GREAT LAKES FISHERY COMMISSION

2000 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 I

by:

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P.W. Sorensen
March 10, 2000

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.

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PROJECT DATES: April 1, 1998- March 31, 2000

PROBLEM STATEMENT & OBJECTIVES AS STATED IN ORIGINAL CONTRACT:
Our previous research has demonstrated that adult sea lamprey possess a highly-sensitive and specialized sense of smell which detects petromyzonol sulfate and allocholic acid, two unique bile acids produced and released by larval lamprey. Recently, we discovered that migratory adult lamprey locate rivers using their sense of smell and that a critical component of river odor is the pheromonal odor of larval lamprey which live in spawning streams. In addition, we have clearly demonstrated that petromyzonol sulfate and allocholic acid are principal components of the larval pheromone. Now we seek to develop a more comprehensive understanding of this system, while developing the technology needed to produce this cue in quantity so that it can be used in biocontrol. In this proposal we seek to collaborate with, and provide critical analytical support to two research groups attempting to develop the means of producing petromyzonol sulfate. We also propose to collaborate with and provide critical support to a biotechnology company seeking to develop a sensitive technique (enzyme immunoassay, or EIA) for measuring the presence of petromyzonol sulfate in natural river waters. Additionally, we propose to conduct the first experiments to determine the utility of using EIA to determine larval abundances while providing background data for eventual pheromone application. Because our earlier studies suggested that the larval pheromone also contains yet-to-be-identified components, we ask for funds to characterize these components so that they can be identified and the entire pheromonal mixture can eventually be synthesized. Finally, we seek to determine whether the larval pheromone is unique to sea lamprey or whether some native lamprey species also produce this extremely important compound. We ask for funds to assist other laboratories attempting to synthesize petromyzonol sulfate, and to address objectives relating to four deliverables:

1) The technical means of measuring petromyzonol sulfate (PS) in natural waters inexpensively.
2) An understanding of whether concentration of PS can be used to determine larval abundance.
3) An understanding of whether native lamprey species release the same pheromone as sea lamprey.
4) An understanding of the chemical complexity of the complete sea lamprey larval pheromone.

PROBLEM STATEMENT & OBJECTIVE IN SUPPLEMENTAL CONTRACT:

In a supplemental request submitted May 1999, we received additional funding to accelerate our research progress. In addition to receiving funds to accelerate progress developing the EIA in the laboratory (deliverable #1 in original contract) we received funding for a new deliverable which I paraphrase below:

5) To determine the most promising means of testing the feasibility of using pheromones to control lamprey distribution in the field. A written report will describe licensing procedures, the next priority areas in testing pheromones, and a possible experimental design (and field site for this work).

SYNOPSIS OF PROGRESS TO DATE.

Over the past two years, we have:

1) Developed the means of measuring petromyzonol sulfate (PS) in natural waters using liquid chromatography- mass spectrometry but have yet to complete development of the means to make this inexpensive through the use of an enzyme immunoassay (EIA).
2) Collected natural river waters to answer deliverable #2 but have not yet measured them (we await completion of the EIA).
3) Have determined that native lamprey release a pheromone that attracts adult sea lamprey.
4) Have ascertained that the larval pheromone contains components other than PS (final confirmation awaits)
5) Have developed an approach for moving the application of pheromones to a field setting and have initiated a survey amongst those concerned with lamprey control to define what form this first step will take. We have also contacted all appropriate government agencies concerned with applying pheromones to natural waters. Policies concerning registration of pheromones are nearly summarized.

The key details of this progress follow below. An additional report will be submitted by the end of April detailing our progress understanding licensing requirements for pheromone application together with a synopsis of the findings of our survey (if we have response from the majority of GLFC-personnel by then).

First objective (not a deliverable): To assist other laboratories attempting to develop alternative means of synthesizing petromyzonol sulfate.

a) Summary. We have provided assistance to three laboratories attempting to develop the means of producing petromyzonol sulfate or analogues in bulk. None have worked, but several appear to have potential. Progress is described below.

b) Collaboration with Dr. Collodi. We have been performing HPLC analysis of lamprey liver cell culture media for Dr. Collodi's group (Purdue). In 1998 we analyzed samples on an approximately bi-monthly basis. In 1999 he sent us samples six times. We analyzed all within a month of receiving them. Talking with him over the telephone, it is our understanding that some of these samples did show cultured liver cells to produce PS. However, despite several requests to share some of his findings or plans with us, he has not done so. We now understand from another telephone conversation with Dr. Collodi that he has requested a 6-month extension to this project. Even though this falls outside the time period of our contract with him and we are no longer routinely running bile acid analyses in the manner he needs, we will do our very best to accommodate his needs during this extension. Clearly, if PS can be produced by cell culture this would be a significant breakthrough. I trust that Dr. Collodi has reported his findings to the GLFC directly.

c) Collaboration with Dr. Maxey and Cayman Chemical Co. We used electro-olfactogram recording (EOG) to test the olfactory activity of two analogues of petromyzonol sulfate (Delta4-petromyzonol, Delta4-petromyzonol-24-phosphate) produced by the Cayman Chemical Company. Unfortunately, neither compound had any olfactory activity at 10^{-8} Molar. We have also employed EOG recording to test the olfactory activity of several analogues of petromyzonol sulfate (multiply-sulfated-5β petromyzonol, and multiply sulfated 5α petromyzonol) produced by Toronto Research Chemical Company. Neither had notable olfactory activity at 10^{-8} Molar. The lamprey olfactory receptor for petromyzonol sulfate is clearly extremely specific;

multiply (nonspecifically) sulfated forms of petromyzonol (which are much less expensive to produce) are not good analogues for petromyzonol-24-sulfate. Cayman Chemical is apparently continuing to develop analogues of PS and we will test them when/if they become available to us.

d) Own initiative. We used electro-olfactogram recording (EOG) to test whether 5β-petromyzonol (5βP) and 5α-petromyzonol (P) interact with the same receptor site and whether the former might be an appropriate and cost effective analogue for petromyzonol that might be used to make PS. Unfortunately, while there was considerable cross-reactivity between these compounds, it was not complete and we can not recommend using 5βP as an analogue for P.

e) Collaboration with Dr. K.V. Venkatachalam. We have been communicating with Dr. K.V. Venkatachalam of Nova Southeastern University, who is proposing to clone the sulfotransferase that attaches the sulfate group to petromyzonol. Having this enzyme would make synthesis of PS much cheaper. We have agreed to assist Dr. Venkatachalam in all ways appropriate including providing standards and analyzing samples.

Deliverable #1: To develop the technical means of measuring petromyzonol sulfate (PS) in natural waters inexpensively.

a) Summary statement.

It had been our initial hope that we could develop an enzyme-linked immunosorbent assay (EIA) for PS that would permit direct, rapid and inexpensive measurement of PS in natural river waters. This, however, proved impractical because we could not develop an

antibody to PS itself as the rabbits being used for antibody development were de-conjugating PS. Consequently, we switched strategies and asked Cayman Chemical Co. to develop an antibody and EIA to petromyzonol (P) itself. This effort was successful, and although a few details still remain to be resolved for this EIA before it can be used on river waters, it is now clear that all these challenges can be overcome, and we look forward to being able to use this assay within a year. Because of our initial difficulties with the EIA, we have investigated another approach as a backup: liquid chromatograph-mass-spectrometry (LC-MS). A pilot experiment has shown this technique to also have great potential and it is outlined below.

b) The EIA

I am pleased to report that an enzyme immunoassay (EIA) for petromyzonol (P) has now been developed with the assistance of the Cayman Chemical Company. The EIA developed by Cayman has a detection threshold that consistently reaches levels of approximately 30 pg/ml (Figure 1), about an order of magnitude below the concentration of PS we expect to be present in lamprey rivers. Further, the antibody is highly specific and discriminates between all other bile acids we have tested (Table 1). 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. Cayman Chemical Company has now lyophilized and packaged enough vials of P tracer and antiserum to perform approximately 100,000 determinations. Half of these vials are stored in the

freezer in the Sorensen laboratory at the University of Minnesota, and the other half are stored in the freezers at Cayman Chemical Company in Ann Arbor, MI.

Quantification of P using EIA in well water has proven to be consistently reliable using pure laboratory waters. Unfortunately, however, direct attempts to measure P (de-sulfated PS) 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 (NOM) present in river water. The identities of these interfering compounds are unknown, but they are almost certainly not bile acids because, as reported above, we have tested them. The solution to this problem is to specifically extract PS from river water so that it can be measured in the absence of interfering compounds. Accordingly, we have developed a technique which uses high performance liquid chromatography to fractionate river water over the course of a 160 min run, collecting only the 5-min window which contains PS (the latter we identify by retention time relative to a known standard). The fraction that contains the PS should be pure because over 97% of the other compounds should be excluded from it, and it can easily be treated with acid solvolysis to produce P (for measurement by EIA). The efficiency of the latter reaction can be precisely quantified by adding estradiol-sulfate as a tracer (estradiol production can be measured by a commercially available EIA kit) and we expect conversion and recovery of PS to be 95% or greater (based on pilot studies). Currently, we are conducting experiments to develop this technique for HPLC purification and de-sulfation. Final validation of the EIA will require us to: 1) add a known amount of PS to river waters and measure it correctly with EIA; and 2) dilute river water extracts and observe a corresponding linear decrease in the amount of PS we measured. We expect these experiments to be complete by the end of the summer.

c) Liquid-Chromatography/ Mass Spectrometry.

Recently, we learned that advances in the field of liquid chromatography / mass spectrometry (LC/MS) make this technique suitable for measuring sulfated steroidal compounds such as PS at low concentrations in solutions containing complex assortments of other compounds. In particular, the advent of new ionization techniques such as electrospray ionization and atmospheric pressure chamber ionization has made it possible to introduce compounds into a mass spectrometry analyzer without conjugation, a process that had previously impeded quantification. Fortunately, we have access to a modern LC-MS at the University of Minnesota because of Dr. Sorensen's affiliation with the Agriculture Experiment Station (which also makes access affordable). We have conducted a single pilot experiment using this machine and find that we can measure PS in methanol carrier in concentrations approaching 100 pg/ml (Figure 2). Even greater sensitivity may be possible because we have not yet attempted to optimize conditions. This technique has the advantage that it can tolerate the presence of some impurities because it measured the molecular ion for PS itself (Figure 3), but samples will still have to be purified by HPLC fractionation. Although a sensitivity of 100pg is slightly less than the EIA, it should be adequate for most samples; however the slope of the dose-response curve for LC-MS is less than for EIA, meaning that we would have less ability to discern small differences in relative concentrations between samples. Nevertheless, LC-MS appears to represent a technique that will almost certainly be suitable for research purposes and to confirm the EIA. We are planing to continue to investigate its true potential by using it to quantify the amount of PS added to extracted river waters known

to not contain larval lamprey, and repeatedly analyzing these extracts after serial dilution to establish sensitivity and instrument error. Parallel measurements of PS in samples by both EIA and LC-MS are also planned.

Deliverable #2. An understanding of whether the concentration of PS can be used to determine larval abundance.

a) Summary. This objective has not been met because we do not yet have an analytical procedure capable of providng measurements of PS with enough resolution to be useful. However, these techniques are now nearly developed (see above). In the meantime we have collected and extracted nearly 5 dozen samples of water which will permit us to answer this question in a definitive manner within the term of the next contract.

b) Water collections. To answer this question we have been collecting and extracting water samples from many locations over the years. Although a few have been analyzed (and they have confirmed the presence of PS), most remain in cold storage until we have a technique in hand which is capable of accurately quantifying the amounts of PS present. Between 1996-1998 we collected (with the help of control agents) and extracted 44 river water samples (Table 2). These samples will permit us to answer two questions:

1) Do waters from rivers that both contain larval lamprey (regardless of species) and attract adult sea lamprey contain higher concentrations of PS than waters from rivers which do not? (water samples were collected in matching sets from the Cheboygan and Ocqueoc Rivers [which have and attract lamprey], and the Lone Pine and Nagel Rivers [which don't]).
2) Is there is a seasonal trend in the concentrations of PS found in river water (water samples were collected on a monthly basis from St. Mary's River for a year).

In addition, during the course of this contract (1998-2000), we, together with the Hammond Bay Staff, collected four other sets of river water samples (Table 3) to answer the following questions:

3) Is there is relationship between the density of sea lamprey larvae and PS concentrations? (Mr. Bergstedt collected water samples upstream and immediately downstream of specific sections of two streams with known populations of sea lamprey larvae – the Misery River (MI) and the Rock River (MI). Flow rates were measured concurrently. Collections were performed two times – during the falls of 1998 and 1999.)

4) Do waters from rivers that contain native species of larval lamprey also contain PS? (Mr. Bergstedt 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.)

5) Does PS concentration vary with the time of day? (Water samples were collected from the Black Mallard River (MI) at 6-hour intervals over the course of 3 days.)

6) Does PS concentration vary on a day-to-day basis over the course of one week? (Water samples were collected daily from the Black Mallard River (MI) for one week in the spring of 1999 at the same time each day.)

Deliverable #3. To determine whether native lamprey species release the same pheromone as sea lamprey.

a) Summary. Two years of behavioral experiments demonstrate that waters collected from tanks of larval sea lamprey, larval American brook lamprey, and larval northern brook lamprey have similar properties: all are attractive at very low concentrations to migratory adult sea lamprey, and all can be repulsive at high concentrations. This apparent lack of species-specificity is consistent with the results of earlier HPLC analysis which shows all three species to release PS to the water (although only sea lamprey appear to release ACA).

b) The Behavioral Experiments

To address this question, behavioral experiments were conducted in the raceway mazes at Hammond Bay Biological Station in both 1998 and 1999. Protocols closely followed those used in previous years. Briefly, migratory sea lamprey were captured in traps in the Cheboygan and St. Mary's Rivers and brought to the Hammond Bay Biological Station. After acclimating to laboratory conditions for several days, groups of 4 animals were placed in a two-choice maze with Huron water flowing down both arms. Different odors (river waters and larval holding waters) were mixed into each arm, and the amount of time lamprey spent in each of the arms was quantified for 20 minutes. To test for preferences, the total amount of time lamprey spent in one arm was calculated as a percentage of the total time spent in both arms, and that percentage was compared to a theoretical no-preference value of 50% using a t-test.

The response towards three different species of larva was compared-- *Lampetra appendix* (American brook), *Ichthyomyzon sp.* (believed to be northern brook based on rivers they were collected in), and *Petromyzon marinus* (sea lamprey). Separate tanks of each species were maintained (details in Table 4), along with an identical control tank containing sand and aeration, but no larvae. 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 experiments by pumping them from these tanks into the raceways.

Experiments were performed in 1998 (N=148 total trials using 4 adult sea lamprey in each; 11 trials per typical experiment) and 1999 (N=131 total trials using 4 adult sea lamprey in each; 12 - 16 trials per experiment). For each trial, water from Nagel Creek, a stream that does not contain any larval lamprey, was pumped into both arms of the maze at a rate of about 4L/min, with larval holding water being pumped into one arm and control holding water pumped into the opposite arm. The pumps were pulsed in order to conserve odor water and because in a natural setting animals are exposed to odors in a pulsatile fashion because of turbulence (see Table 5).

In both 1998 and 1999 we tested odors of the three larval species at a series of dilutions to derive a dose-response relationship. We used higher doses in 1998, the greatest of which caused repulsion, so we changed pumping regimes and dosing protocols in 1999. Thus, in 1998, lower doses were obtained by decreasing the amount of time the odor pumps were pulsed on, while in 1999 the pulse rate was held constant and

the quantity of larval holding water that was mixed with the stream water was adjusted instead (Table 5). Because of these differences in protocol, one must be cautious in making comparisons between years, although all experiments can be 'standardized' by calculating the total amount of larval holding water pumped into the maze during a single trial. The 'residual odor test' conducted in 1998 was performed slightly differently. At the end of each night of experiments, all animals were returned to their cages, the larval odor pumps were turned off, and the river odor pumps remained on. The animals from the first three trials were then released and observed for 10 min. The arm that had the larval odor earlier in the night was termed the 'larval odor arm' even though no larval odor was being added at that time.

In 1998 we found that although the odor of each of the three species of larvae was somewhat repulsive at the highest pulse rate, in each case it became progressively more attractive as the pulse rate was reduced (Figure 4). At the medium pulse rate, *P. marinus* odor was significantly attractive, and the odor all three species were so at the 'residual concentration' ($P<0.05$; Figure 4). This result suggested that lamprey were able to detect and orient to larval odor at concentrations much lower than we had tested to date. The response towards both species of native larvae was nearly identical, while *P. marinus* larvae switched from being repulsive to attractive at a higher pulse rate than native larvae. However, this may have been due to the fact that although the *P. marinus* tank held a similar number of individuals as the native tanks, they were smaller, resulting in the total biomass being only about half that of the native larvae (Table 4).

In 1999 we chose to examine species-specificity again while removing differences in the biomass of larvae tested and our changing pulsing regime as confounding variables

within the experiment. Accordingly, we matched all larval tanks by biomass (instead of number of individuals; Table 4), tested lower concentrations of larval water only, and altered pumping regimes so that concentration, and not pumping rate, varied with dose (note that less larval water was pumped during the 'high' concentration trials in 1999 than the 'low' pulse rate trials in 1998). Our results from 1999 largely confirmed those of 1998; low concentrations (which were lower than any used in 1998) were attractive, with the response being marginally significant for the lowest concentrations of *P. marinus* and *I. spp. L. appendix* was significantly attractive ($P<0.05$) even at the lowest concentration (Figure 5). When the attractiveness of the odor of each species was compared at each dilution, no statistical differences were seen among species or dilutions (2-way ANOVA). Clearly, native larval lamprey are attractive to adult sea lamprey.

In another 1999 experiment we tested whether the odor of *L. appendix* could, when added to natural stream water, make it more attractive than a naturally more attractive stream water. Odor from *P. marinus* holding tanks has previously been shown capable of effecting such a convincing reversal (Sorensen, 1998 Project Completion Report). First, we tested water from the Cheboygan River against the Ocqueoc River; we found adult sea lamprey to prefer the latter ($P<0.05$; Figure 6). However, after *L. appendix* holding water was added to the Cheboygan (at the 1999 high concentration) and control water was added to the Ocqueoc, the Cheboygan became more attractive ($P<0.01$; Figure 6). This demonstrates that the odor of native lamprey species can exert a powerful influence on the relative attractiveness of natural river waters.

Deliverable #4. To Determine the chemical complexity of the complete sea lamprey larval pheromone.

a) Summary. Tests of the potency of larval waters using electrophysiologcial recording (EOG) and behavioral assays both suggest that the larval pheromone may contain compounds which supplement the actions of petromyzonol sulfate (PS) and allocholic acid (ACA). EOG recording has clearly shown that while the entire odorous portion of larval odor can be extracted using C18 columns, that only about half of this odor can be attributable to the presence of PS and ACA: larvae release unknown compounds which adults can detect. Similarly, behavioral studies demonstrated that a low concentration of larval odor was more attractive to adults than a 0.1 nanomolar concentration of PSA and ACA. Although interpretation of the latter experiment is complicated by the fact that we as yet do not have measurements of the amount of PS and ACA present in the larval waters tested, it does suggest that PS and ACA may not explain the full behavioral potency of the larval pheromone.

b) Electrophysiology (EOG)

In these experiments, which were conducted in 1998 and 1999, tanks of larval *P. marinus* were maintained at the University of Minnesota along with control tanks that contained sand only. Lamprey were fed as in the behavioral experiments described above, and 36h after feeding their waters were extracted by C18-column extraction. These extracts and filtrates were then tested as odorants on the olfactory system of the adult sea lamprey using EOG recording. The adult animals used for these experiments were collected at the Cheboygan River trap and shipped

immediately by air to the University of Minnesota where they were held in large holding tanks at cold temperatures until needed. EOG recording followed established protocols (Li & Sorensen 1997), in which we used –5Molar L-arginine as a standard and we only used animals that produced a 1mv response to this stimulus. Four experiments were conducted, two using larval waters, two using river waters. The latter produced somewhat equivocal results and were reported in the 1999 interim report so are not reported here. Instead, we review the key components of two larval odor experiments.

i) Can the odor of larval sea lamprey be extracted by C18 Sep-Paks?

Both in 1998 and 1999 water samples were collected from recently-fed lamprey and control tanks, extracted by C18 columns (Waters Inc., Milford, MA), and methanol extracts tested by EOG recording. In 1998, we found that larval extracts were much more potent than control extracts, and that while the former could be diluted nearly 1000 times and remain detectable by EOG, the latter could only be diluted 100 times and remain detectable. These experiments also demonstrated that when diluted 10-fold (thus removing responses to nonspecific irritating stimuli), the entire potency of larval odor was extracted by C18 Sep-Paks as there was no difference in responses to the filtrate from control and larval tanks at this concentration, while huge difference existed between the eluate of control and lamprey tanks. The size of the latter responses could account for the difference between whole control and larval waters (Figure 7)

In 1999 we conducted a more detailed analysis of the ability of C18 Sep-Pak columns to extract the odor of larval lamprey and whether it could be isolated in a specific fraction. In this experiment, larval waters were extracted by a C18 Sep-pak and then

eluted with 5ml aliquots of a mixture of methanol/water, whose methanol concentration was increased in steps with each elution. The elutes were then dried down and their potency tested by EOG recording and compared to that of C18 Sep-pak washing extracted with 100% methanol. These experiments confirmed that C18 Sep-pak column extracted the entire larval odor and that it all came off in the 70% methanol washing – excellent news if further purification and fractionation is planned (Figure 8).

ii) Are petromyzonol sulfate and allocholic acid the only odorous compounds in larval washings?

Cross-adaptation experiments were performed on adult migratory lamprey to address this question. In this type of experiment, the nose of a test animal is continuously adapted to an odor and test odorants are added to the background flow at concentrations which elicit equipotent EOG responses: if no response is seen it is assumed that the test odorant is recognized by the same receptor(s) as the adapting odor. Alternatively, if a response is seen, it is assumed that responses are associated with olfactory receptors different than those which detect the adapting odor. Of course, if different receptors are involved it can be assumed that different, distinguishable odorants exist. We analyzed our cross-adaptation data by calculating 'Percent Unadapted Response' (PUR) using a formula modified from Li &Sorensen (1997):

$$PUR = \frac{100 \times \text{Adapted Response Magnitude}}{\text{Unadapted Response Magnitude}}$$

A PUR of 100% indicates no cross-reactivity between two samples, and odors occupy separate receptor mechanisms, while a PUR of zero indicates that the two odors likely

share the same receptor mechanism. PUR values obtained were compared to a 100% value using a T- test.

Very briefly, in our experiment, we established dose response relationships to ACA, PS, TLS (taurolithocholic acid, a control bile acid detected but not released by sea lamprey), C18sep-pak eluate from control tanks which contain sand only, and C18-sep-pak eluate from tanks which contain larval lamprey. Having determined this relationship, we then calculated the concentrations for each which elicited a response equivalent to that of our control standard, -5Molar L-Arginine. These concentrations were then used throughout the experiment. Four adult sea lamprey were tested. In each case, the fish was first tested with each of these 7 odors, and then its nose was perfused (adapted with) a continuous flow of a mixture of C18sep-pak eluate from the control lamprey tanks (sand only) plus a mixture of –9Molar ACA and –10Molar PS. Then, while adapted, we tested each of the test stimuli again. Of course we expected EOG responses to C18-spepake eluate from control tanks to disappear as well as those to ACA and PS, while responses to other chemically distinct odorants (arginine, TLS) should have persisted unchanged. This is in fact was what we observed. In addition, however, we noted that while responses to ammocete washings were significantly depressed (as we expected because we knew they contained control tank odor as well as ACA and PS), they were still much greater than 0 (Figure 9). The latter finding clearly demonstrates that ammocete washings contain odorous compounds other than PS and ACA, the identity and behavioral significance of which is unknown.

b) **Behavioral experiments:**

In addition to examining the complexity of larval odor using EOG recording, we conducted a series of behavioral tests to assess whether the larval cue might contain components other than PS and ACA. To accomplish this we directly compared the behavioral preferences for whole larval odor versus purified bile acids to see if there was any indication that whole larval odor might be more attractive. The behavioral assay described in the description of Deliverable #3 was used. First, the lamprey's response to bile acids was tested by itself. Nagel Creek water (a stream without larvae) was pumped into both arms of the maze, while a mixture of petromyzonol sulfate and allocholic acid was pumped into one arm of the maze, resulting in a concentration of bile acids of approximately $1x10^{-10}$ M in that arm (the concentration we estimate to be present in larval waters but have not directly measured in Hammond Bay waters). Next we conducted the same experiment, but added sea lamprey larval waters to the Nagel, to confirm that they, too, were attractive. A concentration corresponding to the 1999 medium concentration (0.32 L larval water/trial) was used. Finally, having confirmed that both odors were attractive, we tested them against each other.

As expected, PS and ACA attracted adult sea lamprey, although somewhat weakly ($P<0.10$) as had been seen in both of the other tests of bile acids conducted in 1997 (Completion report from that year). Larval water odor was strongly attractive ($P<0.05$; Figure 10). The test of larval water versus bile acids alone found the former to be marginally more attractive ($P<0.10$). Although this finding supports the EOG results, it is important to note that we do not know whether the concentration of PS and ACA used actually represents the concentration found in larval waters, and we know that larval odor

becomes repulsive at high concentrations. Thus, before concluding that the larval pheromone must contain compounds other than PS and ACA, we must assess the precise concentrations of bile acids that were present in these waters (we retained a sample and plan to analyze it by EIA as soon the technique is fully operational), and ascertain whether lower concentrations of bile acids might be more attractive to adult sea lamprey, as we saw with larval waters themselves (an experiment planned for 2000).

Deliverable #5. To determine the most promising way of testing the feasibility of using pheromones to control sea lamprey distribution in the field.

a) Summary:

To accelerate the development of the use of pheromones in lamprey control, we have begun to examine how best to test pheromones in the field. We had originally proposed to accomplish this task by describing: 1) the long-term plan for developing pheromones for control; 2) priority research areas; 3) design of field experiments and criteria for an experimental field site; 4) some possible field sites suggested by field agents; and 5) licensing procedures for pheromones. After initiating this process, it soon became apparent that future research could go in many different directions, depending on the specific type of management application that was being developed. We saw a need to first focus our research efforts and realized that the Commission would be most interested in those potential applications that were most pragmatic. To identify the most promising applications, we required the advice and opinions of those most familiar and involved with lamprey control. Accordingly, a survey was developed and distributed to twenty biologists and managers involved with lamprey control in order to identify

the most promising strategies for using pheromones in control, and the most important questions associated with them. Our thinking is that once these strategies are identified, we will then be able to focus on the science needed to facilitate their development.

The completion of several of the objectives listed above requires the results of the survey, and thus they await the survey's return and analysis. However, we have made significant advances with each of the objectives (basically completing numbers 1 and 5, which do not require the survey results), and we here briefly describe that progress. A complete report on all objectives and the findings of the survey will be submitted by the end of April. The final identification of a field site may not be completed by that time, but the search will be actively continued in the following months.

b) The long-term plan for developing pheromones for control

We have now established a working plan for developing the use of migratory pheromones for lamprey control. Theses steps are: 1) investigating biological aspects of the pheromone [largely completed]; 2) identifying the most promising strategies for applying pheromones in lamprey control; 3) determining what issues need to be addressed before the identified control strategies can be implemented with a reasonable chance of success; 4) investigating the aforementioned issues in the best manner possible; and 5) designing a full-scale application to test the utility of the strategy for lamprey control.

The defining features of this plan are the placement of management-based decision-making to guide the research early in the process (step two) and the careful identification and testing of questions (steps three and four) before attempting a full-scale management application. We believe that this will result in the most efficient path towards the goal of obtaining a viable

and useful tool for lamprey control, while also ensuring the greatest chance of successful implementation of a full-scale application.

Up until this point in the development of pheromonal control, we have been almost exclusively focused on the first step--researching the basic biology of lamprey pheromones. We feel that our basic biological understanding of the pheromone has now reached a point where it is now necessary to move onto the next step and have our future research be directed by management input. This is because the next questions to be researched are largely dependent on, and different for, different potential applications.

c) The survey

As discussed above, obtaining the advice of lamprey control agents and scientists was a necessary first step in deciding the direction of future research. A survey was prepared and distributed with the objective of determining the most useful and promising applications and their priority research questions from a management perspective. One of the major accomplishments derived from the survey's preparation was the categorization of all potential applications into a simplified matrix. This matrix consists of three components: 1) how we are attracting (synthetic pheromone or larval source); 2) where we are attracting from (three different geographical scales); and 3) why we are attracting (management goal). The supporting document (see Attachment 1) that accompanied the survey discusses each of these components in detail, including their advantages and disadvantages and corresponding research questions.

The survey (Attachment 2) asks for individual's opinions on the perceived feasibility and utility of the different components. It also inquires about potential shortcomings of the different proposed applications and asks for the identification and prioritization of research questions

specific to the application. Finally, it asks for a numeric ranking of the proposed strategies in order to obtain a clear idea of where we should focus future research efforts. Completed surveys are now being returned and analyzed, and a summary of their findings will be included in the April report.

d) Defining priority research areas which must be addressed.

The areas of research given the highest priority will be dependent upon the opinions obtained through the management survey. However, we have already defined research questions specific to each potential application and expect the survey to prioritize these and produce additional ones (one of the objectives of the survey). It is hoped that this objective will be completed as soon as the returned surveys have provided a consensus on which potential applications in which to focus.

e) Designing experiments and defining specific criteria of an experimental field site

Once again, the field experiment design will be dependent on the identification of the priority research areas which will be decided through the management survey. However, we have already defined field site criteria and outlined potential field experiments for each of the three possible geographical scales at which lamprey might be attracted from. All designs involve the introduction of pheromone to an area previously lacking it and the release and recapture of tagged lamprey. In addition, we have been investigating the technologies of different sonic telemetry companies which could be used if it is decided that it is important to track lamprey's response to a pheromone plume within the lake.

f) Selection of possible field sites

The field site chosen will be dependent on the application (geographical scale) that is identified as the highest priority in the survey. Several managers have agreed to assist us in the location of potential sites. Since this objective requires the cooperation and time of control agents, we are delaying their involvement as much as possible until the highest priority geographical scale is identified through the survey. However, they have all been given the criteria for field sites and have begun to consider the possibilities, with several candidate sites having already been passed on to us. A visit to management stations to further discuss sites and examine historical records is being planned for April. In addition, we have visited several sites on Lake Champlain and have had two meetings discussing the potential for collaborating with the local office of the Fish and Wildlife Service and the Lake Champlain Technical Committee. We have also talked with a fisheries manager on Lake Cayuga about the potential for performing experiments in that basin. Increased cooperation and more active communication is being initiated with control staff in Canada and the U.S.

g) The licensing procedure for pheromones

We have contacted the United States Environmental Protection Agency and Health Canada and have now been assigned to specific registration officers (Brian Steinwand and Sean Muir, respectively). Both have been helpful in multiple conversations. The details of registration will be discussed fully in the final April report, but are summarized here. We have been advised that if a synthetic pheromone is used to control lamprey, it requires registration as a pesticide and a permit for experimental use. However, special, less stringent regulations are in place to cover what are termed "biopesticides." These receive priority review and require less

data to complete the application. Notably, further reductions in data requirements are routinely granted for biopesticides when scientifically justified. Since no aquatic or vertebrate pheromone has ever been registered, we are moving into uncharted bureaucratic territory. Neither advisor could say exactly how our case would be handled, but they offered some predictions based on experience. Applying the pheromone at levels found in nature and using extremely small quantities (grams) for field trials are aspects of the lamprey pheromone that would greatly reduce the data requirements. An aquatic application, especially in flowing waters, is an aspect that could increase the data requirements. Any application will most likely require at least some toxicity tests. It will be important to consider the quantity of pheromone required for such tests when determining the overall cost of registration, as unlike traditional pesticides (TFM), this may be a significant component of the cost. Another point which will need to be considered is that all component chemicals of the pheromone need to be registered. Registration will not be a trivial matter, but it appears that it will be much easier and faster than for traditional pesticides.

We have found that if native larvae are to be used as a source of pheromone in initial field tests or eventual management applications, the cooperation and permission of state and provincial agencies will be required to move native lamprey species. We have contacted the fishery agencies in Michigan and Wisconsin and have received their provisional support and cooperation in such matters. Conversations continue and will be disclosed in our April report.

h) Conclusions on our progress determining how to test pheromones in the field

We have made much progress in the last few months as we have begun to move our research from laboratory studies of the basic biology of pheromones towards the development of applied control techniques in the field. We believe that it was important that the direction of the

research be guided by lamprey managers and scientists early in this process. As such, we await the return of the management surveys before producing the final report on our findings.

We are in a position with most of our objectives to complete a report soon after the surveys have been analyzed, and we will submit it at the end of April. This report will contain a full description of our findings on the procedures for pheromone registration and the permit process required for the movement of native species in various jurisdictions. It will also summarize the priority applications and research questions determined from the numeric rankings of those surveys that have been returned by the beginning of April (a return deadline of March 22 was given on the survey). In addition, possible field trial designs and candidate field sites will be discussed, with further investigations into this important decision continuing in the following months.

Talks given:

L.A. Vrieze. 1998. Direct behavioral evidence that sea lamprey are attracted to spawning streams by bile acids released by larvae. Annual Meeting of the American Fisheries Society, Hartford, CT August 1998.

L.A. Vrieze. 1998. Lamprey control: the search for pheromones. Hamline University biology seminar series, St. Paul, MN

Sorensen, P.W., Vrieze, L.A. 1999. Identification of a Migratory Pheromone in the Sea Lamprey Creates New Possibilities for Lamprey Control. Ninth International Zebra Mussel and Aquatic Nuisance Species Conference, Duluth, MN.

Sorensen, P.W., Vrieze, L.A. 1999. Identification of a migratory pheromone in the sea Lamprey (Petromyzon marinus) creates new possibilities for lamprey control. 129th Annual National Meeting of the American Fisheries Society. Charlotte, North Carolina.

Manuscripts Published:

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. **Canadian Journal of Fisheries and Aquatic Sciences** (in press).

Manuscripts Submitted:

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

TABLE 1. Cross-reactivity of petromyzonol EIA for bile acids show high specificity

Petromyzonol EIA Cross-Reactivity

Compound	Cross-Reaction
Petromyzonol	100%
Petromyzonol Sulfate	10%
Allocholic Acid	<0.01%
Cholic Acid	<0.01%
Cyprinol	<0.01%
Cyprinol Sulfate	<0.01%
Taurocholic Acid	<0.01%
Chenodeoxycholic Acid	<0.01%
B-Estradiol	<0.01%
Estradiol-17-Sulfate	<0.01%

Table 2. 1996-98 River water samples collected and extracted.

Date filtered	River	Site	Date collected	Liters filtered
3/27/96	Cheboygan	Trap site	3/26/96	9.0
3/28/96	St. Mary's	Site 1	3/26/96	9.2
3/29/96	Grand Lake	Outlet	3/26/96	9.2
5/13/96	Grand Lake	Outlet	5/19/96	10.0
5/16/96	Cheboygan	Trap site	5/10/96	9.1
5/23/96	Cheboygan	Trap site	5/22/96	9.4
5/23/96	Grand Lake	Outlet	5/22/96	10.2
6/4/96	St. Mary's	Site 1	5/29/96	10.0
6/21/96	Cheboygan	Trap site	6/17/96	10.2
6/24/96	Grand Lake	Outlet	6/17/96	10.4
7/1/96	St. Mary's	Site 1	6/25/96	9.1
7/1/96	St. Mary's	U.S. landing	6/25/96	8.5
7/3/96	Nagel River	Hwy.23	6/27/96	11.0
7/8/96	Brule River	Above barrier	7/5/96	10.0
7/22/96	Nagel River	Hwy. 23	7/15/96	10.4
7/19/96	Cheboygan	Trap site	7/15/96	10.2
7/31/96	St. Mary's	U.S. landing	7/29/96	8.9
8/1/96	St. Mary's	Site 1	7/29/96	8.2
8/22/96	St. Mary's	U.S. landing	8/19/96	8.2
8/23/96	St. Mary's	Site 1	8/19/96	8.3
9/11/96	Nagel River	Hwy. 23	9/5/96	7.5
9/11/96	Cheboygan	Trap Site	9/5/96	10.2
9/23/96	Nagel River	Hwy, 23	9/21/96	9.1
9/23/96	St. Mary's	U.S. landing	9/20/96	9.3
9/23/96	St. Mary's	Site 1	9/20/96	9.8
9/24/96	St. Mary's	Gros Cap	9/20/96	10.1
9/24/96	Cheboygan	Trap site	9/21/96	10.1
10/18/96	St. Mary's	U.S. landing	10/11/96	8.7
10/23/96	St. Mary's	Site 1	10/11/96	9.8
12/6/96	St. Mary's	Site 1	11/29/96	10.0
1/10/97	St. Mary's	Site 1	1/7/97	10.0
4/17/97	St. Mary's	Site1	4/14/97	10.2
5/20/97	St.Mary's	Site 1	5/13/97	10.0
5/28/97	Nagel	Hwy 23	5/21/97	10.0
5/28/97	Ocqueoc	Bridge Site	5/21/97	10.0
5/29/97	Cheboygan	Trap Site	5/21/97	10.0
6/20/97	St.Mary's	Site 1	6/17/97	10.0
7/18/97	Cheboygan	Trap Site	7/14/97	10.0
7/18/97	Lone Oak		7/14/97	10.0
8/2/97	St. Mary's	Site 1	7/30/97	10.0
9/8/97	St.Mary's	Site1	9/2/97	10.0
9/29/97	St.Mary's	Site 1	9/24/97	10.0
10/31/97	St. Mary's	Site 1	10/28/97	10.0
1/14/98	St. Mary's	Site1	1/7/98	10.0

Table 3. Additional River Water Samples for Questions 3-6.

River Sample	Location	Collection Date	Amount Filtered (L)
Misery	Upstream	9/24/98	19.5
Misery	Downstream	9/24/98	20
Rock	Upstream	9/23/98	28.8
Rock	Downstream	9/23/98	29.1
Big Garlic	Upstream	9/23/98	28.6
Big Garlic	Downstream	9/23/98	29.8
Middle	Upstream	9/24/98	20
Middle	Downstream	9/24/98	29.8
Misery	Upstream	10/7/99	6
Misery	Downstream	10/7/99	6
Rock	Upstream	10/7/99	6
Rock	Downstream	10/7/99	6
Big Garlic	Upstream	10/7/99	6
Big Garlic	Downstream	10/7/99	6
Middle	Upstream	10/8/99	6
Middle	Downstream	10/8/99	6
Black Mallard	Highway 23 Bridge	6/23/99	2
Black Mallard	Highway 23 Bridge	6/23/99	2
Black Mallard	Highway 23 Bridge	6/23/99	2
Black Mallard	Highway 23 Bridge	6/23/99	2
Black Mallard	Highway 23 Bridge	6/24/99	2
Black Mallard	Highway 23 Bridge	6/24/99	2
Black Mallard	Highway 23 Bridge	6/24/99	2
Black Mallard	Highway 23 Bridge	6/24/99	2
Black Mallard	Highway 23 Bridge	6/25/99	2
Black Mallard	Highway 23 Bridge	6/25/99	2
Black Mallard	Highway 23 Bridge	6/25/99	2
Black Mallard	Highway 23 Bridge	6/25/99	2
Black Mallard	Highway 23 Bridge	6/26/99	2
Black Mallard	Highway 23 Bridge	6/27/99	2
Black Mallard	Highway 23 Bridge	6/28/99	2
Black Mallard	Highway 23 Bridge	6/29/99	2

Table 4. The number and biomass of larvae in odor production tanks

	1998		1999	
Species	Number	Total biomass (g)	Number	Total biomass (g)
Petromzyon	966	734	811	393
Lampetra	969	1337	402	390
Ichthyomyzon	829	1335	418	401

Table 5: Concentration of larval odor and river water in dose-response experiments

	Concentration	Odor pulse (sec on/sec off)	Larval water pumped/trial (L)	River water pumped/trial (L)
1998	High pulse	18 on/ 12 off	96	47
	Medium pulse	15 on/ 70 off	28	14
	Low pulse	15 on/ 200 off	8	5
	Residual	--	0	47
1999	High concentration	15 on/ 15 off	3.2	36
	Medium concentration	15 on/ 15 off	0.32	36
	Low concentration	15 on/ 15 off	0.032	36

Figure 1. ELISA Standard Curve - Petromyzonol

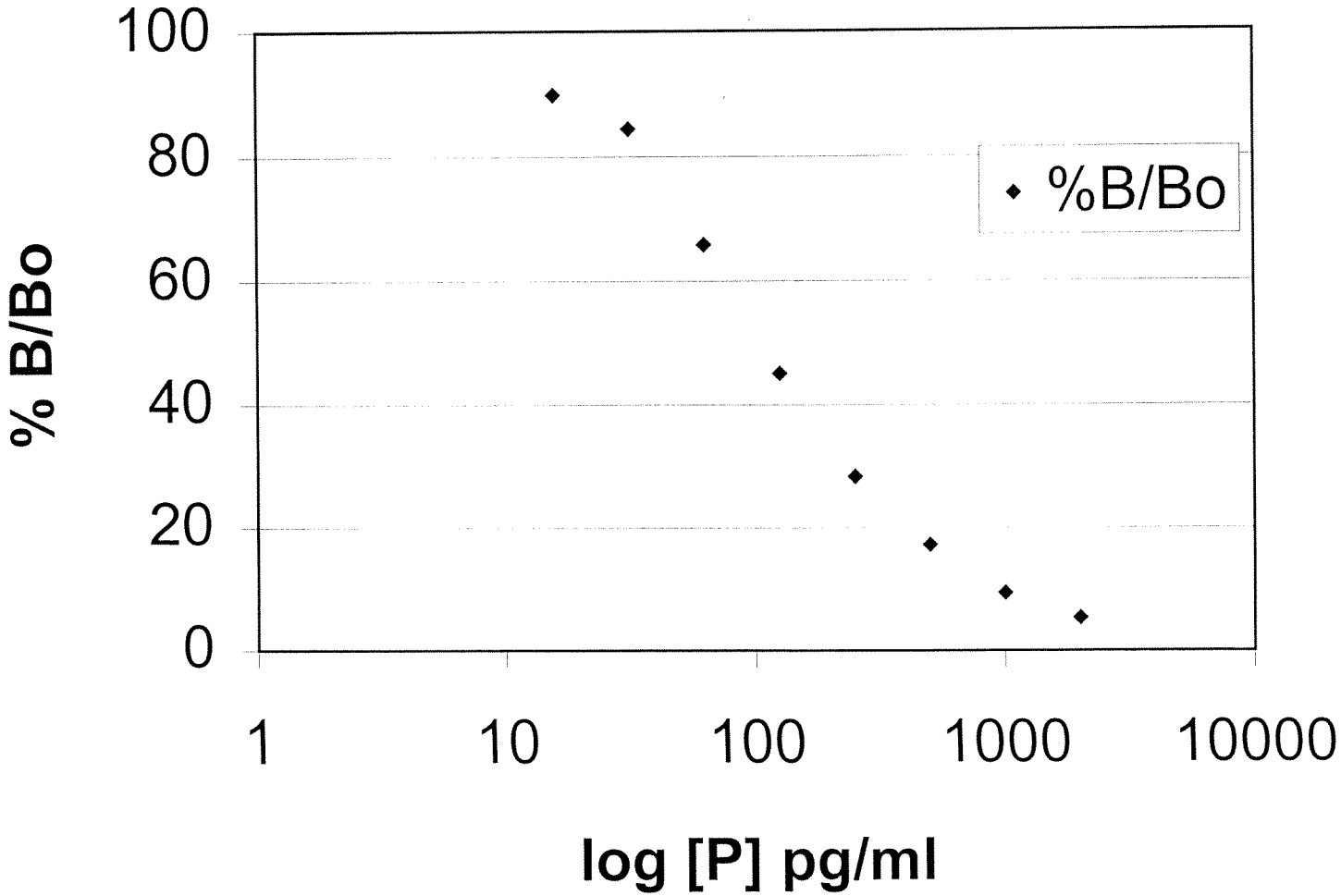
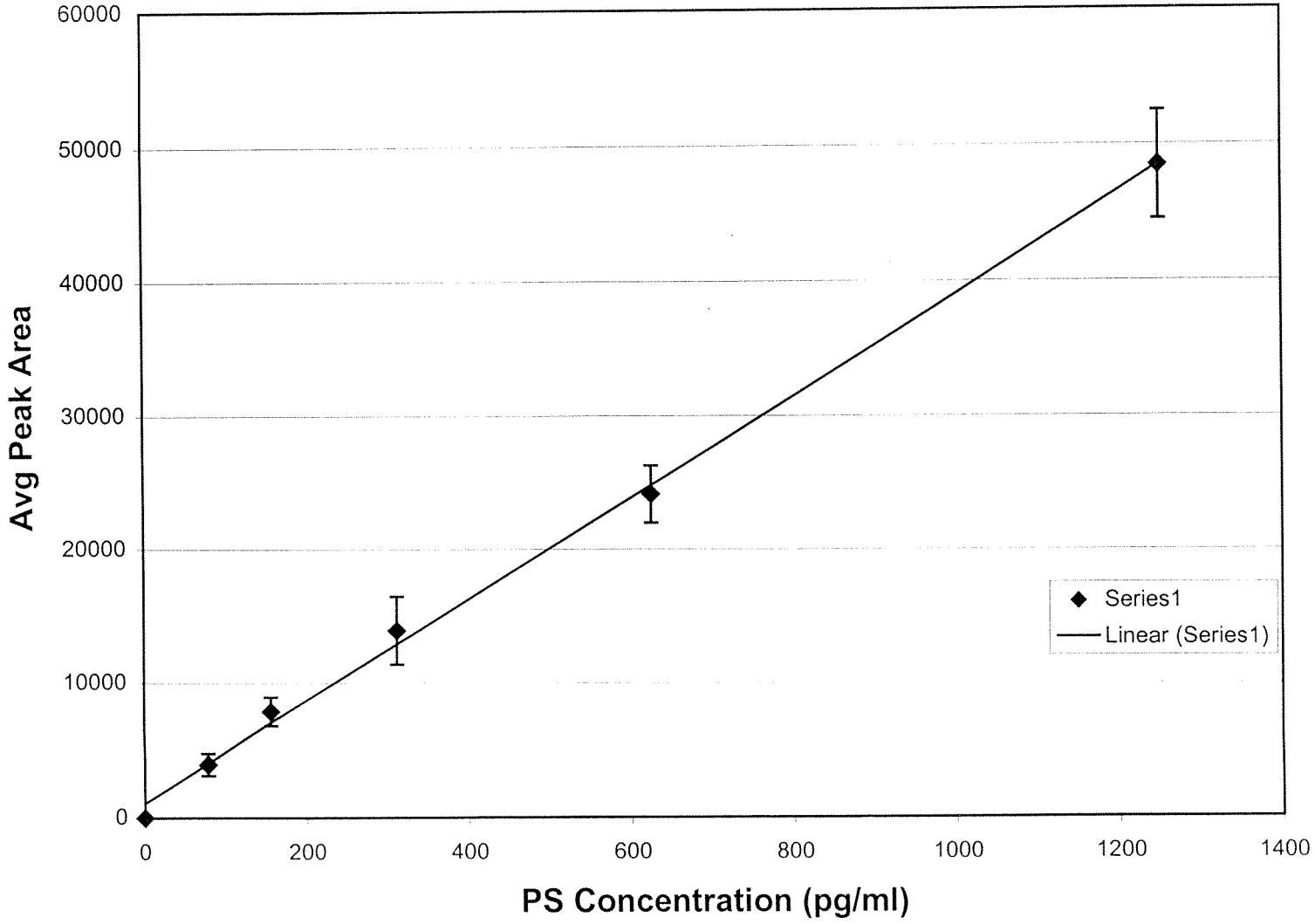
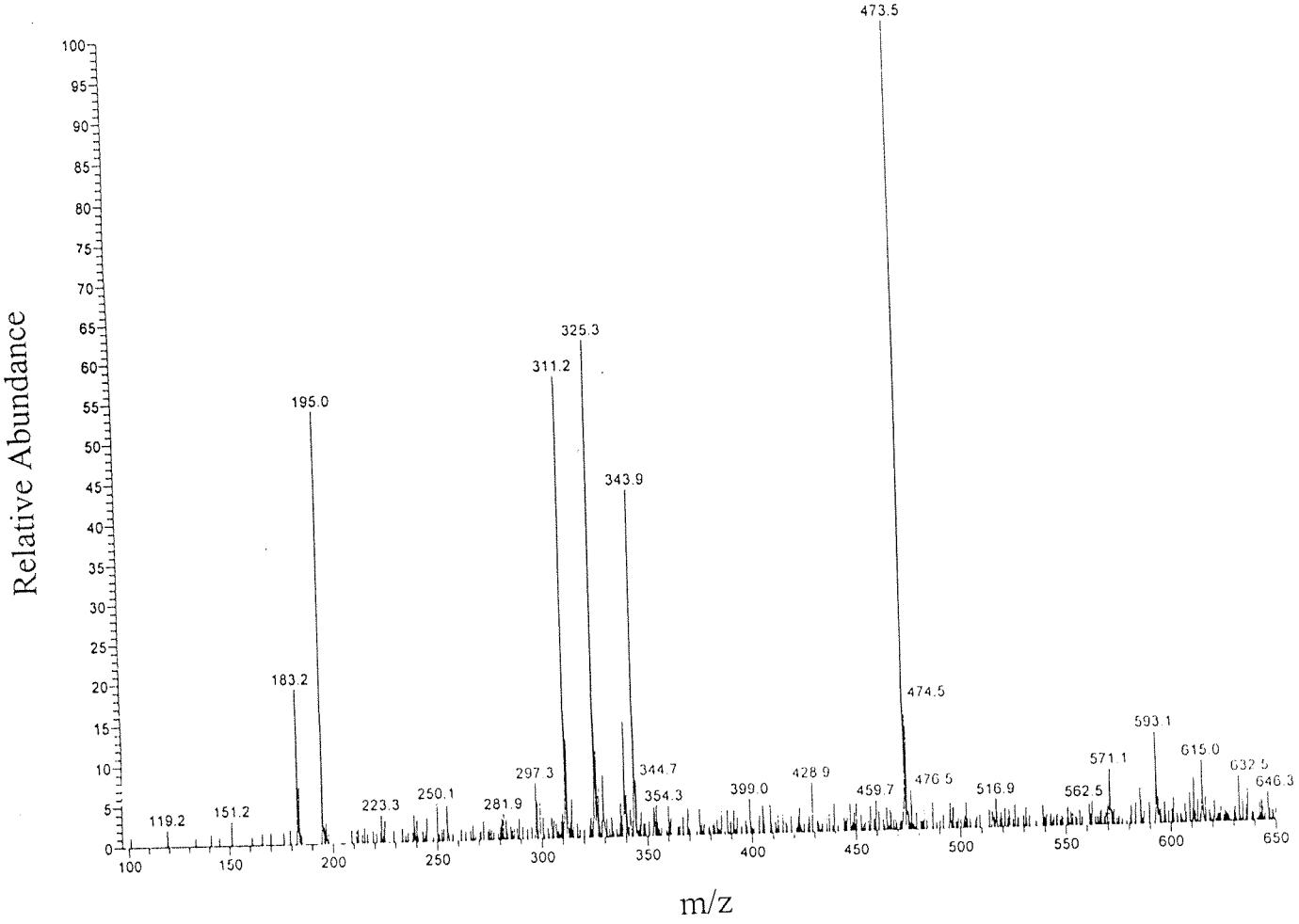


Figure 2. Standard Curve for Petromyzonol Sulfate with Mass Spectrometry



Mass Spectrum for Petromyzonol Sulfate Using Electrospray Ionization



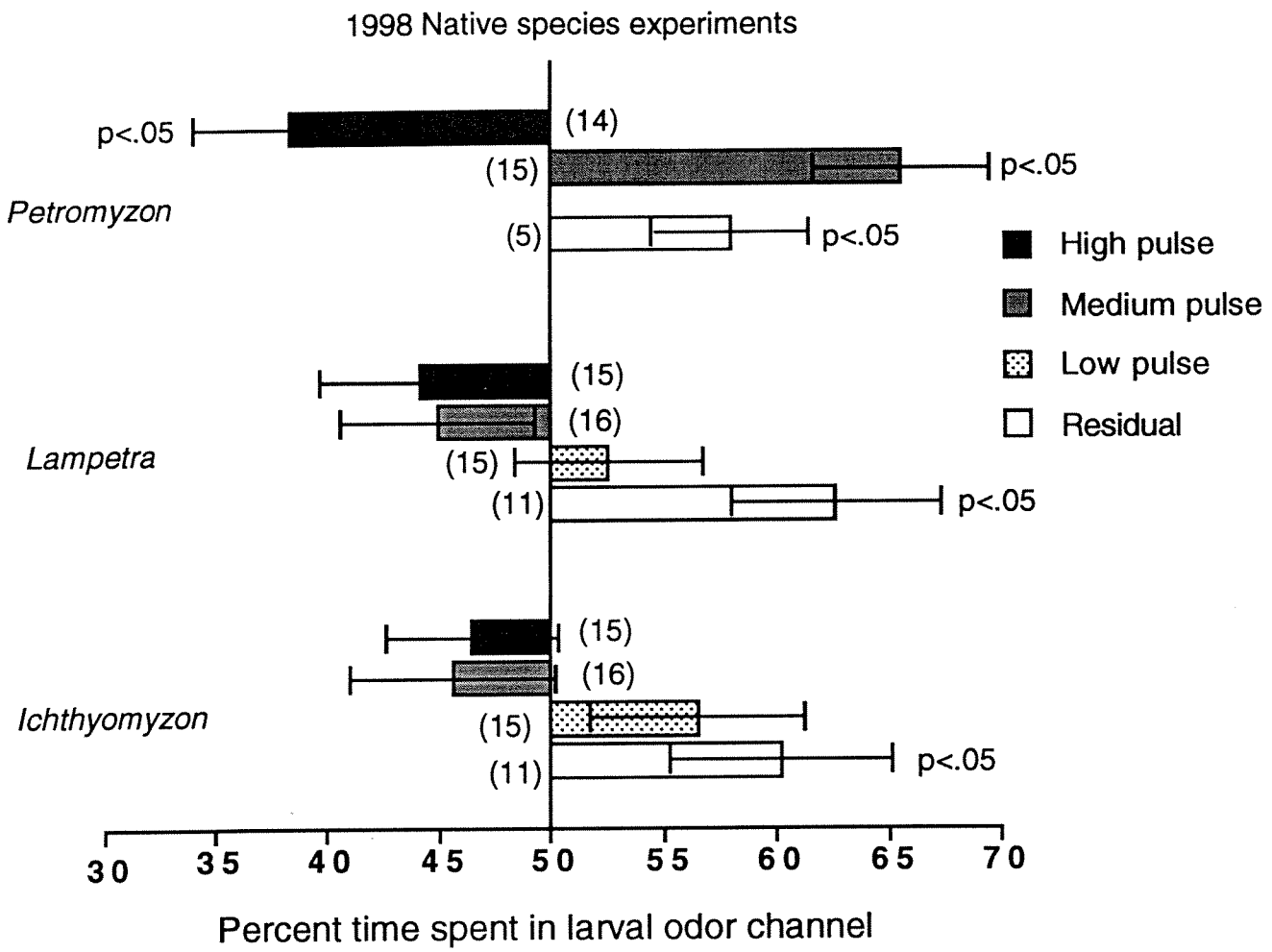


Figure 4. Attraction of adult sea lamprey to larval washings of three species at four different pulse rates. Bars are mean percent time spent in side of maze with larval washings. Error bars are +/- 1 S.E. Sample size in parentheses.

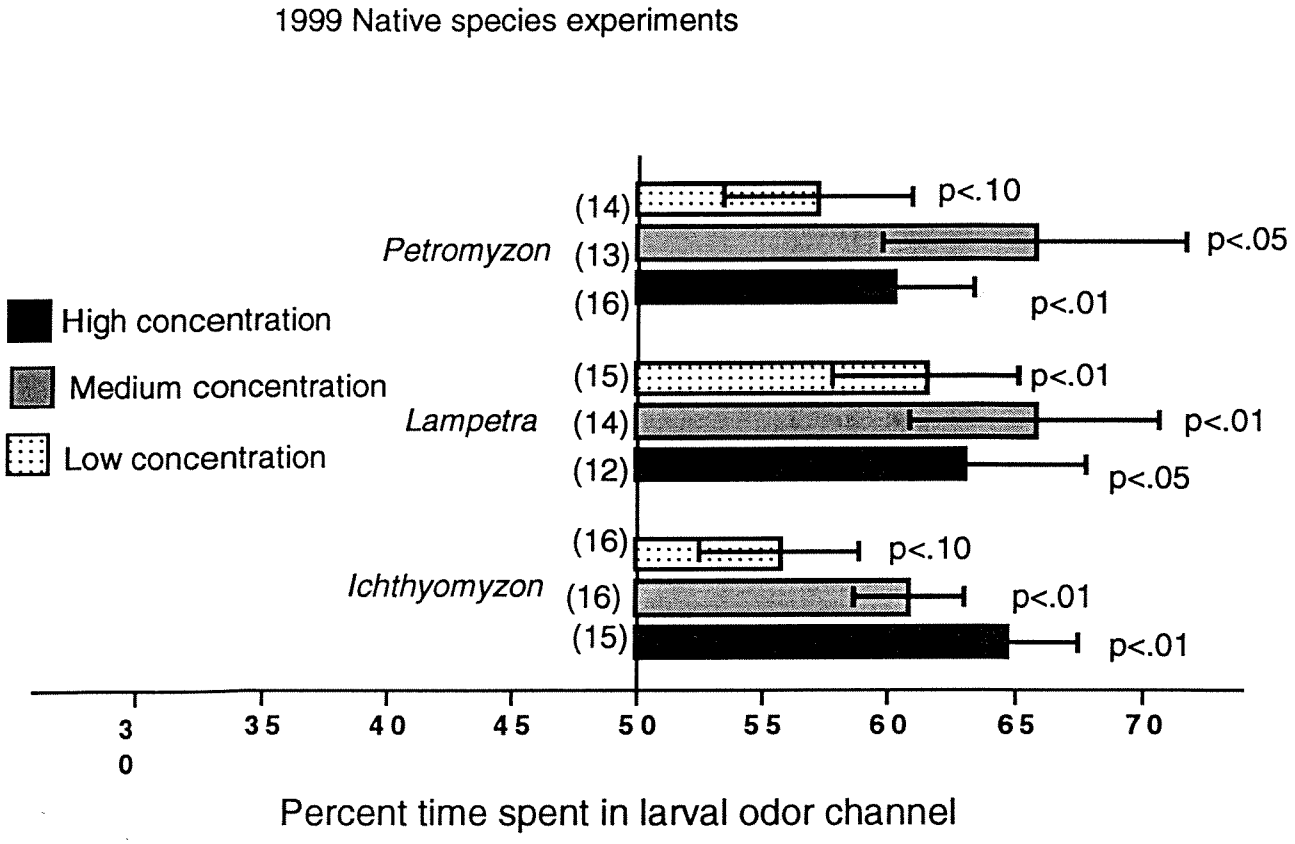


Figure 5. Attraction of adult sea lamprey to larval washings of three species at three different concentrations. Bars are mean percent time spent in side of maze with larval washings. Error bars are +/- 1 S.E. Sample size in parentheses.

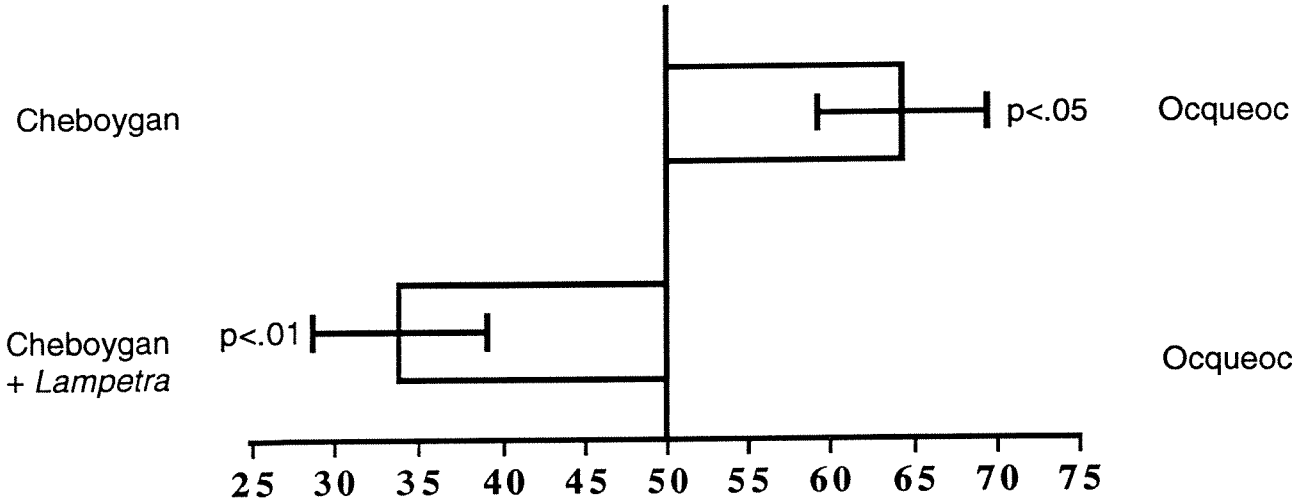


Figure 6. Preference of adult sea lamprey when Ocqueoc and Cheboygan water are directly compared, before and after *Lampetra* holding water is added to the Cheboygan. Bars are mean time spent in Ocqueoc side of maze expressed as a percentage of the total time spent in Ocqueoc and Cheboygan. Error bars are +/- 1 S.E. Sample size in parentheses.

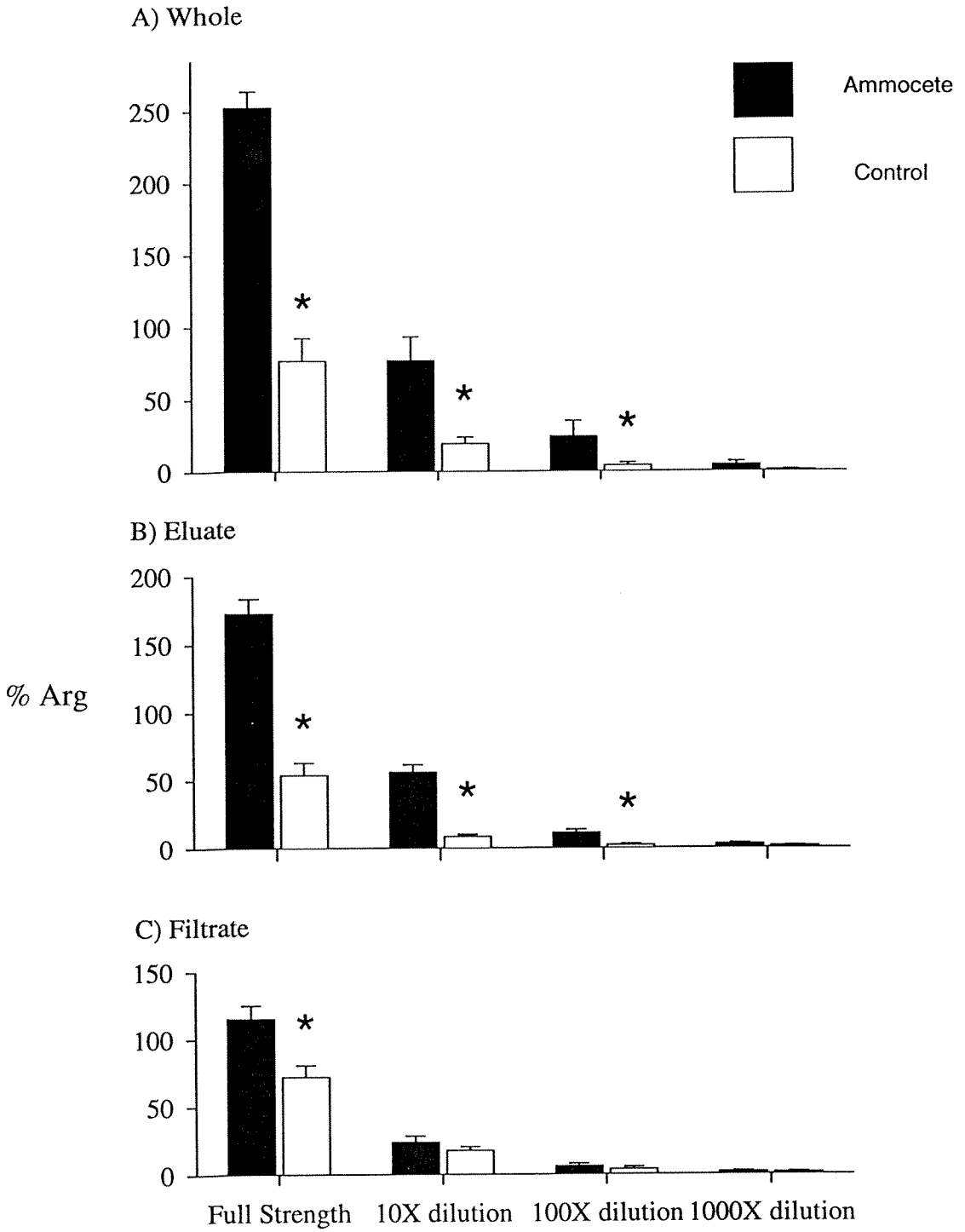


Figure 7 EOG concentration response studies (% Arginine -5 M) of migratory lamprey to A) whole ammocete water and control water (*N*=5), B) C18 solid phase extraction elutions of ammocete water and control water (*N*=4), and C) ammocete and control water samples that had passed through a C18 solid phase extraction cartridge (filtrate). Data are presented as mean + SE (n=5). * = control response was significantly different than response to ammocete water.

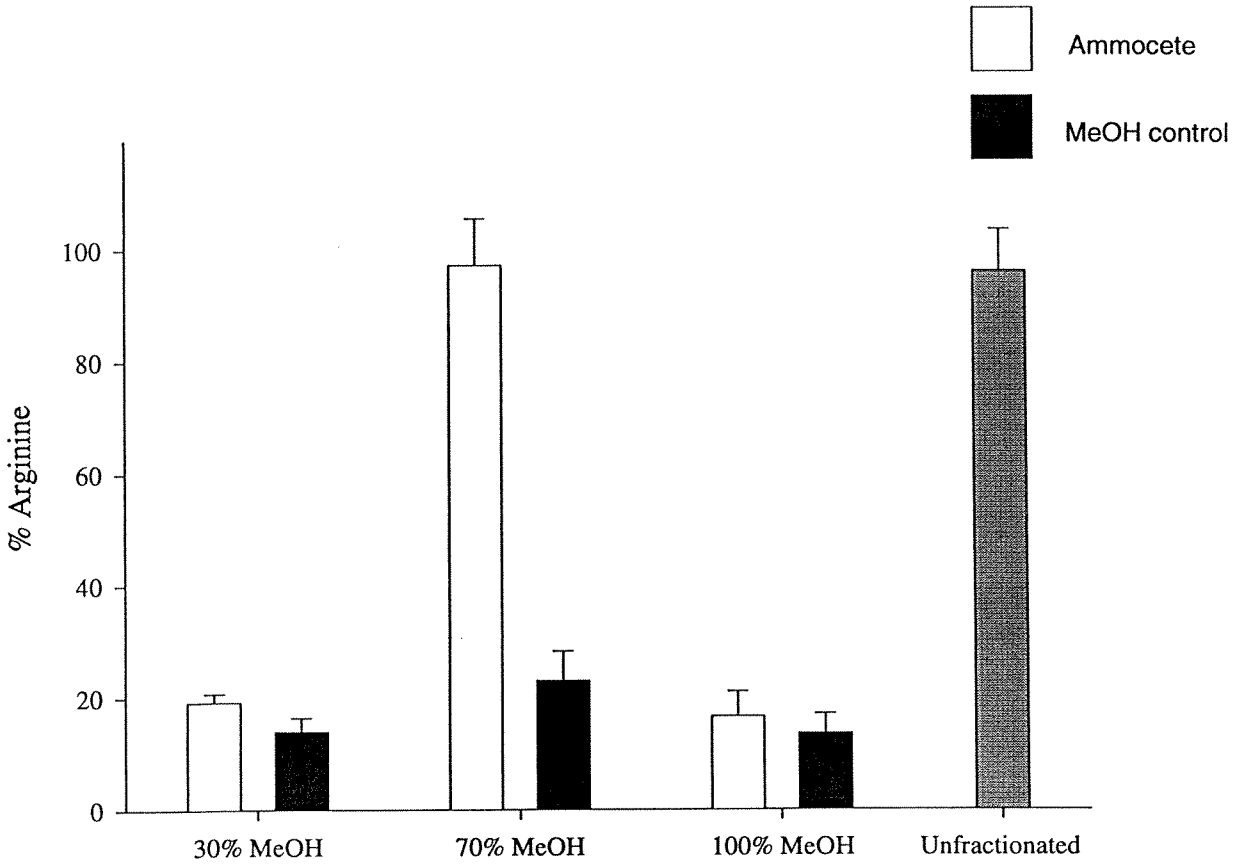


Figure 8 EOG response to fractionated ammocete elutions and control MeOH fractions. *N*=4

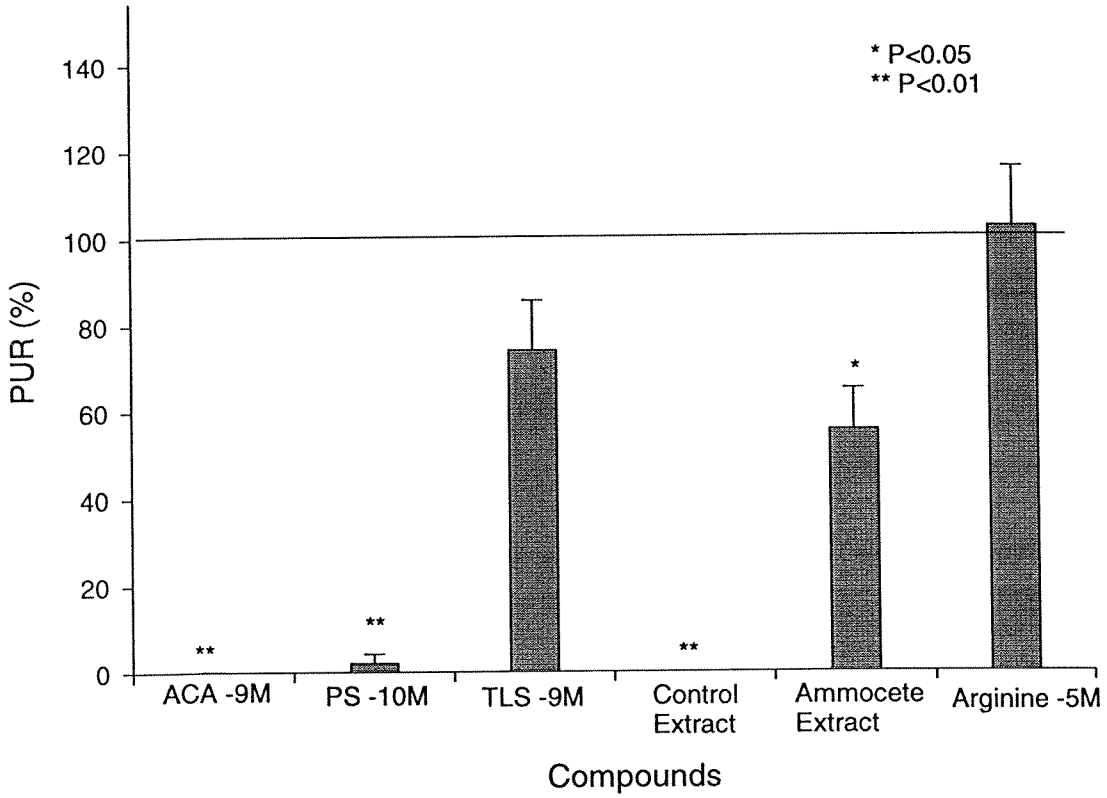

* P<0.05
** P<0.01
PUR (%)
140
120
100
80
60
40
20
0
**
**
**
*
ACA -9M
PS -10M
TLS -9M
Control Extract
Ammocete Extract
Arginine -5M
Compounds

Fig. 9

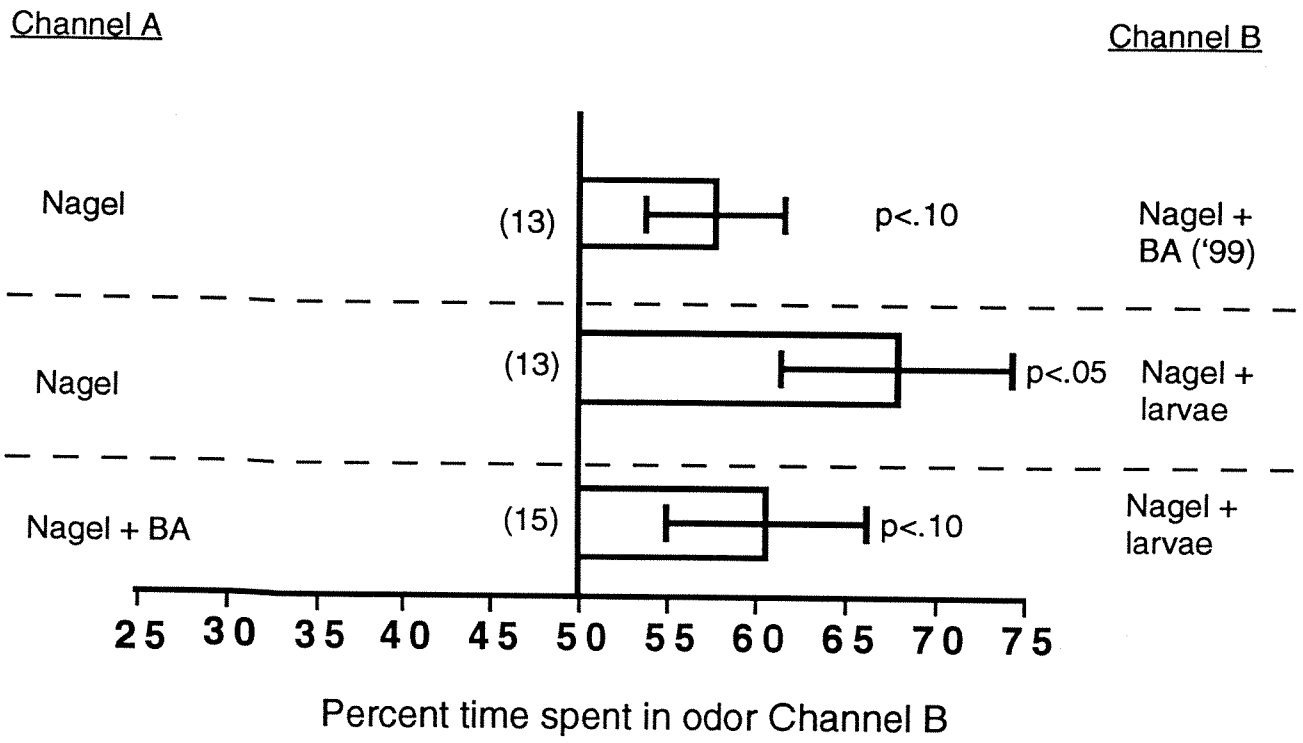


Figure 10. Attraction of adult sea lamprey to larval washings and bile acids. Bars are mean time spent in Channel B of maze expressed as a percentage of the total time spent in Channel A and Channel B. Error bars are +/- 1 S.E. Sample size in parentheses.