GREAT LAKES FISHERY COMMISSION
Research Completion Report *

DETERMINING WHY THE SEA LAMPREY OLFACTORY SYSTEM IS EXTREMELY SENSITIVE TO BILE ACIDS: ARE BILE ACIDS PHEROMONES?

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Project Title: DETERMINING WHY THE SEA LAMPREY OLFACTORY SYSTEM IS EXTREMELY SENSITIVE TO BILE ACIDS: ARE BILE ACIDS PHEROMONES?

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Overall Project Objective: To determine whether bile acids, which we have found to be extremely potent olfactory stimulants for the sea lamprey, function as migratory pheromones for this species.

INTRODUCTION:

A preponderance of evidence strongly suggests that olfactory cues play a fundamental role in the life cycle of the sea lamprey, Petromyzon marinus. First, histological examination has clearly shown that the lamprey olfactory system is extremely well-developed and is likely the dominant sensory system in this species (Kleerekoper 1972). Second, where studied in fish, olfaction has been found to play critical roles in the recognition of food, spawning sites, and mates (Hara 1992). Finally, the few studies which have been conducted on olfactory function in sea lamprey all strongly support the possibility that lamprey also use their olfactory system to find food, mates, and spawning streams. The objective of our study was to determine what olfactory (chemical) cues might be functioning as migratory cues used by adult lamprey to locate spawning streams.

There is excellent reason to believe that adult lamprey employ chemical cues emanating from conspecifics ('migratory pheromones') to locate spawning streams. Adult lamprey tend to enter particular river systems for spawning and when these systems are treated with TFM, spawning runs in these systems frequently drop in subsequent years suggesting that the larvae release a migratory pheromone (Moore and Schleen 1980). Furthermore, preliminary behavioral experiments conducted by Teeter (1980) have shown that dilute washings of ammocoete larvae are attractive to adults (Teeter 1980). It would appear to make sense for an anadromous species to use the odor of its own young as a migratory cue, for it signals the presence of suitable spawning/larval habitat . Migratory pheromones have been postulated for many species of fish (Smith 1985) with the strongest evidence being for Arctic charr, Salvelinus alpinus. Adult Arctic charr have been observed both to stray into newly stocked streams which were previously barren of fish (Nordeng 1971) and to be attracted to conspecific washings in Y-mazes (Selset and Doving 1980). Bile acids are detected by the Arctic charr olfactory system with great sensitivity (threshold 10^{-11} Molar) and have been suggested to function as the migratory pheromone (Doving et al. 1980) because of their species-specific nature, chemical stability, and the great quantity in which they are produced.

If the hypothesis that bile acids function as fish migratory cues is correct it should be true in the lamprey. Larval lamprey, which persist in the streams for extended periods, contain large quantities of a single, unique bile steroid in their gallbladders. This steroid, 3α,7α,12α-trihydroxy-5α-cholane- 24-sulfate, named 'petromyzonol sulfate' by its discoverer Professor Haslewood (1969), differs from all other bile acids because of its 5α-configuration, 24 carbons, and the presence of a sulfate group at the 24 position. Accordingly, the principal objective of the present study is to determine whether this bile acid is released to the water to function as migratory pheromones for this species. As a first step in fulfilling this objective we have spent the past two years performing studies directed towards meeting the following sub-objectives:

Year 1 (1992):

1) To chemically characterize bile acid metabolism and release in sea lamprey to determine which particular bile acids could serve as a species-specific pheromone.
2) To synthesize 3α,7α,12α-trihydroxy-5α-cholane-24-sulfate ('petromyzonol sulfate'), a unique bile acid isolated 20 years ago in ammocoete larvae, both to confirm the chemical analysis described above and to provide adequate material for testing as a pheromone in Year 2.

Year 2 (1993):

3) To determine the olfactory potency of petromyzonol and other authentic lamprey bile acids to determine whether lamprey detect them and to establish at what concentrations and mixtures they should be tested for pheromonal function.

4) To determine whether/how bile acids influence the behavior of adult lamprey.

Sub-Objective 1: To chemically characterize bile acid metabolism and release in sea lamprey to determine which particular bile acids could serve as a species-specific pheromone.

Methods:

Larval and adult sea lamprey were shipped live from Hammond Bay Biological Station (courtesy of Dr. Seelye) to Minnesota where the animals were sacrificed and their gall bladders (ammocoetes only), livers, and intestines dissected out and preserved in ethanol. Liver and intestine samples were lyophilized and sequentially extracted with ethanol/ chloroform:methanol and then partially purified using C18 solid phase extraction cartridges (Bakerbond SPE, NJ). Larval shipping water (50 larvae in 8 L for 24 h)was passed through a paper filter and then extracted with C_{18} solid phase extraction cartridges. A 6-L water sample collected from the St. Mary's River in July 1992 was extracted in the same manner. A sample of 'pheromonally active' adult male urine was also obtained from J. Teeter (Monell Chemical Senses Center, PA) and extracted using a Sep-Pak. Extracted tissue, urine and water samples were frozen at -80^0 C until they could be analyzed.

HPLC was then be used to quantify bile acids present in extracts. This was accomplished using a reverse-phase Nova-Pak C_{18} 4mm cartridge (5 mm x 10 cm) housed in a radial compression module (Waters Chromatography Division, MA). A step-wise gradient elution system was used composed of two mobile phases, ammonium dihydrogen phosphate (25 mmol/L, pH 7.8) and acetonitrile. Samples were prepared for injection by reconstitution in 0.25 mL methanol containing hyocholic acid as an internal standard. Because bile acids do not absorb UV light well, they were detected by a second column (5 cm x 0.5 cm i.d., Alltech, IL) containing 3α-hydroxysteroid dehydrogenase (50 units, Sigma Chemical Co, MO) mounted on glutaraldehyde-treated aminopropyl glass beads (Sigma) (Marshall 1973). A buffer containing NAD (0.1 mol/L Tris-HCl, pH 8.5, 2.7 mmol/L EDTA, 1.63 mmol/L dithiothreitol and 0.01 mmol/L NAD) was introduced by a tee between the first and second columns at a constant rate of 1 mL/min. NADH

produced by the reaction of bile acids and NAD with the immobilized enzyme is then detected fluorometrically (model 121, Gilson Medical Electronics; narrow hand excitation filter at 340 nm and a wide band emission filter with a range of 420-650 nm). Peak areas were calculated using a chromatography software program (712 System Controller, Gilson). Bile acids in samples were identified by comparison to retention times of authentic bile acids synthesized by Toronto Research Chemicals (see below). Several samples were collected using a fraction collector for olfactory testing after treatment with 3α-hydroxysteroid hydrogenase to restore the configuration of the molecule at the 3-position. These sample were tested for olfactory potency using electro-olfactogram recording (EOG, see below) and their chemical identities confirmed using mass spectrometry.

Results:

Considerable quantities of petromyzonol sulfate and small quantities of allocholic acid and petromyzonol were consistently found in the gall bladders of larval sea lamprey (Fig. 1a). Petromyzonol sulfate was also found in all samples of ammocoete livers and intestines which were examined; however, allocholic acid and petromyzonol were not found in every sample (Fig.1b,c; Table 1). Petromyzonol sulfate was the only bile acid detected in ammocoete urinary tracts (Fig. 1d). However, all three bile acids were discovered in larval holding water and water collected from the St. Mary's River (Fig. 2b). Concentrations in St. Mary's River water were estimated to be approximately 5×10^{-10} Molar. Finally, it appears that adult sea lamprey intestines lack the bile acids found in the larvae: only one peak with a retention time in the vicinity of petromyzonol sulfate was clearly evident in liver and intestine tissue but its elution time clearly differed from that of petromyzonol sulfate (Fig. 3a,b).

Sub-Objective 2. To synthesize 3α,7α,12α-trihydroxy-5α-cholane-24-sulfate ('petromyzonol sulfate').

Methods:

Synthetic samples of lamprey bile acids were obtained from two sources. First, an authentic sample (1 mg) of allocholic acid, the precursor of petromyzonol, was obtained as a gift from Professor Elliot (Medical School, University of St. Louis, MI). Second, we contracted Toronto Research Chemicals to synthesize 100 mg of petromyzonol sulfate. The latter company was chosen because it was willing to undertake the project and because its president, Dr. David Dime, received his Ph.D. under the direction of Dr. McLean at the University of Toronto whose

laboratory synthesized petromyzonol in 1987. Dr. Dime was recommended by Dr. McLean who said he was not interested. Although Toronto Research Chemicals has not been willing to release the details of the synthesis protocols they employed it is likely that they followed those reported by Dr. McLean in Zhu et al. (1987).

Sample identity and purity was confirmed by the University of Minnesota Agricultural Station using a Kratos MS-25 mass spectrometer (Ramsey, NJ). Electron impact (EI) spectra of both larval-derived bile acids and synthetic bile acids were determined at an ionization potential of 70 eV. Preliminary EI mass spectra indicated homogeneity of purified and synthetic bile acids but did not show the mass of molecular ions because the molecules were fragmented. Thus, fast atom bombardment (FAB), a soft ionization technique, was used to further analyze the samples. FAB produces little fragmentation of C-C bonds, resulting in mass spectra that provide an insight to molecular structure. The fast atom beams were generated by xenon atoms which were accelerated to 8 KeV. Glycerol (Sigma) and silver nitrate (Sigma) doped glycerol were used as the matrix.

Results:

The synthesis of petromyzonol proved to be more difficult than expected (for reasons never fully disclosed by Toronto Research Chemicals) and the yield was less than originally hoped. Final yield was 50 mg allocholic acid (ACA; 80% pure), 50 mg petromyzonol (P; 95% pure), and 15 mg petromyzonol sulfate (PS; 95% pure). Mass spectrometry confirmed that the larval-derived bile acids are ACA, PS and P. The EI mass spectra of larval-derived PS and synthetic PS are essentially identical, suggesting that the two samples contained identical molecules (Fig. 4A,B). The FAB mass spectra agrees with the EI spectra but provides more information about the structure. First, the FAB mass spectra of larval-derived ACA and the synthetic ACA were basically identical (Fig. 5 A,B): they all had a MH^+ pseudomolecular ion at m/z 409, a glycerol adducted MH^+ at m/z 501, and a silver adducted M^+ at m/z 515, indicating that they all had a molecular mass at 408, the same as predicted from the formula ($C_{24}H_{40}O_5$). In addition, they all had mass peaks at m/z 391, 373 and 355, corresponding to three neutral water losses during FAB ionization, which is consistent with the original molecules having three hydroxyl groups. Dr. Elliot's ACA showed essentially identical spectra. The FAB spectrum of CA (Fig. 5C) resembled that of ACA but had the base peak at m/z 355. Evidently, CA loses water more readily than ACA, suggesting some small structural or configuration difference. Second, FAB mass spectra of both larval PS and synthetic PS (Fig. 6F,G) were also essentially identical to

each other: they had $[M+H+G]^+$ and $[M+G]^+$ at m/z 567 and 581, indicating a molecular mass at 474, the same as predicted ($C_{24}H_{42}O_7S$). Third, the FAB mass spectra of larval and synthetic P (Fig. 5F,G), again, are essentially identical. The mass peaks at m/z 395, 487 and 501 for MH^+, $[M+H+G]^+$ and $[M+G]^+$, respectively indicate a molecule mass of 394, again, as predicted ($C_{24}H_{42}O_4$). Last and notably, the neutral water loss of both PS and P resembled that of ACA but contrasted with that of CA (Fig. 6 D,E). The PS had the base peak at m/z 439 and two peaks at m/z 457 and 421. Similarly, the P had the base peak at 359 and two peaks at m/z 377 and 341. These suggest that PS and P have three hydroxyls oriented the same to those of ACA but different to those of CA. FAB mass spectra with glycerol as the matrix indicated the same results. It thus appears that all larval bile acids have a similar structure around the steroid nucleus and that the different masses are due to differences in the side chains. In conclusion, we are confident in our identification of petromyzonol sulfate, allocholic acid and petromyzonol in larval sea lamprey and that the samples of these compounds synthesized by Toronot Research Chemical are valid.

Sub-objective 3. To determine the olfactory potency of petromyzonol and other authentic lamprey bile acids to determine whether lamprey detect them and to establish at what concentrations and mixtures they should be tested for pheromonal function.

Methods

The olfactory potency of allocholic acid, petromyzonol, and petromyzonol sulfate isolated from sea lamprey and synthesized by Toronto Research Chemicals were determined using established electro-olfactogram (EOG) recording techniques and adult animals shipped from Hammond Bay Biological Station. Olfactory sensitivity to a variety of other commercially available synthetic mammalian bile acids was also determined. Briefly, lamprey were immobilized with a muscle relaxant (100mg/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 enclosed olfactory sac was then exposed by carefully cutting away the dorso-caudal portion of the sac and olfactory tissue associated with it. The exposed olfactory epithelium was perfused with well water into which 5-sec pulses of test odorants were injected using a specially apparatus which minimizes pressure and temperature fluctuations. Electrical responses were recorded differentially using Ag/AgCl electrodes bridged to saline/gelatin-filled glass pipettes (tip diameter 60-80μm) positioned

just above the olfactory epithelium. Responses were amplified with a DC-preamplifier (Grass Instruments Ic., MA; P16 amplifier), digitized, displayed, and stored on an Apple Macintosh CX computer using a MacLab (WPI systems) data collection and analysis system. Responses were compared to those of our standard, 10^{-5}M L-arginine. At least 3 min separated odorant injections to permit complete recovery and each odorant stimulus was tested three times.

Results

As measured by EOG recording, the olfactory sense of migratory sea lamprey was extremely sensitive and specific to allocholic acid and petromyzonol sulfate produced by larval lamprey. These findings were confirmed using authentic standards. Allocholic acid and petormyzonol sulfate both had detection thresholds of 10^{-12} - 10^{-13} Molar (M) (Fig. 7a). Petromyzonol was detected at 10^{-8} M (Fig. 7a). Taurolithocholic acid sulfate, a mammalian bile acid also detected with good sensitivity by the lamprey olfactory system (Fig.7a). Although nearly 30 other bile acids were tested olfactory potency, no other s were found to be highly stimulatory indicating a high degree of specificity (data not shown). Both cross-adaptation and mixture experiments indicated that the lamprey olfactory system has different olfactory receptor mechanisms for petromyzonol sulfate and allocholic acid (Fig. 7 b,c). The detection threshold of both allocholic acid and petromyzonol sulfate were lower than the concentration of these compounds in the St. Mary's River.

Sub-Objective 4. To determine whether/how bile acids influence the behavior of adult lamprey.

Methods:

We chose to conduct this study in the outlet of Grand Lake, Presque Isle Country, Michigan. This location was selected because it lacks lamprey (i.e. should not have a confounding pheromonal background odor), it is accessible by vehicle, and its flow is normally suitable for our studies. Unfortunately, the spring of 1993 was an extremely wet year and this site experienced extensive flooding so were forced to move to the Black Mallard River, (a site which was treated in 1992 with lampricide) for two weeks of the study. Experiments were conducted using migratory adult lamprey captured by the U.S. Fish and Wildlife Service in the Cheboygan River trap. These animals were transported on

the day of capture and placed into plastic wire holding pens which we constructed to one side of the testing area.

Our initial experiments tested the influence of odors on rheotactic behavior of groups of adult lamprey. To accomplish this upstream swimming activity was monitored using two wooden 'traps' which were anchored next to each other in the stream. Test odor(s) were then pumped/added to one of these traps and control stimuli to the other. The traps were constructed from plywood and measured 4.42m x 1.28 m x 0.60 m (LxWxH) and had three chambers with gates separating each chamber. The downstream chamber (into which lamprey were placed) was connected to the middle chamber via an opening which measured (4 cm x 4cm). The purpose of this opening was to restrict the upstream movement of lamprey to those few individuals which were persistent in their efforts (thereby reducing our measure of random movements). The third (upstream chamber) was connected to the middle chamber by a funnel opening and its upstream end screened to permit water movement through the trap. For testing larval odors, plastic garbage cans with screened sides containing sand with ammocoete larvae or nothing (control) were placed immediately upstream of the trap. For testing odorants, test solution(s) were pumped into the upstream chamber using remote battery operated pumps. Experiments commenced when groups of 10 adult lamprey were placed into the downstream chambers. Trials lasted 10-30 min (initial trial were 30 min long but this was shortened to 10 min) at the end of which lamprey position in the traps was noted and the lamprey removed. Lamprey were tested once and lamprey found in the upstream chamber were counted as having exhibited a positive response. All experiments were conducted between 9PM (sundown) and 3AM (migratory lamprey are nocturnal). Washings of ammocoetes and several synthetic odorants were tested in this manner.

Later (after June 10 1993), the experimental apparatus was altered to test whether odors from sexually mature adults were attractive. This was performed by modifying the traps by adding a single (common) downstream chamber to the downstream end of the trap. The downstream sections of the old traps were then opened up so that lamprey placed into the new compartment could enter either of the two traps (i.e. they were now offered a choice). Experiments were conducted as described above with the exception that animals were now placed into the new section at the start of the experiment and trial duration was shortened to 5 min. Odors of several synthetic odorants, mature adults, and larvae were tested again. Our plan/hope was establish a stable and predictable testing regime in which lamprey exhibited strong responses to larval odors and then test the larval bile acids alone (of which we had only a few mg). Unfortunately, owing to the unpredictable water and weather conditions during the short time that adults were responsive to larval odor, and our

inability to control these conditions at a remote field site, we never had the opportunity to test larval bile acids.

Results:

Rinses of larval lamprey elicited strong rheotactic behavior in adult lamprey at the start of the spawning seasons in early May but by late June these responses were no longer in evidence (Table 2). Behavioral responses of mature males and female to each other's odors were also tested. Males were found to be repelled by the odor of other males and attracted to the odor of female (experiment was successfully repeated; $P<0.05$). Females were not observed to respond to any conspecific washings (Table 3). Several synthetic compounds were also tested during periods of time which should have produced meaningful responses. No responses was observed to L-arginine (an amino acid detected by the lamprey olfactory system with great sensitivity), cholic acid, or taurolithocholic acid sulfate when these compounds tested in late May and early June (Table 4). However, trimethylamine inhibited upstream swimming activity on two of the three occasions it was tested (Table 4). The attractiveness of water-borne testosterone was tested on 6 occasions and found to repel males on two of these occasions (Table 5).

GENERAL CONCLUSIONS:

We have confirmed and extended Haselwood and Tokes (1969) discovery that larval sea lamprey produce the unique bile acid petromyzonol sulfate in great quantity. Additionally, our analyses (which used techniques which are both more sensitive and definitive than those employed by Haslewood 25 years ago) now indicate that in addition to producing large quantities of petromyzonol sulfate, larval lamprey also produce allocholic acid and petromyzonol. Intriguingly, we have discovered that none of these compounds are produced by adult lamprey (which lack gall bladders) suggesting that the production of these cues is life-stage specific. This supports the hypothesis that these cues are migratory pheromones and are not associated with sexual behaviors. We are confident in our identification of these extremely unusual bile acids both because we were able to use modern spectrometric technologies to positively confirm the molecular structures of these compounds, and because the properties of natural lamprey acids corresponded perfectly with synthetic standards synthesized by Toronto Research Chemicals (see below). Ours is

the first definitive biochemical analysis of bile acid production by any fish and it will now be important to extend these findings to other species found in the Great Lakes to fully appreciate how bile acids might be functioning as chemical cues.

In addition to confirming that larval sea lamprey produce bile acids in large quantities we have also demonstrated for the first time in a fish that natural bile acids are released to the water in quantities large enough to be of biological significance. Intriguingly, these compounds are released in the urine of larval lamprey, a condition considered pathological in higher vertebrates. Our discovery of measurable quantities of bile acids in St. Mary's River water is particularly significant because it clearly demonstrates that these cues are present in quantities which migratory lamprey can detect: they meet a fundamental criterion of a migratory pheromone. It is our hope that future research will clarify what physiological and ecological phenomena control the levels of bile acids found in lamprey spawning streams and so that we can assess whether measurement of these compounds could serve as a useful indicator of lamprey abundance to lamprey control. Also, of course, it is essential that the physiological and ecological processes controlling bile acid release be understood if we are to attempt to use bile acids to manipulate the behavior of wild animals by adding these compounds into streams. Research is presently planned to address these questions.

We succeeded in our efforts to have petromyzonol sulfate, allocholic acid, and petromyzonol synthesized: authentic standards are now available for behavioral and olfactory testing. Although chemical synthesis of these compounds proved difficult it is feasible. New techniques such as cell culture of liver cells, extracting livers from native lamprey, or alternative synthetic procedures must now be explored for the production of larger quantities of these compounds in the future. We are presently investigating the second option but have some ideas for the other two.

Using electrophysiological recording we have demonstrated for that the olfactory system of migratory adult sea lamprey is acutely and specifically sensitive to naturally-occurring water-borne bile acids. Sensitivity to petromyzonol sulfate, the principal bile acid produced and released by larval lamprey, is most notable. Both the sensitivity and specificity of the lamprey olfactory system to natural bile acids is extraordinary and supportive of the hypothesis that these cues function as migratory pheromones which could be useful in lamprey control. The sensitivity of the lamprey olfactory system is clearly adequate to detect bile acids which are naturally released by their larvae into streams. Evidence also clearly suggests that allocholic acid and petromyzonol sulfate are detected by different olfactory receptor mechanisms suggesting these cues are individually recognized and could comprise a species-specific pheromone. Future research efforts must now be

expended to determine what the precise behavioral function of these cues is and what other species produce them.

Finally, we have completed pilot behavioral studies of the effects of lamprey odors on the behavior on migrating adults. These experiments produced somewhat mixed results and were greatly impeded by extremely poor weather conditions and the lack of an appropriate testing facility. Although lamprey bile acids were not tested (a result of poor testing conditions and a scarcity of the compounds), we did test the odor of larval lamprey on several occasions and found it to be elicit a migratory response. This appears to confirm Teeter's (1980) earlier findings and because we now know that larval lamprey release bile acids supports the possibility that the latter compounds have an important role in migratory behavior. Interestingly, the odor of conspecific females was attractive to males which were repelled by the odor of other males. These findings also lend support to Teeter's (1980) hypothesis that these animals use sex pheromones. They could important factors in understanding (and controlling) trapping efficiencies of riverine traps and clearly warrant future investigation. We were unable to confirm Teeter's (1980) findings that the odor of mature males is attractive to females. This could be related to maturational state of the females and males tested. Trimethylamine and testosterone appeared to function as repellents. The repulsive actions of the later compound which Teeter (1980) found to be attractive to females, and we have found to lack olfactory potency, is puzzling.

Taken together, our results clearly establish that authentic bile acids produced larval sea lamprey have the potential to functioning as migratory pheromones/attractants. Additional steps which must now be taken to determine the potential of bile acids as cues for use in bio-control are: 1) determining their behavioral actions under controlled (laboratory) conditions; 2) determining whether they are produced by other species of larval lamprey, 3) determining whether other species of fish detect and respond to them, and 4) determining whether olfactory and behavioral sensitivity to these compounds is influenced by season and/or gonadal maturity. These questions will be addressed in the next two years.

PUBLICATIONS AND AWARDS RESULTING FROM THIS RESEARCH

Publications

Li, W.M., P.W. Sorensen, and D. D. Gallaher. 1993. The olfactory system of sea lamprey is highly sensitive and specific to bile acids produced by fish. Chemical Senses 18(5) abstract #158, p.589.

Li, W., Sorensen, P.W. and Gallaher, D.D. 1994. The olfactory system of adult sea lamprey (Petromyzon marinus) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. J. General Physiology (in review).

Presentations which received 'best paper' awards:

Li, W., Sorensen, PW, and Gallaher, DD. 1993. The olfactory system of sea lamprey is highly sensitive to bile acids produced by larval sea lamprey. Minnesota Chapter of the American Fisheries Society (best paper award)

Li, W., Sorensen, PW, and Gallaher, DD. 1993. The olfactory system of sea lamprey is highly sensitive and specific to bile acids produced by fish. XVI Annual meeting of the Association for Chemoreception Senses, Sarasota, FL. (Don Tucker Memorial Award for best student paper)

Li, W., Sorensen, PW, and Gallaher, DD. 1993. Unique bile acids of larval lamprey may offer an alternative way to control sea lamprey in the Great Lakes. Annual Meeting of the American Fisheries Society, Portland, Oregon. (Best student paper)

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2444-2447.

TABLE 1. Bile acid concentrations in gallbladder, liver, and intestine of larval lamprey:

Petromyzonol sulfate	Gallbladder (mg/g)	Liver (mg/g)	Intestine (mg/g)
Ammocoete #1	49.9	2.0	0.03
Ammocoete #2	42.6	1.1	0.24
Ammocoete #3	35.5	0.66	0.06
Allocholic acid			
Ammocoete #1	1.4	0.12	0
Ammocoete #2	5.4	0	0
Ammocoete #3	0.3	0	0
Petromyzonol			
Ammocoete #1	0.77	0	0
Ammocoete #2	0.44	0	0.09
Ammocoete #3	0.38	0.11	0.02

TABLE 2. Behavioral responsiveness of migratory sea lamprey to the odor of larvae

Date:	5/14	6/2	6/22*
Expt Site:	Black Mallard	Grand Lake	Grand Lake
Sample Size:	14	28	16
P values:	<0.001	<0.05	not sig.
GSI(%):	13.81%	17%	24.73%
Temp(^{O}C):	8-12	12-15	20-21

sample size: n = groups of 10 lamprey

GSI: gonado-somatic index (gonad weight/body weight)

* Note: This was a preference test, prior to this time rheotaxis was tested.

TABLE 3. Behavioral responsiveness of migratory sea lamprey to the odor of adults (These were preference tests)

Date	Odor source	Test Subjects	Sample size	P-value
6/14	Females	Males	20	not sig.
6/14	Females	Females	20	not sig
6/14	Males	Males	20	<0.05 Repulsive
6/19	Males	Females	10	<0.05 Attractive
6/14	Males	Females	20	not sig.
6/20	Males	Females	10	<0.05 Attractive

TABLE 4. Behavior responsiveness to some synthetic compounds

Date	Compound	(log M)	Sample size	Response
5/29	Cholic Acid	-9	4	not sig
5/29	Cholic Acid	-8	4	not sig
6/7	L-arginine	-7	16	not sig
6/9	Trimethylamine	-7	6	$p<0.05$, Repulsive
5/30	Taurolithocholic Acid sulfate	-9	4	not sig
6/7	L-arginine	-7	16	not sig
6/9	Trimethylamine	-7	6	$p=0.031$, Repulsive
6/10*	Trimethylamine	-7	6	not sig
6/10*	Taurolithocholic Acid sulfate	-10	3	not sig
6/10*	Cholic Acid	-8	6	not sig
6/11*	Taurolithocholic Acid sulfate	-10	6	not sig

* preference test (prior to these tests, experiments examined rheotaxis)

TABLE 5. Behavioral responsiveness to testosterone (Preference Test)

Date	Subjects	log M	Sample size	p-value
6/16	Male	-11	10	Not sig
6/16	Male	-10	10	< 0.001
6/18	Male	-10	10	<0.01
6/16	Male	-9	10	Not sig
6/18	Male	-9	10	Not sig
6/16	Female	-10	10	Not sig

Figure 1a. Representative chromatogram of bile acids extracted from larval lamprey gallbladder. The peak at 59 minutes is petromyzonol sulfate, the peak at 89 minutes is petromyzonol, and the peak at 32 minutes is allocholic acid. The internal standard peak (hyocholic acid) is at 24 minutes.

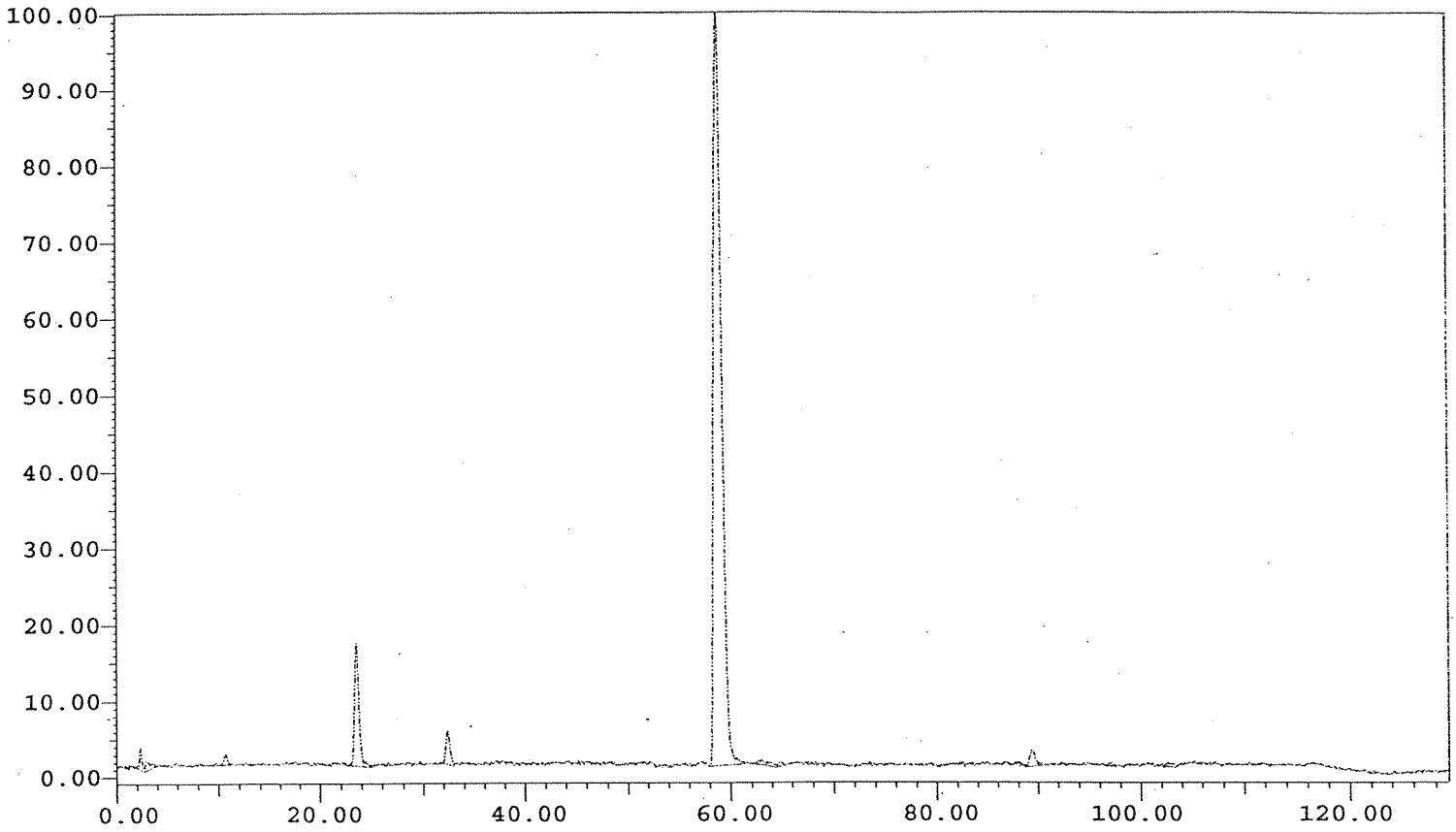


Figure 1b. Representative chromatogram of bile acids extracted from larval lamprey liver. The peak at 59 minutes is petromyzonol sulfate and the peak at 32 minutes is allocholic acid. There was no petromyzonol detected in this sample. The internal standard peak (hyocholic acid) is at 24 minutes.

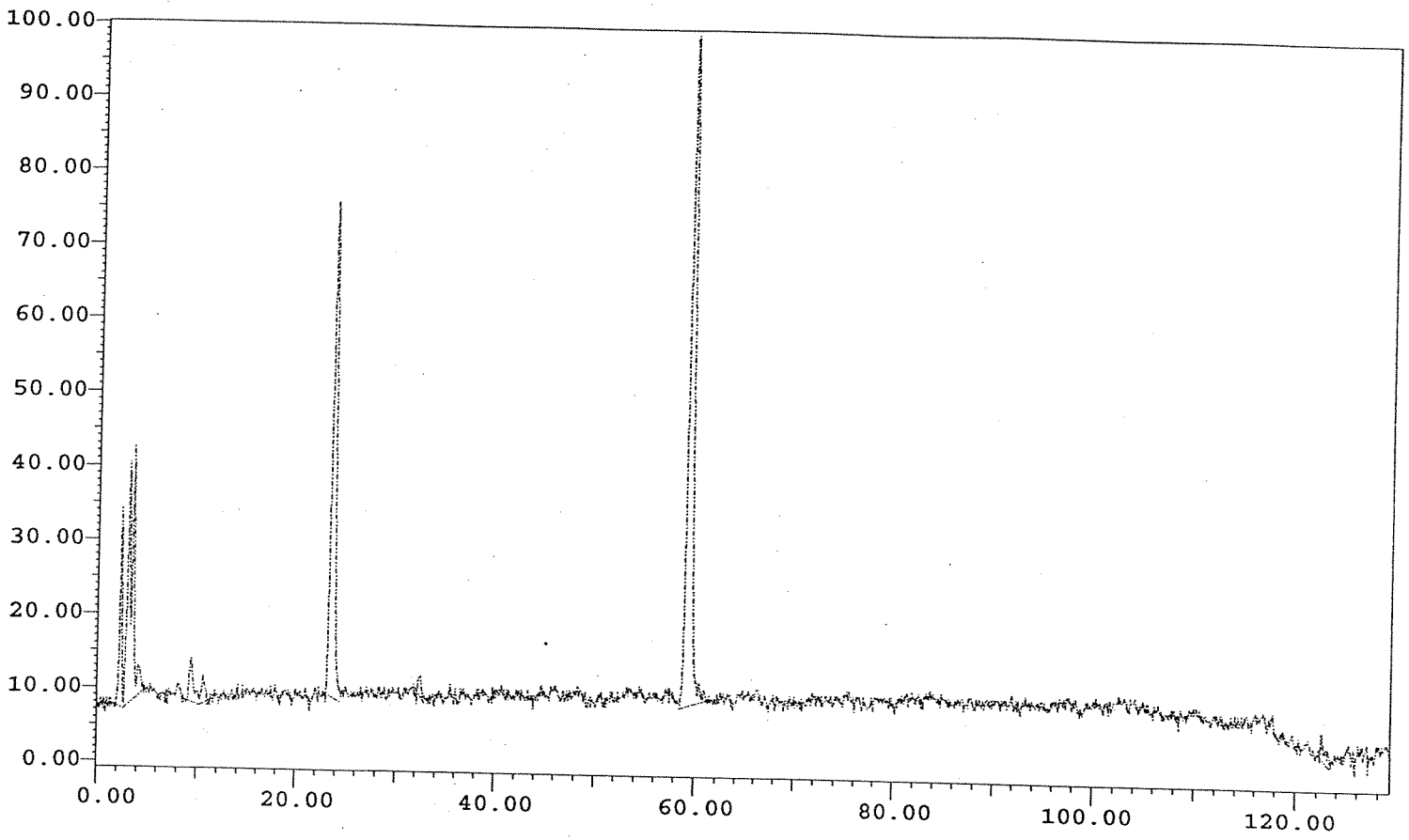


Figure 1c. Representative chromatogram of bile acids extracted from larval lamprey intestine. The peak at 59 minutes is petromyzonol sulfate, the peak at 89 minutes is petromyzonol, and the peak at 32 minutes is allocholic acid (barely detected). The peaks at 42 minutes and at 92 minutes are unknown and may represent bacterial degradation products of petromyzonol sulfate. The internal standard peak (hyocholic acid) is at 24 minutes.

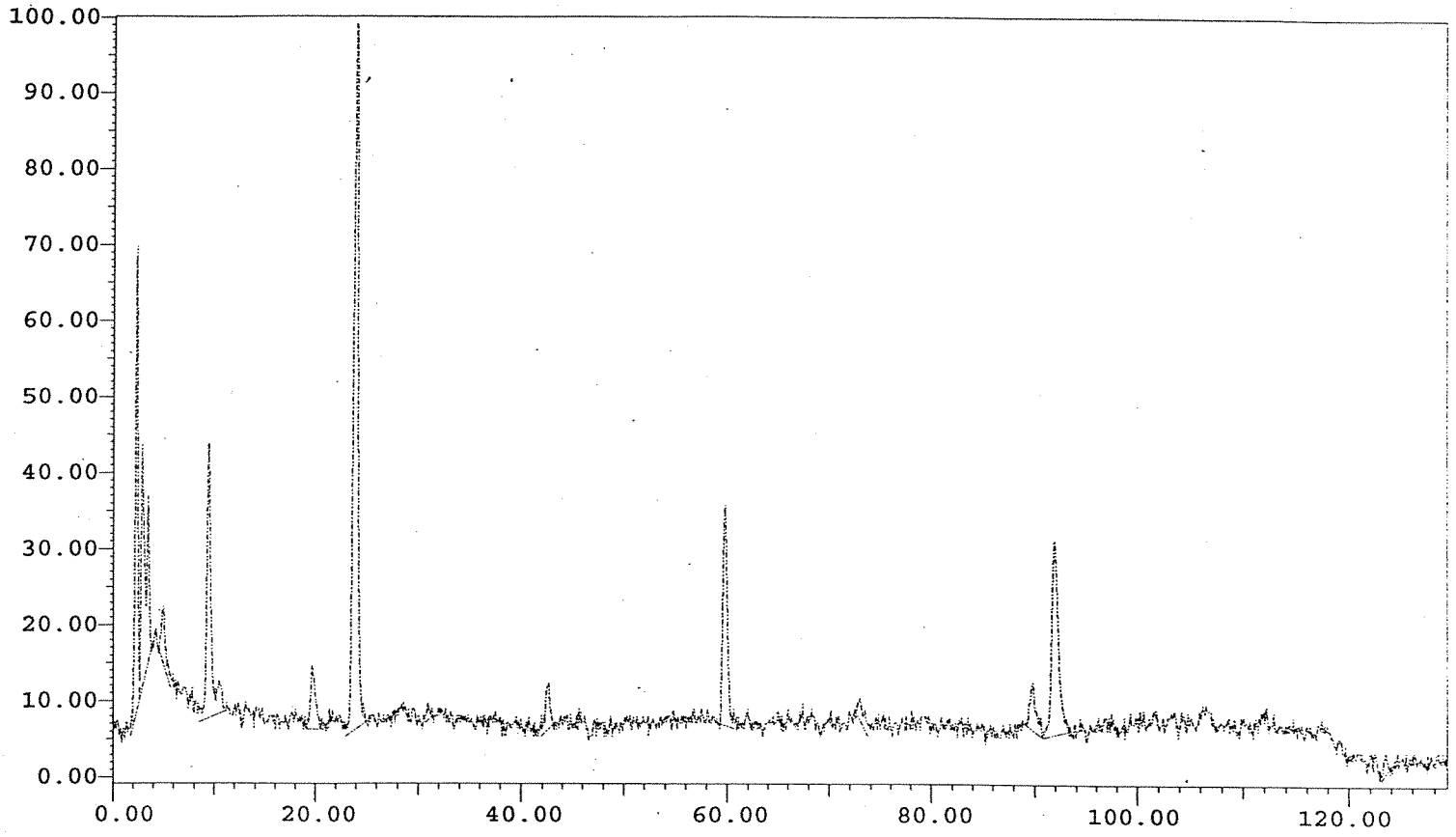


Figure 1d. Chromatogram of bile acids extracted from a pool of larval lamprey urinary tracts. The peak at 99 minutes is petromyzonol sulfate. The peaks at 42 minutes and 133 minutes may be allocholic acid and petromyzonol, respectively. The internal standard peak (hyocholic acid) is at 30 minutes. The bile acids have different retention times than in Fig. 1a-c because a different mobile phase gradient was employed.

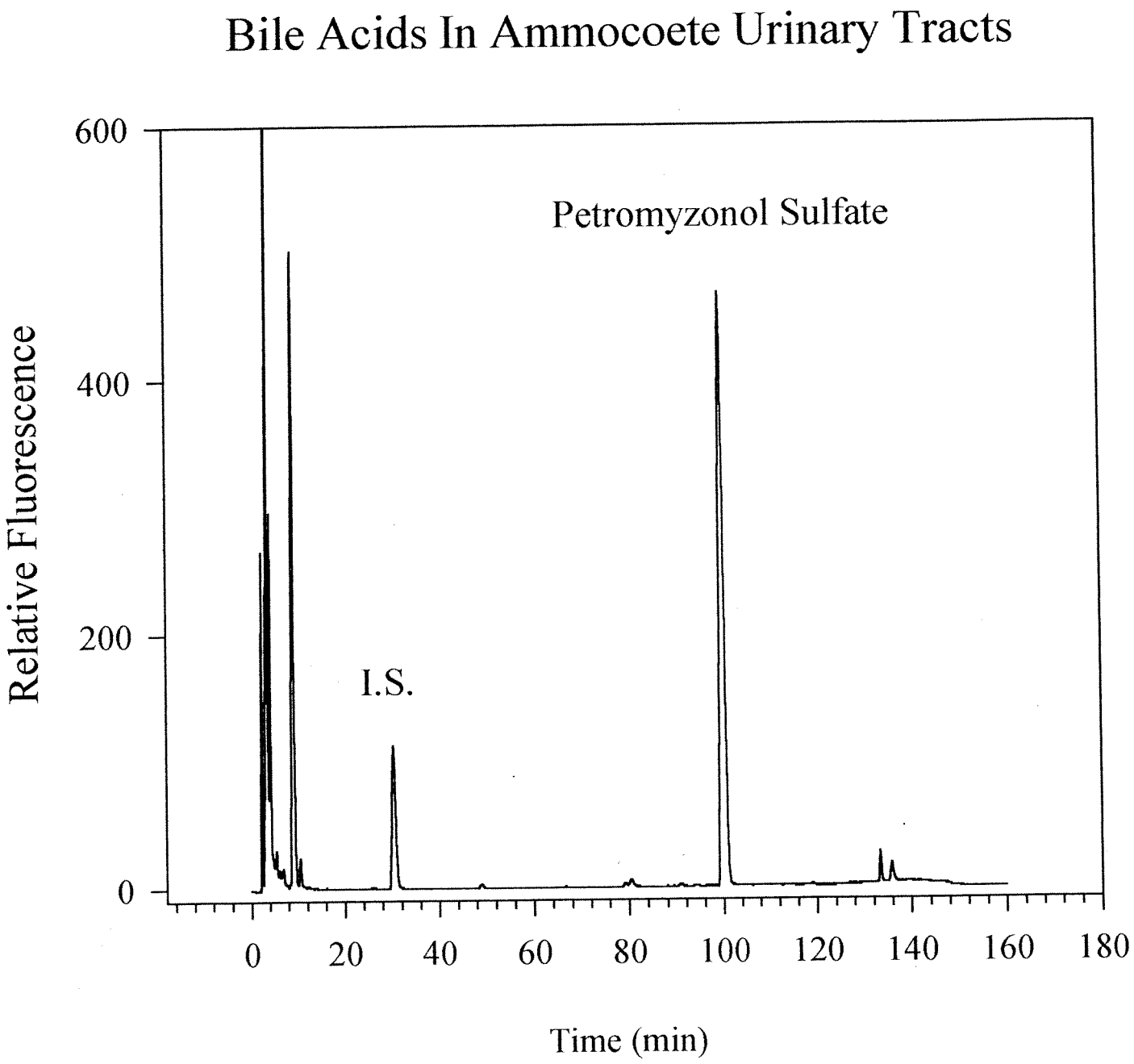


Figure 2a. Bile acids present in larval lamprey holding water. The peak at 99 minutes is petromyzonol sulfate. The peaks at 42 minutes and 133 minutes may be allocholic acid and petromyzonol, respectively. The bile acids have different retention times than in Fig. 1a-c because a different mobile phase gradient was employed.

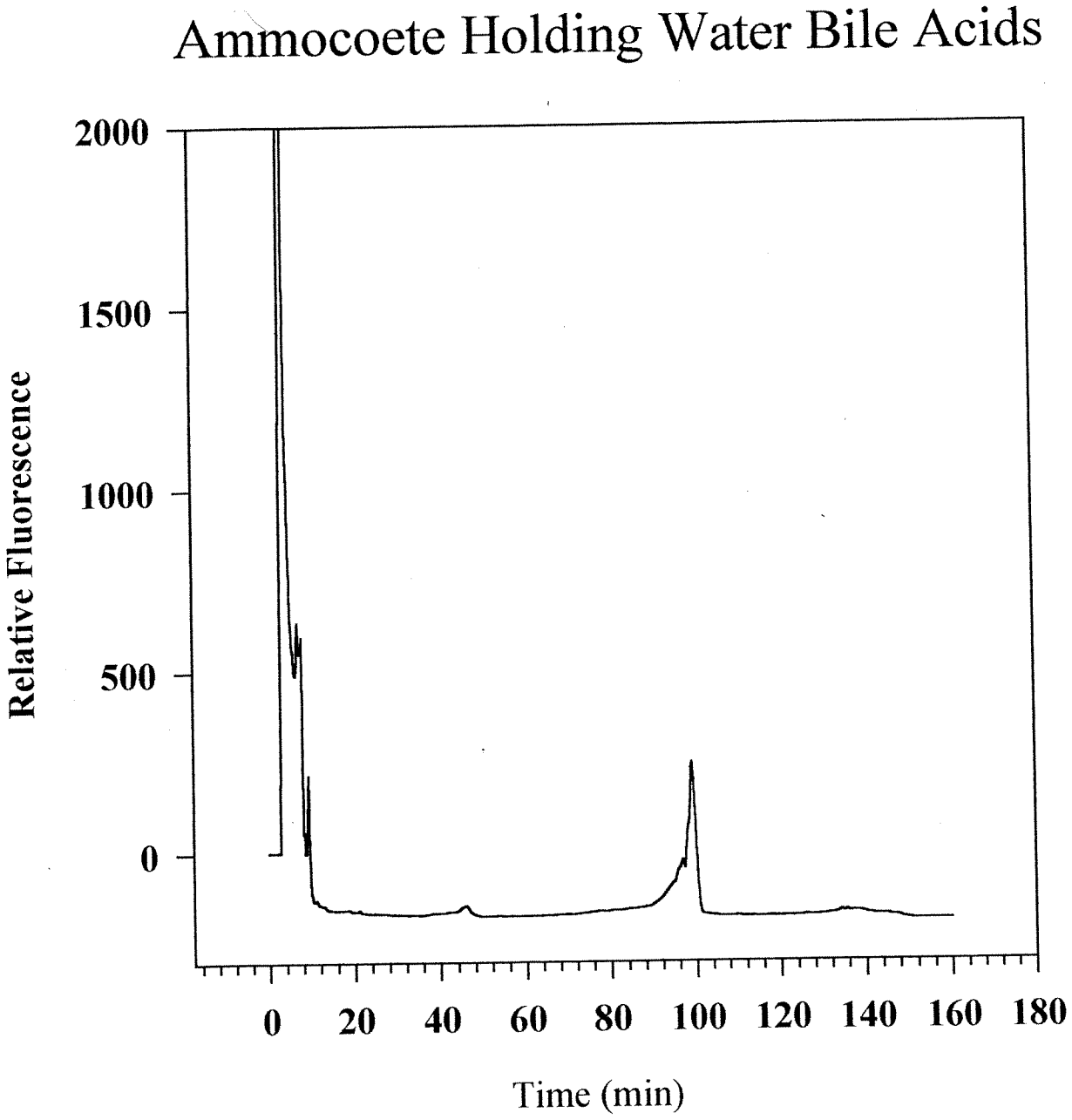


Figure 2b. Bile acids present in St. Mary's River water. The peak at 99 minutes is petromyzonol sulfate. The peak at 42 minutes is allocholic acid. The peak at 133 minutes may be petromyzonol. The bile acids have different retention times than in Fig. 1a-c because a different mobile phase gradient was employed.

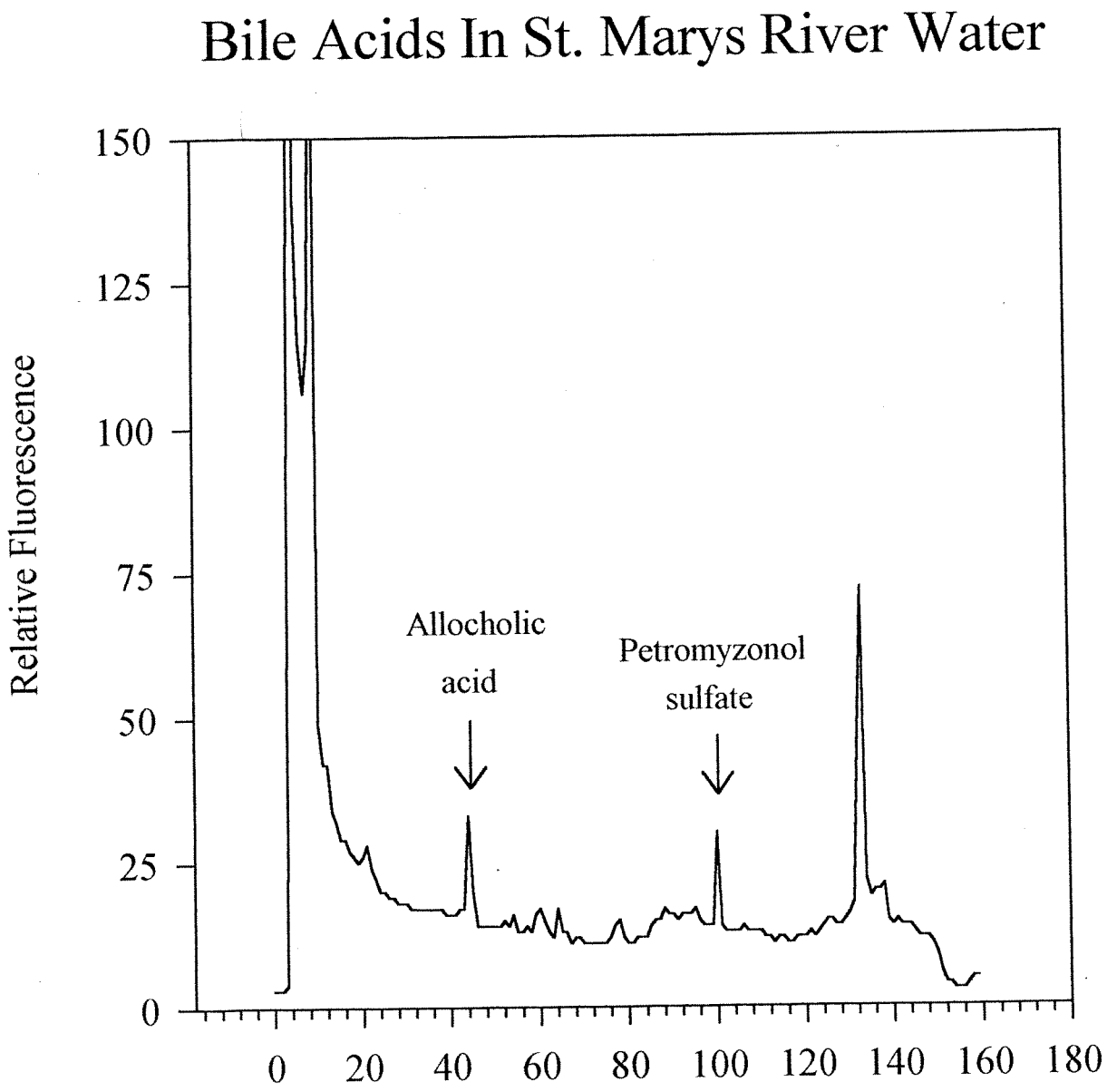


Figure 3a. A representative chromatogram of bile acids in an adult male liver. The peaks found did not correspond to allocholic acid, petromyzonol sulfate, or petromyzonol. The peak at 26 minutes is the internal standard (hyocholic acid). The method of detection will detect any steroid with a 3α-hydroxy group; therefore, the compounds seen are not necessarily bile acids.

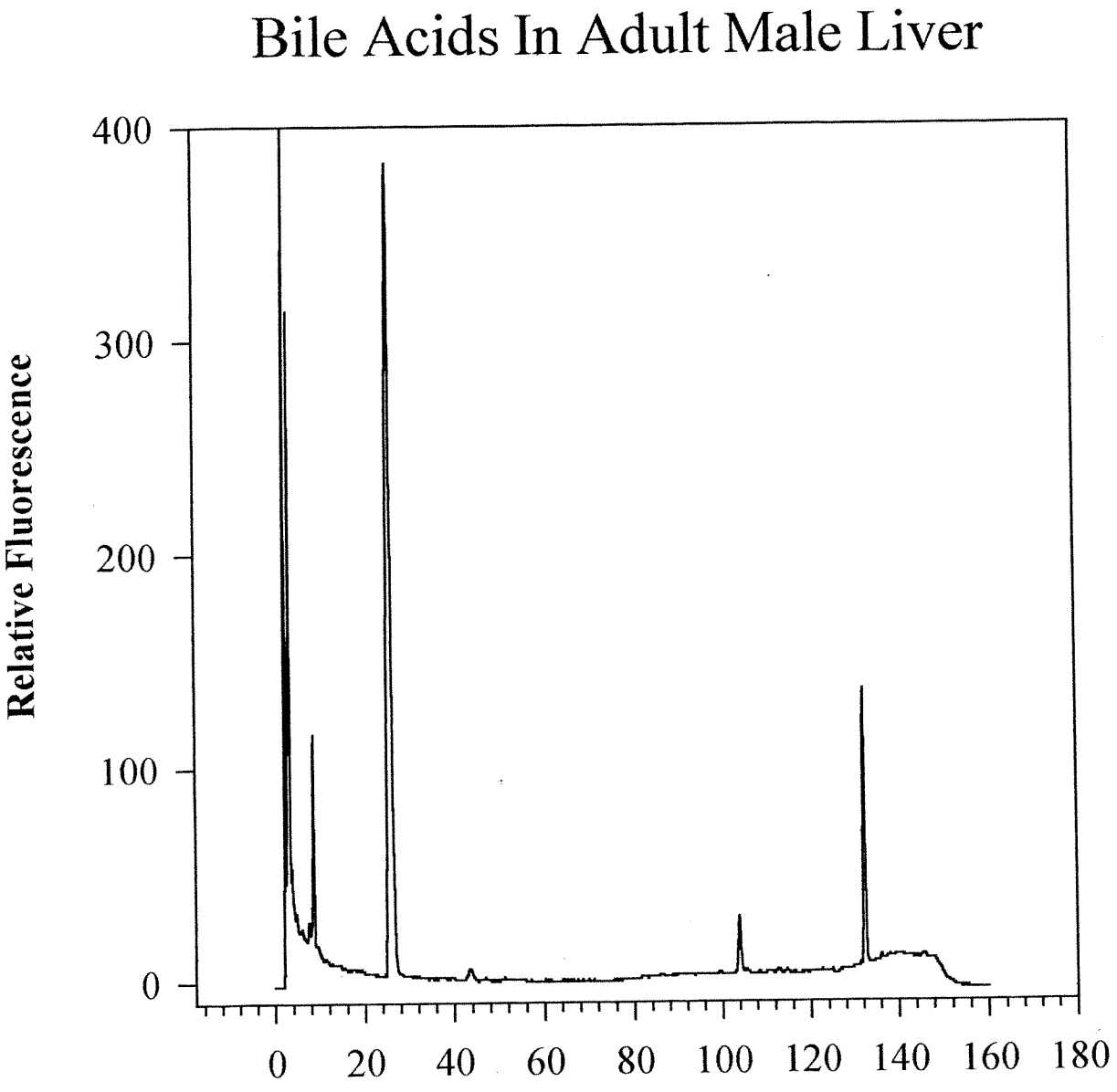
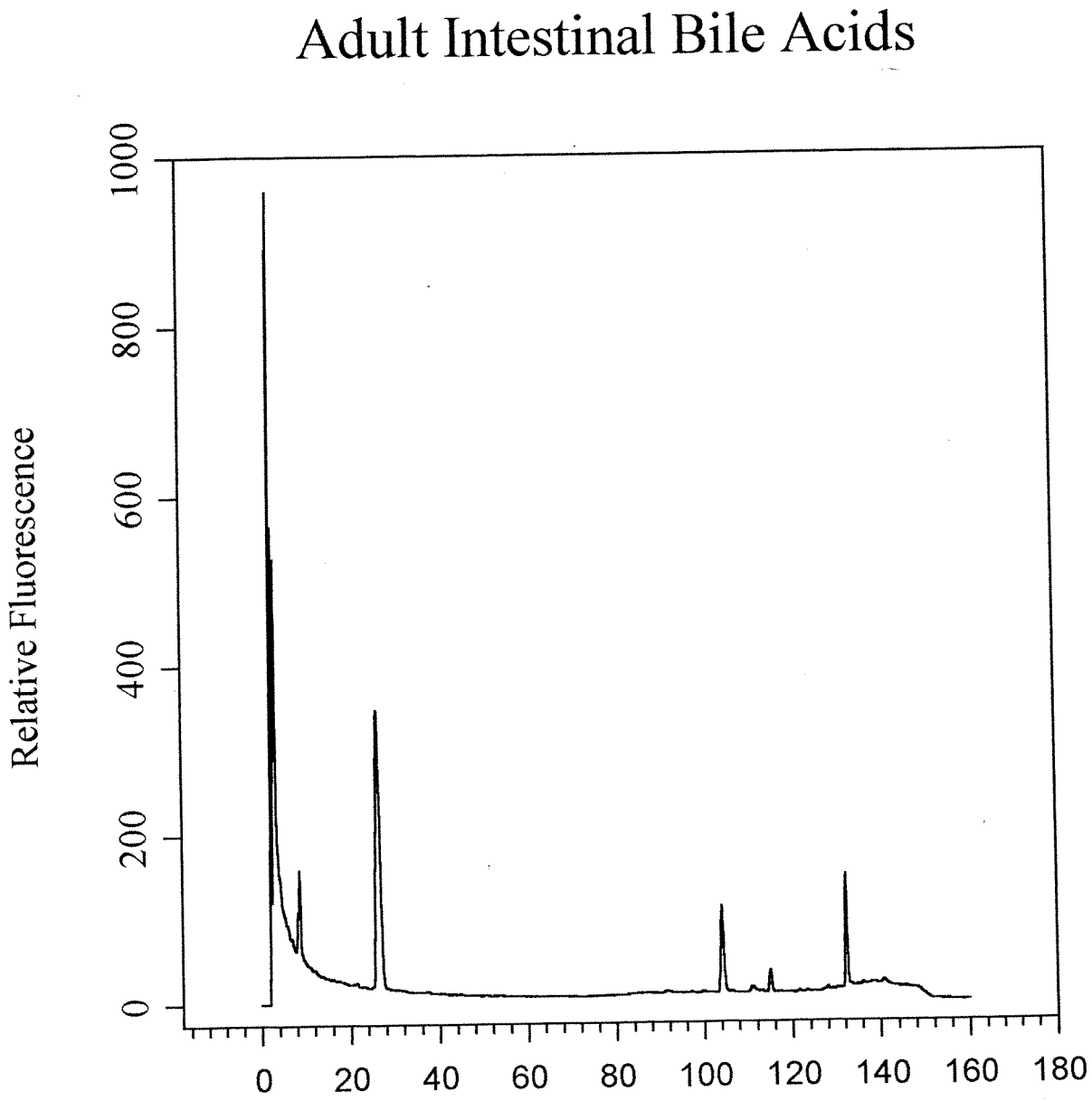


Figure 3b. A representative chromatogram of bile acids in an adult intestine. The peaks found did not correspond to allocholic acid, petromyzonol sulfate, or petromyzonol. The peak at 26 minutes is the internal standard (hyocholic acid). The method of detection will detect any steroid with a 3α-hydroxy group; therefore, the compounds seen are not necessarily bile acids.



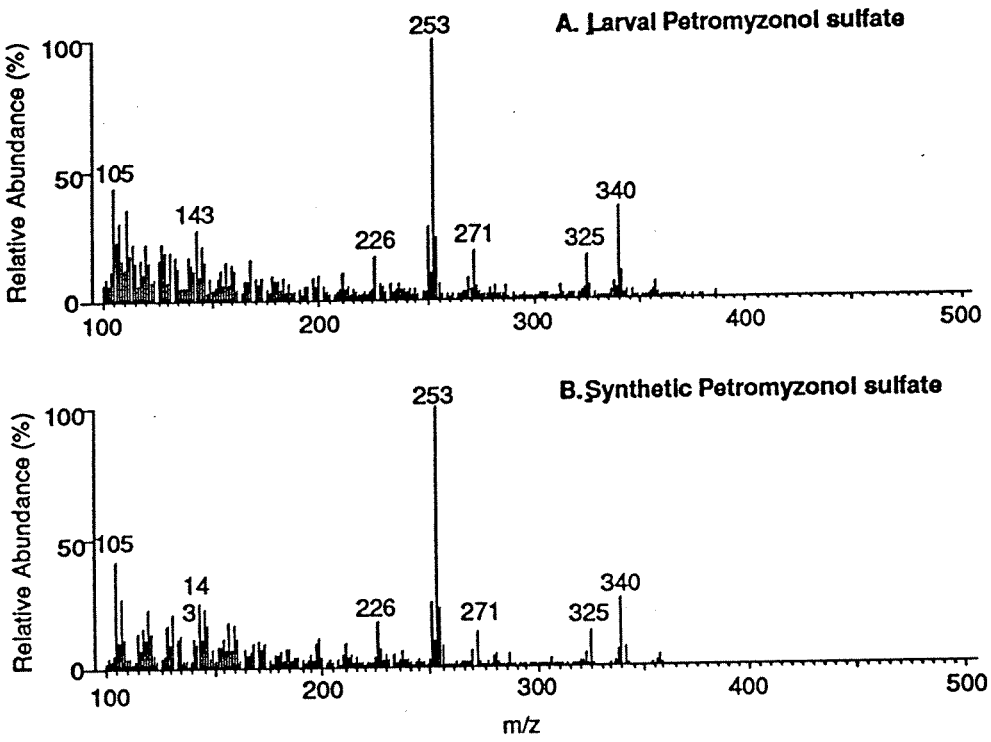


Figure 4. Electron impact (70 eV) mass spectra of: a) larval sea lamprey-derived petromyzonol sulfate; b) chemically synthesized petromyzonol sulfate.

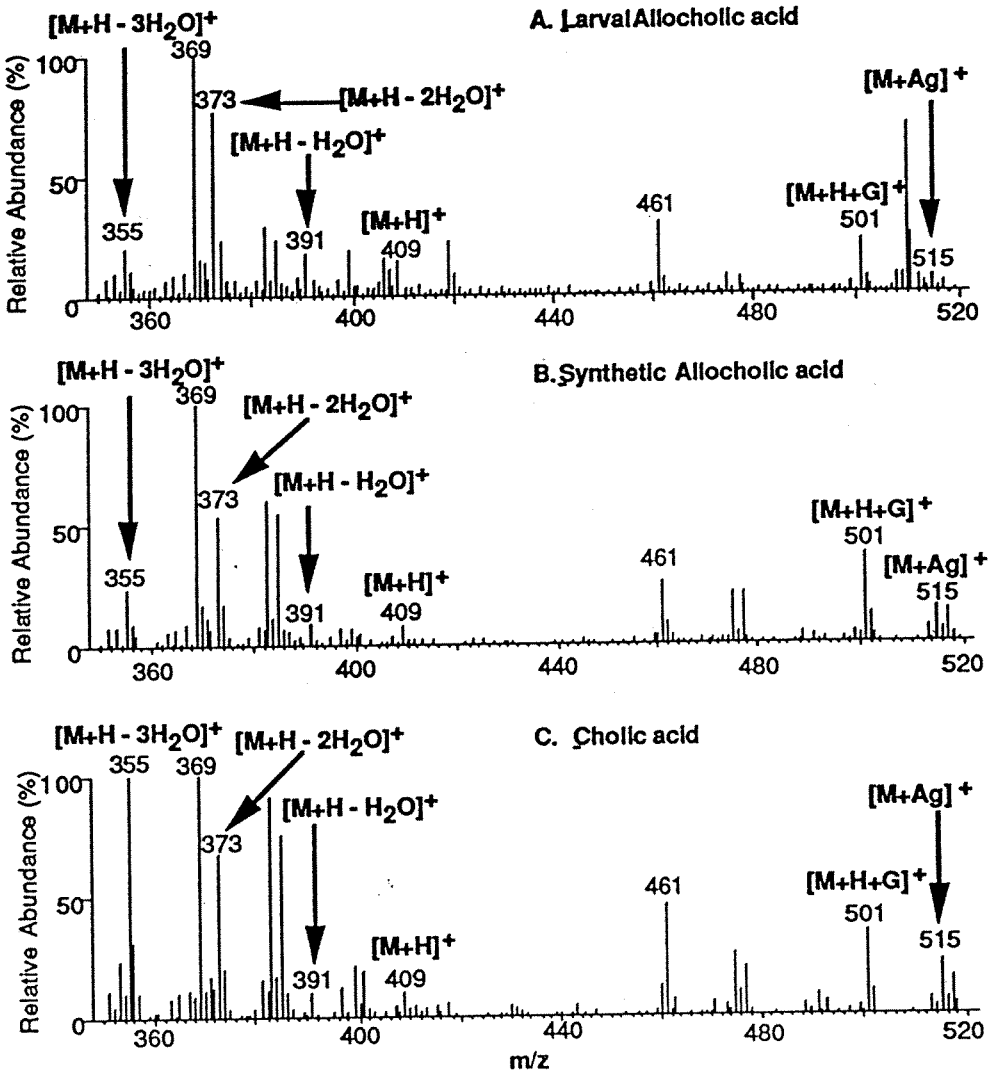


Figure 5. Fast atom bombardment (FAB) mass spectra of: A) larval sea lamprey-derived allocholic acid; (B) chemically synthesized allocholic acid; cholic acid (Sigma); Matrix: glycerol doped with silver nitrate. The glycerol peaks, at m/z 369 and 461, were not marked.

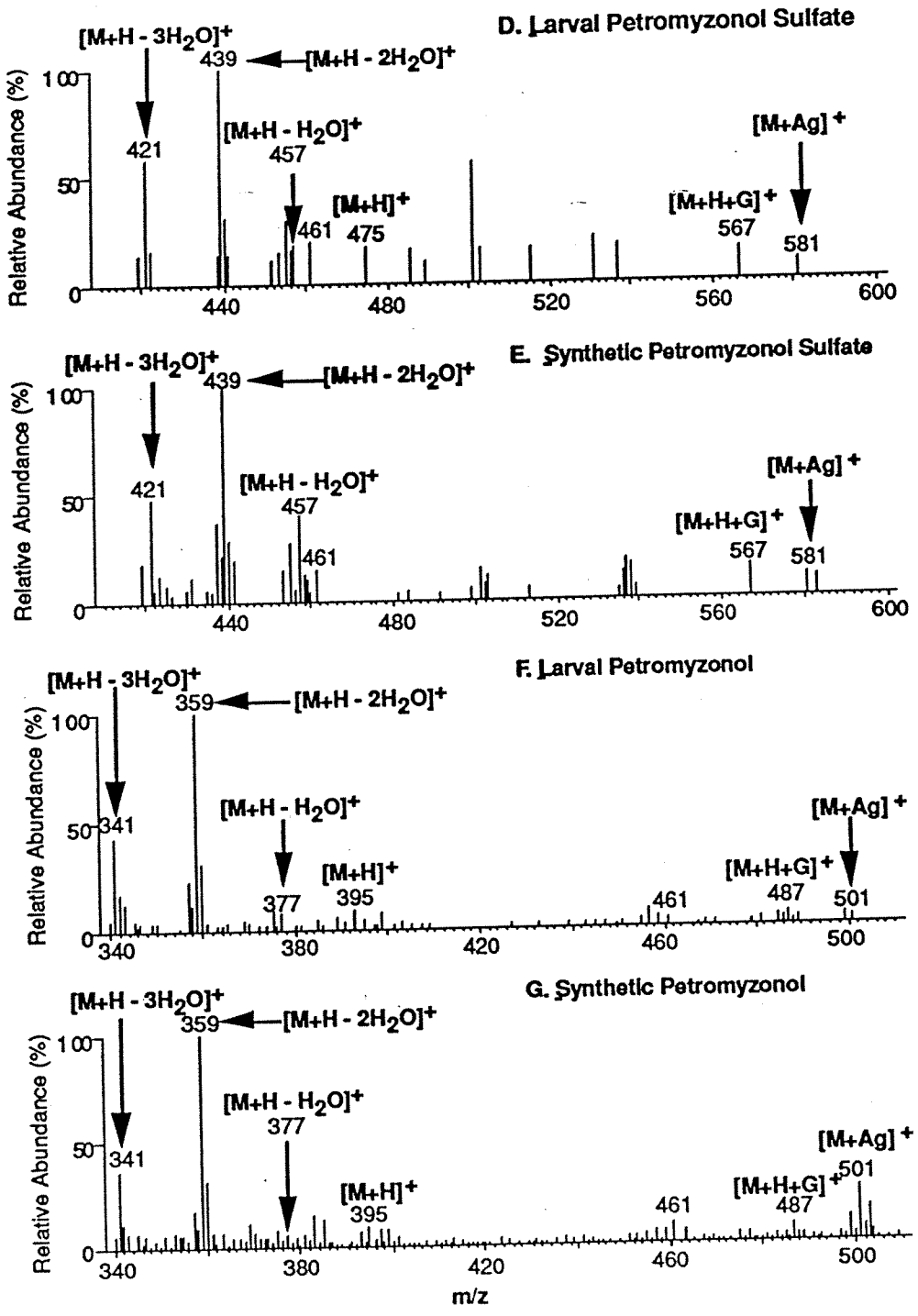


Figure 6. Fast atom bombardment (FAB) mass spectra of: (D) larval sea lamprey-derived petromyzonol sulfate; (E) chemically synthetic petromyzonol sulfate; (F) larval sea lamprey-derived petromyzonol; (G) chemically synthesized petromyzonol. Matrix: glycerol doped with silver nitrate. The glycerol peaks, at m/z 369 and 461, were not marked.

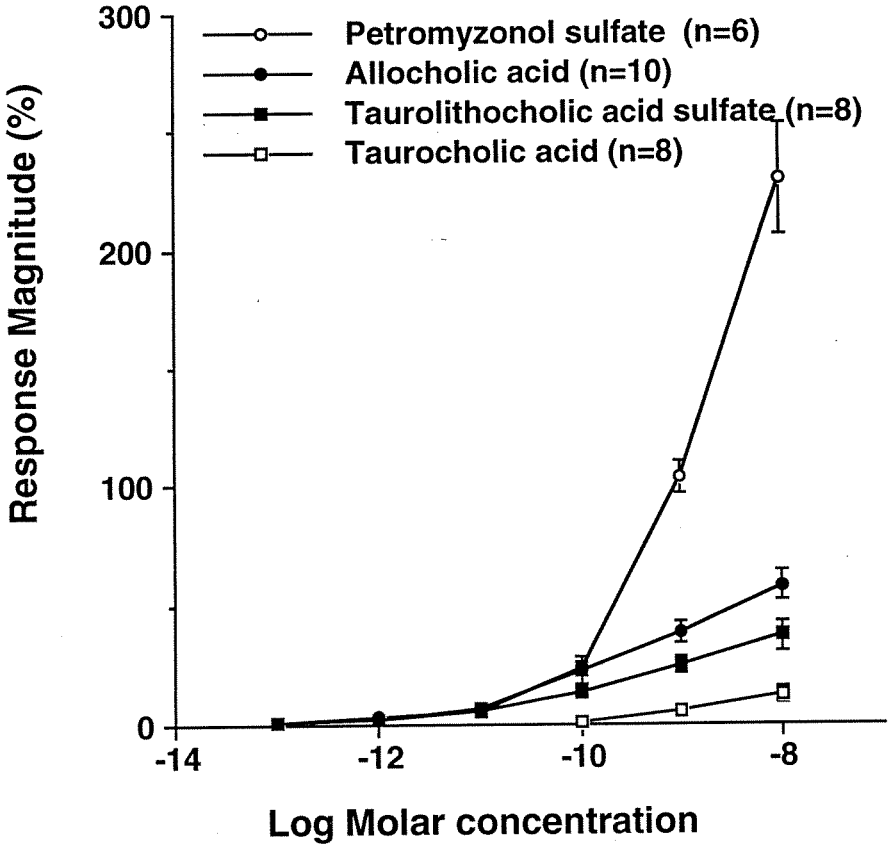
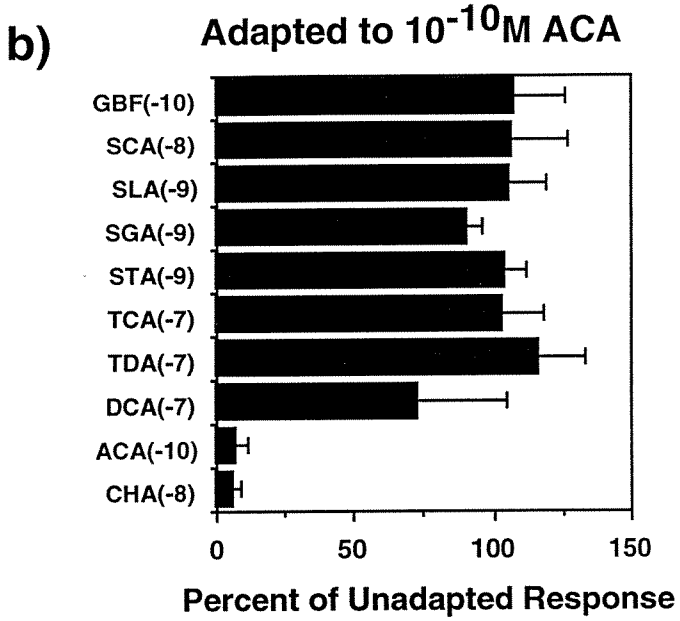


Figure 7a. Relative EOG responses of migratory adult lamprey to the four most potent bile acids. Responses are calculated relative to our standard, 10-5 Molar L-arginine. Bars represent standard error. No differences betwen male and female lamprey were evident.

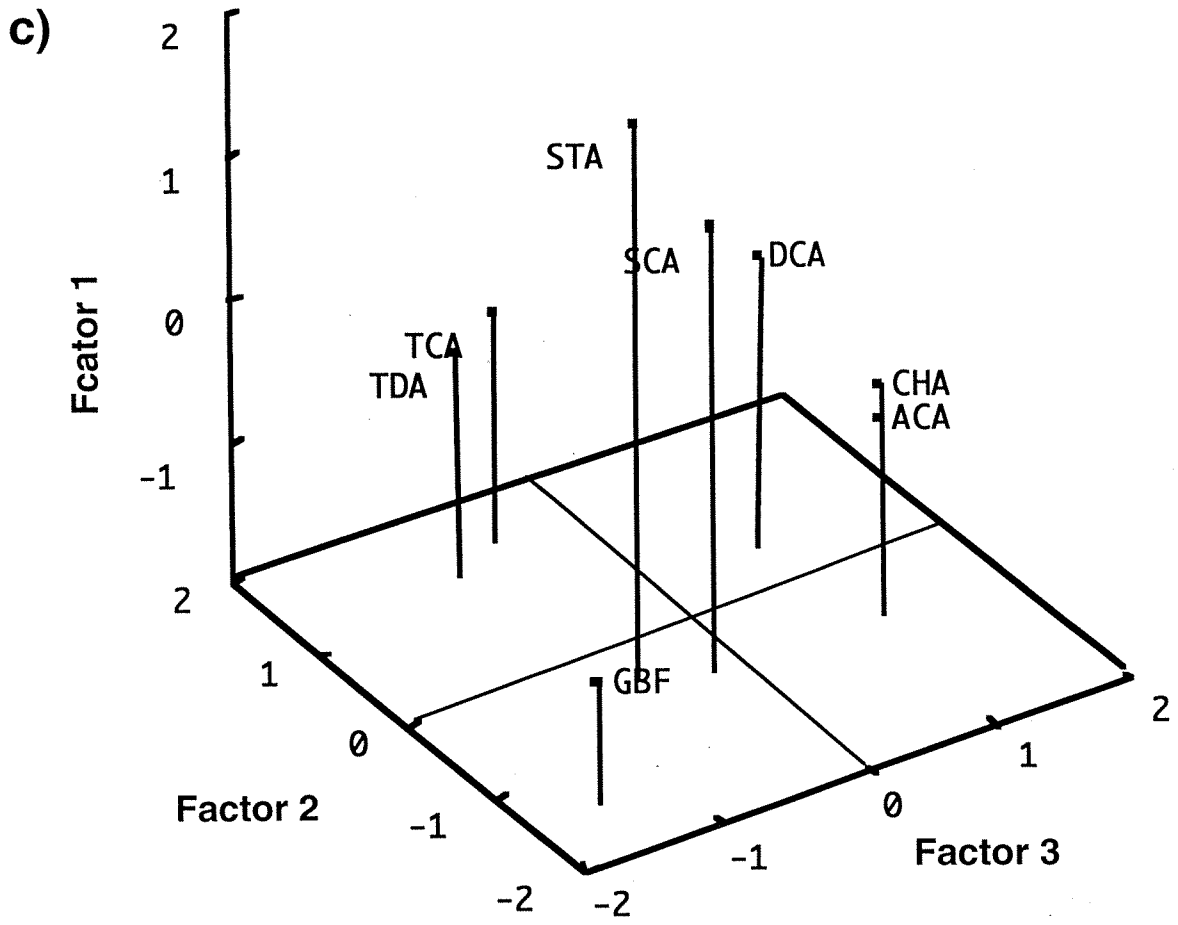
Figure 7. Further **Analysis of Lamprey EOG Data (cont.)**



Key to Figs. b and c

ACA Allocholic Acid
CHA Cholic Acid
DCA Deoxycholic Acid
GBF Gall Bladder Fraction 2 (Sulfopetromyzonol)
SCA Sulfocholic Acid
SGA Sulfoglycolithocholic Acid
SLA Sulfolithocholic Acid
STA Sulfotaurolithocholic Acid
TCA Taurocholic Acid
TDA Taurodeoxycholic Acid

c) A representative principal-component ordination (with Varimax rotation) relating all "adapting" bile acids to each other . There appear to be four receptor types.



b) Electro-olfactogram (EOG) responses of adult sea lamprey to bile acids during adaptation to 10^{-10} Molar allocholic acid (represented as the percentage of unadapted responses). Solutions were tested at the concentrations which elicited responses the same as that of the adapting solution. These concentrations are shown in parentheses.