GREAT LAKES FISHERY COMMISSION

2001 Project Completion Report[1]

Determining the Sources and Complete Chemical Composition of the Lamprey Larval Pheromone, and Assessing the Merit of Measuring One of its Principal Components in River Waters – Phase II

by:

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P.W. Sorensen
March 8, 2001

Completion Report for the Great Lakes Fishery Commission

PROJECT TITLE: Determining the sources and complete chemical composition of the lamprey larval pheromone, and assessing the merit of measuring one of its principal components in river waters - Phase II.

PRINCIPLE INVESTIGATOR: Dr. Peter W. Sorensen, Professor, U. of Minnesota
PROJECT DATES: April 1, 2000 - March 31, 2001

PROBLEM STATEMENT AND OBJECTIVES AS STATED IN THE CONTRACT: A decade of research has proven that adult sea lamprey locate spawning streams using a pheromone released by stream-resident larvae, and that two unique bile acids, petromyzonol sulfate (PS) and allocholic acid (ACA) are components of this pheromone. We have spent the past two years developing the means to produce and measure petromyzonol sulfate, and to ascertain its precise role and importance in the larval pheromone. These studies are not yet complete: while they have lead to the development of a sensitive enzyme immunoassay (EIA) assay for petromyzonol, we still do not have the means to measure the sulfated pheromonally-active form, petromyzonol sulfate. Similarly, although we have shown that we can attract adult lamprey using a mixture of the two lamprey bile acids, PS and ACA, several studies suggest that the 'whole' larval pheromone may be more potent than the bile acids, perhaps because they contain other components which enhance its activity. Here, we propose to resolve these issues by addressing four objectives, all of which are directly derived from those in our current contract. They are:

1) Aid in the development of means to produce petromyzonol sulfate by providing analysis of bile acid products.
2) Develop the means to measure petromyzonol sulfate using the EIA developed for petromyzonol.
3) Determine if the concentration of petromyzonol sulfate is a good index of larval abundance that can be used in the management of sea lamprey.
4) Characterize the complexity of the larval pheromone and the role of petromyzonol sulfate and allocholic acid in it.

1) Aid in the development of means to produce petromyzonol sulfate by providing analysis of bile acid products.

We have been in active communication with three laboratories (Dr. Collodi of Purdue University, Cayman Chemcial Company of Ann Arbor, MI; and Dr. K.V. Venkatachalam of Nova Southeastern University) which have been attempting to develop the means of producing petromyzonol sulfate or analogues. Advice has been provided to all three, and especially to Dr. K.V. Venkatachalam who is cloning the sulfotransferase enzyme that attaches the sulfate group to petromyzonol (P). Having this enzyme would make synthesis of PS much less expensive. We have agreed to assist Dr. Venkatachalam in all ways appropriate including providing standards and analyzing samples. Plans have been made to analyze samples for him in the upcoming years.

2) Develop the means to measure petromyzonol sulfate (PS) using the EIA developed for petromyzonol.

Summary.
As reported previously, an enzyme immunoassay (EIA) for petromyzonol (P, desulfated form of PS) has been developed with the assistance of the Cayman Chemical Company. Although the assay is sensitive enough to measure desulfated PS (P) at concentrations expected to occur in river water, direct attempts to measure P in natural river water using the EIA have not proven practical because we have encountered a high level of cross-reactivity between the antibody and natural organic matter present in river water. Because of these difficulties, we have taken the initiative of investigating another approach: electrospray ionization-mass spectrometry (ESI-MS). Initial experiments have shown this technique to have great potential and some recent results are outlined below. Our plan is now to use ESI/MS to first determine whether it would be useful to learn the natural concentrations of PS in natural river waters (e.g. does the concentration of PS reflect larval abundance), and if it is, return to the EIA and develop it to it's full potential.

The EIA.
An EIA for P has been developed, and is capable of measuring P at concentrations as low as 30 pg/ml; about the concentration of PS we expect to find in river waters (details in previous progress report). The EIA was developed for P rather than PS because the PS was breaking down in the rabbit blood, making the development of an antibody impossible. However, conversion of PS to P (via a technique known as acid solvolysis) yields approximately 90% conversion rates, making quantification of PS in river water possible using the EIA for P. Unfortunately, however, direct attempts to measure P in natural river water using the EIA have not worked because we have encountered a high level of cross-reactivity between the antibody and natural organic matter present in river water. The identities of the interfering compounds are unknown, and purification steps

must be taken to further purify the sample before the EIA will be useful. Although we believe we can overcome this challenge by utilizing HPLC to isolate the PS in river waters, it is very time consuming and cumbersome (especially as it must be followed by desulfation), so we have decided to experiment with ESI/MS as described below.

Electrospray Ionization/ Mass Spectrometry (ESI/MS)

We recently learned that advances in ESI/MS allow for quantitative measurement of steroids and have developed the means of applying this technology to problem at hand. In particular, we have found that the use of an electrospray ionization (ESI) chamber allows for ionization of PS and ACA in a liquid matrix by concurrently ionizing the molecules and evaporating the solvent. These ions are then injected into the mass spec analyzer where they are identified by their mass/charge ratio ($\pm$ 0.1 mass/charge units) and can be quantified. This technique not only has great sensitivity but is definitive because the parent ion can be identified and if necessary further broken up into distinctive components. Also, it is easier that EIA because desulfation is not needed.

The procedure we employed used 1 L samples of river water. The river water was collected (if necessary with preservative), and then extracted through reverse phase C18 sep-pak cartridges (Waters, MA) with hexane washes and an increasing methanol gradient. Next, this extract was subjected to further purification by injecting it onto an HPLC and collecting the 4 minute window known to contain PS using a fraction collector. This window was identified by adding a radiolabeled bile acid standard (C14-chenodeoxycholic acid) to the sample with has an elution time exactly 11 min before PS. The PS fraction was then dried down and reconstituted into 50 µl methanol for ESI/MS analysis; 20,000 times more concentrated than the original 1 L of water.

To identify PS using ESI/MS, samples were compared to a known PS standard using the 'zoomscan' mode which yields a profile of 10 mass/charge (m/z) units surrounding the PS peak (473.5 m/z, Fig. 1). Samples were simultaneously quantified using selected ion monitoring (SIM), in which only ions with m/z ranging from 473.5 + 0.5 m/z are measured, and peak areas were measured and compared to a standard curve. The points for the standard curve were prepared by subjecting river water samples collected from a stream (Valley Creek, MN) lacking lamprey (and PS) and then spiking it with known amounts of PS before analysis with ESI/MS. The standard curve was prepared in this manner to precisely correct for background and ionization effects that occur in a river water background. We have found the detection threshold of PS in river water to be 6×10^{-13} M (Fig. 2) using this technique, nearly that of the EIA. Clearly, this technique has enormous potential.

3) An understanding of whether the concentration of PS can be used to determine larval abundance.

Summary.

This objective has not yet been completely met because we have only recently optimized the ESI/MS for measuring PS in river waters. However, we have already succeeded in demonstrating our first sub-objective: we found that PS is present in rivers that are known to contain larval sea lamprey and absent in rivers that do not. Also, within the last year we have collected (with the assistance of the Hammond Bay Biological Station) and extracted more water samples that will permit us to address the question of whether concentrations of PS correlate with larval abundance in a definitive manner. We expect to have an answer to these questions within the year now that ESI/MS is functional. In a closely related study, we have also determined that larvae of native lamprey species are producing and releasing PS at rates similar to sea lamprey larvae, suggesting that PS measurement may be complicated by native species and temperature effects that will need more study.

Presence/ absence of PS in river waters.

Using the ESI/MS technique described in objective 2, we have now measured significant concentrations of PS in 3 lamprey rivers (St. Mary's River (MI), Cheboygan River (MI), and Ocqueoc River (MI). Further, we were unable to measure any PS in 3 non-lamprey rivers (Valley Creek (MN), Lone Pine (MI), Nagel Creek (MI)). PS was present at concentrations of approximately $1 - 3 \times 10^{-12}$ M in all three lamprey rivers, within the olfactory detection threshold of adult sea lamprey close to the value we earlier estimated to be present based on larval release rates (Polkinghorne et al. 2001). We will analyze another dozen samples during the next contract period to precisely determine how well PS might serve to identify lamprey streams.

Other questions and water collections.

We have now collected and extracted water samples from well over a dozen locations over the past 4 years. Although a few have been analyzed by high performance liquid chromatography (HPLC) and ESI/MS, most remain in cold storage and await analysis which is underway. Tables of water samples collected prior to 2000 are listed in our previous completion report, and samples collected in the past year are listed here (Table 1). We will use these stored samples to answer the following questions in addition to the one described above:

a) Is the concentration of PS found in streams directly correlated with the number of sea lamprey larvae living in it? To answer this question, Mr. Bergstedt collected water samples upstream and immediately downstream of specific sections of two streams with known populations of sea lamprey larvae in the Misery River (MI) and the Rock River (MI). Flow rates and temperatures were measured concurrently. Collections were performed two times during the falls of 1998 and 1999.

b) Does water from streams which contain native species of larval lamprey also contain PS? To answer this question we collaborated with Mr. Bergstedt who collected water samples upstream and immediately downstream of specific sections of two streams with known populations of native lamprey - the Middle River (WI) and the Big Garlic River (MI). Flow rates were measured concurrently. Collections were performed two times - during the falls of 1998 and 1999).

c) Is there is a relationship between time-of-day and PS production in lamprey streams? To answer this question water samples were collected on a 4 h basis for 2 days from the Black Mallard River.

d) Is there a relationship between time-of-year and PS production in a lamprey stream? To answer this question water samples were collected on a monthly basis from St. Mary's and Black Mallard Rivers for a year. The Hammond Bay Biological laboratory and the Sea Lamprey Control Centre were our collaborators on this project.

Specificity of bile acid production by other species of Great Lakes lamprey.
Although previous studies (Sorensen and Vrieze, submitted) have shown that adult sea lamprey are attracted to the odor of native lamprey species, bile acid production by native species has not yet been characterized. Because this is an important question that will influence our interpretation of any PS that might be measured in lamprey rivers, we investigated this question although it was not specified in the contract proposal. HPLC was employed to determine bile acid production profiles in the gallbladders of all 5 species of Great Lakes lamprey species while ESI/MS was used to examine larval holding water for *P. marinus, L. appendix*, and *I. fossor* (a representative of each genus). Briefly, gall bladders were removed from freshly captured ammocetes and the bile acids extracted and partially purified according to previously established methods (Polkinghorne et al, 2001). Samples were then analyzed as previously described in the Sorensen lab. We found that all Great Lakes lamprey species produced PS in similar quantities, and small and variable quantities of ACA were found in *L. appendix* and *P. marinus* (Table 2). The presence and identity of ACA in these samples was confirmed by MS-MS,

Being more sensitive than HPLC, ESI/MS was used to measure PS and ACA concentrations in larval holding waters. Water samples were collected and extracted following established protocols from 9 tanks of larvae that we held in the laboratory and fed on a regular cycle. We measured PS and ACA in the holding water for all three species. Release rates of all three species were similar (Fig. 4). Holding temperatures changed during the course of these experiments, affording us to examine the effects of temperature on release rate. Interestingly, we described a relatively tight linear relationship between temperature and release rate for all 3 species (Fig. 5). Although this relationship will need further study to discern the relative roles of bile acid production and decay, they do suggest that measurement of riverine concentrations of PS will have to factor in temperature to correctly assess larval population abundance. Because temperatures were recorded when we sampled river waters we will be able to examine this in greater detail in the future.

4) Characterize the complexity of the larval pheromone and the role of petromyzonol sulfate and allocholic acid in it.

Summary.
Recent tests of the potency of larval waters using electrophysiological recording (EOG) and behavioral assays have now demonstrated that the larval pheromone contains compounds that supplement the actions of PS and ACA. EOG recording has clearly shown that while the entire odorous portion of larval odor can be extracted using C18 columns, only about half of the odor's potency is attributable to the presence of PS and ACA: larvae release unknown compounds which adults can detect. Further, fractionation of larval holding water by HPLC shows that there are 5 fractions with considerable olfactory potency to adults, 4 of which are not associated with PS or ACA. Similarly, behavioral studies have demonstrated that larval odor was more attractive to adults than PS and ACA at the same concentrations as those in the larval water, suggesting that PS and ACA may not explain the full behavioral potency of the larval pheromone. Importantly, however, we discovered that the entire pheromone (as defined by its behavioral actions) can be extracted and concentrated using C18 columns, meaning that we will be able to obtain enough of this cue for identification.

i) Confirming the chemical complexity of larval sea lamprey odor and the role of petromyzonol sulfate and allocholic acid in it using chemical fractionation and EOG recording.

In these experiments tanks of larval *P. marinus* were maintained at the University of Minnesota along with control tanks that contained sand only. Larval and control odors were created by shutting off the water to the tanks and feeding the animals with yeast because we know feeding to stimulate bile acid release. Twenty-four hours later residual yeast was flushed from the tanks by turning the water on for 12h, after which it was shut off again to concentrate the odor. After sitting for another 12h these waters were then used for our experiments, and 36h after feeding their waters were extracted by C18-column extraction. Extracts representing 1 L aliquots of larval and control waters were then fractionated by injecting them onto an HPLC. The HPLC run lasted for 60 minutes and 20 fractions were carefully collected in clean tubes. Pilot studies with radiolabeled bile acids demonstrated this separation technique to yield clear precise separation of bile acids. Each resulting fraction was then tested on the lamprey nose to determine olfactory responses by electro-olfactogram recording (EOG).

EOG recording followed established protocols (Li & Sorensen 1997), in which we used 5 Molar L-Arginine as a standard. Newly migrating adult animals were collected from the Tittabawassee River and shipped to Minnesota by air. We only used animals that produced at least a 1 mV response to this stimulus. All 20 fractions of control and experimental water extracts were tested along with PS and ACA. PS and ACA were also quantified within each fraction by ESI/MS and values measured matched with their EOG potency.

We found larval waters to be much more potent than control waters, with 5 distinct fractions (#1, #7, #9, #10, and #11) having notable potency (Fig. 6). Results were highly repeatable although there was some indication that fractions #10 and #11 may contain the same compound(s). Interestingly, PS only accounted for about 10% of the potency of the fraction in which it was found in (#11), while ACA could not account for any significant activity. Together, these results prove that larval lamprey release PS and at least 4 as yet unidentified odorous compounds. It is very important to note that the behavioral significance of these fractions is as yet unknown (and not all may have pheromonal activity) and will be examined in the coming year. Further chemical characterization is also currently underway and will continue into the next granting cycle.

ii) Testing the behavioral potency of various concentrations of PS and ACA to clearly test whether these compounds can fully explain the potency of larval odor (the 'whole' pheromone).

a-1) Can the entire pheromone be extracted by C18 columns?

Although it had previously been demonstrated by EOG recording that the entire odor of larval holding water could be extracted by C18 columns (previous reports), it had yet to be confirmed by a behavioral assay. Here we conducted this important test which compliments and confirms the chemical fractionation/EOG studies described above. It was not originally described in the contract. Larval odor was collected in the same manner described above for EOG experiments by holding 660 sea lamprey larvae (total mass = 373g) in a tank at Hammond Bay Biological Station along with a second control tank that contained sand but no larvae. Yeast was added to the larval (and control) tank to stimulate larval bile acid release through feeding. After 24 hr, the water was turned on for 12 hours to flush out remaining yeast, and then water was again shut off for 12 hours. At the end of this time the water was collected from the control and larval tanks and passed through C18 columns. The filtrate was frozen and the material retained by the C18 columns was extracted with methanol.

Behavioral tests were then performed in a two-choice maze constructed in large concrete raceways supplied with flowing Lake Huron water at Hammond Bay Biological Station (see Vrieze 1999). Animals recently captured in nearby rivers were observed during a 20 min trial at night as they were allowed to choose between two different odors pumped into the raceway. During all tests, water from Nagel Creek, a small stream that does not contain ammocetes of any species was pumped along with the odors into both arms of the maze. Preferences were determined by noting the position of the animals every 30 sec and testing whether their distribution in one experimental odor was significantly greater than the 50% expected by chance (two-tailed one-sample student t-test). First, fresh whole larval water (removed from the larvae tank immediately before tests) was tested to confirm once again that larval holding water was attractive to adult lamprey. Next, the C18 extract of larval water was tested against control. Finally, the larval filtrate was thawed and tested against control filtrate to see if any attractant was not extracted. In addition, a control test was also performed using previously frozen whole

larval water (not passed through C18 filter) to determine that freezing did not influence the activity of larval pheromone.

Behavioral results confirmed EOG recording. As expected, adult lamprey were attracted to fresh whole larval water ($P<0.01$; Fig. 7). Very strong attraction was also seen to the C18 extract of larval holding water. ($P<0.01$; Fig. 7). However, lamprey showed no preference for the larval filtrate over the control filtrate ($P>0.10$; Fig. 7). Frozen, thawed larval water retained its potency ($P<0.05$; Fig.7), establishing that freezing could not account for the loss of activity seen with the filtrate. We conclude that the entire larval pheromone can be easily and efficiently extracted using C18 columns and safely frozen for future analysis.

a-2) To determine if the ability of PS and ACA to attract lamprey is influenced by concentration and to determine the optimal concentration.

Previous behavioral tests conducted in 1997-1999 have shown strong ($P<0.001$) attraction to larval holding water and weak attraction to purified PS and ACA ($P<0.05$ or $P<0.10$). One possibility for this observation was that we had only tested one concentration of bile acids. To determine if we might have simply been testing the 'wrong' concentration of PS and ACA, last summer we tested 3 concentrations of PS and ACA using the same maze described above in 2000. A 7:1 PS:ACA mixture (approximately the ratio we measure in release waters) was added to one arm of the maze, and methanol control was added to the other with background odor of Nagel Creek odor being added to both (as above). The three concentrations were $1x10^{-10}$M (what had been tested in previous years), $1x10^{-12}$M, and $1x10^{-13}$M. A significant ($P<0.05$) attraction for PS/ACA was only seen to $1x10^{-12}$M concentration (Fig. 8). Although this concentration is about 100 times lower than that previously tested and similar to that which we have now measured in river water by ESI/MS; it nevertheless was inconsistent (responses to larval odor have always been very consistent and strong), leading us to conclude that although PS and ACA are indeed part of the pheromone, they do not comprise the entire signal.

b) To determine if an optimal concentration of PS and ACA is as attractive as larval odor with the same optimal/real concentration of PS and ACA.

In previous years we had directly tested whole larval water in one arm of the maze against PS and ACA in the other arm. While we had found PS and ACA to be less attractive than larval odor, these experiments were confounded by our not knowing the concentration of PS and ACA larval water. However, this past year we knew that we could safely freeze larval release waters (see above) and we had the ability to rapidly measure PS and ACA using ESI/MS to precisely match the bile acid concentrations: an appropriate test could be performed. In the summer of 2000, our experiment had two replicates. Each night we tested a sample of diluted frozen larval water against a matched concentration of the PS and ACA we knew it to contain. Accordingly, one night we were testing PS at a concentration of $4.7x10^{-14}$M and the other of $8.8x10^{-14}$M. When the matched concentration of purified PS and ACA was directly paired with bile acids,

lamprey showed a significant preference for the arm containing the larval water ($P<0.05$). We conclude that the larval pheromone is not made up entirely of PS and ACA but must contain other unknown products.

c-1) If bile acids are as attractive as larval washings when concentration is considered, determine if they, like larval odor, will reverse the preference of adult lamprey streams.

This experiment was not conducted because experiment b found PS/ACA to lack the activity of the whole larval pheromone – instead we proceeded to experiment c-2 (below).

c-2) If bile acids are not as attractive as the larval washing when concentration is considered, determine if other bile compounds make parts of the pheromone.

One additional test was conducted to see if other compounds found in larval gall bladders could perhaps contribute to the complete pheromone. A crude extract of *P. marinus* gall bladder was tested in a manner similar to that specified above for PS and ACA. The extract was tested so that the concentration of PS in the raceway was $1x10^{-11}$M. Adult lamprey were only weakly attracted to this extract, spending $59.5 \pm 5.3\%$ (mean $\pm$ S.E) of their time in the channel containing extract ($p<0.10$; $n=16$). This was the same level of response repeatedly seen to PS and ACA, leading us to conclude that this missing component does not come directly from the gall bladder. The possibility that unknown component(s) are metabolite(s) of bile produced further down the digestive tract or even in the water will be tested this coming year.

References.

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Polkinghorne, C.A., Olson, J.M., Gallaher, D.G., and P.W. Sorensen. 2001. Larval sea lamprey release two unique bile acids to the water at a rate which is sufficient to produce a detectable pheromonal plume. Fish Physiol. Biochem. (in press)

Sorensen, P.W., and L.A. Vrieze. Current understanding of the migratory pheromone employed by sea lamprey: biochemical origins, behavioral function, and promise for application. Journal of Great Lakes Research. (in review)

Vrieze, L.A., and P.W. Sorensen. Migratory adult sea lamprey are attracted to the odor of river waters, a principal component of which is a bile-acid derived pheromone released by larval conspecifics. Can. J. Fish. Aquat. Sci.. (in review)

Published articles that acknowledge this contract:

Bjerselius, R., Li, W., Teeter, J.H., Seelye, J.G., Maniak, P.J., Grant G.C., Polkinghorne, C.N. and P.W. Sorensen. 2000. Direct behavioral evidence that unique bile acids released by larval sea lamprey function as a migratory pheromone. Can. J. Fish. Aquat. Sci. 57: 557-569

Polkinghorne, C.A., Olson, J.M., Gallaher, D.G., and P.W. Sorensen. 2001. Larval sea lamprey release two unique bile acids to the water at a rate which is sufficient to produce a detectable pheromonal plume. Fish Physiol. Biochem. (in press)

Articles in review which acknowledge this contract.

Vrieze, L.A., and P.W. Sorensen. Migratory adult sea lamprey are attracted to the odor of river waters, a principal component of which is a bile-acid derived pheromone released by larval conspecifics. Can. J. Fish. Aquat. Sci.. (in review)

Sorensen, P.W., and L.A. Vrieze. Current understanding of the migratory pheromone employed by sea lamprey: biochemical origins, behavioral function, and promise for application. Journal of Great Lakes Research.

Twohey, M., Sorensen, P.W., and Li, W. Possible applications of pheromonesan integrated program of sea lamprey control. Journal of Great Lakes Research.

Presentations that acknowledged this contract.

Sorensen, P.W., and L.A. Vrieze. 2000. The migratory pheromone in the sea lamprey, *Petromyzon marinus*. Sea lamprey International Symposium-II, Sault Ste. Marie. MI. (article to be published).

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migratory pheromone is not species-specific. Sea lamprey International Symposium-II, Sault Ste. Marie. MI.

Vrieze, L.A., and Sorensen, P.W. 2000. Interactions of temperature and odor as attractants for adult sea lamprey. Midwest Fish and Wildlife Conference, Minneapolis, MN.

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Sorensen, P.W., Vrieze, L.A., and W. Li. 2000. A migratory pheromone in sea lamprey: biochemical origins, behavioral function, and effects of sexual maturity on olfactory sensitivity. IV. International Symposium on Fish Endocrinology. Seattle, Washington.

Table 1: Stream waters collected, 2000-2001

Name	Date Collected	Amount	Experiment
Laperell Cr.	5/10/00	2 L	Before and after TFM treatment
Laperell Cr.	5/15/00	2 L	"
Trout River	5/28/00	2 L	"
Trout River	6/2/00	2 L	"
Elliott Cr.	5/15/00	2 L	Larval presence
Upper Grand Lake Outlet	5/28/00	2 L	"
Lower Grand Lake Outlet	5/29/00	2 L	"
Greene	5/20/00	2 L	Larval presence / To trap in 2001
Mulligan	5/20/00	2 L	"
Lone Pine	5/20/00	2 L	"
Three	5/20/00	2 L	"
Grace	5/20/00	2 L	"
Cheboygan	5/24/00	2 L	Continue time series and behavior
Lower Ocqueoc	5/24/00	2 L	"
Upper Ocqueoc	5/24/00	2 L	"
Schmidt Creek	5/28/00	2 L	"
Nagel Creek	5/28/00	2 L	"
Black Mallard	6/2/00	2 L	"
Upper Rock River	9/18/00	6 L	PS corresponds to larval abundance?
Lower Rock River	9/18/00	6 L	"

Table 2: Bile Acid Content in Larval Lamprey Gallbladders

			Total Bile Acid Present in Gall Bladder (mean+SE)			Relative Contribution to Total Bile Acid Content (mean+SE)		
Species	n	Mean Gallbladder Mass (g)	ACA (ug)	PS (ug)	P (ug)	ACA (%)	PS (%)	P (%)
P. marinus	7	4.7±1.3	5.0±1.9	102.6±17.6	0.7±0.4	3.8	95.7	0.5
I. fossor	7	5.7±2.4	N.D.	65.1±12.5	0.3±0.2	<0.05	99.7	0.3
I. unicuspis	4	2.8±0.3	N.D.	79.1±11.7	N.D.	<0.04	100	<0.04
I. castaneous	5	4.8±0.8	N.D.	73.9±14.2	1.6±0.5	<0.04	97.6	2.4
L. appendix	7	7.9±3.0	1.8±0.9	218.1±36.5	4.1±0.9	1	97.1	1.9

N.D. = Not Detectable (5% total tissue injected and detection threshold was 0.03 ug)

Mass Spectrometry Zoomscan of PS

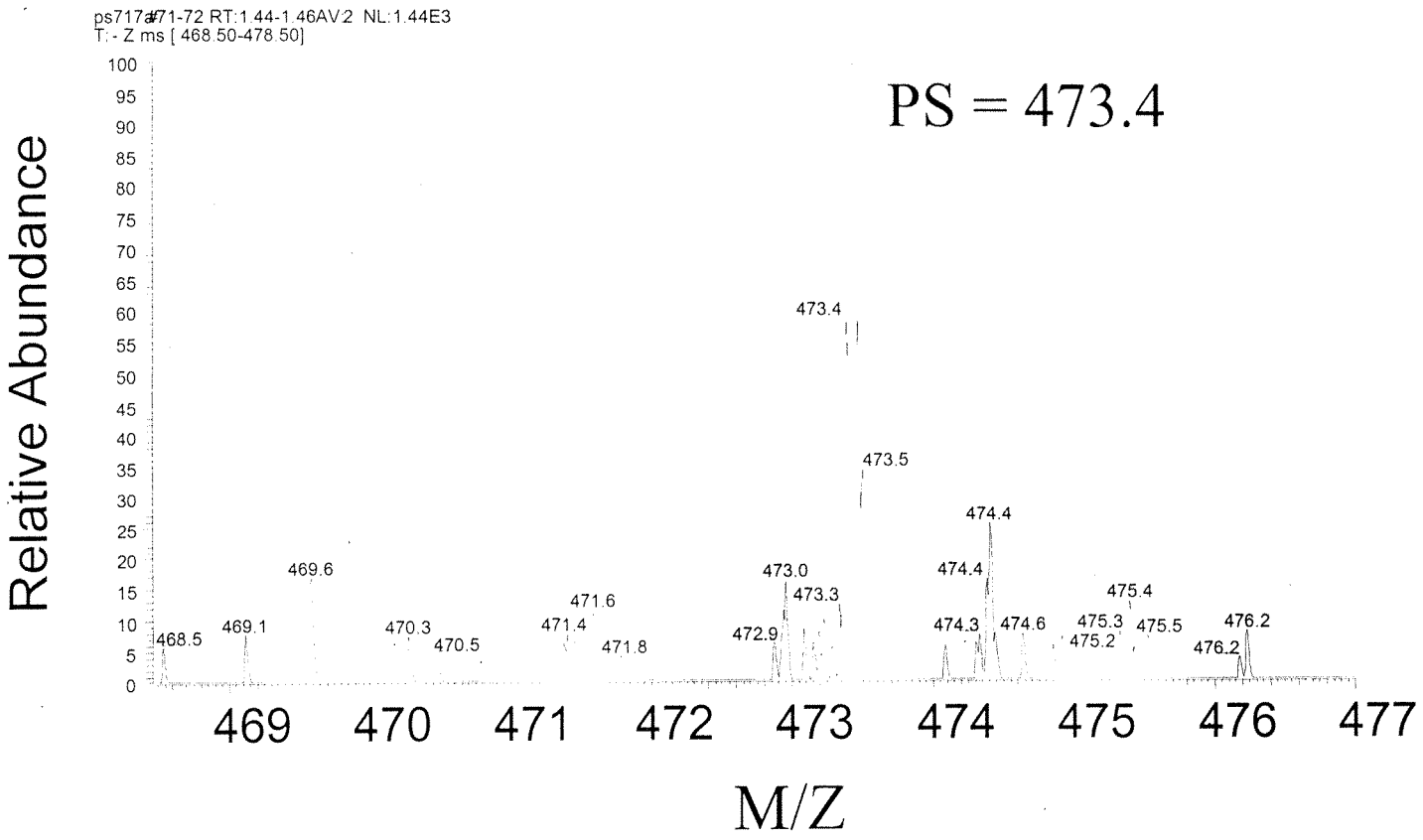

Figure 1: Electrospray ionization/mass spectrometry zoom scan (profile) of petromyzonol sulfate standard. Used for identification of PS.

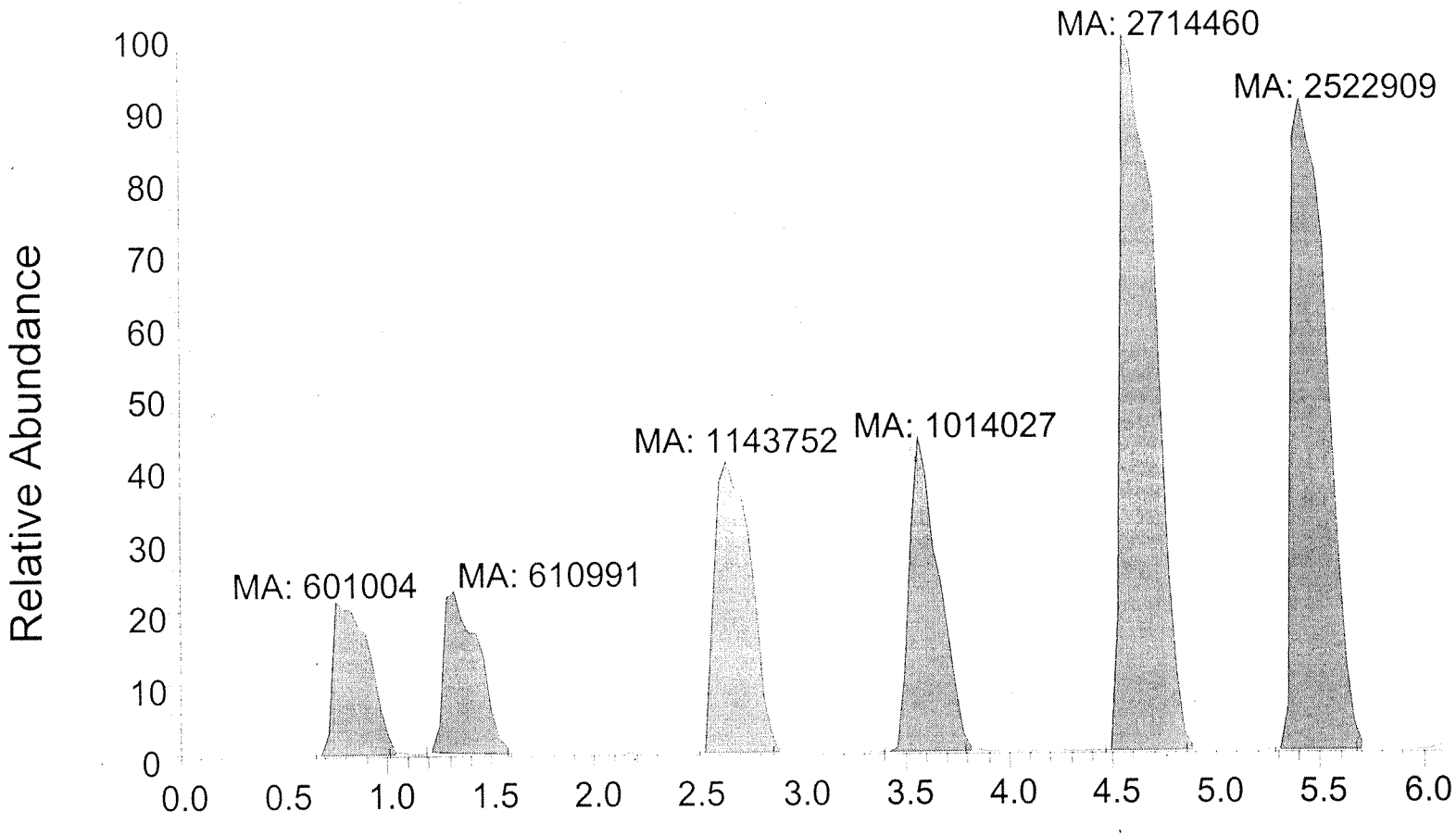

Figure 2: Selected ion monitoring (SIM) of petromyzonol sulfate by electrospray ionization mass spectrometry. Peaks shown represent 3 linear points of the standard curve made by putting known amounts of PS into a nonlamprey stream (Valley Creek - 2 injections for each point of the standard curve). The standard curve was then used for quantitation of PS in river water. PS concentrations are 180 ng/ml, 540 ng/ml, and 1,620 ng/ml.

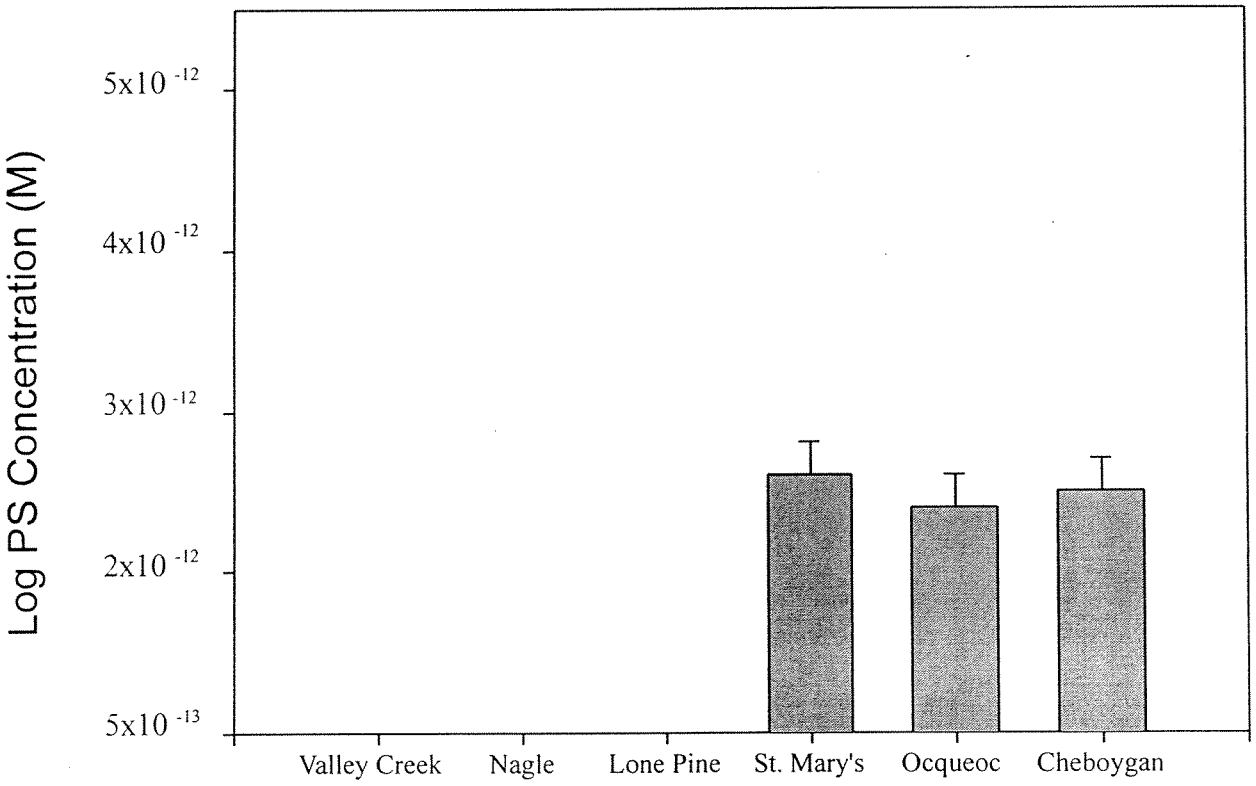

Figure 3: Concentrations of PS in 6 rivers. Quantitations were done with electrospray ionization/mass spectrometry (ESI/MS). The detection limit for PS is 6x10^-13 M. Valley Creek (MN), Nagle (MI), and Lone Pine (MI) were collected in the summer of 1999. St. Mary's (MI), Ocqueoc (MI), and Cheboygan (MI) samples were all collected in summer 1997.

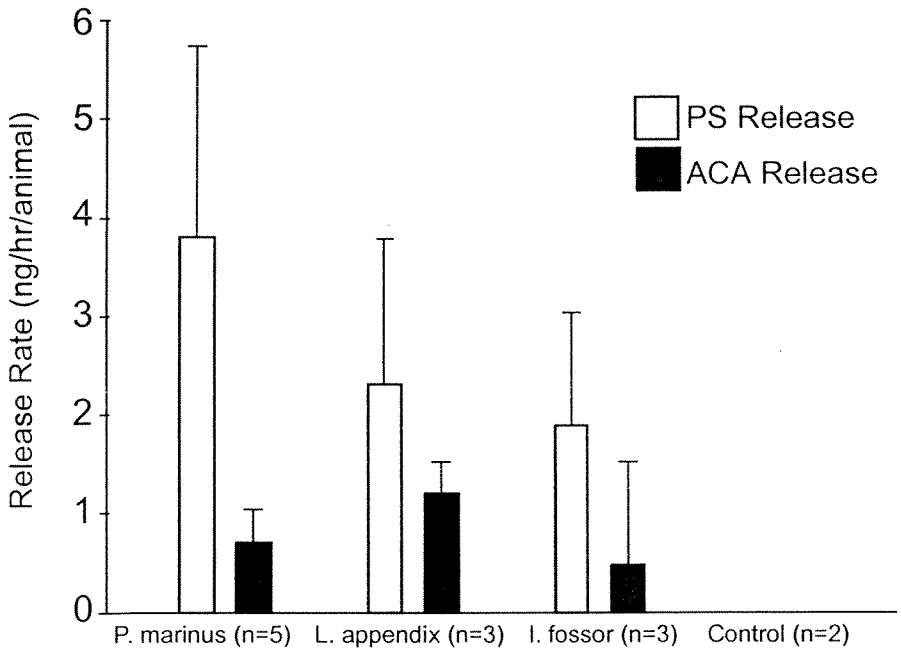

Figure 4. Release Rates of PS and ACA from larval *P. marinus*, *L. appendix*, and *I. fossor*. PS and ACA were purified by HPLC and measured with ESI/MS. Error bars are standard error.

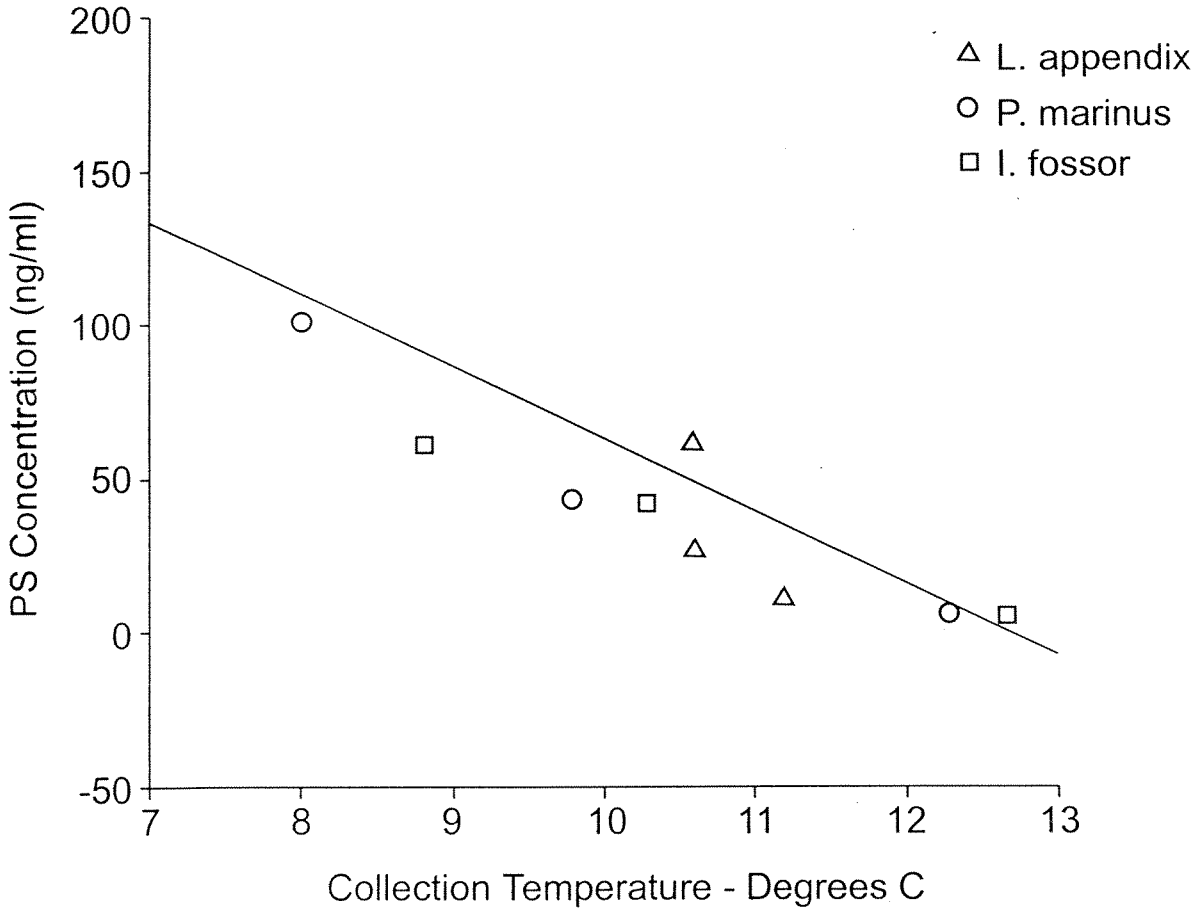

Figure 5: Correlation between PS concentration (ng/ml) in larval holding water and water temperature at time of collection for *L. appendix, P. marinus*, and *I. fossor*. Statistical analysis shows the regression line to be significant (model utility test, $p < 0.001$). Data shown excludes one outlier data point.

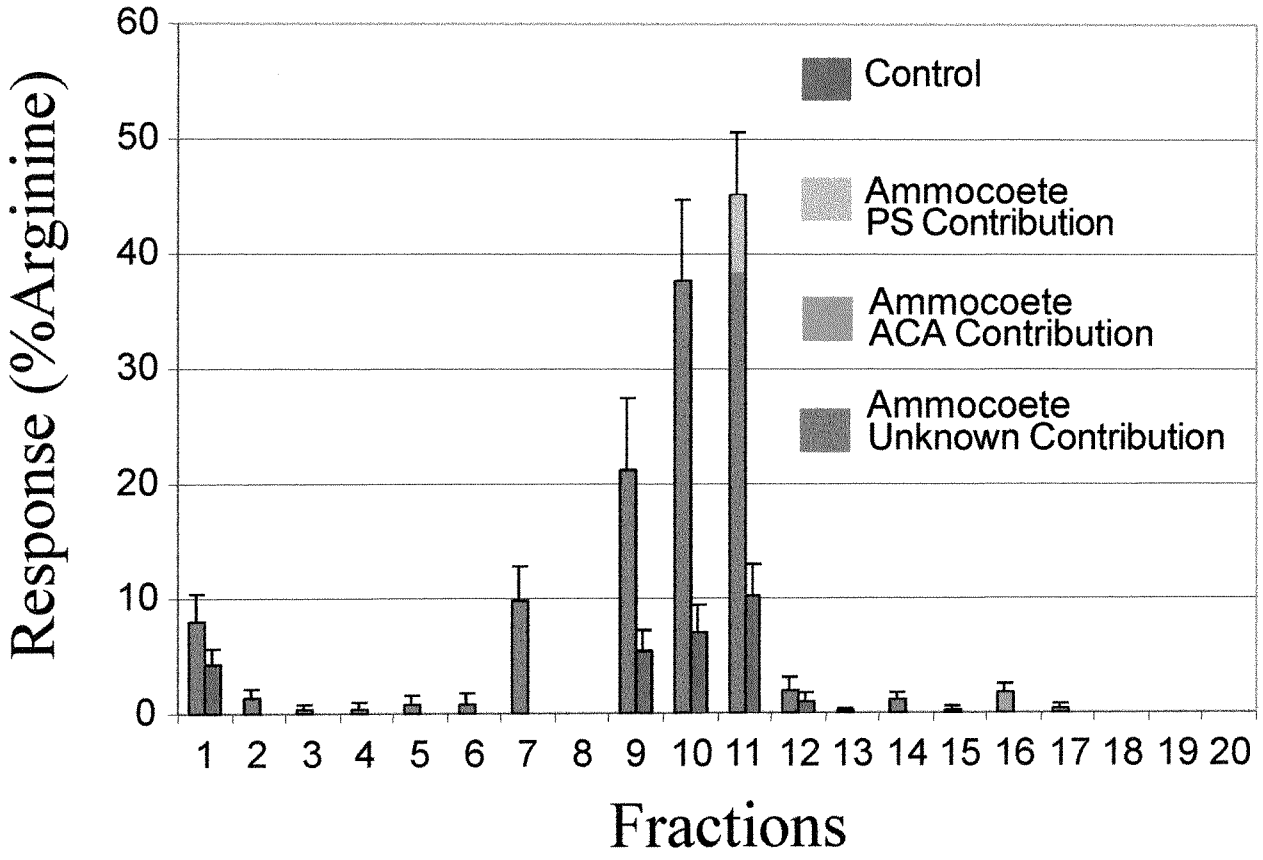

Figure 6. EOG responses (%Arginine –5M) to 1 L ammocoete holding water and control water that have been fractionated into 20 fractions by HPLC with an increasing methanol/ water gradient. Contributions from PS and ACA in ammocoete holding water (based on MS quantitation and EOG dose response curves) can be seen in fractions 11and 16, respectively.

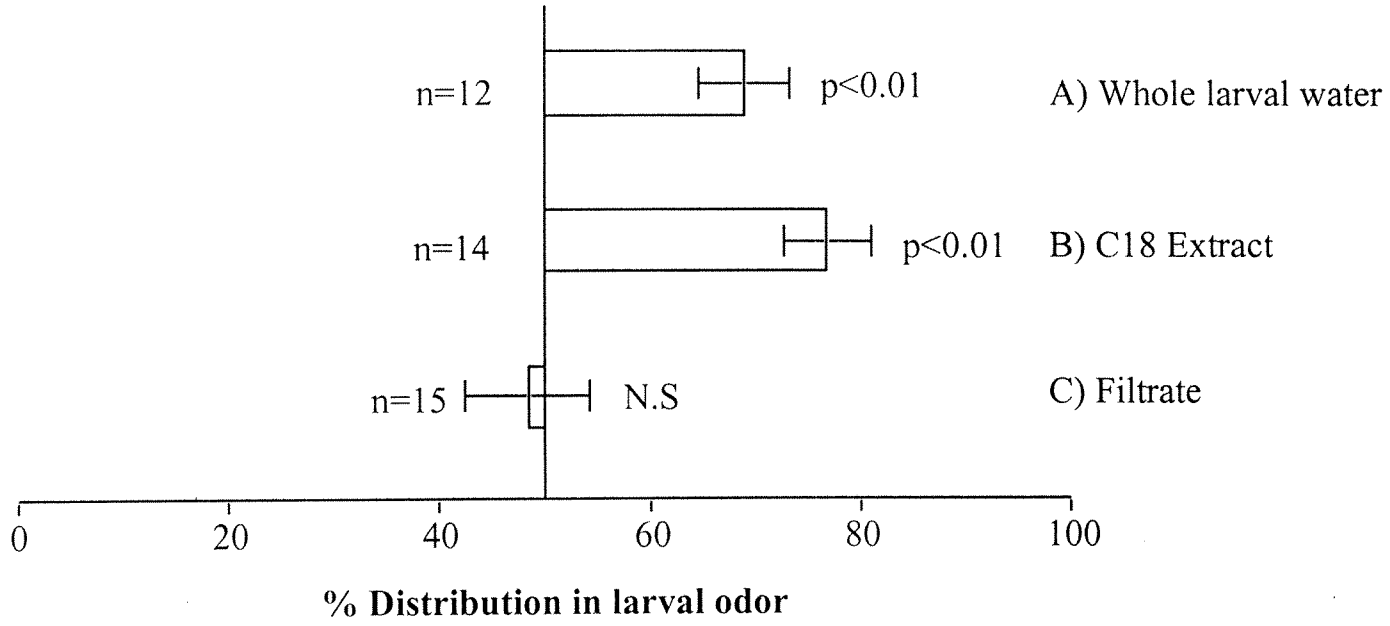

Figure 7. Behavioral preferences of adult sea lamprey in a 2-choice preference maze. Test A is a direct comparison of whole larval water vs. control water, test B is the C18 extract of larval water vs. the C18 extract of control water, and C is a test of the C18 filtrate of larval water vs. the filtrate of control water. Columns represent mean time spent in the experimental channel; error bars are standard error. Means are compared to a 50% expected no-preference value (two-tailed one-sample student t-test.)

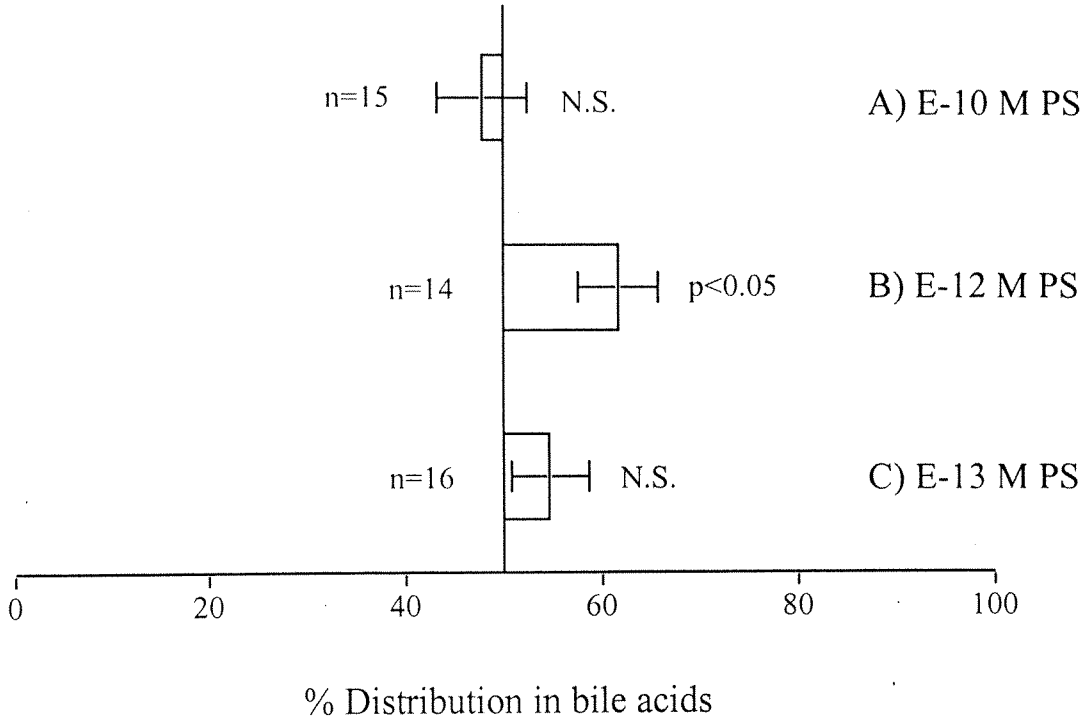

Figure 8. Behavioral preferences of adult sea lamprey in a 2-choice preference maze. Tests A, B, and C are tests of the bile acids PS and ACA at three different concentrations; -10 M, -12 M, and -13 M, respectively. Columns represent mean time spent in the experimental channel; error bars are standard error. Means are compared to a 50% no-preference value(two-tailed one-sample student t-test).

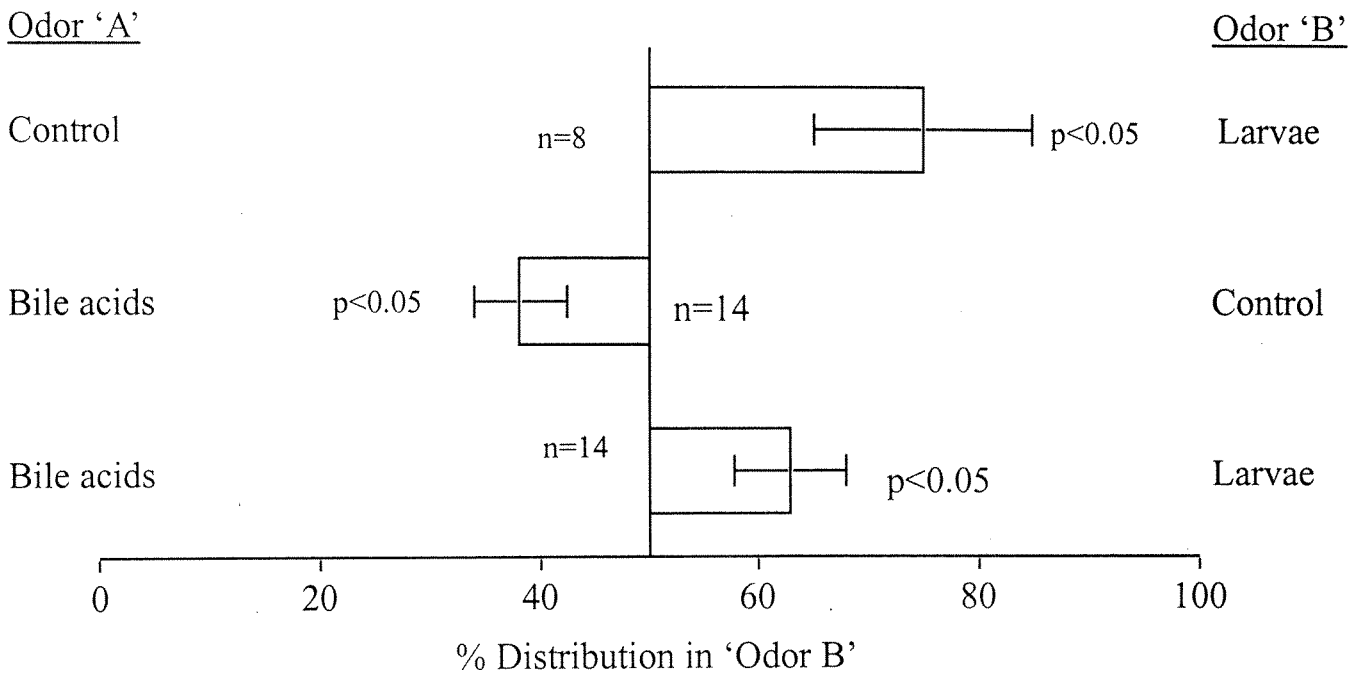

Figure 9. Behavioral preferences of adult sea lamprey in a 2-choice preference maze. These tests include direct comparisons of larval water vs. control, bile acids vs. control, and bile acids vs. larval water. Columns represent mean time spent in the channel B; error bars are standard error. Means are compared to a 50% expected no-preference value (two-tailed one-sample student t-test.)

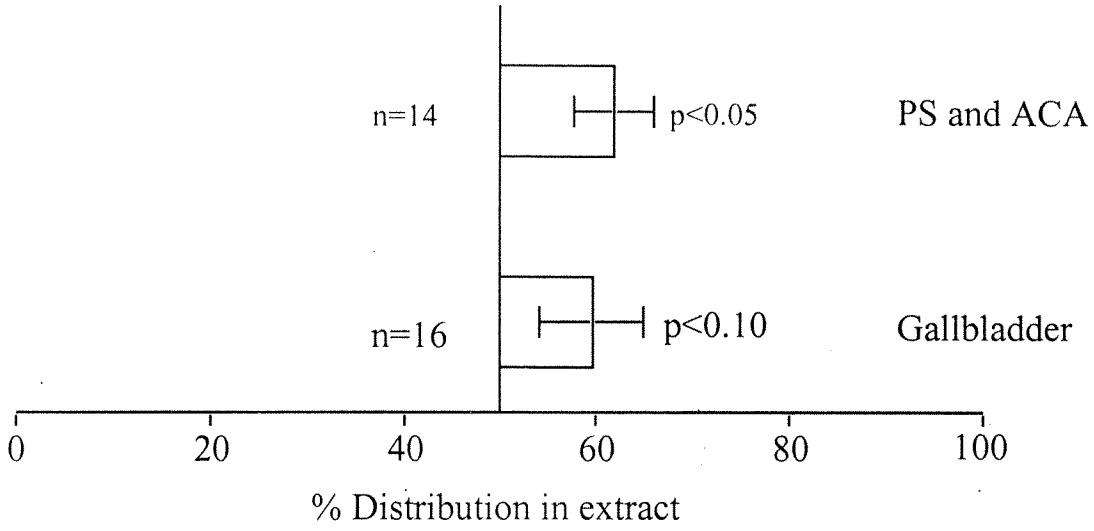

Figure 10. Behavioral preferences of adult sea lamprey in a 2-choice preference maze. These tests include the bile acids PS and ACA vs. control, and larval lamprey gallbladder vs. blank methanol. Columns represent mean time spent in the experimental channel; error bars are standard error. Means are compared to a 50% expected no-preference value (two-tailed one-sample student t-test.)