GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Evaluating the seasonality of olfactory function of migratory adult sea lamprey and the distribution of water-borne lamprey bile acids in the Great Lakes to determine whether bile acids function as the lamprey migratory pheromone

by:

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Final Report for the Great Lakes Fishery Commission

Project Title: Evaluating the seasonality of olfactory function of migratory adult sea lamprey and the distribution of water-borne lamprey bile acids in the Great Lakes to determine whether bile acids function as the lamprey migratory pheromone.

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PROBLEM STATEMENT AND OBJECTIVES:

We have hypothesized that larval lamprey produce unique bile acids which they then release into the water to function as a migratory pheromone which attracts migrating adult lamprey into spawning streams. Prior to this study we knew that larval sea lamprey produce two unique bile acids which adults detect with great sensitivity and specificity (Li et al. 1995). However, we did not know whether larval lamprey released these compounds in adequate quantities to explain either the correlation between runs of adult lamprey and the presence of larvae (Moore and Schleen 1980) or Teeter's (1980) observation that washings of larval lamprey attract migratory adults. Accordingly, we designed a series of experiments designed to answer these questions. These experiments took the form of seven objectives which are listed below. This report summarizes the progress we have made towards meeting these objectives during the last two years of support.

1) To optimize our protocols for measuring bile acid content in stream waters.

2) To determine if the quantity of bile acids in river waters exhibits a seasonal cycle.

3) To determine if the presence of larvae bile acids in river waters of the Great Lakes is correlated with the number of sea lamprey larvae in these systems.

4) To determine if the olfactory sensitivity of sea lamprey to bile acids is influenced by life stage and/or relative sexual maturity.

5) To determine how metamorphosis influences the production of bile acids in larval sea lamprey.

6) To determine the release rate of bile acids by larval sea lamprey.

7) To determine whether/how larval bile acids influence the behavior of adult lamprey during the initial stages of their migration.

Objective 1: To optimize our protocols for measuring bile acid content in stream waters. In 1992 we successfully measured bile acids in the St. Mary's River. Several attempts to measure bile acids in other rivers in 1992 and 1993 subsequently failed. Both sample degradation and the relative insensitivity of our analytical technique were suspected; the latter was about 100-1000 times less sensitive than the lamprey nose and considerably less specific (see below). Accordingly, we have developed new techniques to improve our ability to extract and measure these compounds in river waters. Our new technique (the development of which is described below) gives us a 10-100 fold increase in sensitivity (depending on background), still about 10 times less than th sensitivity of the lamprey nose but adequate to confirm the existence of lamprey bile acids in lamprey spawning streams including the St. Mary's River. Specifically, we have accomplished the following: a) Insured the stability of the bile acids in the water samples between the time of collection and the time of isolation of bile acids from the samples; b) Increased the sensitivity of detection of bile acids in our high performance liquid chromatography (HPLC) method; c) Confirmed that bile acids detected by HPLC analysis are in fact the bile acids of interest and not co-eluting peaks. Further explanation follows.

a) Insuring the stability of bile acids in the water samples. Pilot studies using river water samples collected in 1993 and then stored for several months failed to reveal the presence of bile acids. We suspected that bacterial degradation could be partially responsible and in the spring of 1994 tested an anti-bacterial agent (sodium azide) on the stability of petromyzonol sulfate and allocholic acid in river water held at room temperature

for a period of 4 weeks. Briefly, petromyzonol sulfate and allocholic acid (10 mg/L) were added to 10 liter water samples collected from a local river. Four containers contained 1 g/L sodium azide and one contained no sodium azide. These containers were sampled at weekly intervals and the content of petromyzonol sulfate and allocholic acid determined by high performance liquid chromatography (HPLC -- according to procedures described in Li et al. 1995). The results, shown in Figure 1, demonstrated that water-borne bile acids rapidly decay between 7-14 days after being placed into containers lacking preservatives but take 3-4 weeks in the presence of sodium azide. This has several ramifications. First, it demonstrates that 1993 analyses were flawed because all of the riverine bile acids would have been degraded by the time we analyzed them. Second, it actually lends support to our pheromone hypothesis as the week long period of time required for breakdown is long enough for these cues to make it to a river mouth. Third, these findings have caused us to modify (correct) our collection procedures so that all river samples collected in 1994 and 1995 have both sodium azide and sodium benzoate (another preservative added for extra protection) added to them.

b) Increasing the sensitivity and specificity of the detection method. Having ascertained how to collect river water bile acids, we next wished to maximize our ability to detect these compounds. The limits of detection for petromyzonol sulfate using our original (enzymatic) HPLC method is approximately 100 ng per sample. However, only about 10 L of river water at a time because of problems associated with water-borne organic compounds which get excessively viscous, our detection threshold in river waters is about 10^{-10}M, roughly 100 times greater than the threshold of the lamprey olfactory system. Further complicating this scenario has been the high and variable quantities of organic compounds which are naturally found in river waters which fluoresce and frequently (and not always predictably) create a high background, masking our ability to measure bile acids reliably. It is highly likely that these compounds are humic substances, a complex mix of dark brown acidic polymers derived from natural sources.

After searching the relevant literature, we focused on derivatizing water-borne bile acids with a fluorescent label, danzyl hydrazine. We have been successful in this endeavor and have realized an approximate 100 fold increase in the sensitivity of detection of petromyzonol sulfate and allocholic acid when measured in clean (blank) water. Unfortunately we have encountered problems with background fluorescence so our gain in sensitivity in samples extracted from a typical river water is generally only about 10-fold. Nevertheless this has been adequate to confirm the existence of bile acids in the St. Mary's

River. Here, we describe the technique which we have developed and presently use to analyze river waters using dansyl hydrazine, then we describe our unsuccessful efforts to alleviate the problems with background contamination to allow us to realize the full potential of this technique, and finally we describe the technique we presently employ to prove that our identification of petromyzonol sulfate is correct.

River water samples are extracted by activated C18 columns (one per liter), eluted with 5 ml of methanol and concentrated under nitrogen and reconstituted with methanol (MeOH) containing non-interfering bile acid(s) which serves as an internal standard. Samples are then incubated 30 min at 37° C with 3α hydroxysteroid dehydrogenase and NAD buffer to convert the 3α hydroxyl group of any bile acids to a keto group. After incubation, samples are dried under nitrogen and dissolved in 100 µl of acidified MeOH (65 ml HCl/100 ml MeOH) to which 100 µl of 0.05% dansyl hydrazine/MeOH is added. These samples are then incubated for 20 minutes, dried, and reconstituted. For HPLC analysis, 50 µl injections of the concentrated sample are run on a non-linear 78 minute gradient. The gradient starts at 58% MeOH with 1.5 mM KH_2PO_4 buffer, pH 3.5. After 58 min the gradient reaches 100% MeOH, and then returns to 58% MeOH over the remaining 20 min period. Detection is with a fluorometer equipped with an excitation filter of 340 nm. We have tried to further increase the sensitivity of this technique by treating the sample with 7α-hydroxysteroid dehydrogenase to free up another site for conjugation with our fluorescent label but it did not work, likely because of steric hindrance. The present technique works well but has the problem of being too sensitive at times; organic compounds naturally found in river waters sometime create high background fluorescence. Another problem has been shifting retention times. Although we have not actually solved either problem, we have successfully modified the technique in such a way that we obtain highly credible results from streams with low organic loads. We will now discuss these solutions/modifications

Three approaches were explored to reduce background interference but none has worked satisfactorily to date. Consequently, we have elected to analyze lessor of quantities of river waters (2 L). Although in doing so we have sacrificed overall sensitivity, it has been adequate to confirm the presence of bile acids in several streams, our principal objective. Briefly, the first technique we examined as a possible means of 'cleaning' up our samples was based on the fact that bile acids derived from mammalian sources are known to bind to albumin rather specifically. Thus, we attempted to extract river water bile acids using albumin columns with the hope that many of the contaminating compounds would pass through. Preliminary work using river water with radiolabeled cholic acid and petromyzonol sulfate appeared promising (extraction rates were on the order of 70%) but

the technique was unfortunately plagued by contaminating peaks from the albumin itself. Because of limited time we have not pursued this further although we believe that this problem may be surmountable by cleaning the albumin. The second approach we have explored has been to remove the riverine contaminants using molecular weight cut-off filters. Unfortunately, riverine bile acids appeared to stick to the columns as recovery rates were low (less than 33%). A third approach we have examined has been to treat samples with a compound which conjugates with free carbonyl groups, thus removing such contaminating compounds from the pool of material which might react with our label. Briefly, extracted river waters were incubated with 0.5 mg of dinitrophenylhydrazine (DNPH) in 5 ml of acidified ethanol (9% H_2SO_4) overnight in the dark. Reacted samples were then reconstituted in 10% MeOH, extracted using a C18 solid column which was then washed with dichloromethane (to remove un-reacted DNPH), and eluted with MeOH. Unfortunately, we found that this technique also desulfated petromyzonol sulfate, reducing our ability to measure it. In summary, at present, we have no real solution to this problem, the severity of which varies with the source of the water, and simply use smaller quantities of water.

c) Confirming our identification of riverine bile acids. Finally, one of the problems we have experienced with HPLC analysis of river waters has been some shifting retention times, apparently a consequence of high and variable organic loads. We have successfully circumvented this problem by splitting each river water sample into 3 aliquots, one of which is spiked with standard (to confirm retention times), another of which is desulfated to verify identity of the petromyzonol sulfate peak (if present) because such a treatment should both cause the elimination of this peak and lead to the appearance of petromyzonol, and a third of which is untreated. Desulfation (solvolysis) is accomplished by treating a dried bile acid sample with a water- acidified dioxane solution for 18 hours at 37°C, then evaporating it to dryness with nitrogen. Although this process is somewhat labor-intensive it gives us great confidence in our identification some idea of quantity present. Quantification is achieved by comparing the size of the petromyzonol sulfate peak in the spiked sample after subtracting out that quantity in the untreated spike sample (thus producing a value for how big of peak a known quantity of petromyzonol sulfate produced). This value is then compared to the difference in size of the petromyzonol sulfate in the untreated sample minus that which does not de-sulfate in the de-sulfated sample (in 2/4 samples nothing was left). Because we know the relationship between peak size and petromyzonol sulfate is linear from testing using standards, we can estimate the amount present using a single point. However, these numbers should be interpreted

carefully, and we now plan to verify them more accurately by extracting a large volume of water from a local river and spiking it with varying amounts of bile acid this year. Likewise, although we estimate our detection threshold to be approximately 10^{-11}M in a 2 L sample (and less in those samples with high background fluorescence), we plan to get a better grasp of the situation in the upcoming year as part of the aforementioned experiment.

<u>Objective 2:</u> To determine if the quantity of bile acids in river waters exhibits a seasonal cycle.

Approximately five dozen 10 L water samples were collected on a regular basis from both the Ocqueoc and St. Mary's Rivers during the ice-free portion of the year in 1994 and 1995 (Samples collected in 1994 are shown in Table 1, those in 1995 in Table 2). These samples were collected in clean plastic jugs with preservative and immediately shipped to the University of Minnesota where they were extracted within 2 weeks of collection using activated C18 columns (Sep-paks). Most of these samples were used in methods development but about a dozen remained and have been analyzed by the dansyl hydrazine technique described above. Of these, only three samples were available which had been collected from a single site and thus could be used to address seasonality. These samples were from Site A on the St. Mary's River, just below the Lamprey Control Centre. Petromyzonol sulfate was measurable in all three instances with the highest quantity being present in June, the time of our first sampling and the time that the run commences (Table 1). Approximately 3.5 x 10^{-10} Molar petromyzonol sulfate was measured at this time (Figure 2a), approximately 100 times the detection threshold of adult sea lamprey. Only about one sixth as much was petromyzonol sulfate was measured in July and about half as much in August. Although this is suggestive of a seasonal trend, we lack a clear idea of measurement error, and there are too few samples to make definitive conclusion. We have however arranged for new collections of water samples to be made in 1996 to answer this question.

<u>Objective 3:</u> To determine if the presence of larvae bile acids in river waters of the Great Lakes is correlated with the number of sea lamprey larvae in these systems.

As mentioned above, 14 river water samples have been assayed using our new technique whose sensitivity is about 10 times less than that of adult lamprey. It is somewhat difficult to compare these values because of the limited number of samples available and the fact that many are not matched across time. Of the 14 riverine samples analyzed, we have measured petromyzonol sulfate in 4. Three of these are from site A of the St. Mary's (described above), and the fourth from the Ocqueoc River in June, 1994 (Fig 2b) -- a stream which contains many ammocetes and attracts adults. We have also tested 2 non-lamprey streams to date (Grand Lake Outlet and the Brule River) and have been unable to measure petromyzonol sulfate in either one. Somewhat perplexing has been our inability to measure petromyzonol sulfate in the Ocqueoc River and Cheboygan in May 1994 or allocholic acid in any stream. However, our technique is still not sensitive enough to give us an accurate picture of the distibution of this cue at all concentrations detected by the adult lamprey and it is because of this that we propose to develop an immunoassay in 1996 to increase our sensitivity.

Objective 4: To determine if the olfactory sensitivity of sea lamprey to bile acids is influenced by life history stage and/or relative sexual maturity.

Methods: This study was completed in 1994. We chose the Ocqueoc River as a model system for this study because both its mouth and upstream sections are reasonably accessible, thereby permitting us the opportunity to collect animals through out their migratory cycle. A fyke net trap was constructed at the mouth of the Ocqueoc River and maintained by personnel from Hammond Bay on a regular basis from late March until animals were no longer captured entering this river (early June). As the capture of these early-migrants waned, we arranged for the collection of later migrating animals from the permanent trap located mid-stream at the barrier. These animals were captured through late June at which time we obtained sexually-mature animals from Hammond Bay who were using them in spawning experiments. In this manner we were able to obtain healthy lamprey throughout their migratory and spawning cycle. Later, we also obtained parasitic-phase and larval animals to compare their responsiveness with the migratory adults.

A range of 32 odorants representing all classes of odorants to which lamprey are known to respond was tested. These included: 4 bile acids (petromyzonol sulfate and allocholic acids were tested at three concentrations), L-arginine (the only amino acids which

lamprey detect well which thus functioned as our standard), water from spermiated males (putative sex pheromone), water from immature males, water from ovulated females, water from immature females, water from ammocetes, water from two streams (including the Ocqueoc), two gonadal steroids which we believe may have pheromonal function, and trimethylamine. All odorants were made up fresh every day. EOG responsiveness of lamprey to these stimuli was tested and their olfactory epithelia preserved for histological analysis by Dr. Zeilinski (described below). EOG recording followed established protocol. Briefly, fish were immobilized with a muscle relaxant (10mg/kg body weight; gallamine triethiode), positioned in a flow-through trough, and their gills perfused with well water via a tube inserted into their oral cavity. The olfactory sac was then exposed by carefully cutting away the dorsal skin and perfused with 10°C well water into which 5-sec pulses of odorants were injected. Electrical responses were recorded differentially using Ag/AgCl electrodes bridged to saline/gelatin-filled glass pipettes (tip diameter 400υm) positioned just above the olfactory epithelium. Responses were amplified with a DC-preamplifier (Grass P16), digitized, displayed, and stored on an Apple computer using a MacLab (WPI systems, FL) data collection and analysis system.

The protocol for histological examination of the olfactory epithelia removed from tested fish followed that developed by Dr. Zielinski and was accomplished collaboratively with her assistance. Briefly, olfactory epithelia were fixed in Karnovski's fixative and at weekly intervals these specimens run through cacodylate buffer, dehydrated in ethanol washes, immersed in propylene oxide and embedded in Epon epoxy resin. Blocks of tissue were then sectioned at a thickness of 1um and stained in Richardson's stain prior to examination under the microscope. Selected sections were also stained with uranyl acetate and lead citrate for examination under a transmission electron microscope if deemed necessary.

Results: We tested approximately 10 animals a week for 10 week period by EOG. There were several major findings. First, when the olfactory sensitivities of different life stages of sea lamprey were compared, the migratory adults were found to be approximately 5 times more sensitive than either the ammocete larvae or spawning adults (Fig. 3) Migratory animals were also more sensitive than parasitic animals. These differences were dramatic and evident for all odorants. Because of the consistency of our testing regime and a lack of correlation between lamprey size and EOG response, we have great faith in this finding which strongly suggests that the principal function of olfaction in lamprey is detection of migratory cues.

A second major finding was that the olfactory sensitivity of migratory adults decreases dramatically and consistently during the course of their several week spawning migration (Fig. 4).

In fact, by the time they are spawning, they were virtually anosmic! These findings strongly support earlier behavioral studies conducted by Teeter (1980) and ourselves (Sorensen Gallaher, last year's report) suggesting that responsiveness to olfactory cues drops off in late-migrating animals. Less-systematic studies of animals collected from the Cheboygan and St. Mary's River in 1992 and 1993 also support this trend (Sorensen, unpublished). No sexual dimorphism was noted and this decrease in olfactory sensitivity was observed for all 32 odorants. We know of no precedent for this finding which we believe is related to sexual maturity as a strong negative relationship was found between gonadal development and EOG responsiveness. Interestingly, however, no relationship was seen between EOG responsiveness and the density of olfactory receptor cells as measured by Dr. Zeilinski -- the change must be at the molecular level (Fig. 5). These findings are not only of scientific interest but of extreme practical importance: clearly stocking of sterile males should be done in a way which minimizes their need for an intact sensory system. Also, odorant based bio-control programs should be designed to focus on early migrating animals. An encouraging facet of our finding is that if migratory animals can be drawn to inappropriate traps/rivers using introduced larval odors/bile acids, they will probably not be able to correct their 'mistake' owing to a degenerating olfactory system and their reproductive potential will be wasted

A third major finding which is not one of our defined objectives is that spermiated male fish release a potent odorant. This not only confirms earlier behavioral work by Teeter (1980) but clarifies that this pheromone is restricted to mature males whose odor was thousands of time more potent than that of mature females or immature males (Fig. 6). EOG recording and chemical characterization suggest that this cue has two component(s), one of which is sulfate, and that their release is promoted by GnRH treatment. We strongly believe that this cue is a male pheromone and may be useful in bio-control as short-range attractant.

Objective 5: To determine how metamorphosis influences the production of bile acids in larval sea lamprey.

Larval sea lamprey were captured in Michigan streams and shipped to the University of Minnesota in 1994. Some animals were sacrificed immediately for analysis (see below) while other were maintained in the laboratory for several months and allowed to metamorphose at which time they were sampled. Parasitic lamprey were received from the Lake Huron fishery and adults from the Cheboygan River. Life stage was determined according to the criteria defined by Manion and Stauffer (1970). Lamprey were not fed for

at least a week prior to sampling so that all life stages would be comparable (adults do not feed). Animals were sacrificed by overdose of MS-222, and their gall bladders and/or livers dissected out and stored in 95% methanol. Tissue was then extracted according to the methods of Locket and Gallaher (1989). Extracts were analyzed by HPLC to identify which bile acids were present in the tissues using the enzymatic method of Gallaher et al. (1992).

As reported by Yamamoto (1986), we found that larval lamprey lost their gall bladders and ducts during metamorphosis; stage 4 transformers had neither structure. Larval lamprey had extremely high levels of bile acids in their gall bladders (about 140 μg/mg of tissue or 530 μg for an average gall bladder). The vast majority of bile acid (99%) was in the form of petromyzonol sulfate, with allocholic acid and petromyzonol present in much smaller and roughly equivalent amounts (Table 3). Gall bladders from metamorphosing Stage 1 animals contained far less bile acid (less than 10 μg, most as petromyzonol sulfate and no measurable allocholic acid). All three bile acids were also measured in larval livers, in about the same ratio seen in their gall bladders. Notably, no bile acids were found in livers removed from Stage 4 transformers, parasitic-phase animals, or sexually mature adults. Bile acid synthesis is thus extremely high in larval lamprey but ceases during metamorphosis.

Objective 6: To determine the release rate of bile acids by larval lamprey.

Sea lamprey ammocetes were collected by Mr. Sidney Morkert (US Fish and Wildlife Service) from streams in Michigan. These ammocetes were packed in coolers on ice and transported to the University of Minnesota via overnight Express Mail. Upon arrival, 50 ammocetes were placed into each of three 80 L plastic containers with flowing and aerated 15°C water and washed sand. Larvae were allowed to acclimate for a month, during which time they were fed a mixture of BioKyowa (0.03 g/ammocete) and yeast (0.27 g/ammocete) biweekly. One container had sand only as a control. To test the possibility that bile acid release may be associated with onset of feeding in the spring, we measured release rate to the water after a period of starvation and later after having been fed. To accomplish the first measurement, larvae were not fed for a period of 14 days following their acclimation period. The in-flowing water was then shut off and a 10 liter sample taken immediately as the zero hour control sample. Samples were then taken at 6 and 24 h intervals. Each water sample was filtered through Whatman paper filters to remove debris

and then extracted using C18 columns. Following the last sample, the flow of water was turned back on and the animals given three days to recover. Next, to determine the effect of feeding, the flow of water was discontinued and water samples taken. Each container was then supplied with a mixture of BioKyowa and yeast (0.03 g/ammocete and 0.27 g/ammocete, respectively) and waters samples taken 6 and 24 hours later. All samples were filtered and extracted as described above.

Only recently fed larvae released significant quantities of petromyzonol sulfate and allocholic acid -- the two bile acids which adults detect with greatest sensitivity (Fig. 7). Interestingly, starved animals released relatively large quantities of petromyzonol, an extremely ineffective olfactory stimulant (Li et al. 1995). Factors which remain to be addressed are the effects of continuous feeding, diet, and temperature on these estimates which nevertheless clearly demonstrate that ammocetes release great quantities of biologically-relevant bile acids.

Objective 7: To determine whether/how larval bile acids influence the behavior of adult lamprey during the initial stages of their migration.

General Introduction: The primary purpose of these studies was to test whether bile acids released by larval sea lamprey influence the behavior of adult lamprey in a manner which is consistent with their function as a migratory cue. We met this objective and are now prepared to move to testing in large raceways in 1996. Although we had originally proposed to conduct initial experiments in 1994 in the laboratory and then move to the field in 1995, we opted instead to conduct behavioral experiments in both years in St. Paul. This was done primarily because we wished to confirm our positive results from experiments conducted in standing water in 1994 in a controlled, flowing water scenario in 1995: thus providing a 'spring board' for larger-scale test in the future. Thus, the following section is divided into a description of our 1994 results conducted in standing water and our 1995 results conducted in flowing water.

Phase I, 1994: Does the odor of larval sea lamprey bile acids influence the distribution and activity of migrating sea lamprey in still water?

Migratory animals were captured in the Manistique and St. Mary's Rivers and held at 12° C. The experimental tanks used in this study consisted of two large circular tanks (3 m in diameter) divided into four equal sized sections by plywood walls which extended in from the sides. Each quadrate had a line subdividing the section into equal halves for monitoring

locomotor activity. For testing, groups of 10 animals were placed into these tanks during the morning of the experiment and allowed to acclimate. All tests were conducted after sunset (2100h) and ran until 0100h, a time frame which pilot studies demonstrated to include peak migratory activity (Sorensen, unpublished). The experiment started with a 30 min pre-test during which time a control odor (alcohol) was pumped into the tank and the distribution and locomotor activity (line crossings) of the animals noted by 2 observers. This was immediately followed by another period of either another control odor or experimental odor. This procedure was then repeated on the second tank. Observers were unaware of the identity of the odor added. The experimental odor was comprised of a mixture of petromyzonol sulfate and cholic acid. Cholic acid was used as a substitute for allocholic acid because it cross-reacts with cholic acid as measured with EOG (unpublished), and we had little allocholic acid available. Test substances were pumped into the tanks at a rate of 100 ml/min to give a final concentration of 5×10^{-10} M for petromyzonol sulfate and 5×10^{-8} M for cholic acid in the tank. The odor of ammocetes (150 animals held in 50 L for 24 h) and adult males (3 animals held in 30 L for 4 h) were also tested in a similar manner.

Adult sea lamprey became significantly more active when exposed to water-borne bile acids ($P<0.01$) (Fig. 8a). Similar responses were also seen to larval washings (Fig. 8b). No change in distribution was noted (data not shown). Adult lamprey exposed to odor of spermiated males also stimulated enhanced locomotor activity ($P<0.05$; Fig. 9). Behavioral activity had thus been established for these odorants and the next step will be to find out their actions in a flowing water environment, more similar to that of a stream

Phase II, 1995: Does the odor of larval bile acids influence the distribution and activity of migrating sea lamprey in flowing water?

We were interested in determining whether bile acids influence adult sea lamprey behavior in a flowing water in a manner similar to that seen to larval odors in a natural stream (Teeter, 1980; Sorensen, unpublished). We also wanted to test whether we could motivate lamprey to swim into an elevated concentration of these compounds. Several designs were attempted but we will only describe the one which we conducted our tests with. Once again, recently captured migratory animals from the Cheboygan and St. Mary's Rivers were transported to St. Paul and held at 12°C prior to testing. All tests were conducted at night under infrared illumination using a low light camera and video system. Fish were tested as groups of five in semi-square glass-fiber tanks (244 cm a side) which had two separate channels built into from plexiglass sheets. The flow rate of water (13°C, 15 cm in depth) in these channels was 38 l/min and 19 l/min; a scheme based on Kleerekoper (1967) who hypothesized that differential flows assist animals attempting to

locate odors. Fish were allowed to acclimate and explore the test area from 1200h until 2200h when the experiment commenced. A mixture of petromyzonol sulfate and allocholic acid was then pumped into one channel (at a concentration designed to create a final concentration of 5 x 10^{-10} M) and methanol carrier into the other. Odorant delivery was pulsed (15 sec on/ 15 sec off) to enhance contrast and save stimulus. Fish were observed for two 15 min period, the first one during which time the flow was high in the odor side, and second during which the situation was reversed. Upstream swimming activity was measured during these periods as the number of times that fish crossed two lines drawn on the floor of each channel.

Adult sea lamprey exhibited more upstream swimming activity in the channel with the bile acids than in that channel without this stimulus (Fig. 10). A posteriori tests of responses to the odorant in high flows versus responses in the low flow demonstrated that flow rate itself had no influence on this behavior. These responses in flowing water closely resembled that measured by us earlier in flowing stream waters to ammocete odor in 1993. It can now be concluded that lamprey bile acids stimulate swimming behavior in migratory adult lamprey.

General conclusions:

Our results from 1994 and 1995 lend extremely strong support to our hypothesis that bile acids released by larval lamprey function as a migratory pheromone for this species. We have now firmly established that larval lamprey produce and release significant quantities of bile acids to the water. Release is confined to the larval stage and associated with feeding. Furthermore, we are now able to definitely confirm the presence of the principal lamprey bile acid, petromyzonol sulfate, in natural river waters inhabited by this species. It is also now clear that the sensitivity of the olfactory system of adult to larval bile acids peaks during the early phases of migration. Finally, we now have clear evidence that exposure to water-borne bile acids stimulates migratory behavior in adults. Yet to be determined is the distribution of bile acids in the Great Lakes and their precise role and importance relative to other sensory cues effecting stream selection. This will require the development of an ELISA which is not hindered by the presence of organic compounds naturally found in streams. More direct tests of the behavioral actions of these bile acids on a large scale are also needed (and planned).

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TABLE 1.

1994Riverwaters Collected and Extracted.

Date filtered	River	Site	Date collected	Liters filtered
4/25/94	Ocqueoc	DNR Launch	4/18/94	7
4/25/94	Cheboygan	Trap site	4/18/94	7.7
5/10/94	Ocqueoc	DNR Launch	5/2/94	7.6
5/19/94	St. Mary's	Site 1	5/11/94	9.6
5/23/94	St. Mary's	Site 1	5/12/94	9.4
5/23/94	Ocqueoc	DNR Launch	5/17/94	6.4
5/23/94	Grand Lake outlet	Weiming collected	5/18/94	11.6
5/23/94	Cheboygan	Weiming	5/18/94	9.9
5/23/94	Ocqueoc	Weiming	5/18/94	10.4
6/5/94	Ocqueoc	DNR Launch	6/1/94	9.35
6/5/94	Ocqueoc	Bridge Site	6/1/94	9.7
6/16/94	St. Mary's	Site 1	6/10/94	10.3
6/22/94	Ocqueoc	Launch site	6/14/94	7.8
6/23/94	Cheboygan	Trap site	6/14/94	8.5
6/23/94	Ocqueoc	Road Bridge	6/14/94	9
7/7/94	Ocqueoc	DNR Launch Site	6/29/94	7.55
7/7/94	Ocqueoc	Bridge	6/29/94	8.3
7/19/94	Ocqueoc	DNR Launch	7/13/94	8
7/19/94	Ocqueoc	Bridge	7/13/94	7.7
7/29/94	Ocqueoc	DNR Launch	Jul-94	7.5
7/29/94	Ocqueoc	Bridge	Jul-94	7.35
8/1/94	St. Mary's	Site 1	7/21/94	10.5
8/1/94	St. Mary's	Site 2	7/21/94	10.7
8/2/94	St. Mary's	Site 3	7/21/94	10.5
8/2/94	St. Mary's	Site 4	7/21/94	10.7
8/3/94	St. Mary's	Site 5	7/21/94	10.7
8/31/94	Well water	Lab	8/31/94	7.3
8/31/94	St. Mary's	Site 5	8/26/94	10.9
8/31/94	St. Mary's	Site 4	8/26/94	10.9
8/31/94	St. Mary's	Site 3	8/26/94	10.9
8/31/94	St. Mary's	Site 2	8/26/94	10.9
8/31/94	St. Mary's	Site 1	8/26/94	10.9
9/6/94	Ocqueoc lower	DNR Launch Site	8/29/94	7.2
9/7/94	Ocqueoc upper	Bridge	8/29/94	7.6
9/7/94	Cheboygan	Trap Site	8/29/94	7.6
9/7/94	Holding water w/yeast	quaculture 150 ammo	9/7/94	2
9/8/94	St. Mary's	Site 1	8/25/94	10.6
9/9/94	Water and yeast	St.Paul well water	9/9/94	6
9/9/94	Water blank	St. Paul well water	9/9/94	6
9/13/94	Water and yeast	St. Paul well water	9/13/94	6
9/13/94	Water blank	St. Paul well water	9/13/94	6
10/3/94	Upper Ocqueoc	Bridge	9/27/94	8.25
10/3/94	Lower Ocqueoc	DNR Launch	9/27/94	8.6
10/4/94	Cheboygan	Trap	9/27/94	7.3
11/2/94	Cheboygan	Trap	10/25/94	7.4
11/7/94	Lower Ocqueoc	DNR Launch	10/25/94	7.7
12/5/94	Cheboygan	Trap Site	11/30/94	8
12/6/94	Lower Ocqueoc	DNR Launch	11/30/94	7.6
12/7/94	Upper Ocqueoc	Bridge	11/30/94	7.8

Table 2

Petromyzonol Sulfate Concentration in River Water from Various Sites

RIVER	SITE	COLLECTION DATE	PETROMYZONOL SULFATE
Cheboygan	Trap site	5/18/94	N.D.[2]
Ocqueoc	Launch site	5/18/94	N.D.
Grand Lake	Outlet	5/18/94	N.D.
St. Mary's	Site 1[1]	6/10/94	3.50×10^{-10} mol/L
Ocqueoc	Bridge	6/29/94	0.89×10^{-10} mol/L
St. Mary's	Site 1	7/21/94	0.53×10^{-10} mol/L
St. Mary's	Site 1	9/19/95	1.80×10^{-10} mol/L
St. Mary's	Site 2	9/19/95	N.D.
St. Mary's	Site 3	9/19/95	N.D.
St. Mary's	Site 4	9/19/95	N.D.
St. Mary's	Site 5	9/19/95	N.D.
St. Mary's	Site 6	9/19/95	N.D.
St. Mary's	Site 7	9/19/95	N.D.
Brule	Unknown	11/22/95	N.D.

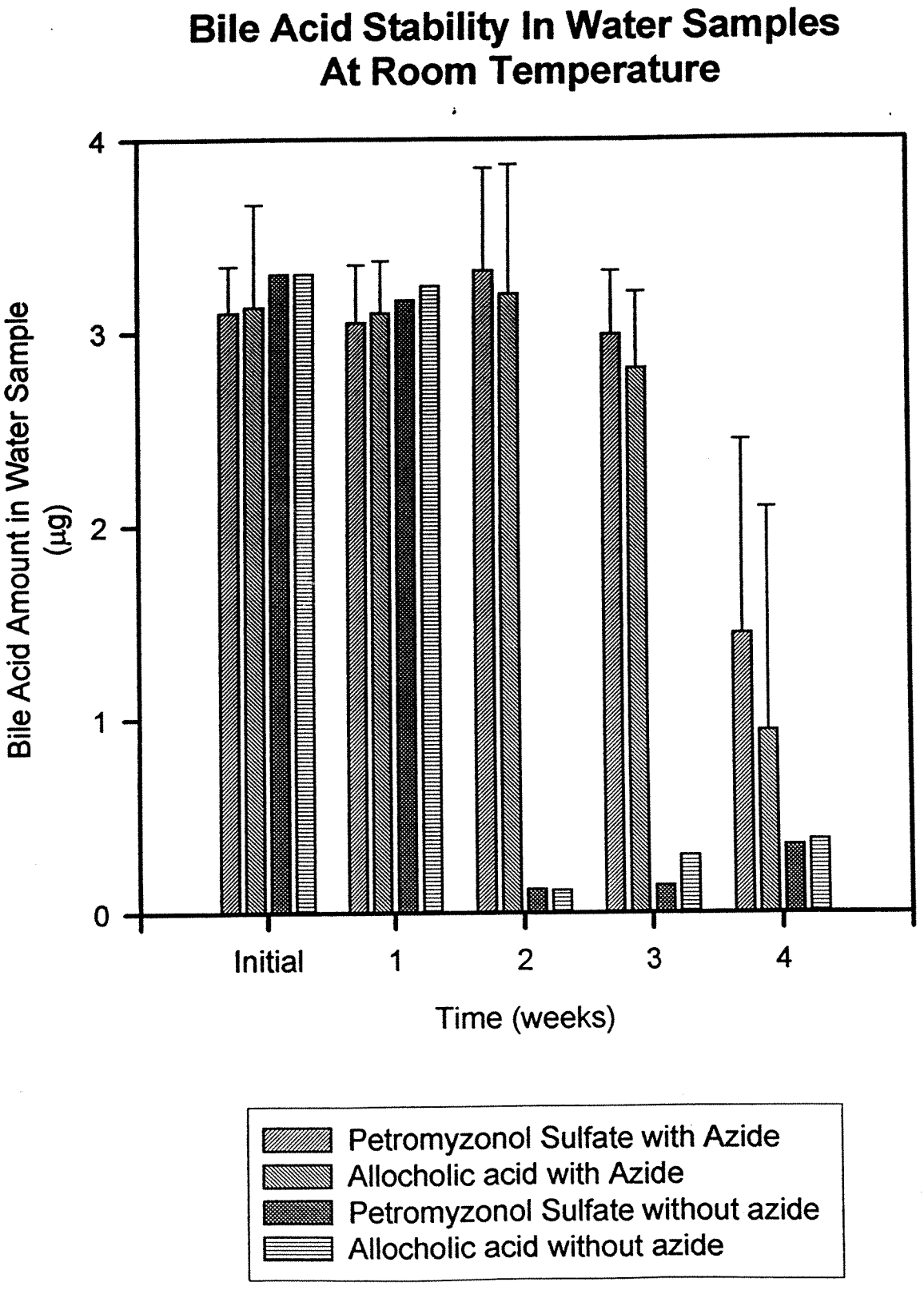
[1] Denotes different transects in the river.

[2] N.D., not detected. Threshold of detectability $\geq 2.0 \times 10^{-11}$ mol/L from 2 L of river water.

TABLE 3: BILE ACID CONTENT IN LAMPREY LIVERS AND GALL BLADDERS.

Life stage	Number examined	Avg. gall bladder weight (mg)	Gall bladder bile acids (mean±SD)			Avg. Liver weight (mg)	Liver bile acids (means±SD)		
			µg/gall bladder ACA	µg/gall bladder PS	µg/gall bladder Pet		µg/mg tissue ACA	µg/mg tissue PS	µg/mg tissue Pet
Larvae	3	4.17	3.6±.90	529.7±155.6	1.9834±.846	12.07	0.092±.158	14.39±22.38	0.033±.047
Stage 1	3	unknown	<0.1µg/mg	8.88±8.14	1.154±.814	not examined	not examined	not examined	not examined
Stage 4	3	NA	NA	NA	NA	approx. 18	<0.1µg/mg	<0.1µg/mg	<0.1µg/mg
Parasitic	4	NA	NA	NA	NA	2225	<0.1µg/mg	<0.1µg/mg	<0.1µg/mg
Adult - F	5	NA	NA	NA	NA	3282	<0.1µg/mg	<0.1µg/mg	<0.1µg/mg
Adult - M	4	NA	NA	NA	NA	4231	<0.1µg/mg	<0.1µg/mg	<0.1µg/mg

Approximately 20% of the parasitic livers were extracted, with half of that amount being injected onto the HPLC.
Approximately 14% of the adult livers were extracted, with half of that amount being injected onto the HPLC.


Bile Acid Stability In Water Samples
At Room Temperature
Bile Acid Amount in Water Sample
(µg)
4
3
2
1
0
Initial
1
2
3
4
Time (weeks)
Petromyzonol Sulfate with Azide
Allocholic acid with Azide
Petromyzonol Sulfate without azide
Allocholic acid without azide

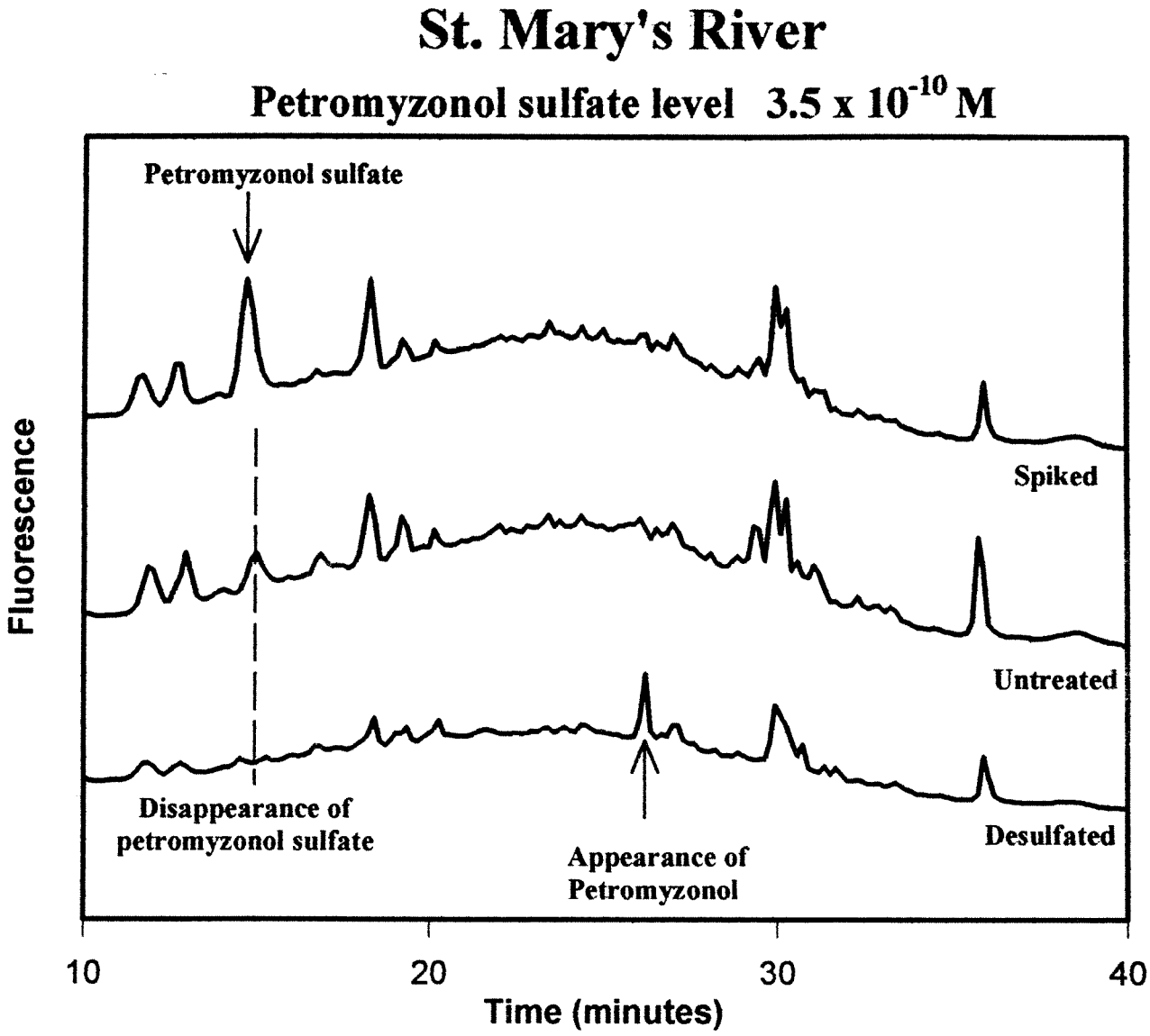


Fig. 2A. Chromatogram of River Waters showing Petromyzonol Sulfate Peak. Dansyl hydrazine technique was used and bile acid confirmed by desulfation.

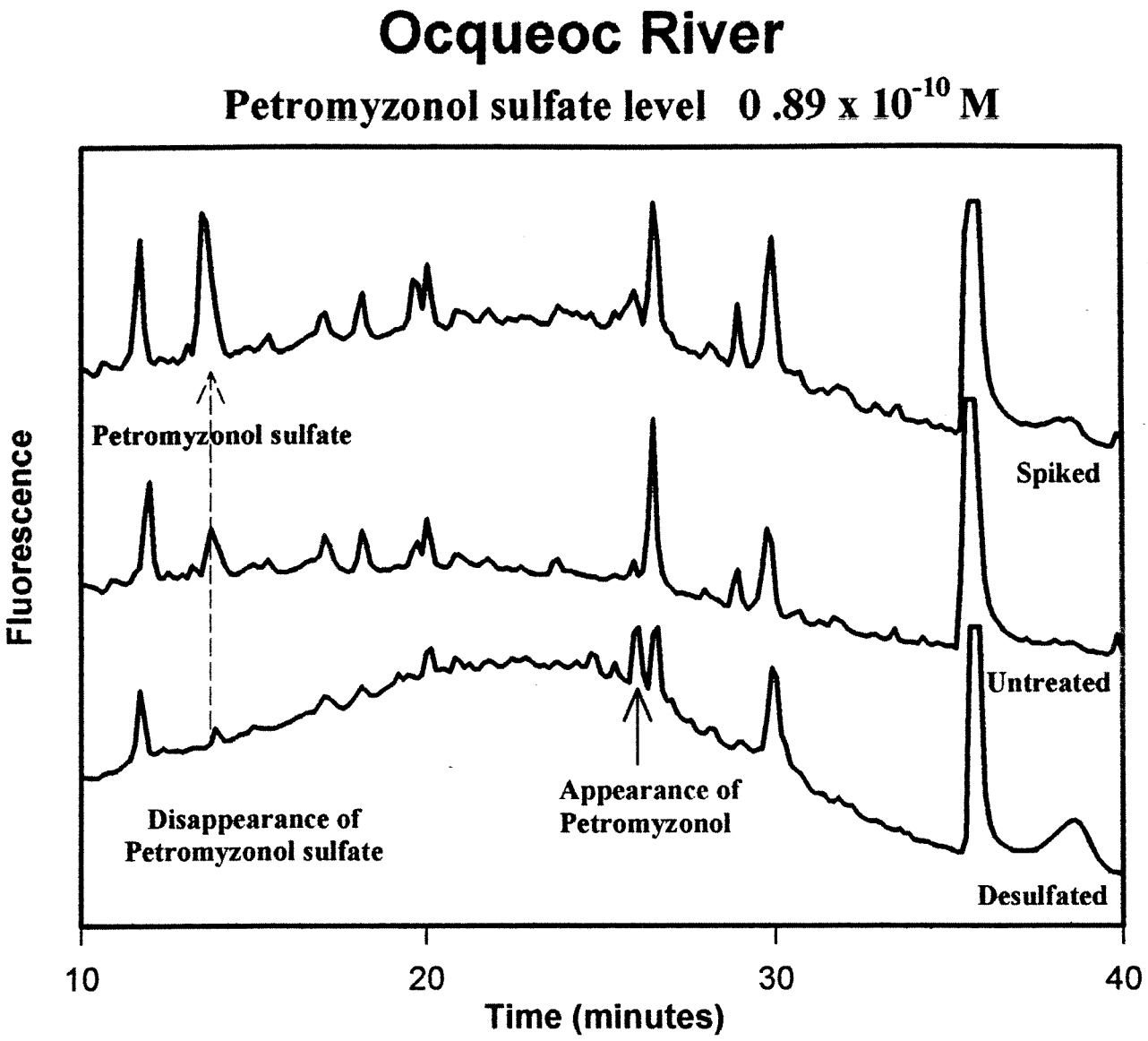


Fig.2B. Chromatogram of River Water with Bile Acid

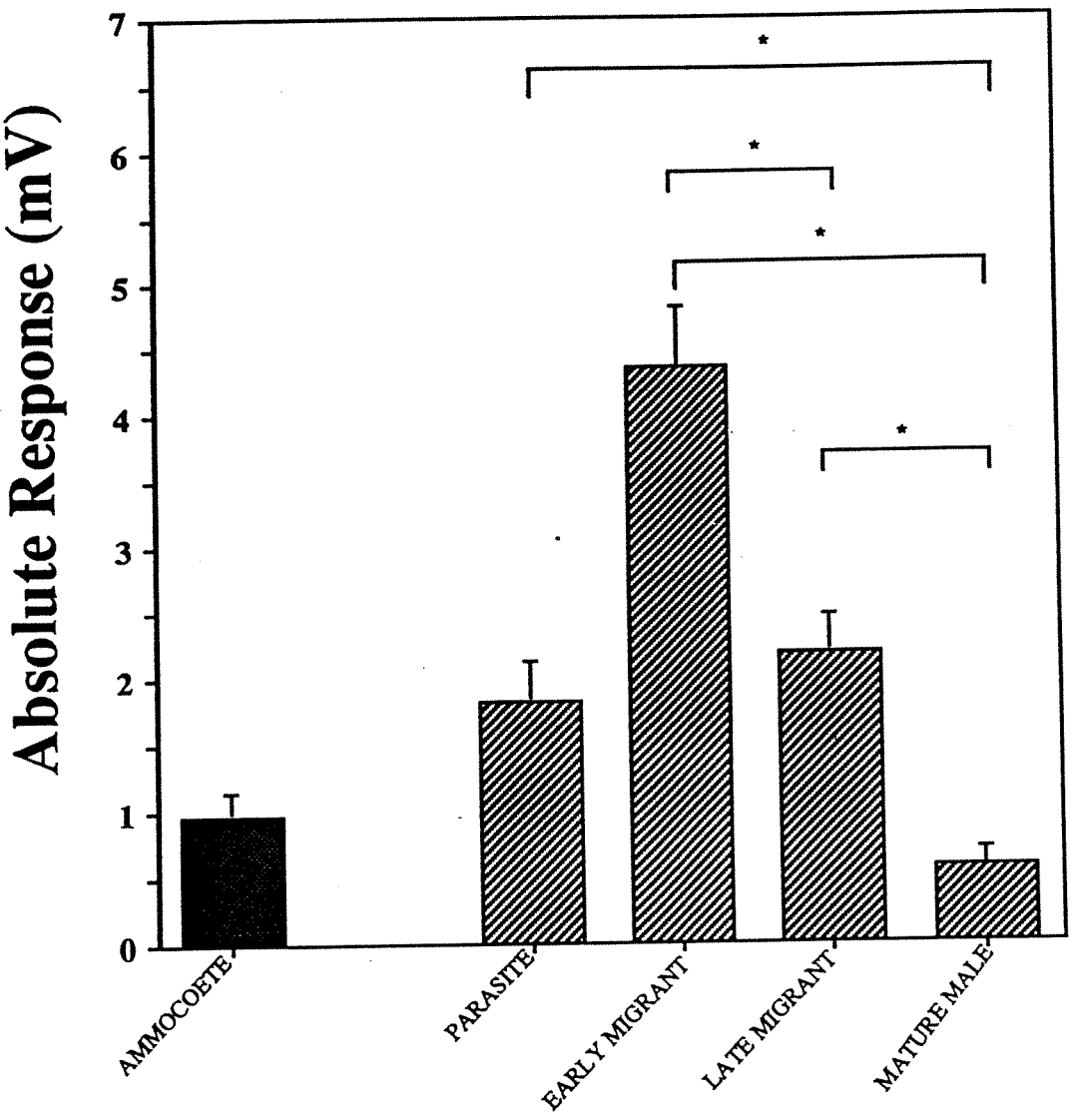


Figure 3. Olfactory responsiveness (EOG) of different life stages of sea lamprey to -5 M arginine (if compared, responses of ammocoetes is significantly less than that of early migrants). * $P<0.05$

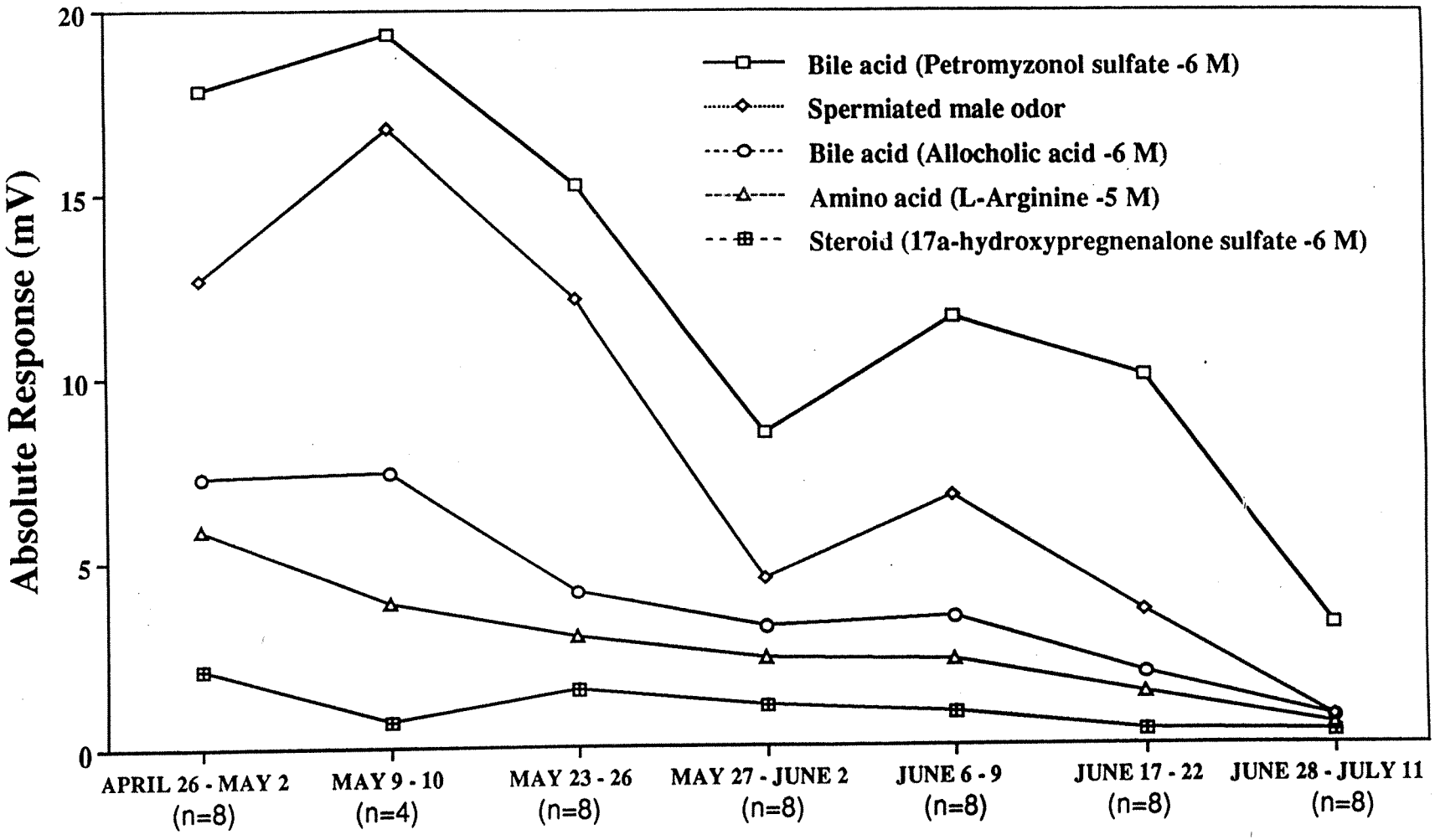


Figure 4. Olfactory responsiveness (EOG) of migratory sea lamprey from the Ocqueoc River to various synthetic and natural compounds at different stages of their spawning migration.

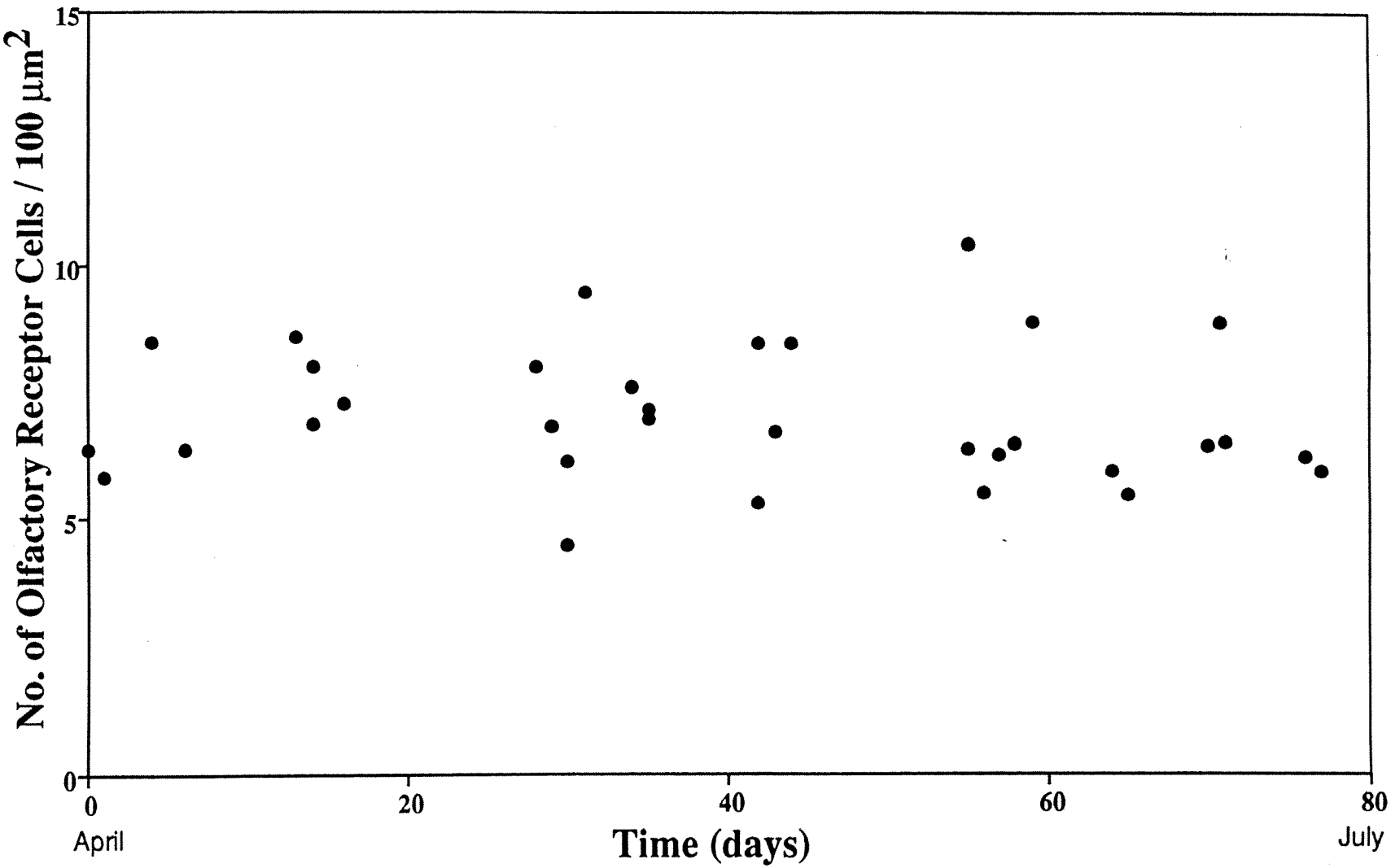


Figure 5. Density of sea lamprey Olfactory Receptor Cells / 100 μm^2 at different stages of their spawning migration. The animals were caught in the Ocqueoc River and were tested first for olfactory responsiveness (EOG) to various synthetic and natural compounds.

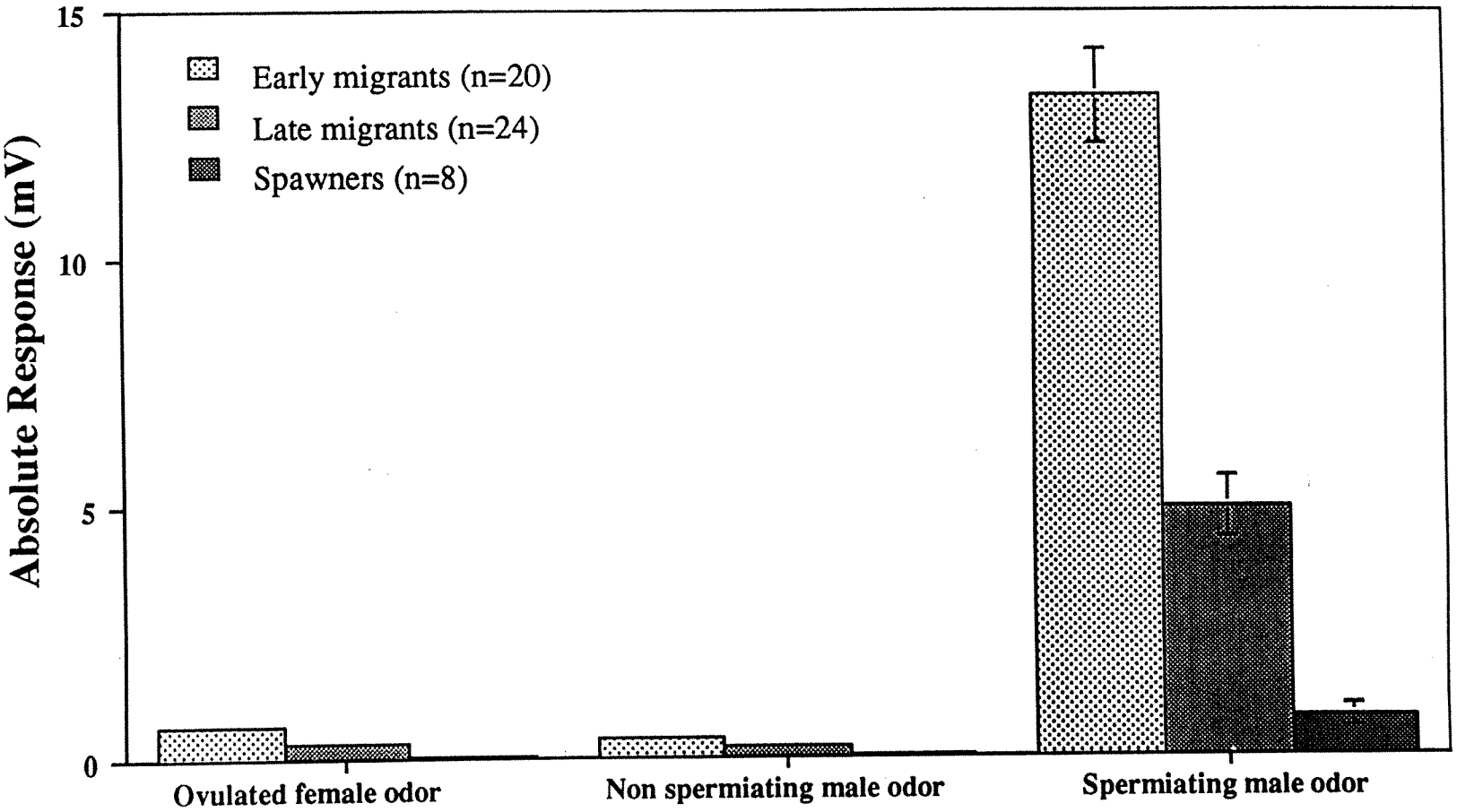


Figure 6. Olfactory responsiveness (EOG) of migratory sea lamprey to conspecific odors (note the high response to spermiating male odor) at different stages of their spawning migration.

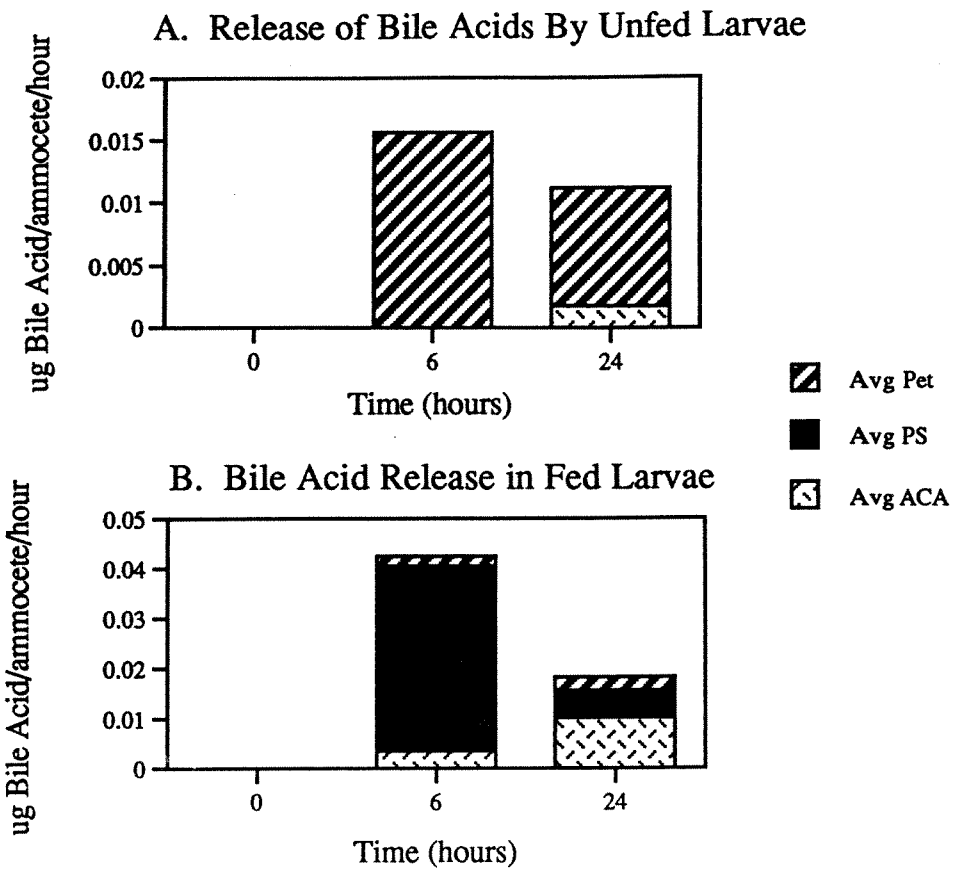
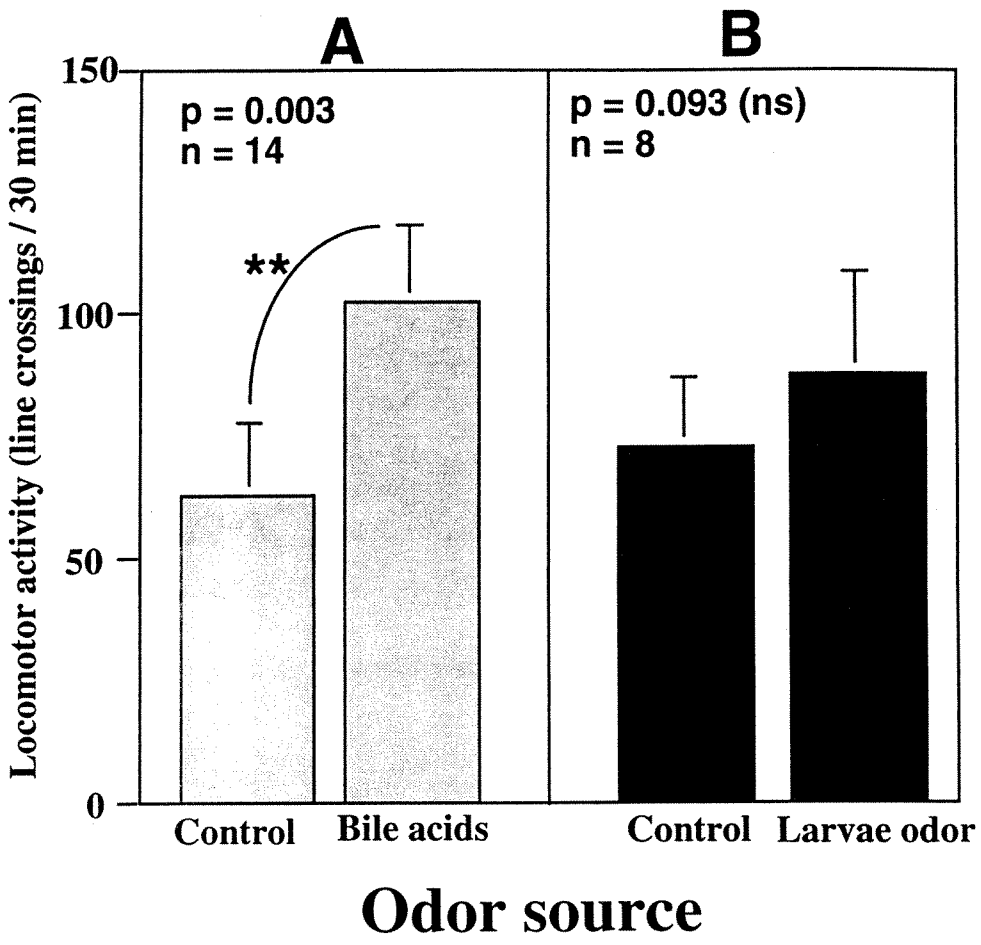


Figure 7. Bile acid release rates of A) starved, and B) fed sea lamprey larvae

(Pet= petromyzonol; PS= Petromyzonol sulfate; ACA=allocholic acid)



The Swimming Activity of Migratory Sea Lamprey is Stimulated by Petromyzonol sulfate and Cholic acid in Still Water

Figure 8. Behavioral responses of adult lamprey to: A) bile acids and, B) larval odor in still water

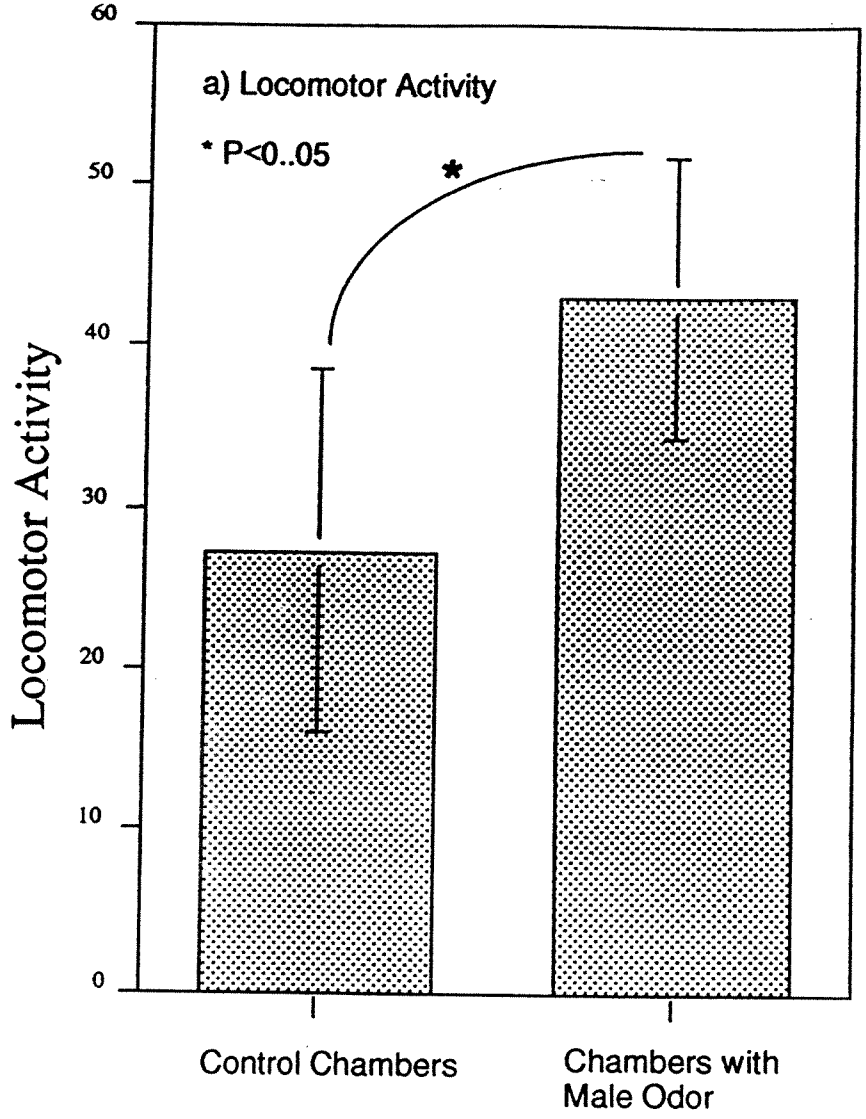


Figure 9.

The Swimming Activity of Female Lamprey is Stimulated by Exposure to the Odor of Spermiated Males

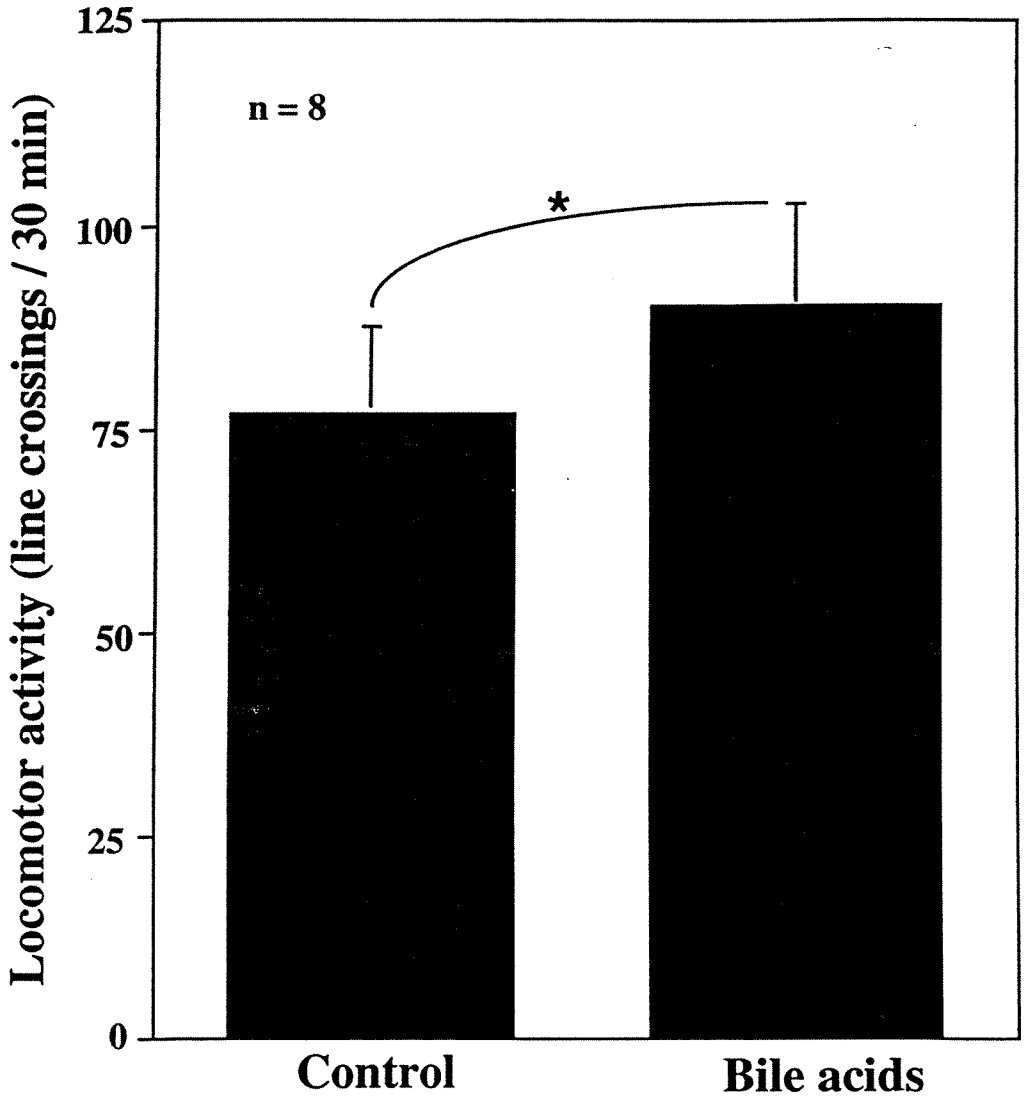


Figure 10. Behavioral responses of adult lamprey to bile acids in flowing water