363.

5. Bird, D.J. and Potter, I.C. 1979. Metamorphosis in the paired species of lampreys, *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch). Zoological Journal of the Linnean Society **65**: 127-143.

6. Davis, W. Comparative susceptibility of three genera of larval lampreys to a lampricide. M.Sc. Thesis. 1970. University of Guelph.

7. Docker, M. F. Labile sex determination in lampreys: the effect of larval density and sex steroids on gonadal differentiation. Ph.D.Thesis. 1992. University of Guelph.

8. Docker, M.F., Youson, J.H., Beamish, R.J., and Devlin, R.H. 1999. Phylogeny of the lamprey genus *Lampetra* inferred from mitochondrial cytochrome *b* and ND3 gene sequences. Canadian Journal of Fisheries and Aquatic Sciences **56**: 2340-2349.

9. Fuiman, L.A. 1982. Family Petromyzontidae, lampreys. *In* Identification of larval fishes of the Great Lakes basin with emphasis on the Lake Michigan Drainage. *Edited by* N.A. Auer. Great Lakes Fisheries Commission Special Publication, pp. 23-37.

10. Gillespie, G.J. and Fox, M.G. 2003. Morphological and life-history differentiation between littoral and pelagic forms of pumpkinseed. Journal of Fish Biology **62**: 1099-1115.

11. Hall, G.E. and Moore, G.A. 1954. Oklahoma lampreys: their characteristics and distribution. Copeia **1954**: 127-135.

12. Hardisty, M.W. 1961. Oocyte numbers as a diagnostic character for the identification of ammocoete species. Nature **191**: 1215-1216.

13. Hardisty, M.W. 1963. Fecundity and speciation in lampreys. Evolution **17**: 17-22.

14. Hardisty, M.W. 1964. The fecundity of lampreys. Archiv Fur Hydrobiologie **60**: 340-357.

15. Hardisty, M.W. 1971. Gonadogenesis, sex differentiation and gametogenesis. *In* The Biology of Lampreys. *Edited by* M.W. Hardisty. Academic Press, London pp. 295-359.

16. Hardisty, M.W. and Huggins, R.J. 1970. Larval growth in the river lamprey *Lampetra fluviatilis*. Journal of Zoology: London **161**: 549-559.

17. Hardisty, M.W. and Potter, I.C. 1971. The behaviour, ecology and growth of larval lampreys. *In* The biology of lampreys. Academic Press, London pp. 5-120.

18. Hubbs, C.L. and Trautman, M.B. 1937. A revision of the lamprey genus *Ichthyomyzon.* Miscellaneous Publications of the Museum of Zoology.

19. Huggins, R.J. and Thompson, A. 1970. Communal spawning of brook and river lampreys, *Lampetra planeri* Bloch and *Lampetra fluviatilis* L. Journal of Fish Biology **2**: 53-54.

20. Hughes, R.L. and Potter, I.C. 1969. Studies on gametogenesis and fecundity in the lampreys *Mordacia praecox* and *M. mordax* (Petromyzonidae). Australian Journal of Zoology **17**: 447-464.

21. Kott, E. 1974. A morphometric and meristic study of a population of the American brook lamprey, *Lethenteron lamottei* (LeSueur), from Ontario. Canadian Journal of Zoology **52**: 1047-1055.

22. Lanteigne, J. The taxonomy and distribution of the North American lamprey genus *Ichthyomyzon.* M. Sc. Thesis. University of Ottawa. 1981.

23. Lanteigne, J. 1988. Identification of lamprey larvae of the genus *Ichthyomyzon* (Petromyzontidae). Environmental Biology of Fishes **23**: 55-63.

24. Lanteigne, J. 1991. Status of the Northern brook lamprey, *Ichthyomyzon fossor*, in Canada. Status Report on Endangered Wildlife in Canada.

25. Lanteigne, J. 1992. Status of the Chestnut Lamprey, *Ichthyomyzon castaneus*, in Canada. Canadian Field-Naturalist **106**: 14-18.

26. Leach, W.J. 1940. Occurrence and life history of the northern brook lamprey, *Ichthyomyzon fossor*, in Indiana. Copeia **1**: 21-34.

27. Lyons, J., Cochran, P., and Sneen, M. 1997. Taxonomic status and distribution of the lamprey *Ichthyomyzon* cf. *gagei*. The American Midland Naturalist **138** : 69-76.

28. Macdonald, T.M. 1959. Identification of British lampreys. Glasgow Naturalist **18**: 91-95.

29. Malmquist, B. 1978. Population structure and biometry of *Lampetra planeri* (Bloch) from three different watersheds in South Sweden. Arch. Hydrobiol. **84**: 65-86.

30. Mandrak, N. E., Docker, M. F., and Heath, D. D. Heath 2002. Native *Ichthyomyzon* lampreys of the Great Lakes: development of genetic markers and a morphological key to ammocoetes. Great Lakes Fishery Commission Annual Progress Report.

31. Manion, P.J. 1972. Variations in melanophores among lampreys in the upper Great Lakes. Transactions of the American Fisheries Society **101**: 662-666.

32. Neira, F.J., Bradley, J.S., Potter, I.C., and Hilliard, R.W. 1988. Morphological variation among widely dispersed larval populations of anadromous southern hemisphere lampreys (*Geotriidae* and *Mordaciidae*). Zoological Journal of the Linnean Society **92** : 383-408.

33. Parsons, K.J., Robinson, B.W., and Hrbek, T. 2003. Getting into shape: an empirical comparison of several traditional truss-based morphometric methods with a newer geometric method applied to New World cichlids. Environmental Biology of Fishes 417-431.

34. Piavis, G.W. , Howell, J.H., and Smith, A.J. 1970. Experimental hybridization among five species of lampreys from the Great Lakes. Copeia **1970**: 29-37.

35. Potter, I.C. , Lanzing, W.J.R., and Strahan, R. 1968. Morphometric and meristic studies on populations of Australian lampreys of the genus *Mordacia*. Journal of the Linnean Society of

London **47**: 533-546.

36. Potter, I.C. and Osborne, T.S. 1975. The systematics of British larval lampreys. Journal of Zoology **176**: 311-329.

37. Potter, I.C. and Strahan, R. 1968. The taxonomy of the lampreys *Geotria* and *Mordacia* and their distribution in Australia. Journal of zoology: Proceedings of the Linnean Society of London **179**: 229-240.

38. Scion Image for Windows. Rasband, W. 2000. Frederick, Maryland.

39. Renaud, C. 1997. Conservation status of Northern hemisphere lampreys (Petromyzontidae). Journal of Applied Ichthyology **13**: 143-148.

40. Richards, J. E. The freshwater life history of the anadromous Pacific lamprey, *Lampetra tridentata.* M. Sc. Thesis. University of Guelph. 1980.

41. Richards, J.E., Beamish, R.J., and Beamish, F.W.H. 1982. Descriptions and keys for ammocoetes of lampreys from British Columbia, Canada. Canadian Journal of Fisheries and Aquatic Sciences **39**: 1484-1495.

42. tpsRegr. Rohlf, F. J. 97. State Univeristy of New York, Stony Brook: Department of Ecology and Evolution.

43. tpsDIG. Rohlf, J. F. 96. SUNY at Stony Brook.

44. tpssmall. Rohlf, J. F. 2000. SUNY at Stony Brook.

45. tpsUtility. Rohlf, J. F. 2002. SUNY at Stony Brook.

46. Schuldt, R.J. and Goold, R. 1980. Changes in the distribution of native lampreys in Lake Superior tributaries in response to sea lamprey (*Petromyzon marinus*) control, 1953-1977. Canadian Journal of Fisheries and Aquatic Sciences **37**: 1872-1885.

47. Scott, W.B. and Crossman, E.J. 1973. Freshwater Fishes of Canada. Fisheries Research Board of Canada, Toronto, Ontario.

48. Vladykov, V. 1950. Larvae of Eastern American lampreys (*Petromyzonidae*). Le Naturaliste Canadien.

49. Vladykov, V. 1951. Fecundity of Quebec lampreys. Canadian Fish Culturist **10**: 1-14.

50. Vladykov, V.D. 1960. Description of young ammocoetes belonging to two species of lampreys: *Petromyzon marinus* and *Entosphenus lamottenii*. Journal of the Fisheries Research Board of Canada **17**: 267-288.

51. Vladykov, V.D. 1973. North American nonparasitic lampreys of the family Petromyzonidae must be protected. Canadian Field-Naturalist **87**: 235-239.

52. Vladykov, V.D. and Kott, E. 1980. Description and key to metamorphosed specimens and ammocoetes of Petromyzontidae found in the Great Lakes region. Canadian Journal of Fisheries and Aquatic Sciences **37**: 1616-1625.

53. Willey, R.L. 1971. Microtechniques: A Laboratory Guide . The Macmillan Co., New York.

54. Winans, G.A. 1984. Multivariate morphometric variability in Pacific salmon: technical demonstration. Canadian Journal of Fisheries and Aquatic Sciences **41**: 1150-1159.

APPENDIX 3

Effects of ethanol and formalin preservation on pigmentation and length in larval lampreys

Introduction

To prevent protein breakdown, biological samples are often fixed and stored in formalin or, alternately, fixed in formalin and subsequently stored in ethanol. Although both types of preservation techniques have been found to influence pigmentation (Taylor 1981) and body shape (Starrett et al. 1960; Kristoffersen and Salvanes 1998) it is often assumed that the characteristics of preserved specimens do not differ significantly from those of living specimens (Kristoffersen and Salvanes 1998). Taxonomic keys often utilize small differences in pigmentation and morphometric characteristics in preserved specimens (e.g. Vladykov and Kott 1980; Lanteigne 1988), but fail to consider that these characteristics may have been altered by preservation. Ideally, keys would to be applicable to both live and preserved specimens. Larval lampreys, called ammocoetes, differ very little in appearance among species; therefore the use of detailed keys is essential for their identification. Due to a high degree of similarity between these species, ascertaining the potential effects of preservation on the characters which are used in these keys is critical. In this study, I examined the effects of ethanol and formalin preservation on larval lampreys. I performed this research in association with a study which investigated the feasibility of creating an identification key for ammocoetes of *Ichthyomyzon* species (Chapter Two).

Few studies have examined the effects of preservatives on pigmentation in fishes. Fading of preserved lampreys has been documented (Hubbs and Trautman 1937;

Vladykov 1950); however it has been claimed that identification of larval lampreys is facilitated by preservation in formalin, due to increased visibility of pigmentation differences (Vladykov 1950). Different preservation techniques have varied effects on colour patterns of preserved specimens (Taylor 1981). Effects of preservation have been reduced by adding antioxidants (e.g. liquid ionol), which can reduce bleaching of some colours (Fraser and Freihofer 1971; Taylor 1981), but long-term effects of these techniques are not well documented. Furthermore, these antioxidants cannot be used with ethanol (Taylor 1981). Melanin, the pigment which is the basis of the majority of colour patterns, can also by degraded by exposure to light (Taylor 1981).

In contrast, many papers have compared the effects of ethanol and formalin preservation on total lengths of fish (Kruse and Dalley 1990; Hjorleifsson and Klien-Macphee 1992; Distefano et al. 1994; Fisher et al. 1998), with a wide range of results. One study found greater shrinkage in formalin than ethanol in winter flounder (*Pleuronectes americanus*) (Hjorleifsson and Klien-Macphee 1992); another found negligible effects for both in central stonerollers (*Campostoma anomalum*) (Distefano et al. 1994). The most common result was greater shrinkage in ethanol than in formalin, such as found in yellow perch (*Perca flavescens*) (Fisher et al. 1998) and capelin (*Mallotus villosus*) (Kruse and Dalley 1990).

Ethanol and formalin, the most common methods of preserving lampreys, were compared in the current study. The two techniques examined were ammocoete fixation and storage in 10% formalin, and short-term fixation (48 hours) in 10% formalin followed by preservation in 95% ethanol. As the majority of the effects induced by preservation have been shown to occur within a relatively short time period (Morkert and

Bergstedt 1990; Treasurer 1992; Fisher et al. 1998), I focused initial efforts on examining short-term effects, and used multiple measures of pigmentation and shape characters of the ammocoetes. Long-term effects were explored separately using total body pigmentation and total length as representative measurements. The objective of this study is to quantify the potential effects of preservatives on pigmentation and morphological characters. This will allow for determining the applicability of existing taxonomic keys to specimens in different preservatives and to identify potential biases that should be considered when developing a new ammocoete key.

Methods

Specimen Collection

Over 1200 ammocoetes ranging from five to 18 cm in length were collected from 25 streams tributary to Lake Superior, Lake Erie and Lake Huron (Table 1). I included both silver lamprey (*Ichthyomyzon unicuspis)* and northern brook lamprey (*I. fossor)* ammocoetes, as identification of the two species could not be confirmed and morphological and pigmentation differences between species were found to be negligible (Chapter 2). The presence of a single dorsal fin and a low myomere count enabled identification to genus (Vladykov 1950). The ammocoetes were collected between May and September in 2002 and 2003. Specimens were collected using an Advanced BackPack-2 (ABP-2) electrofisher, and shipped live to SLCC, Sault Ste Marie in coolers containing natal resident stream water and approximately 10 cm of mixed silt and sand. Ammocoetes were maintained in flow-through tanks containing St. Mary's River water and were digitally imaged within 2 to 3 weeks.

I systematically divided ammocoetes from 15 streams into two approximately equally sized groups, and each group was preserved in one of two ways. All specimens were fixed in 10% formalin. Of these specimens, 271 were left in formalin for approximately 48 hours, and subsequently transferred to 95% ethanol. The other 1016 ammocoetes remained in 10% formalin throughout the duration of the study.

Table 1. Sources and numbers of ammocoetes by preservation type used in this study.

Source	Ethanol	Formalin
Big Cr.	15	15
Bray R.	15	15
Coldwater Cr.	45	45
Cool Cr.	15	15
Grand R.	9	6
Gratiot R.	15	75
Hog R.	19	71
Little Buffalo R.	15	15
Manistee R.	33	20
Manitou R.	6	6
Big Sauble R.	4	24
Otter R.	30	0
Pearl R.	15	15
Platte R.	15	15
Saugeen R.	10	10
Sibley R.	10	10
Betsie R.	0	18
Black R.	0	90
Cayuga R.	0	90
Fox R.	0	90
Menominee R.	0	90
Peshtigo R.	0	90
Pilgrim R.	0	43
Pine R.	0	58
Upper Middle R.	0	90
Total	271	1016

Digital Imaging

I took digital images of all specimens using an Olympus C-3020 digital camera, with the lens placed 20.5 cm above the subject with the macro setting enabled. Specimens were placed on a 20 cm x 30 cm wax tray which contained 600 mL of stream water. I anaesthetized ammocoetes using a low concentration of clove oil (20 parts per million) to prevent movement during the imaging process, and then submerged them in water during imaging to mitigate the effects of glare (Smith and Smith 1975). I used a sheet of Rite in the Rain All-Weather Copier Paper to provide a white background to minimize background colour influences on measurements of transparent fins. Halogen lights (200 watts) were angled at approximately 45° onto the specimen, at a distance of 30 cm above, and across from, the ammocoete. A ruler was included in the photograph to provide scale. When required, I straightened and stabilized ammocoetes with insect mounting pins, and the centre of the cloaca was indicated by the insertion of an insect pin before taking the photo. All ammocoetes were then sacrificed and stored in 200 millimetre screw-top glass tubes to maintain their shape.

Camera settings were an F-stop of F4, with a shutter speed of 1/100. No flash was used. I increased the whiteness from standard levels by one setting to maximize capture of pigmentation levels. The macro setting was enabled, and auto-focus was used, with the ammocoete in the middle of the field of view. I employed the timer feature to minimize movement of the camera during the imaging to maximize image clarity. The camera resolution setting was 1200 x 1600 pixels. Digital photos were converted from jpeg files to bitmap files for individual image analysis in an image analysis software

package, Scion Image for Windows (Rasband 2000). I standardized the scale before any measurements were made, using a ruler which had been placed in each photo.

Short-Term Effects

Five morphometric measurements and ten pigmentation measurements were made on each image (Fig 1). All measurements were made using Scion Image for Windows (Rasband 2000). I used the Scion measuring tool to conduct morphometric measurements to the nearest hundredth of a cm, and pigmentation levels of different areas were also analyzed with the measure option, which I used to assess the mean grey value of selected areas of the image. The mean grey value range can range from 1-255, and represents the sum of the grey values of all the pixels divided by the total number of pixels. I assumed this measure to represent relative intensity of pigmentation. After 3 weeks the lampreys were individually imaged and measured again in the manner outlined above.

To determine if there were significant changes in characters between live and preserved specimens, a paired t-test was performed on each character, using live versus preserved values of each measurement. Ethanol and formalin preserved specimens were treated separately throughout the analyses.

To determine if the changes were significantly different between formalin and ethanol specimens, first I calculated changes in characters from the live specimens using a percentage change from the initial, live value. I then compared the percent change of each character with a nested analysis of variance (ANOVA) using preservation type as the independent variable and measurement type as the dependent variables. Homogeneity of variance was tested with a Levene's test, and the Welch statistic was employed due to heterogeneity of variance. To quantify effects of preservation on each variable, I

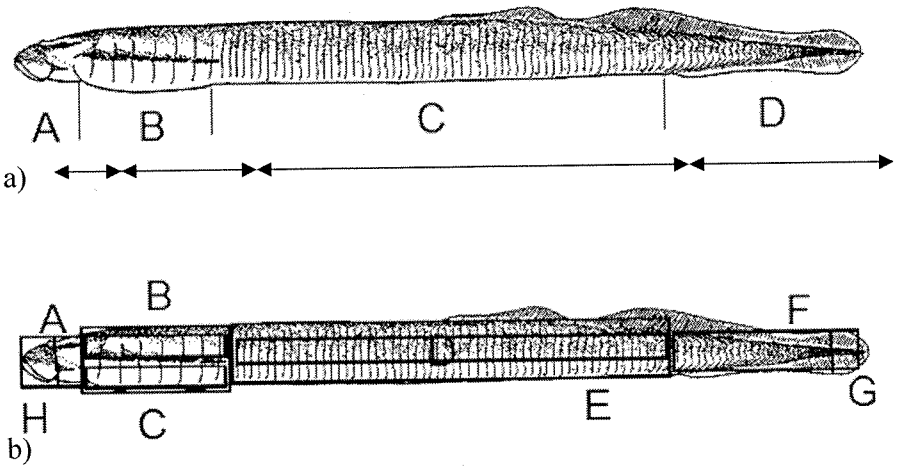


Figure 1. Morphometric (a) and pigmentation (b) measurements used in this study. See Table 2 for descriptions of the measurements.

Table 2. Morphometric and pigmentation measurement descriptions corresponding to Figure 1. Measurements not displayed on Figure 1 are labelled ND.

A. Morphometric Measurements	
A	Head length: from anterior edge of snout to anterior edge of first brachial pore
B	Branchial length: from anterior edge of first branchial pore to anterior edge of last branchial pore
C	Trunk length: from anterior edge of last branchial pore to centre of cloaca
D	Tail length: from centre of cloaca to posterior edge of caudal fin
ND	Suprabranchial non-pigmented band height: height of non-pigmented band anterior to the third and fourth branchial pores
B. Areas for Pigmentation Measurements	
A	Head
B	Suprabranchial area
C	Subbranchial area
B+C	Branchial area
D	Lateral line area on trunk
E	Trunk area
F	Caudal area
G	Caudal fin edge
H	Oral hood
ND	Caudal fin

regressed live pigmentation and morphometric values against preserved values by preservation type.

Long-term effects

I further examined 166 lampreys from the short-term study by measuring total length and total body pigmentation after preservation durations of 3 weeks, 6 weeks, 3 months, and 6 months. Half of the lampreys (83) were originally preserved and left in formalin; the other half (83) were fixed in formalin for 48 hours and then transferred to ethanol. Measurement techniques were as outlined above, but were limited to total length and total body mean grey value. To test if measurements varied with time or between preservation types, I conducted two repeated measures ANOVAs; one for length data and one for pigmentation data. In each ANOVA, the between-subjects effect was preservation type, and the within-subject effect was length or pigmentation. Variances of differences were tested with Mauchly's test of sphericity.

Results

Short Term Effects

Both ethanol and formalin had significant effects on each of the 15 characters ($p<0.025$ in all cases). Mean changes of each characteristic by preservation type are listed in Table 3, which shows the large changes and variation in mean grey values (ranges in value from an increase of pigmentation of 4% to a 30% reduction in pigmentation). Mean changes in morphometric measurements are shown in Table 4. Results ranged from a very slight

increase in branchial length (<1%) for one character in ethanol to a 7% reduction in head length in ethanol.

The ANOVA which tested for differences between ethanol and formalin by character found significant differences among the preservation types ($p<0.001$ for pigmentation measurements, $p=0.026$ for length measurements) and among characters ($p<0.001$ for pigmentation measurements, $p<0.001$ for length measurements). All regression equations for morphometric data were highly significant with high correlation coefficients in both formalin and ethanol (0.72 to 0.99), except for the suprabranchial band height (Table 5). All regression equations for pigmentation variables were also significant ($p<0.01$) but were accompanied by low correlation coefficients ($r^2<0.5$) (Table 6).

Long Term Effects

There was a substantial decrease in both the pigmentation and length measurements during the initial three week period, and this decrease was more pronounced in ethanol than formalin (Fig. 2, Fig. 3). These decreases stabilized following their initial decline. The repeated measures ANOVA for total body pigmentation revealed significant effects for both time and preservation type for both total length and total body pigmentation (Table 7). Interaction effects were significant for the total length measurement, suggesting that there were differential effects of preservation on length over time. The interaction effect was not significant for the pigmentation measurement. Mauchly's test of sphericity was significant for both measurements (p <0.001), therefore the degrees of freedom were adjusted using the Greenhouse-Geisser correction method for the within-subject effects.

Table 3. Mean percent decreases and standard deviations of pigmentation characters after 3 weeks in 10% formalin and 95% ethanol.

	Ethanol		Formalin	
	Mean	SD	Mean	SD
Head	19.62	14.83	10.80	11.38
Suprabranchial	25.01	11.61	20.36	9.99
Sub-branchial	26.54	14.14	19.45	10.81
Total Branchial	25.12	12.70	17.43	9.84
Lateral Line Area	30.72	10.55	26.86	10.42
Trunk	27.13	10.57	24.14	10.26
Tail	14.24	11.20	11.25	9.70
Whole Body	22.60	10.80	19.05	9.58
Caudal Edge	-4.39	17.74	-3.28	15.50
Oral Hood	6.03	16.85	0.26	13.07

Table 4. Mean percent decreases and standard deviations of morphometric characters after 3 weeks in 10% formalin and 95% ethanol.

	Ethanol		Formalin	
	Mean	SD	Mean	SD
Total Length	3.73	1.56	3.34	3.79
Head Length	6.34	7.48	4.09	6.25
Branchial Length	-0.79	5.08	1.42	4.52
Trunk Length	3.87	2.78	2.83	2.32
Tail Length	5.67	4.53	5.27	4.12
Suprabranchial Band Height	-0.03	0.06	-0.01	0.06

Table 5. Regression equations and correlation coefficients for morphometric characters between live specimens and specimens preserved in ethanol and formalin for 3 weeks.

	Ethanol	r^2	Formalin	r^2
Head Length	y=0.061 + 0.857x	0.720	y=0.068 + 0.874x	0.804
Branchial Length	y=0.069 + 0.953x	0.930	y=0.040+ 0.954x	0.950
Trunk Length	y=0.085 + 0.977x	0.980	y=0.071+ 0.959x	0.993
Tail Length	y=0.010 + 0.947x	0.949	y=0.027 + 0.938x	0.969
Suprabranchial Band Height	y=0.041 + 0.498x	0.069	y=0.034 + 0.223	0.015

Table 6. Correlation coefficients of pigmentation characters between specimens measured live and preserved in ethanol and formalin for 3 weeks.

	r^2	
Region	Ethanol	Formalin
Head	0.134	0.250
Suprabranchial	0.005	0.337
Subbranchial	0.008	0.149
Total Branchial	0.246	0.230
Lateral Line	0.219	0.376
Trunk	0.158	0.363
Tail	0.271	0.457
Whole Body	0.158	0.359
Caudal Fin Edge	0.127	0.227
Oral Hood	0.008	0.376

Table 7. Results of the ANOVA showing effects of time and preservation type on total body pigmentation and total length, including the number of degrees of freedom (F) and *p* value.

Measurement		F	*p*
Total Mean Grey Value	Time	55.6	<0.001
	Preservation Type	169.9	<0.001
	Time/Preservation Interaction	5.5	0.005
Total Length	Time	33.7	<0.001
	Preservation Type	15.4	<0.001
	Time/Preservation Interaction	0.4	0.674

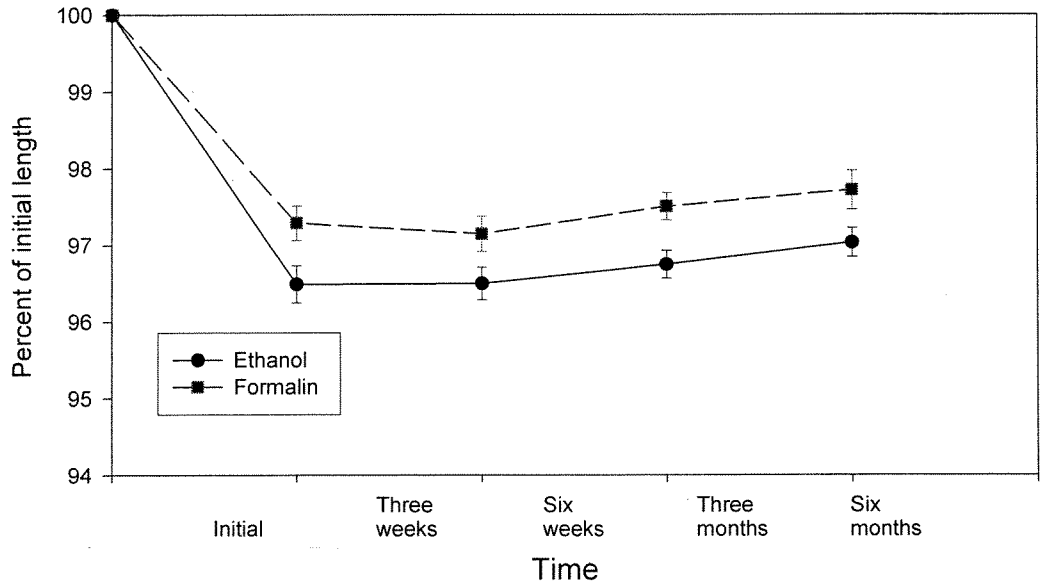


Figure 2. Mean change in length over time of larval lampreys preserved in 10% formalin and 95% ethanol. Bars represent 95% confidence intervals of the mean.

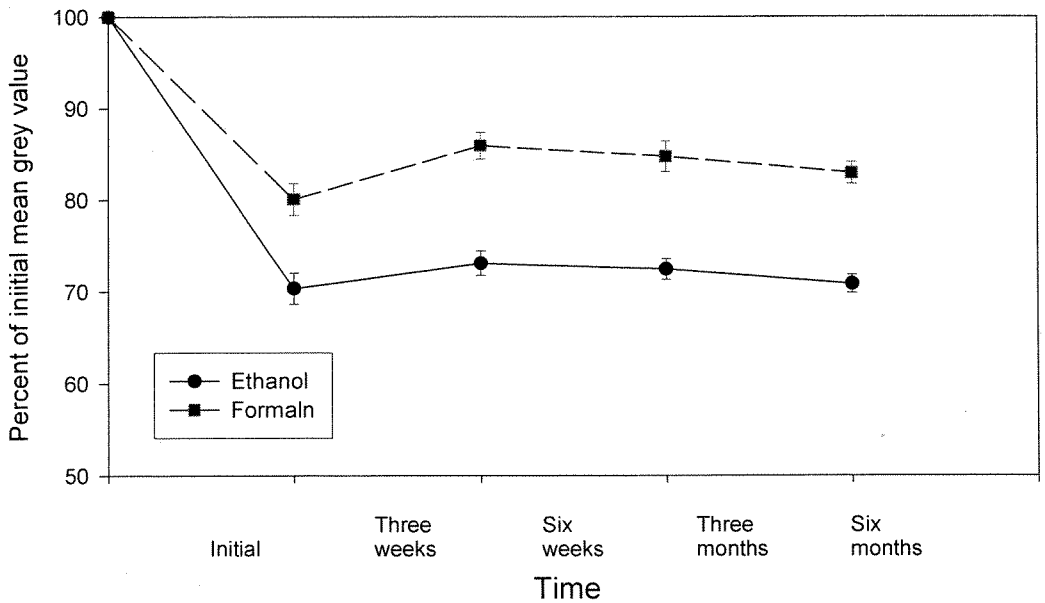


Figure 3. Mean change in pigmentation over time of larval lampreys preserved in 10% formalin and 95% ethanol. Bars represent 95% confidence intervals of the mean.

Discussion

The results of the current study demonstrated that both ethanol and formalin affected the appearance and form of ammocoetes. Both types of preservatives reduced pigmentation levels and caused shrinkage to varying degrees in external shape measurements. As predicted, formalin had less of a detrimental effect than ethanol. Almost all effects occurred within the first 3 weeks after preservation, and effects stabilized after the initial three week period.

The reduction in lengths are consistent with the findings of Fisher et al. (1998), who published similar results when comparing the effects of ethanol and formalin on total lengths of yellow perch (*Perca flavescens*) larvae. Specifically, they found ethanol caused more shrinkage than formalin, as did Shields and Carlson (1996) and Kristoffersen and Salvanes (1998). The average ammocoete total length shrinkage rate of 3.3 – 3.7% found in this study corresponded closely with other studies, including those which found similar shrinkage rates in formalin, such as of 3% (Morkert and Bergstedt 1990; Churchill 1947); 3-5% (Vladykov 1960); and 5% (Schuldt et al. 1987). The small variation of the preservation effects is likely influenced by minor variation in concentrations of active ingredient in the preservative employed in each of the different studies.

To date, few studies have examined the effects of preservation on pigmentation on lampreys, as most taxonomic keys for other taxa rely on morphometric features. Ethanol was found to have more marked effects on pigmentation, and this was supported by Taylor (1981), who recommended the use of formalin rather than ethanol to minimize effects on pigmentation. Vladykov (1950) claimed that ammocoete identification using

pigmentation characteristics was enhanced after preservation in formalin. This claim may depend on the type of diagnostic character being considered. The reduction of mean grey values found in the current study does not necessarily mean the loss of pigmentation pattern; it is more reflective of a change in intensity of colouration. The presence or absence of melanophores is not altered, but their intensity is. This is important because although this study showed overall reductions in mean grey values due to preservation, it was assumed that the pigmentation was still present, but was less contrasting in appearance. Specimens in the current study certainly became lighter in colour, but that would not necessarily preclude the use of pigmentation features in a key, provided the patterns were visible, regardless of the intensity of the pattern. Although the pigmentation may have appeared lighter, it was still visible. Most keys use pigmentation features as either present or absent, such as pigmented lateral lines (Vladykov and Kott 1980), and hence the type of preservation is not important, provided all pigmentation is not lost. However, if the diagnostic features are simply a relative degree of pigmentation, such as descriptions of "heavily pigmented" versus "weakly pigmented" head and tail areas (Vladykov and Kott 1980), then the effects of preservation may become confounding. The influence of preservation may render some pigmentation characters ineffective if lampreys were stored in different preservatives, as the relative pigmentation levels could be distorted by the differential effects of the different techniques.

The effects of preservation on morphometry are much less substantial than those of pigmentation. Average changes were 2-4%. These, although variable from character to character, would likely not affect the usage of these characters in a taxonomic key. The low r^2 value of the suprabranchial band height measurement is likely related to the small

length of the measurement, which varied only from 0 to 0.19 cm. One can see that if the changes in the fluctuation from live to preserved values, when measured as percentages would fluctuate much more than the other measurements given the relative size of the other measurements (i.e. the other length measurements are considerably longer).

An unexpected result of the long term analysis was a reversal of mean size changes over time. After the initial substantial shrinkage, there was a gradual increase in length. This trend was minor, and is worth mentioning only because other studies have found similar results. Kristoffersen and Salvanes (1998) found similar reversals over time in pearlsides (*Maurolicus muelleri*) and glacier lanternfish (*Benthosema glaciale*) over similar time periods. They attributed this increase to measurement error, but the similarities are striking, and I speculate that there is a mechanism that functions to slowly negate the initial shrinkage. Lee's (1982) study of the influence of preservatives on toads also found that after initial short-term effects, the shrinkage then proceeded in the reverse direction to a significant degree. One potential explanation of this phenomenon is that of tissue degradation over time. Formalin causes the initial shrinkage by inducing protein cross linkage between tissue cell walls. Despite the presence of preservatives, cells are still subject to breakdown, although at a diminished rate. A weakening of the cross-linkage over time could cause a re-lengthening of the organism (Simons 1993), as the cells are still subject to lysis and may result in this observed gradual progression of organisms back toward their initial lengths.

There has been a recent trend toward storing of specimens directly in ethanol. Ethanol does not restrict the use of tissues in genetic analysis, i.e. this preservation technique does not interfere with DNA structure like formalin does (Giannella et al.

1997). Preserving lampreys using only ethanol (i.e. without formalin fixation) is worth investigating if the specimens might subsequently be used in genetic analyses.

Some loss of pigmentation and shape changes may be caused by death, rather than the preservative. Post-mortem shrinkage has been observed to occur in fishes which were not subjected to any type of preservation (Morison et al. 2003). Therefore, preservation is not the sole cause of these length changes. Similarly, pigmentation is affected by stress and death of the individual (Manion 1972). To fully contrast the effects of ethanol and formalin, it would be beneficial to investigate whether the small changes induced by preservatives differ from changes that occur without any type of preservative.

The conclusions of this study could be used to benefit lamprey taxonomic, growth, or production studies. Should ammocoetes have been preserved differently, or are measured live, the use of the correction factors presented here could serve to provide a more accurate representation of their true lengths.

A potential confounding factor in this study was inconsistency in the evaluation of pigmentation characters using the Scion Imaging software. Despite an obvious overall trend of a pigmentation decrease followed by stabilization in the long-term study, there were unexpected fluctuations in mean grey values of some individuals after the initial change. All ammocoetes underwent substantial initial pigment decrease, but many individuals were found to then show subsequent random increases and decreases in pigmentation levels. For example, in one ammocoete measurements of the total body pigmentation over time consisted of a decrease of 31% of the original value in the first three week period. This was followed by subsequent reductions of 22, 15 and finally 26% of the original live value at 6 weeks, 3 months and 6 months, respectively (Figure 4).

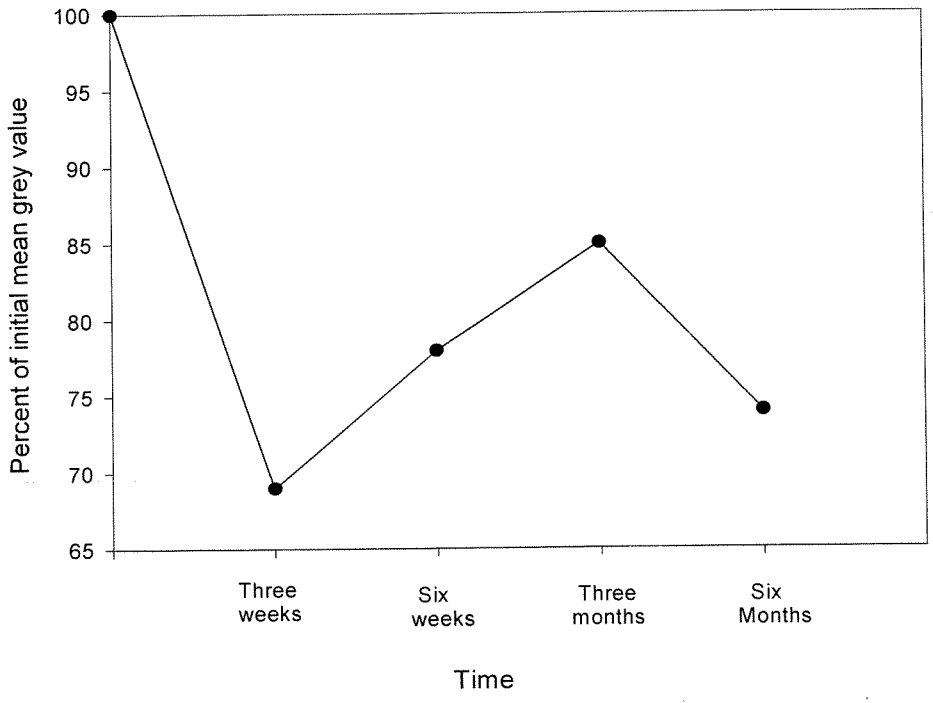


Figure 4. Variation over time of mean grey values of one specimen. Mean grey values are expressed as a percentage of initial live value.

These values indicated a substantial initial pigment loss of the ammocoete, followed by a darkening closer to its original value over time (a darkening of 15%), and finally another loss in colouration. This was in contrast to expected values, which would consist of the initial decrease in pigmentation, followed by a relative stabilization.

The variability of the pigmentation methods is reflected in the regression analysis. The morphometric data showed that the length characteristics could be reliably predicted from the live values. High regression coefficients showed that, except for the suprabranchial band height, the preserved lengths could be accurately predicted from the live values. In contrast, the pigmentation measurements had markedly different regression co-efficients; on average they were less than half that of the morphological measurements. This indicates that the pigmentation measurements after preservation were not linearly related to the live values. This is unexpected, as concentrations of preservative were held constant, and one would not anticipate irregular fluctuations over time. Several possibilities could account for this variation. Pigmentation is known to be sensitive to light (Taylor 1981), so differential exposure of ammocoetes to light might have induced this variability. However, the ammocoetes in this study were stored in closed boxes during the study, and light exposure was minimized. Another potential explanation is that differential exposure to water may have caused some degree of variability, as re-submergence times in water during imaging were subject to variation.

Measurement precision was tested before the commencement of the study and was conducted multiple times on the same images. These tests showed high precision rates (standard deviation <2.3%). However, the irregular fluctuation of mean grey values of individual ammocoetes over time suggested that the software utilized in this study may

have been susceptible to external influences. Images were taken at different dates, times and locations; therefore the influence of subtle changes in external lighting may have contributed to the unexpected changes in mean grey values. Other contributing factors may have been the repeated take down and set up of equipment, which may have resulted in small variation in angles of light sources. Another potential explanation is that changes occurred in the colouring of the background. Small variations of the colour of the white paper used as background material were observed (after the completion of the study), and were due to the effects of water absorption over time. These changes could have influenced mean grey values by altering colouration through fins or reflection from the surface of the body of the ammocoete. It seems unlikely that these differences could account for the observed fluctuations, but combinations of these factors may be sufficient to explain the variation.

Further precision analysis should be conducted on the environmental effects of different imaging environments. No literature is available regarding the validation of this technique, and the results of this study do not encourage further use of this image analysis software without such an analysis. The existence of significant trends in the data indicated that this concern was not of high importance in this particular study, but these findings should be taken into account in future studies.

In summary, this study showed that both ethanol and formalin induced significant changes in larval lampreys, and further illustrated that the two preservatives are significantly different in their effects. Almost all of the changes occurred within the first 3 weeks, and the degree of change was not consistent among characters. Preservation with formalin was less deleterious to external characters than ethanol, so researchers

attempting to maximize similarities of characters of preserved specimens with their initial state should take this into consideration. The most important conclusions are that external characters of ammocoetes are significantly affected by fixation and preservation, and that colouration measurements, as performed in this study, were found to be highly variable.

References

1. SPSS for Windows. 2001. Chicago, SPSS Inc.

2. Churchill, W.S. 1947. The brook lamprey in the Brule River. Trans. Wisconsin Acad. Sci. **37**: 337-346.

3. Distefano, R.J., Roell, M.J., Wagner, B.A., and Decoske, J.J. 1994. Relative performances of four preservatives on fish and crayfish. Transactions of the American Fisheries Society **123**: 817-823.

4. Fisher, S.J., Anderson, M.R., and Willis, D.W. 1998. Total length reduction in preserved yellow perch larvae. North American Journal of Fisheries Management **18**: 739-742.

5. Fraser, T.H. and Freihofer, W.C. 1971. Trypsin modification for Sihler technique of staining nerves for systematic studies of fishes. Copeia 574-576.

6. Giannella, C., Zito, F.A., Paradiso, A., Marzullo, F., Alaibac, M., and Schitulli, F. 1997. Comparison of formalin, ethanol, and Histochoice fixation on the PCR amplification from paraffin-embedded breast cancer tissue. European Journal of Chemistry and Clinical Biochemistry **35**: 633-635.

7. Hjorleifsson, E. and Klien-Macphee, G. 1992. Estimation of live standard length of winter flounder *Pleuronectes americanus* larvae from formalin-preserved, ethanol-preserved and frozen specimens. Marine Ecology Progress Series **82**: 13-19.

8. Hubbs, C.L. and Trautman, M.B. 1937. A revision of the lamprey genus

Ichthyomyzon. Miscellaneous Publications of the Museum of Zoology.

9. Kristoffersen, J.B. and Salvanes, A.G. 1998. Effects of formaldehyde and ethanol preservation on body and otoliths of *Maurolicus muelleri* and *Benthosema glaciale*. Sarsia **83**: 95-102.

10. Lanteigne, J. 1988. Identification of lamprey larvae of the genus *Ichthyomyzon* (Petromyzontidae). Environmental Biology of Fishes **23**: 55-63.

11. Lee, J.C. 1982. Accuracy and precision in anuran morphometrics: artifacts of preservation. Systematic Zoology **31**: 266-281.

12. Manion, P.J. 1972. Variations in melanophores among lampreys in the upper Great Lakes. Transactions of the American Fisheries Society **101**: 662-666.

13. Morison, A.K., Brown, I.W., and Jones, G.K. 2003. Post mortem shrinkage of four species of temperate and tropical marine fishes, without freezing or preservation. Journal of Fish Biology **62**: 1435-1449.

14. Morkert, S.B. and Bergstedt, R.A. 1990. Shrinkage of sea lamprey larvae preserved in formalin. North American Journal of Fisheries Management **10**: 484-486.

15. Scion Image for Windows. Rasband, W. 2000. Frederick, Maryland.

16. Schuldt, R.J., Heinrich, J.W., and Fodale, M.F. 1987. Prespawning characteristics of lampreys native to Lake Michigan. Journal of Great Lakes Research **13**: 264-271.

17. Shields, R.A. and Carlson, S.R. 1996. Effects of formalin and alcohol preservation on lengths and weights of juvenile sockeye salmon. Alaska Fishery Research

Bulletin **3**: 81-93.

18. Smith, H.M. and Smith, R.B. 1975. Submerged-subject photography for preserved material. Bulletin of the Chicago Herpetological Society **10**: 10-13.

19. Starrett, W.C., Harth, W.J., and Smith, P.W. 1960. Parasitic lampreys of the genus *Ichthyomyzon* in the rivers of Illinois. Copeia 337-346.

20. Taylor, W.R. 1981. On preservation of color and color patterns. Curation Newsletter **3**: 2-10.

21. Treasurer, J.W. 1992. Length and weight changes in 0+ perch, *Perca fluviatilis* L., following fixation in formalin. Journal of Fish Biology **41**: 1033-1036.

22. Vladykov, V. 1950. Larvae of Eastern American lampreys (*Petromyzonidae*). Le Naturaliste Canadien.

23. Vladykov, V.D. 1960. Description of young ammocoetes belonging to two species of lampreys: *Petromyzon marinus* and *Entosphenus lamottenii*. Journal of the Fisheries Research Board of Canada 17: 267-288.

24. Vladykov, V.D. and Kott, E. 1980. Description and key to metamorphosed specimens and ammocoetes of Petromyzontidae found in the Great Lakes region. Canadian Journal of Fisheries and Aquatic Sciences **37**: 1616-1625.