GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

THE INFLUENCE OF PHEROMONES ON THE DISTRIBUTIONAL BIOLOGY OF ADULT SEA LAMPREY

by:

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P.W. Sorensen,
February 18, 1997

Completion Report for the Great Lakes Fishery Commission

Project Title: The influence of pheromones on the distributional biology of adult sea lamprey

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PROBLEM STATEMENT AND OBJECTIVES AS STATED IN CONTRACT:
'Our previous research has determined that adult sea lamprey possess a highly sensitive and specialized sense of smell which is extremely sensitive to four bile acids, two of which are unique to larval lamprey (allocholic acid and petromyzonol sulfate). We have hypothesized that these bile acids function as a migratory pheromone because we know larval lamprey release these bile acids and that the odor of larvae is attractive to migratory adults. Further, our earlier studies have shown that these lamprey bile acids (petromyzonol sulfate and allocholic acid) will elicit increased swimming activity in the lamprey held in the laboratory. However, the significance of this activity to free-ranging animals in the Great Lakes has been extremely difficult to evaluate making it equally difficult to assess the potential utility of bile acid cues in lamprey control. Because the cost of conducting a field trapping study with synthetic odorants was quite high, we were funded to conduct an alternative set of large-scale laboratory studies to address 6 sub-objectives to address the behavioral potency of lamprey bile acids, the first two of which were slated to be completed this year. These objectives are:

Year One:

1) To determine the importance of olfactory cues (i.e. pheromones) to adult lamprey attempting to locate spawning streams.

2) To determine if recently caught migratory adult lamprey can be attracted to bile acid odor plumes in large raceways in manners consistent with their functioning as a migratory pheromone.

Year Two:

3) To determine if native species of lamprey also release petromyzonol sulfate and allocholic acid.

4) To determine if water-borne bile acids from another lamprey species attract migratory sea lamprey collected during their most sensitive stage.

5) To develop an enzyme-linked immunoassay (ELISA) for measuring petromyzonol sulfate.

6) To use the aforementioned ELISA and high performance liquid chromatography (HPLC) to determine if the quantity of bile acids found in a small selection of Great Lakes streams demonstrates a seasonal trend and correlates with the numbers of larval lamprey they contain and the number of adult lamprey which they attract.'

Objective 1: To determine the importance of olfactory cues (i.e. pheromones) to adult lamprey attempting to locate spawning streams.

INTRODUCTION: This objective was addressed in both years of the study using a mark-recapture study of nose-plugged lamprey released into Lake Huron. The results conclusively demonstrate that adult free-ranging sea lamprey depend on their sense of smell to locate, and then migrate up spawning streams. It is now clear that olfactory cues are absolutely fundamental to spawning stream location and thus could be used to control the behavior of sea lamprey in the Great Lakes. These conclusions were also confirmed by laboratory tests (objective 2). Here, we briefly describe some of the highlights of these experiments.

1a) 1996: Initial tests in the Cheboygan and St. Mary's Rivers.

In 1996, two experiments were conducted: 1) a mark-recapture study of migrating olfactory-ablated adult sea lamprey caught and released in the Cheboygan River (MI); 2) a mark-recapture study of migrating olfactory-ablated adult sea lamprey caught and released in the St. Mary's River. For the first part of this study, migrating adult sea lamprey were captured by Fish and Wildlife Service personnel at the Cheboygan River trap between May 20-25 1996, marked with a distinctive fin clip and color-coded plastic tag. Their olfactory organ was then either filled with dental impression material ('3M Express'; an innocuous polymer which sets in several minutes) or gelatin (which animals were able to force out, thus serving as a control). These animals were then returned to one of two locations, one 0.5 km from the river mouth (3.1 km downstream from the trap), the other about 3.5 km offshore (i.e. approx. 6.0 km downstream from the trap). In all, a total of 849 sea lamprey were treated, tagged and released in the Cheboygan River study, with approximately equal numbers being released at each site and the ratio of control to experimental animals being 1:1 with 75% of all animals being female (males were saved for sterilization). Fish and Wildlife Service personnel manning lamprey traps throughout the Great Lakes Basin were informed of the presence of these marked animals and were asked to set them aside for us to examine when they were recaptured. For the second part of this study, 896 migratory adult sea lamprey were captured in the St. Mary's River traps by Department of Fisheries and Oceans Canada (D.F.O.) personnel between July 2-8, marked, and their noses either 'plugged' or flushed with gelatin. These were then released at St. Joseph's Island, 60km downstream of the trap site. Similarly, D.F.O. personnel were requested to put these marked animals aside for us as they were recaptured.

Results from 1996 demonstrated that lamprey are very good at locating rivers when displaced outside their mouths (recapture rates of about 50%), but require a functional olfactory organ to do so -- very few olfactory-ablated animals were recaptured. There was no difference in ability of males and females to re-locate rivers and there was tendency for recapture rates to be slightly lower at the end of the season (data not shown), supporting our earlier work that olfactory function deteriorates in late-run animals. The difference in recapture rates of olfactory blocked and control animals was highly significant in all three cases ($P<0.001$; Fig.1). There was no indication that the marking procedure itself had any influence on these results because there was no illness or mortality of tagged, nose-plugged animals held in the laboratory. Further, olfactory-ablated animals were not seen to exhibit 'unusual' behaviors in laboratory testing paradigms, and daily fluctuations in the capture rate of control animals matched that of 'wild', untreated animals.

1b) 1997: Extending the 1996 Findings to the Ocqueoc River and Demonstrating that Stream Odor is Innately Recognized.

In 1997, we conducted another set of experiment to both confirm the importance of olfaction in lamprey migration and to extend these findings to determine whether: 1) lamprey exhibit an affinity for the stream they are captured (i.e. is stream odor influenced by experience?) and 2) whether olfactory cues are also important to lamprey after they enter rivers. Only the first experiment described here because of space limitations. The second experiment demonstrated that lamprey continue to require a functional olfactory system to orient upstream once within a river. For the first experiment, adult migratory sea lamprey were caught at the Cheboygan and Ocqueoc River traps marked, and subjected to one of three olfactory treatments (no treatment (sham); a gelatin flush of their olfactory system (control); or olfactory blocking with 3M dental adhesive material(nose-block; as in 1996)). Four treatment groups of 75 animals each were then released 0.5 km outside the mouths of the Cheboygan and Ocqueoc Rivers. These treatment groups were: 1) animals trapped in the Cheboygan, no nose treatment, 2) animals trapped in the Ocqueoc, no nose treatment, 3) animals trapped in the river to which they were about to be released, gelatin-flushed nose, and 4) animals trapped in the river to which they about to released, with dental impression material in their nose.

Results from 1997 confirmed and extended those of 1996. Recapture rates of sham and control (gelatin-treated) lamprey were equivalent and high (35-45%): lamprey are very good at finding rivers. However, only about 10% of all nose-plugged animals are recaptured ($P<0.001$) (Fig. 2). Further, the rate of recapture rate of lamprey at both rivers was the same whether the lamprey were originally caught at that location or not (about 45% after 2 weeks). Only a few (4) animals strayed to other streams: lamprey with plugged noses seem unable to locate any stream!

CONCLUSION: Lamprey use (and require) their sense of smell to locate spawning streams. Recognition of stream odor appears not to be influenced by experience and is thus likely innate -- consistent with the idea of a pheromone being involved.

Objective 2: To determine if recently caught migratory adult lamprey can be attracted to bile acid odor plumes in large raceways in a manner consistent with their functioning as a migratory pheromone.

INTRODUCTION

The purpose of these studies (which were conducted in both 1996 and 1997) was to determine if larval bile acids and larval odor attract migratory sea lamprey in a manner consistent with their functioning as a migratory pheromone. Recently caught migratory sea lamprey were tested at night in large raceways through which natural waters (lake waters and river waters) were pumped while various test odorants were added to them. These experiments represent an extension of our earlier work which demonstrated that sea lamprey are attracted to larvae and bile acids in laboratory tanks supplied with well water. Over 30 experiments using over 2,000 lamprey were conducted in the summers of 1996 and 1997 at the Lake Huron Biological Station -- here we present our most compelling results that conclusively demonstrate that petromyzonol sulfate is a significant component of the larval pheromone which plays a major role in stream choice by migratory lamprey.

GENERAL METHODS

Animals: Migrating lamprey were captured in the Cheboygan, Ocqueoc, and St. Mary's Rivers by the U.S.F.W.S. or D.F.O. Lamprey from the Cheboygan and Ocqueoc were transported daily to the Lake Huron Biological Station where they were acclimated to lake water and temperatures for three days before testing, while animals from the St. Mary's were transported to the biological station approximately once a week and held at least 24 hours before use. Each lamprey was used in only one trial.

Apparatus: Two concrete raceways at the Lake Huron Biological Station were modified to create identical 9 x 1.8 meter two-choice mazes. Ambient Lake Huron water constantly flowed through the raceways at 1000 L/ min. Each maze had a 4.5 x 1.8 meter acclimation and holding area containing four mesh cages (1 m diameter) (Fig. 3). The holding area was located immediately above a 4.5 x 1.8 meter experimental choice area that was divided into three channels--a central "lake" channel containing only slow-flowing Lake Huron water flanked by two faster-flowing "river" channels that river odors were added to. Experimental animals could swim from the holding area through the central lake channel and then enter one of the two river channels. For each experiment, recently caught lamprey (caught three days before at traps and acclimated to lake temperatures) were placed into 1 m screened holding cages placed in the holding area of the maze during the day. Then, after sunset, each holding cage was lifted (one a time), and the behavior of the released animals tracked for 20 min time period (see below). Control tests using no odor indicated no inherent bias in the apparatus.

River odors: Water from four streams in the vicinity of the Lake Huron Biological Station were used as odor sources in these experiments. The Cheboygan and Ocqueoc Rivers are large rivers which contain larvae and attract a large number of migrants. Nagel and Lone Pine Creek are small, do not contain ammocoetes of any species, and are not known to attract any adult sea lamprey. River water needed for a night's experiments were pumped from rivers that day and transported to the biological station where the water was then pumped into large holding tanks. The temperatures of different river waters were equalized by heating and/or chilling. For experiments using Lake Huron water as an odor source, a holding tank was filled with lake water and heated to match river water temperatures. During experimental trials, these waters were pumped into the top of the river odor channels in a pulsatile fashion (12 sec on, 18 sec off), and were diluted approximately 20 times into the lake water flowing through the channels. Other odors were often added to the raceway with the river waters as noted below.

Testing: Approximately 10 hours before trials began, four lamprey were placed in each of the eight mesh holding cages. The room was completely darkened, illuminated only by infrared light at wavelengths undetectable by lamprey. One half hour after sunset, trials commenced when one cage in each raceway was lifted (releasing four animals) and river odors were pumped into each of the experimental odor channels. The released lamprey were then observed continuously during a 20 min trial via an infrared sensitive camera connected to a video monitor and video recorders. Every 30 sec the positions of all released lamprey were scored as being either in river odor channel A, river odor channel B, or outside both odor channels. At the completion of each 20 min trial, animals were removed from the raceway and another holding cage was lifted, starting a new trial. Each experimental series typically consisted of 16 trials completed over four nights. The odors in each channel were reversed every night, creating a balanced design. In order to determine if lamprey spent significantly more time in one odor over the other, the mean percentage of observations of lamprey within odor A was compared to the mean observations within odor B using the Wilcoxon signed-ranks test (95% significance level).

FIVE SELECTED (REPRESENTATIVE) EXPERIMENTS

QUESTION 1: Are lamprey attracted to river water using their sense of smell?

If lamprey use a larval pheromone to locate spawning streams, then removing their ability to smell the water should remove their ability to be attracted to it in the laboratory (as it did in the lake itself). Preference for water from a larval river over Lake Huron water was compared in animals that had either an intact (Exp 1a: Cheboygan vs Huron, intact nose, n=7) or blocked (Exp. 1b. Cheboygan vs Huron, anosmic, n=10) olfactory sense. To block olfaction, lampreys' nasal cavities were filled with dental impression material approximately twenty-four hours before testing. Control animals' nasal cavities were filled with gelatin, which they could squeeze out themselves, leaving olfaction intact. Lamprey with intact noses exhibited strong attraction ($p < 0.05$) towards the river water, spending 64± 6% (mean ± SE) of their time within the river water channel and only 24 ± 4% of their time within the lake water channel. However, anosmic lamprey did not discern between river and lake water ($p > 0.20$), spending 47 ± 4% of time in river water and 41 ± 3% of time in lake water (Fig. 4). Anosmic animals did not exhibit altered rates of movement, just a lack in orientation: we do not believe they were stressed in any way.

QUESTION 2: Are lamprey attracted to river water by organic compounds?

If larval bile acids are a significant component of river odor, then we reasoned that removing the organic portion of water (which includes bile acids) should reduce the attractiveness of river water. Accordingly, an experiment was conducted in which lamprey were first offered the choice of water from a river with larvae (the Ocqueoc) and Lake Huron water, (Exp 2a: Ocqueoc vs Huron, n=16), and then and in an independent experiment, Ocqueoc River water which had been filtered through a medical grade filter and lake Huron water (Exp 2b: Filtered Ocqueoc vs Huron, n=16). Adults showed a strong preference for un-filtered Ocqueoc River water, spending significantly more time ($p0<.05$) in the river odor channel (53± 3%, mean ± SE) than the lake odor channel (13 ± 2%). However, when organic materials were removed from Ocqueoc River water by filtration, lamprey showed no preference ($p>0.30$) for the river water (20 ± 2 % time spent) over the lake water (23 ± 2% time spent) (Fig. 5). These data demonstrate river waters are attractive because of the organic odorants which they contain. Notably, temperature was ruled out as a variable in river choice in an earlier experiment in which we found cooling river water to have no influence on its attractive properties.

QUESTION 3: Are some rivers more attractive to lamprey than others, and is attractiveness related to larval density?

If larval odor is important in stream selection, lamprey should not only prefer river water to lake water (Exp. 1a and 2a), but should they also more attracted to rivers that contain larvae than those that do not. Accordingly, we tested two rivers containing larvae against two rivers lacking larvae (Exp 3a: Ocqueoc vs Nagel, n=14; Exp 3b: Cheboygan vs Lone Pine, n=15), and we also paired the two rivers with larvae (Exp 3c: Ocqueoc vs Cheboygan, n=18) to see if the Ocqueoc, with higher larval densities, would be more attractive. Migrating lamprey exhibited a strong preference for the streams containing larvae over the streams lacking larvae, spending significantly more time in Ocqueoc than Nagel water (38 ± 3% and 14 ± 3%, respectively; $p<0.05$). Similarly lamprey spent more time in Cheboygan than Lone Pine water (29 ± 4% and 13± 2%, respectively; $p<0.05$). (Fig. 6).

Question 4: Does the addition of larvae make a river more attractive?

To directly test if larval odor is the significant attractive component of river water, we performed two experiments. First, we added larval odor to stream water lacking larvae and compared its attractiveness to the unaltered stream water (Exp 4a: Nagel + larvae vs Nagel, n=17). We also added larval odor to Lone Pine water and compared it to Cheboygan water (Exp 4a: Lone Pine + larvae vs Cheboygan, n=9). This allowed a direct comparison to the results of Exp 3b in which the Lone Pine lacked larval odor. To produce larval odor, approximately 1000 sea lamprey ammocoetes were held in an aerated tank with 10 cm sand. To stimulate bile acid release, inflowing water to the tank was turned off and the larvae were fed yeast. After the larvae were held 24 hours with the inflowing water off, experiments were begun and the larval tank holding water was pumped into the raceway odor channel along with the appropriate river water. Holding water from an identical tank containing sand and yeast, but lacking larvae, was pumped into the opposite odor channel as a control. Adding larval odor to Nagel Creek water greatly increased its attractiveness. Lamprey spent 31 ± 4% of the time in the Nagel channel with larval odor added, while only spending 17± 2% of the time in the non-larval Nagel channel ($p<.05$). When larval odor was added to Lone Pine water, the Lone Pine was made more attractive than the Cheboygan (24.75 ± 3.93% and 13.80 ± 3.18% time spent, respectively; $p<.05$), effectively reversing the preference shown towards the Cheboygan in Exp 3b. (Fig. 7)

Question 5: Does the addition of bile acids make a river more attractive?

Finally, to directly determine if petromyzonol sulfate (PS) and allocholic acid (ACA) are a significant part of the larval cue, we performed two experiments. We first added PS and ACA to Nagel Creek water to see if bile acids would make the water more attractive (Exp 5a: Nagel + bile acids vs Nagel, n=17; comparable to Exp 4a) and then repeated the experiment using Lone Pine Creek water (Exp 5b: Lone Pine + bile acids vs Lone Pine, n=17). Bile acids were added to stream water at a ration of &:1, PS: ACA (the ratio we generally find in larval release waters). The concentration of PS after dilution into the raceway channel was $2x10^{-10}$ M (a concentration that has been found in natural larval river waters). PS was obtained from larval gallbladders and then purified with HPLC; ACA was synthesized. The addition of bile acids to non-larval waters increased the stream waters' attractiveness. Lamprey spent 27 ± 3% of the time in the bile acid enhanced Nagel Creek channel, but spent only 18 ± 2% of their time in the non-enhanced Nagel channel ($p<0.07$) (Fig. 7). Similarly, lamprey preferred the bile acid enhanced Lone Pine water (23 ± 3% time spent) over the Lone Pine water lacking bile acids (16 ± 2% time spent, $p<0.05$) (fig. 7). Even though attraction to Nagel Creek water with bile acids was found to be only marginally significant, a comparable response to bile acid enhanced waters was shown in both experiments (Fig. 8).

CONCLUSIONS:

We now have strong behavioral evidence that migrating adult lamprey use the odor of larvae and the bile acids they release to locate spawning streams. The sea lamprey's complete dependence on olfaction for river location has been confirmed in the field and lab, suggesting that if the olfactory cue could be manipulated or disrupted that we would have a powerful tool for manipulating migratory behavior. Further, it is clear that the larval lamprey bile acids, petromyzonol sulfate and allocholic acid are a principle component of this olfactory cue. In addition to consistently preferring water from the stream with higher larval concentrations, lamprey found stream water into which larval odor had been added to be much more attractive. A non-lamprey stream that had larval odor added became more attractive than the previously preferred larval stream. Since lamprey spent more time in stream water into which purified petromyzonol sulfate and allocholic acid had been added, it is also evident that bile acids are a component of the larval cue. However, the behavioral response to bile acids was never as strong as the response to whole larval odor. This suggests that there may be unknown components to the larval pheromone (a possibility we will investigate in the coming year), or the ratio of the bile acid mixture may be important and was not tested at the critical ratio.

Objective 3: To determine if native species of lamprey ammocetes release petromyzonol sulfate and allocholic acid.

INTRODUCTION:

Much anecdotal trapping evidence suggests that native species of lamprey may also release a chemical cue which attracts sea lamprey. American brook lamprey (*Lampetra appendix*) in particular appear to be attractive (D. Cuddy, D.F.O., personal communication and described in our proposal). Accordingly, we hypothesized that native species should also release petromyzonol sulfate. To answer this question bile acid release studies were conducted on sea lamprey and two native species, American and Northern brook lamprey ammocetes (We have been unable to obtain silver lamprey ammocetes but we are still working on finding a source).

METHODS

Experiments followed the protocol developed for sea lamprey larvae (Polkinghorne, M.S. . Thesis, 1997, University of Minnesota). American brook, Northern brook (*Ichthyomyzon fossor*, and sea lamprey were obtained from the Luddington U.S.F.W.S. Station. Each species was held in 50 liter buckets of well water, 50 ammocetes per bucket. They were fed twice weekly with yeast only. To measure release, water flow was turned off, the ammocetes were fed, and a 10 liter water sample taken from each bucket a for 0-time analysis. Another sample was then taken 12 hours post feeding. All samples were extracted with C18 Sep-Paks and analyzed by HPLC according to established protocol. This experiment was conducted twice, once in each year, using different groups of animals.

In addition to the above two experiments above, we did another experiments to confirm the presence/ identity of PS released by each species. As in the first experiment, all three species of lamprey were held and fed in manner described above, their water extracted 12 h

after feeding, extracted by Sep-Pak, and each extract divided into four aliquots which were then treated as follows:

1. One aliquot of each water extract was prepared 'as is' (no 'special treatment') and analyzed by HPLC following established protocols for enzyme-detectectin (Li et al. 1995). This sample was then used as the standard with which to compare the following:

2. An internal bile acid standard was added to the second aliquot and treated with 3α–dehydrogenase (HSDS; which converts bile acids to their 3 ketone forms, making them undetectable), and analyzed by HPLC (thus confirming bile acid identity in sample #1).

3. An internal standard was added the third aliquot and the extract was desulfated by acid solvlysis following established protocol and analyzed by HPLC after addition of standards (this procedure specifically confirmed identity of petromyzonol sulfate as it should desulfate).

4. An internal standard was added to the last aliquot of the extract, after which it was both desulfated and treated with 3α HSDS , before being analyzed by HPLC (thus serving as a cross-check of both techniques as all bile acid peaks should have disappeared).

RESULTS:

All three species of lamprey released petromyzonol sulfate with the American brook releasing more petromyzonol sulfate than sea lamprey which in turn released more than the Northern brook (Fig. 9). The identity of the petromyzonol sulfate was confirmed by pre-treatment with the enzyme and desulfation. The sea lamprey ammocetes also released small and variable amounts of allocholic acid (on average less than 5 ug; identity confirmed; data not shown). Notably, neither of the native species released any detectable allocholic acid.

CONCLUSIONS:

American brook lamprey release large quantities of petromyzonol sulfate, which in certain stream systems may represent a source of the lamprey pheromone. However, before this can be concluded several questions must be addressed. These include: 1) Is the odor of American brooks is attractive to migratory adult sea lamprey; 2) Is the allocholic acid released by sea lamprey (only) a fundamental part of the pheromonal cue?; and 3) Is the natural degradation rate of bile acids during their passage downstream high? (if decay rate is significant as laboratory data indicate then only larval populations in lower stream sections serve to attract adults -- information which will be directly useful in management). The latter questions we will address in out new contract.

Objective 4: To determine if water-borne bile acids from another lamprey species attract migratory sea lamprey collected during their most sensitive stage.

This has not yet been addressed because we decided (with the permission of the Commission) to conduct spend the time available to conduct more extensive tests of whole river waters and bile acids (outlined above). This objective is now part of the newly funded contract for 1998-2000.

Objective 5: To develop an enzyme-linked immunoassay (ELISA) for measuring petromyzonol sulfate

Unfortunately, this project has not progressed as rapidly as we would have liked. After initial attempts to produce an antibody to petromyzonol sulfate failed (apparently owing to its breakdown by sulfatase enzymes in the rabbit's blood; see below), we have changed our strategy to produce an antibody to petromyzonol instead. We are very optimistic about this approach and are pursuing it with Cayman Chemical Company (MI) with funding from the Great Lakes Fishery Commission in our new contract for 1998-2000. The details are described in that proposal, but I will briefly outline the history of this work below:

a. Results of initial attempts to develop an ELISA to petromyzonol sulfate.

We arranged to have nearly 1,400 sea lamprey ammocetes shipped to Minnesota by the Luddington Fish & Wildlife Station in 1996. Gall bladders were removed from 1,000 of these animals and about half of these were then extracted and purified by HPLC, to collect 50 mg of 99% pure petromyzonol sulfate. The PS was then reduced to its 3-keto form and its purity checked by mass spectrometry and HPLC and found to be at least 95% pure. Subsequently, we sent it to Cayman Chemical Co (with whom we have a contract) where it was conjugated with carboxymethane and then injected into rabbits as an antigen. Cayman has been injecting rabbits with this material monthly since May 1997 (and continues today) but has been unable to measure an antibody for this compound. Several experiments and a literature search have recently suggested that problem is attributable to the sulfate group being hydrolyzed *in vivo* -- possibly producing an antibody to petromyzonol instead. This possibility is now being tested.

b. Results of ongoing efforts to produce an ELISA to petromyzonol

The aforementioned results have lead us to consider the possibility of producing an antibody to petromyzonol instead, a route we are now pursuing with the assistance of Cayman Chemical. This new approach has several very significant advantages (described below) but adds one step to how this assay might be used to analyze river water for pheromone -- extracted water will have to be briefly treated with acid to desulfate the petromyzonol sulfate it contains (described below). The technical advantages of developing an antibody to petromyzonol are as follows. First, unlike petromyzonol sulfate, petromyzonol is a very robust compound and lacks conjugating groups which might be cleaved by the rabbit's blood-borne enzymes. Second, petromyzonol can be attained in good (100 mg) quantity and quality, and at moderate cost from Toronto Research Chemicals which has synthesized it for us in the past, and is now making some more. Thus, availability of antigen is not a factor in developing the assay. Third, it is relatively easy to desulfate petromyzonol sulfate in river waters (see below) and we have much experience with this. Finally, we have developed means of measuring petromyzonol in the waters (see objective 6) so that we can assist directly in assay development and confirmation.

We have begun redirecting our efforts to producing an assay to petromyzonol (note: Cayman is still injecting rabbits with petromyzonol sulfate anyway). In January 1997 we synthesized and provided the Cayman Research Chemical lab with 12mg of 3-keto petromyzonol for conjugation and antibody production. They have just started injecting rabbits with this compound and we are all very optimistic. In addition, we are currently having more petromyzonol synthesized to ensure that this project can proceed uninterrupted. As mentioned above, this approach will however require an added step to the river water analysis: petromyzonol sulfate must be hydrolyzed (desulfated) to convert it to petromyzonol. We are familiar with several techniques to accomplish and presently working towards optimizing this procedure.

Objective 6: To use the aforementioned ELISA and HPLC to determine the quantity of bile acids in a small selection of Great Lakes streams.

In our previous contract we demonstrated that rivers with sea lamprey populations (St. Mary's, Ocqueoc, Cheboygan) had measurable quantities of petromyzonol sulfate while rivers lacking lamprey did not (Nagel, Grand Outlet). However, measurement was laborious, difficult and not precise, leading us to attempt to develop the ELISA described above. We have however continued to extract and save monthly water samples from the St. Mary's River and several other lamprey and on-lamprey rivers (Table 1) for eventual analysis. Also, since it became obvious that the ELISA was going to take longer than expected, we decided to resume efforts to make our HPLC analysis method more sensitive and accurate -- to provide an interim backup technique until we have an operational ELISA. We have made excellent progress. In particular, we have succeeded in surmounting the two most difficult problems associated with measuring petromyzonol: 1) inconsistent peak size, and 2) background fluorescence in the samples from humics which at times 'swamps' the bile acid signal. We have done this by instituting three changes in our protocol:

1) We now hydrolyze all extracted river waters, converting petromyzonol sulfate to petromyzonol. This is desirable because of the relatively efficience with this can be performed and the fact that petromyzonol can be measured with much greater sensitivity than petromyzonol sulfate using dansyl hydrazine (see #3 below). Also, as mentioned, petromyzonol is very stable.

2) We also now partially purify river waters to remove the humics before analysis usiong danzyl hydrazine. To accomplish this, desulfated river water samples are split into several aliquots, injected onto the HPLC in the aceteonitrile/buffer system used for enzyme analysis, and the fraction which elutes in the position of petromyzonol collected. This takes only a few hours and has the advantage of removing most background autoflourescing humics from the sample and is accomplished with high efficiency. (this step could be omitted in 'clean' river waters).

3) We now analyze the final purified extract using a modified dansyl hydrazine method. The above extract is first reacted (conjugated) with dansyl hydrazine for 1 hour (40 C) and the resulting conjugate injected onto the HPLC column which is heated. Heating greatly improves the efficiency of the reaction. The resulting peaks for petromyzonol are consistently large (8-10 times larger than those for petromyzonol sulfate) and easily quantified against what is now a relatively clean and stable background (see Fig 10).

We will continue to develop this method and believe it will provide an invaluable means to accurately measure petromyzonol sulfate in river waters for the purpose of our experimental until an ELISA can be developed. Presently we are developing means to improve the efficiency of sample extraction, de-sulfation, sample fractionation, and conjugation with danzyl hydrazine. It also may help us perform the necessary steps associated with verifying an ELISA. Detection thresholds appear to be below 0.05ug -- which in 10L of river water is approximately 10-11 Molar, about 10 times lower than we have been able to achieve in river water to date, and the approximate olfactory threshold of lamprey to petromyzonol sulfate (the concentrations relevant to lamprey and which we need concern ourselves with).

Presentations pertaining to this work and for which GLFC Funding was Acknowledged

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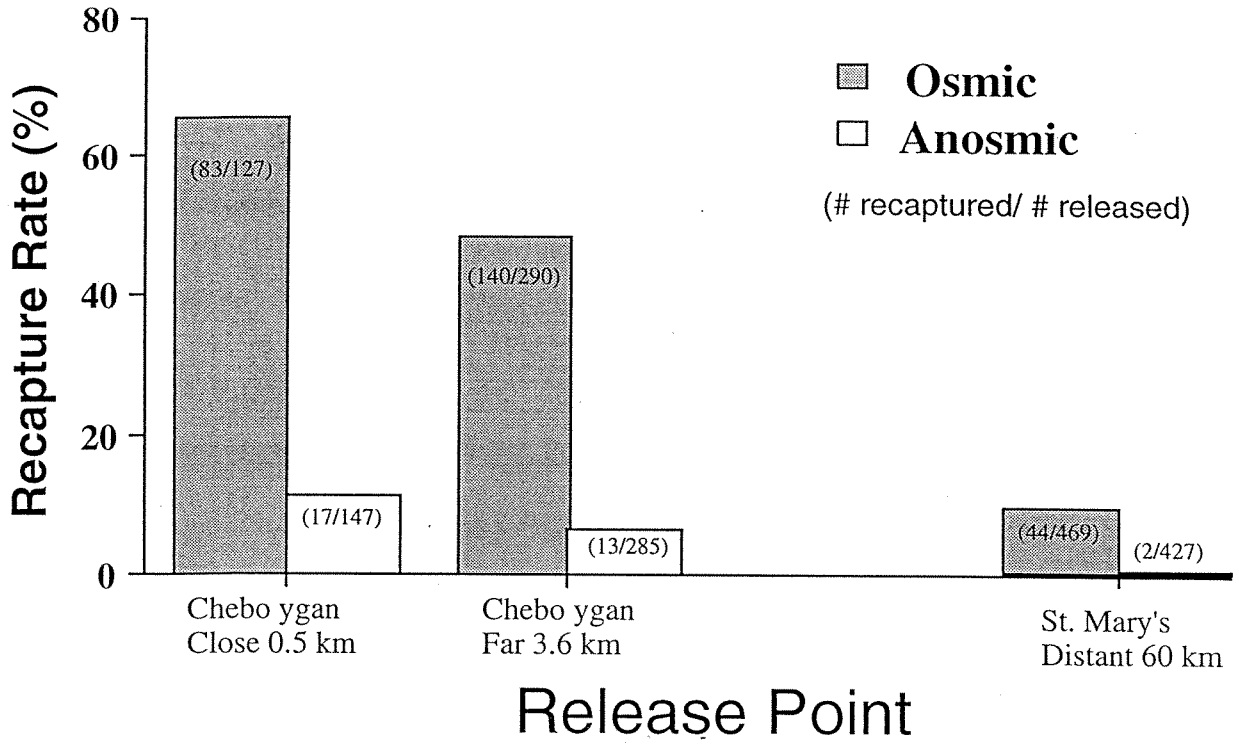
Publications:

Li, W., and P.W. Sorensen. 1997. Highly independent olfactory receptor sites for conspecific bile acids in the sea lamprey, Petromyzon marinus. Journal of Comparative Physiology A. 180(4): 429-438.

Polkinghorne, Christine N. (M.S., Fisheries, 1997). Thesis title: Determining whether bile acids released by larval sea lamprey and other fishes may be functioning as species-specific migratory cues.

Sorensen, P.W., Bjerselius, R., and L. Vrieze. 1997. Spawning stream selection by free-ranging migratory sea lamprey is dependent upon olfactory cues of which conspecific bile acids appear to be an important component. Chemical Senses 22 (6): abstract #532, p.799

Displaced adult sea lamprey are highly successful at re-locating rivers but only if their olfactory sense is functional



(Note: Ratio of osmic to anosmic animals recaptures increased with distance)

Figure 1. Recapture rates of sham- and nose-plugged sea lamprey in streams after their release in Lake Huron in 1996.

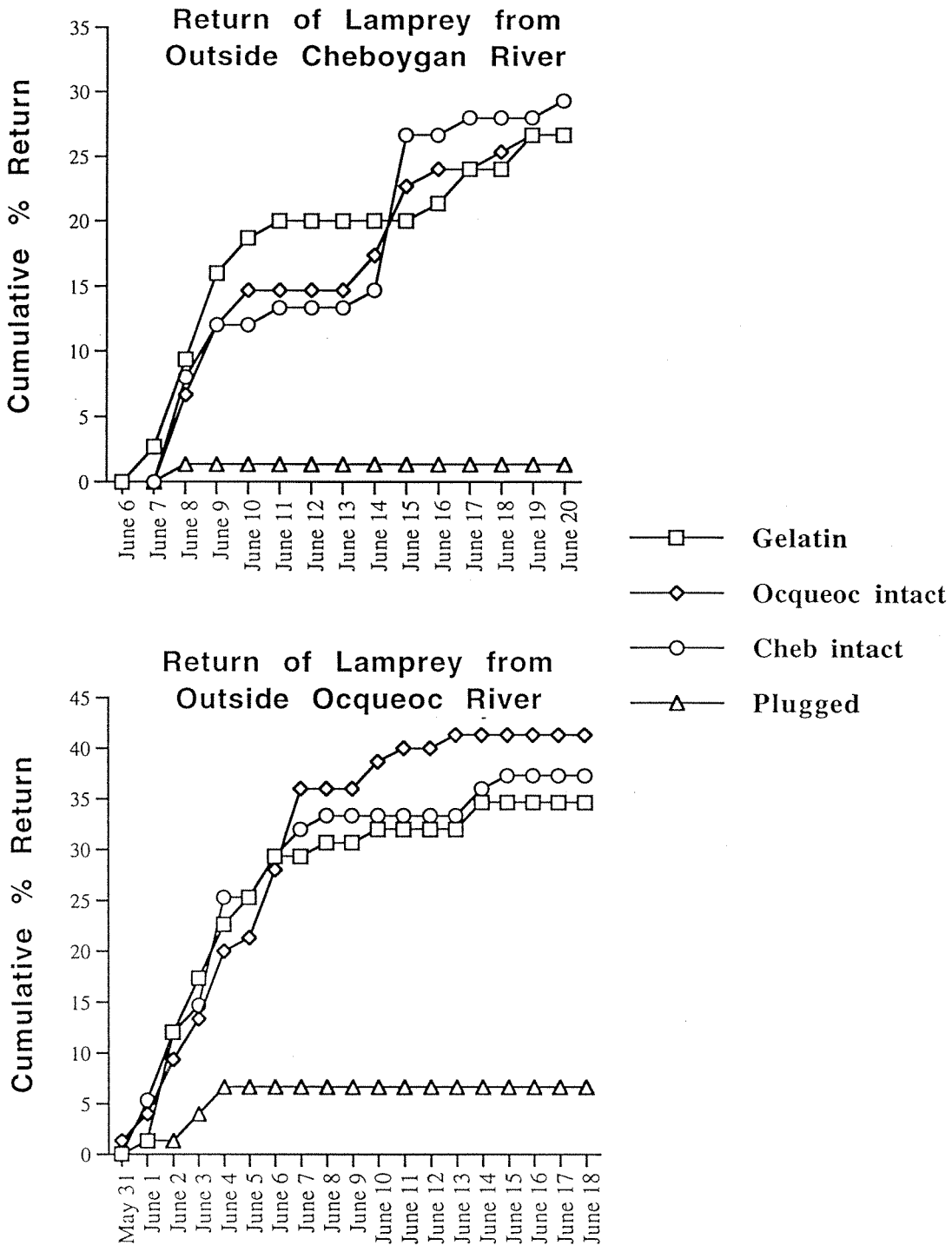


Figure 2: Return to river traps of lamprey released 0.5 km from the mouth of the Cheboygan and Ocqueoc Rivers in the summer of 1997. Animals were given one of three treatments: no treatment of nose (intact), nose flushed with gelatin (gelatin), or nose plugged with dental impression material (plugged). Intact animals originally captured in both the Cheboygan and Ocqueoc were released at both sites.

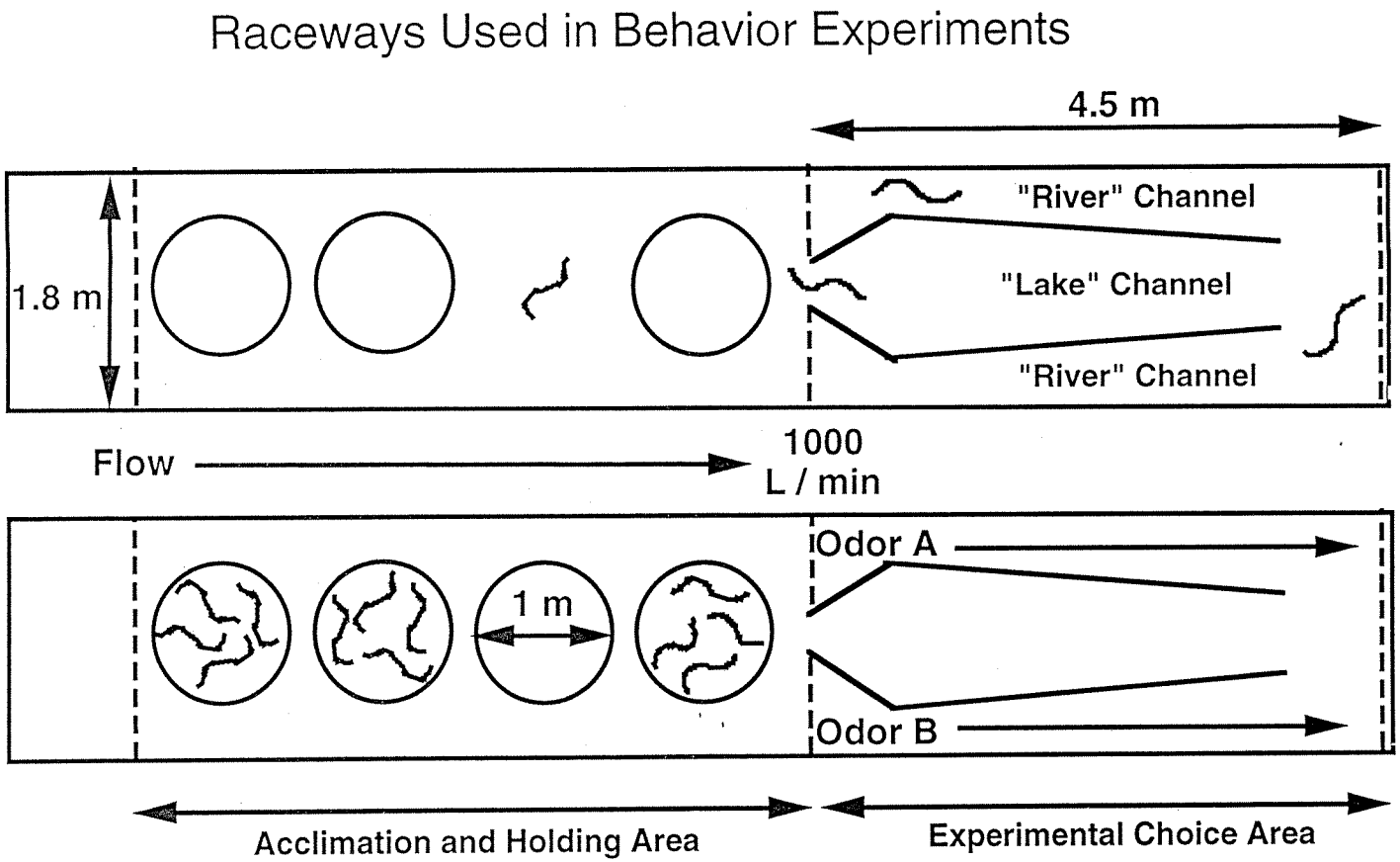


Figure 3: Paired identical raceways modified into two-choice mazes for behavioral testing. Various river odors could be added to the river channels in the experimental choice area. Time spent by lamprey within the two different odors was compared.

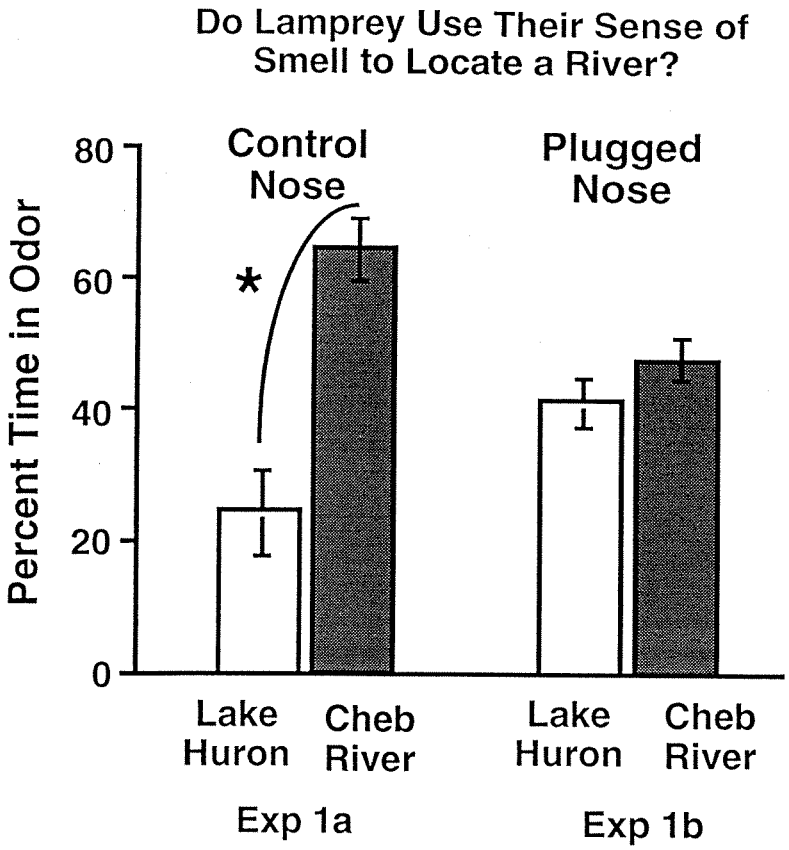


Fig 4. Migratory lampreys' attraction to Cheboygan River water is eliminated after their noses are plugged. Lamprey were offered the choice of river or lake water in a large maze. Animals had their noses plugged with dental impression material. * = $p<.05$, Wilcoxon signed-ranks test. Error bars = plus/minus 1 S.E.

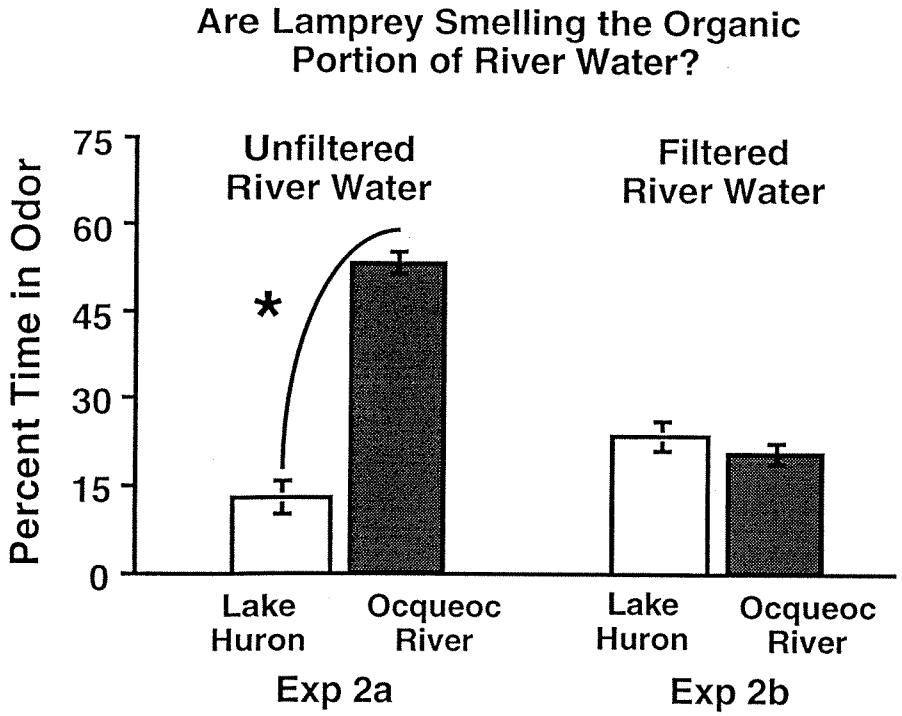


Fig 5. Preference for river water over Lake Huron water is eliminated after organic compounds are filtered from river water. Migratory lamprey were given the choice between Lake Huron and Ocqueoc River water in a large maze. * = $p < .05$, Wilcoxon signed-ranks test. Error bars= plus/minus 1 S.E.

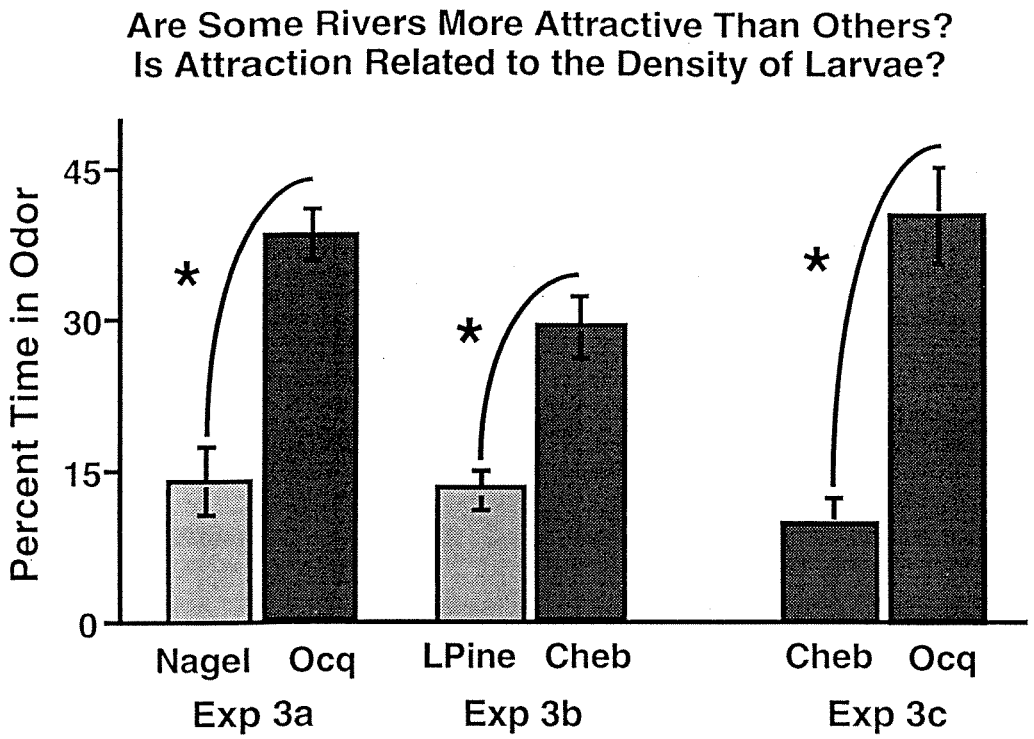


Fig 6. Preference of migratory lamprey for rivers which contain conspecific larvae. Lamprey were offered the choice of two river waters in a large maze. Nagel=water from Nagel Creek, does not contain larvae. Ocq=water from Ocqueoc River, contains high density of resident larvae. LPine=water from Lone Pine Creek, does not contain larvae. Cheb=water from Cheboygan River, contains resident larvae. * = $p < .05$, Wilcoxon signed-ranks test.

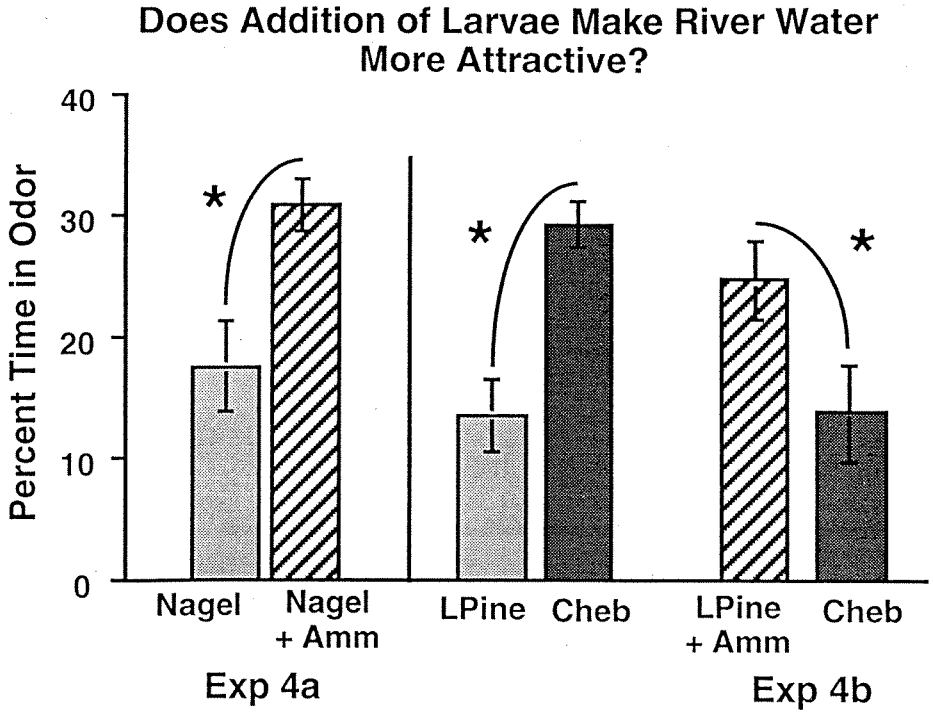


Fig 7. Preference of migratory sea lamprey for river waters into which conspecific larval odor has been added. Lamprey were offered the choice of two different river waters in a large maze. Nagel= water from Nagel Creek, does not contain larvae. LPine= water from Lone Pine Creek, does not contain larvae. Cheb= water from the Cheboygan River, has resident larvae. Amm= ammocoete holding water added to river water. *= $p < .05$, Wilcoxon signed-ranks test. Error bars = plus/minus 1 S.E.

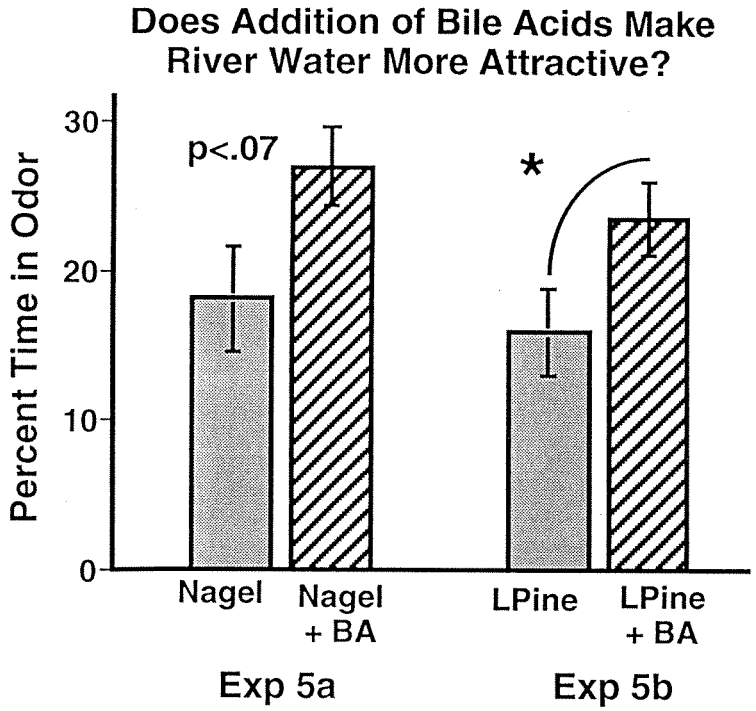


Fig. 8 Preference of migratory lamprey for river water into which the conspecific larval bile acids petromyzonol sulfate and allocholic acid have been added. Lamprey were offered the choice of two different river waters in a large maze. LPine=water from Lone Pine Creek, which does not contain larvae. Nagel=water from Nagel Creek, which does not contain larvae. *= $p<.05$, Wilcoxon signed-ranks test.

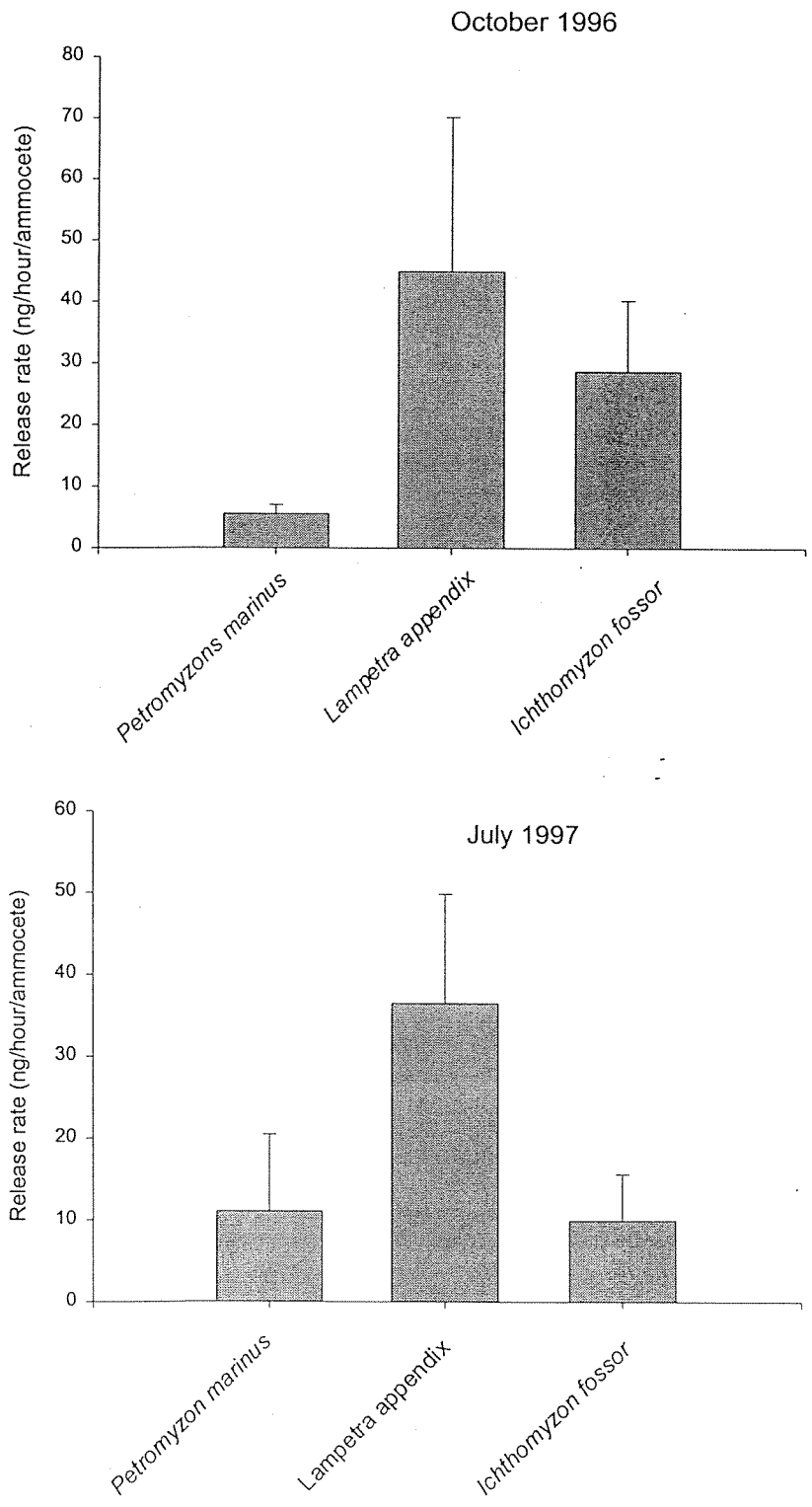


Figure 9. Rate of release of petromyzonol sulfate by three species of larval Great Lakes lamprey. Experiment was repeated on two occasions.

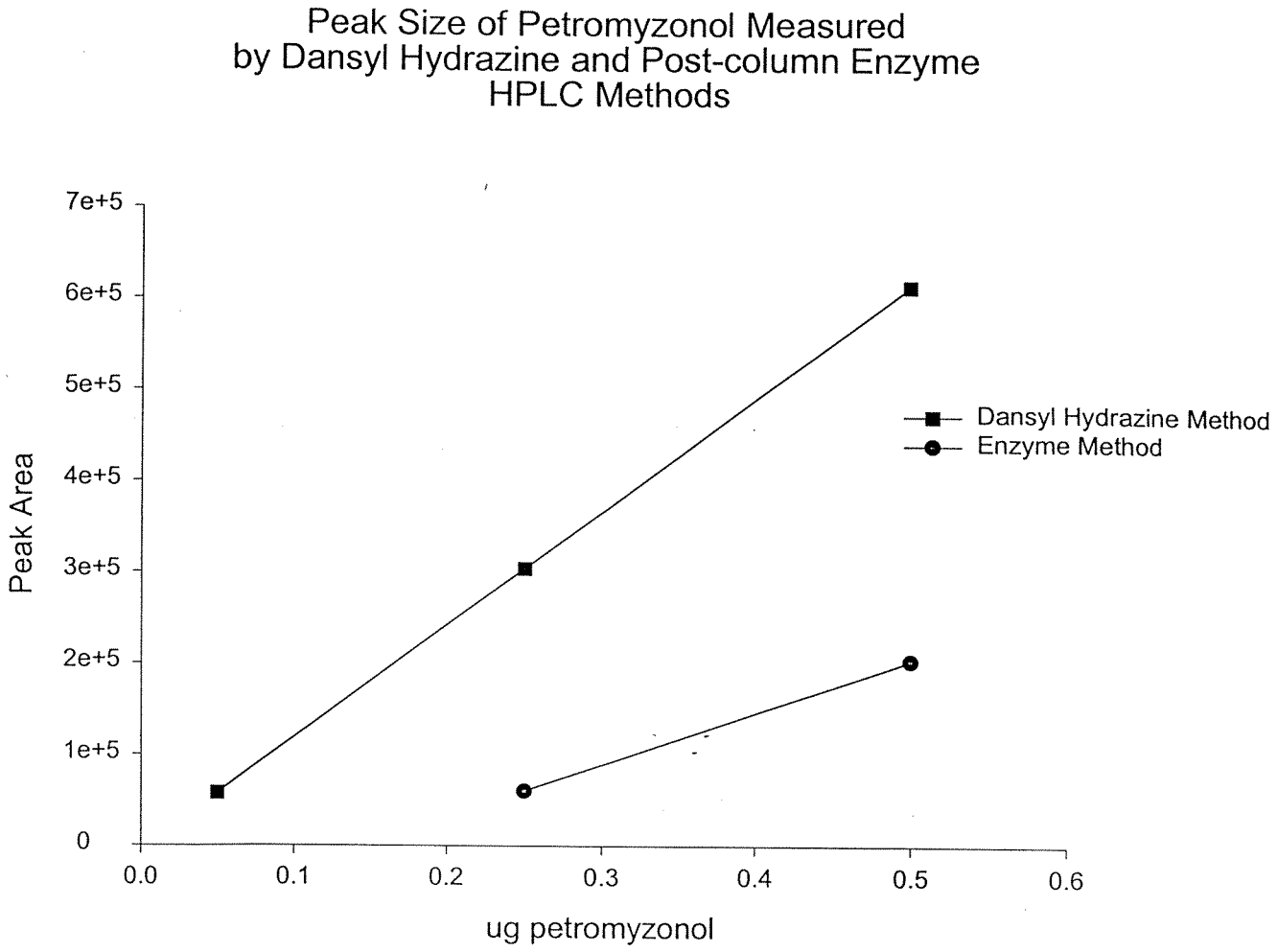


Figure 10: Comparison of reaction efficiencies for petromyzonol using two different methods.

Table 1. 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