

The Olfactory System of Migratory Adult Sea Lamprey (*Petromyzon marinus*) Is Specifically and Acutely Sensitive to Unique Bile Acids Released by Conspecific Larvae

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ABSTRACT Larval sea lamprey inhabit freshwater streams and migrate to oceans or lakes to feed after a radical metamorphosis; subsequently, mature adults return to streams to spawn. Previous observations suggested that lamprey utilize the odor of conspecific larvae to select streams for spawning. Here we report biochemical and electrophysiological evidence that this odor is comprised of two unique bile acids released by larvae. High performance liquid chromatography and mass spectrometry demonstrated that larval sea lamprey produce and release two unique bile acids, allocholic acid (ACA) and petromyzonol sulfate (PS). Electro-olfactogram (EOG) recording also demonstrated that the olfactory system of migratory adult sea lamprey is acutely and specifically sensitive to ACA and PS; detection thresholds for these compounds were $\sim 10^{-12}$ M. ACA and PS were the most potent of 38 bile acids tested and cross-adaptation experiments suggested that adult sea lamprey have specific olfactory receptor sites associated with independent signal transduction pathways for these bile acids. These receptor sites specifically recognize the key substituents of ACA and PS such as a 5 α -hydrogen, three axial hydroxyls, and a C-24 sulfate ester or carboxyl. In conclusion, the unique lamprey bile acids, ACA and PS, are potent and specific stimulants of the adult olfactory system, strongly supporting the hypothesis that these unique bile acids function as migratory pheromones in lamprey.

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INTRODUCTION

Although it is well established that many species of anadromous fish rely on olfactory cues to identify spawning habitats (Hasler and Scholz, 1983; Smith, 1985; Stabell, 1992), the chemical identities of these cues are unknown. Two schools of thought have emerged to explain the nature of these cues for salmonid fishes. The imprinting hypothesis states that young Pacific salmon learn to recognize a bouquet of unknown compounds from their natal stream; later, adults recognize and orient to these odors during their homeward migration (Hasler and Wisby, 1951; Hasler and Scholz, 1983; Scholz, Horrall, Cooper, and Hasler, 1976). The pheromone hypothesis, on the other hand, states that anadromous fish respond to pheromones released by conspecific larvae living in their spawning streams (Nordeng, 1971, 1977). Doving, Selset, and Thommesen (1980) proposed that bile acids might be a key component of migratory pheromones for salmonids. However, this hypothesis has yet to be formally tested: bile acid production and release have not been directly linked to either the olfactory function or behavior of conspecific individuals.

Several synthetic bile acids have been found to be potent olfactory stimuli which modify the behavior of fishes. Electro-encephalogram (EEG) recording has indicated that particular bile acids are highly stimulatory to the olfactory systems of Arctic char, *Salvelinus alpinus*, grayling, *Thymallus thymallus* (Doving et al., 1980), and rainbow trout, *Oncorhynchus mykiss* (Hara, Macdonald, Evans, Marui, and Arai, 1984). Further, electro-olfactogram (EOG) recording has demonstrated that taurocholic acid is a potent odorant for the goldfish, *Carassius auratus* (Sorensen, Hara, and Stacey, 1987), white sucker, *Catostomus commersoni*, and longnose sucker, *C. catostomus* (Cardwell, Dulka, and Stacey, 1992). However, multiunit recording from the olfactory epithelium of channel catfish, *Ictalurus punctatus* (a nonmigratory species), exposed to three synthetic bile acids, has failed to confirm the potency measured by EOG recording and the biological relevance of these compounds has been questioned (Erickson and Caprio, 1984). Nevertheless, behavioral studies have demonstrated that young freshwater eels, *Anguilla anguilla* (Sola and Tosi, 1993), young Arctic char (Jones and Hara, 1985) and cod, *Gadus morhua* (Hellstrom and Doving, 1986) are attracted to particular bile acids and that Arctic char (Selset and Doving, 1980) and Atlantic salmon, *Salmo salar* (Stabell, 1987) are attracted to larval intestinal contents. Notably, only commercially available bile acids and/or tissue extracts have been tested to date. Two critical tests of the hypothesis that bile acids function as conspecific migratory signals have yet to be performed. First, it has yet to be determined whether bile acids uniquely associated with a species are released into the water in large quantities. Second, it has yet to be determined whether the fish olfactory system can discriminate endogenous bile acids from other bile acids.

Only a few studies have chemically characterized bile acids in fish gall bladders, and they describe biliary bile acid content only. In hagfish, lamprey, cartilaginous fish, and primitive bony fish, sulfate esters of various polyhydroxy steroid alcohols appear to be the major biliary components (Haslewood, 1967, 1978; Tammar, 1974). For teleost fish, either sulfated bile alcohols or taurine conjugated bile acids appear to be the major biliary components (Haslewood, 1978; Denton and Yousef, 1975).

Recently, bile acids conjugated with cysteinolic acid were identified in the red seabream, *Pagrosomus major* (Une, Goto, Kihira, Kuramoto, Hagiwara, Nakajima, and Hoshita, 1991). However, only bile acids released to the water can function as an olfactory cue, and evidence from higher vertebrates shows that bacteria in the lower intestine, the route by which bile acids are normally released, often modify biliary bile acids (Cowen and Campbell, 1977). Release of bile acids to the water by fish has not been characterized.

The sea lamprey, *Petromyzon marinus*, is an excellent model for testing the hypothesis that endogenous bile acids function as conspecific olfactory cues. The sea lamprey has three phases in its life cycle: larval, parasitic, and adult (Hardisty and Potter, 1971; Smith, 1971). The larvae, which inhabit freshwater streams, migrate into oceans or lakes to parasitize fish after a radical metamorphosis. Later, adult lamprey migrate back to streams to spawn. Migratory adults are attracted to larva washings (Teeter, 1980), suggesting that a larval attractant exists. Paper chromatography suggests that larval sea lamprey produce large amounts of 3 α , 7 α , 12 α , 24-tetrahydroxy-5 α -cholan-24-sulfate (petromyzonol sulfate, PS) which is thought to be unique to lamprey larvae (Haslewood and Tokes, 1969). Strikingly, larval sea lamprey lose their gall bladders at metamorphosis (Yamamoto, Sargent, Fisher, and Youson, 1986) so PS is likely to be uniquely produced by the larvae. Post-larval sea lamprey have a well-developed olfactory organ which is thought to play an essential role in mediating their behaviors (Kleerekoper, 1972; Kleerekoper and Mogensen, 1963; Teeter, 1980). The olfactory sensitivity of adult sea lamprey, however, has not been characterized.

In this study we hypothesized that PS, possibly with some other unidentified bile acids released by larval sea lamprey, functions as an olfactory cue for conspecific migratory adults. Based on this hypothesis, four hypotheses were tested: (a) larval lampreys produce unique bile acids; (b) these bile acids are released into the water; (c) the olfactory system of migratory adults detects these unique bile acids; and (d) the lamprey olfactory system distinguishes larval bile acids from other bile acids. Our experiments which used high performance liquid chromatography (HPLC), mass spectrometry and EOG recording demonstrate for the first time in a migratory fish that unique bile acids released to the water by larvae are detected by conspecific migratory adults with great sensitivity and specificity.

MATERIALS AND METHODS

Characterizing Larval Bile Acid Production and Release

To characterize and quantify bile acids produced and released by larval sea lamprey, larval gall bladders and water samples were collected, extracted, and analyzed by HPLC. Larvae (mean weight of 2.1 ± 0.2 g) were caught by electrical shocking in tributary streams of the Great Lakes and shipped to St. Paul, Minnesota. The water in which these animals were shipped was then analyzed to examine the bile acids released into the water: 54 larvae were held for ~24 h in a plastic bag containing 8.7 liters of Lake Huron water. This water sample was filtered with qualitative filter paper (Whatman, Maidstone, England), then extracted with activated C18 solid phase extraction (SPE) cartridges (Bakerbond SPE, J.T. Baker, Phillipsburg, NJ, 1,000 ml water/cartridge). Bile acids were eluted from these cartridges with 5 ml of methanol. The larvae

were killed by a high dose of MS222 (tricaine methanesulfonate, Syndel, Vancouver, BC, Canada), and their gall bladders removed and stored at -20°C . The isolation and purification of biliary bile acids followed the protocol developed by Locket and Gallaher (1989). Briefly, tissues were lyophilized, sequentially extracted with ethanol and chloroform:methanol and then partially purified using C18 SPE cartridges.

Lamprey bile acids were characterized by HPLC using a reverse-phase Nova-Pak C₁₈ 4- μm column (4 mm \times 10 cm) and eluted with a step-wise gradient of ammonium dihydrogen phosphate (25 mM, pH 7.8) and acetonitrile (Gallaher, Locket, and Gallaher, 1992). Eluted bile acids were passed through a second column (5 cm \times 0.5 cm i.d., Alltech, Deerfield, IL) containing 3 α -hydroxysteroid dehydrogenase (EC 1.1.1.50, 50 U, Sigma Chemical Co., St. Louis, MO) and bound to glutaraldehyde-treated aminopropyl glass beads (Sigma, Chemical Co.). A buffer containing NAD (0.1 M Tris-HCl, pH 8.5, 2.7 mM EDTA, 1.63 mM dithiothreitol and 0.01 mM NAD) was introduced using a tee between the first and second columns at a constant rate of 1 ml/min. This enzyme oxidizes 3 α -hydroxyl bile acids into 3 α -keto bile acids and reduces NAD to NADH which is subsequently detected by a fluorescence detector (model 121, Gilson Medical Electronics, Middleton, WI) with a narrow band excitation filter of 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, WI). Bile acids in tissue and water samples were initially characterized by comparing their retention times with the retention times of synthetic ACA, PS, and petromyzonol (P), produced by Toronto Research, Inc. (Toronto, Ontario, Canada).

Identifying Larval Bile Acids by Mass Spectrometry

Lamprey bile acids were further characterized by mass spectrometry (MS). The HPLC fractions of gall bladder extracts were loaded onto C18 SPE cartridges which were washed extensively with 10% methanol followed by double distilled water, eluted with 5 ml methanol, dried under N_2 , and redissolved in 200 μl methanol. These samples were mixed with 2.5 ml 0.05 M potassium phosphate buffer (pH 6.1) containing 1.25 mg/ml NADH and 300 μl 3 α -hydroxysteroid dehydrogenase solution (1.5 U/ml 0.01 M KH_2PO_4 , Sigma Chemical Co.), and incubated for 30 min at 35°C . At this pH, 3 α -hydroxysteroid dehydrogenase reduces 3 α -keto bile acids back to 3 α -hydroxyl bile acids. Converted samples were loaded onto prewashed C18 SPE cartridges, washed with 10% methanol and double distilled water, eluted with methanol, dried under N_2 , and redissolved in methanol for MS analyses.

The mass spectra of these larval bile acids were determined using a Kratos MS-25 mass spectrometer (Ramsey, NJ). Electron impact (EI) spectra of both larvae-derived bile acids and synthetic bile acids were determined at an ionization potential of 70 eV. 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 insight into molecular structure (Watson, 1985). The fast atom beams were generated by xenon atoms which were accelerated to 8 KeV. Glycerol and silver nitrate doped glycerol were used as the matrix.

Electro-olfactogram (EOG) Recording

To determine if the olfactory organ of migratory adult sea lamprey is specifically sensitive to larval bile acids, EOG responses to ACA and PS were recorded. Migratory adult sea lampreys (weight of 238 ± 8.5 g, length of 50.7 ± 0.6 cm, mixed sex) were trapped in the Cheboygon River, Michigan, and the St. Mary's River, Ontario, Canada, early in their spawning migration

(May for the former and July for the later). Animals were then shipped by air to St. Paul, Minnesota where they were held in plastic tanks with flowing well water chilled to 5–9°C and a natural photoperiod. Before being used for EOG recording, animals were gradually warmed to 11°C (the temperature of the water supplying the EOG apparatus), anaesthetized with an intramuscular injection of metomidate hydrochloride (Syndel, Vancouver, Canada, 3 mg/kg body weight), immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; Sigma Chemical Co., 150 mg/kg body weight), and secured to a stand in a flow-through trough. The gills were perfused with water by way of a plastic tube placed in the animal's oral disc. The olfactory lamellae were exposed by removing the surface skin and the top part of the nasal capsule, and then perfused with water at a rate of 6 ml/min. The same water was used to maintain the sea lampreys, to perfuse their gills and nose, and to formulate the odorants.

Differential EOG responses were recorded using two Ag/AgCl electrodes (type EH-1S; WPI, Sarasota, FL) filled with 3 M KCl and bridged to saline gelatin (8%) filled glass capillaries (tip diam $472.59 \pm 18.75 \mu\text{m}$). The reference electrode was placed on the skin near the naris. The recording electrode was placed between two lamellae and adjusted to maximize responses to the standard odorant, 10^{-5} M L-arginine and to reduce background noise. L-arginine was chosen as the standard because it is the most potent amino acid for sea lamprey (Li and Sorensen, 1992). Electrical signals were either amplified by a DC-preamplifier (Model P16, Grass, Quincy, MA), digitized by MacLab/4 (Analog Digital Instruments Pty Ltd., Castle Hill, Australia), displayed and stored on a Macintosh IICx computer, or amplified by a 7P1 DC-preamplifier (Grass) and displayed on a 7B Polygraph pen recorder (Grass). Odorant solutions were delivered by a device which minimizes the pressure and temperature differences between the background flow and odorant solutions. Stimulus duration was 5 s unless otherwise indicated. 3-min interstimulus intervals were employed to permit reproducible, nonadapted responses.

Each lamprey was tested with the L-arginine standard, then blank well water, and then the odorant solution. Ethanol or methanol controls were tested randomly. The L-arginine standard was tested every 1–2 h to ensure that the olfactory responsiveness remained constant. When the concentration-response relationship of an odorant was determined, the L-arginine standard was tested, followed by well water, then the odorant in order of increasing log molar (M) concentrations, and finally the standard again. Each concentration of each odorant was tested at least twice on each animal. If the size of the EOG responses were not similar, a third test was conducted.

Both larval-derived and chemically synthesized lamprey bile acids were used for EOG recording. In addition to ACA, PS, and P (petromyzonol, the desulfated form of PS), 35 nonlamprey bile acids and related compounds were tested to characterize the structure-activity relationships of bile acids as odorants for sea lamprey. These bile acids, purchased from either Sigma Chemical Co. or Steraloids, Inc. (Wilton, NH), were selected either because they are common to many vertebrates and might therefore be present in lamprey habitats, or because they differ from lamprey bile acids by certain structural characteristic(s). Table I lists all the tested bile acids along with the abbreviations which are used throughout this report. Our nomenclature follows that of Hofmann, Sjoval, Kurz, Radominska, Schteingert, Tint, Vlahcevic, and Setchell (1992) and Moss (1989). A schematic representation of ACA is shown in Fig. 1 whose legend briefly explains bile acid nomenclature and typical substituents. Stock solutions of synthetic bile acids were made at concentrations of either 10^{-3} M or 0.5×10^{-3} M with ethanol or 50% ethanol and kept at -20°C . Natural bile acids were dissolved in methanol because this was the solvent used for extraction. Control solutions of both ethanol and methanol were thus tested. An L-arginine stock solution of 10^{-2} M was made with well water every other week and stored at 4°C .

TABLE I
Compounds Tested

Abbre	Common name	Chemical name
PS	Petromyzonol sulfate	3 α , 7 α , 12 α , 24-Tetrahydroxy-5 α -cholan-24-sulfate
ACA	Allocholic acid	3 α , 7 α , 12 α -Trihydroxy-5 α -cholan-24-oic-acid
P	Petromyzonol	3 α , 7 α , 12 α , 24-Tetrahydroxy-5 α -cholan
5 β -P	5 β -Petromyzonol	3 α , 7 α , 12 α , 24-Tetrahydroxy-5 β -cholan
CA	Cholic acid	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid
CAS	Cholic acid 3-sulfate	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid-3-sulfate
TCA	Taurocholic acid	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)-amide
GCA	Glycocholic acid	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(carboxymethyl) amide
CAH	Cholic acid hydrazide	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid hydrazide
CAM	Cholic acid methyl ester	3 α , 7 α , 12 α -Trihydroxy-5 β -cholan-24-oic-acid methyl ester
CDC	Chenodeoxycholic acid	3 α , 7 α -Dihydroxy-5 β -cholan-24-oic-acid
GCD	Glycochenodeoxycholic acid	3 α , 7 α -Dihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(carboxymethyl) amide
TCD	Taurochenodeoxycholic acid	3 α , 7 α -Dihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)- amide
UDC	Ursodeoxycholic acid	3 α , 7 β -Dihydroxy-5 β -cholan-24-oic-acid
TUD	Tauroursodeoxycholic acid	3 α , 7 β -Dihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)- amide
KCA	12-Keto-Chenodeoxycholic acid	3 α , 7 α -Dihydroxy-12-one-5 β -cholan-24-oic-acid
TDC	Taurodeoxycholic acid	3 α , 12 α -Dihydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)- amide
KDC	7-Ketodeoxycholic acid	3 α , 12 α -Dihydroxy-7-one-5 β -cholan-24-oic-acid
DOC	Deoxycholic acid	3 α , 12 α -Dihydroxy-5 β -cholan-24-oic-acid
HCA	α -Hyocholic acid	3 α , 6 α , 7 α -Trihydroxy-5 β -cholan-24-oic-acid
HDC	Hyodeoxycholic Acid	3 α , 6 α -Dihydroxy-5 β -cholan-24-oic-acid
LCA	Lithocholic acid	3 α -Hydroxy-5 β -cholan-24-oic-acid
TLC	Tauroolithocholic acid	3 α -Hydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)-amide
GLC	Glycolithocholic acid	3 α -Hydroxy-5 β -cholan-24-oic-acid <i>N</i> -(carboxymethyl) amide
LAM	Lithocholic acid methyl ester	3 α -Hydroxy-5 β -cholan-24-oic-acid methyl ester
GLM	Glycolithocholic acid ethyl ester	3 α -Hydroxy-5 β -cholan-24-oic-acid methyl ester <i>N</i> -(carboxymethyl) amide
TLS	Tauroolithocholic acid 3-sulfate	3 α -Hydroxy-5 β -cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)-amide 3-sulfate
GLS	Glycolithocholic acid 3-sulfate	3 α -Hydroxy-5 β -cholan-24-oic-acid <i>N</i> -(carboxymethyl) amide 3-sulfate
LCS	Lithocholic acid 3-sulfate	3 α -Hydroxy-5 β -cholan-24-oic-acid 3-sulfate
CLS	Cholesterol 3-sulfate	3 β -Hydroxy-5-cholesten 3-sulfate
DCL	Dihydrocholesterol 3-sulfate	5 α -Hydroxy-5-cholesten 3-sulfate
CLA	5 β -cholanic acid	5 β -Cholan-24-oic-acid
TCL	Taurocholanic acid	5 β -Cholan-24-oic-acid <i>N</i> -(2-sulfoethyl)-amide
GCL	Glychocholanic acid	5 β -Cholan-24-oic-acid <i>N</i> -(carboxymethyl) amide
DHC	Dehydrocholic acid	3, 7, 12-Trione-5 β -cholan-24-oic-acid
CLO	5 α -cholanic acid 3 β -ol	3 β -Hydroxy-5 α -cholan-24-oic-acid
CDH	Chenodeoxycholic acid 3 hemisuccinate	3 α , 7 α -Dihydroxy-5 β -cholan-24-oic-acid 3-hemisuccinate
DCH	Deoxycholic acid 3-acetyl methyl ester	3 α , 12 α -Dihydroxy-5 β -cholan-24-oic-acid 3-acetyl methyl ester

Characterizing Olfactory Receptor Specificity (Cross-adaptation)

To determine whether EOG responsiveness to bile acids is mediated by one or more olfactory receptor sites, cross-adaptation experiments were performed. In cross-adaptation, responsiveness to a test odorant is first measured, then the olfactory epithelium is perfused with an adapting odorant, and responsiveness to pulses of the test odorant made up in the adapting odorant measured. Reductions in EOG responsiveness during adaptation are believed to reflect the extent to which the test and adapting stimuli interact with the same receptor site and associated signal transduction pathway. Bile acids were tested at concentrations which induced similar sized EOG responses to reduce the possibility of nonspecific interactions (10^{-10} M PS,

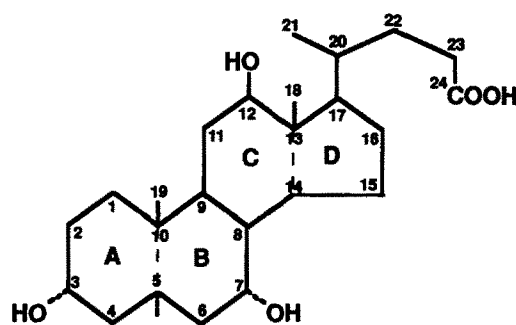


FIGURE 1. Allocholic acid (3α , 7α , 12α -trihydroxy- 5α -cholan-24-oic acid, ACA), a bile acid produced by larval sea lamprey. A bile acid is composed of a cyclopentenophenanthrene nucleus (the A, B, C, and D ring), an alkyl side chain at C-17, two methyl groups at C-10 and C-13, and other substituents (Hofmann et al., 1992). The structure that combines the nucleus and the side chain is termed cholan (without oic acid on carbon 24). The

5-hydrogen of ACA is in the α (allo) configuration. ACA has three hydroxyls attached to C-3, C-7 and C-12 positions, all in an α -configuration and all in an axial orientation. The orientation of hydroxyls may be either equatorial (in the plane) or axial (out of the plane) (Carey, 1985). For 5α -bile acids, 6α and 7β hydroxyls are equatorial, whereas the 3α , 7α , and 12α hydroxyls are axial. 5β -bile acids differ from 5α -bile acids in that their 3α hydroxyl is equatorial. Petromyzonol (P) is similar to ACA except that the keto group attached to C-24 is replaced by two hydrogens. Petromyzonol sulfate (PS) is identical to P except it has a sulfate ester at C-24 (Haslewood and Tokes, 1969). The only difference between cholic acid (CA) and ACA is that the 5-hydrogen of CA has a β -configuration. When the 5-hydrogen is in the α -configuration, the A, B, C, and D rings are virtually all in the same plane: the nucleus is planar. When the 5-hydrogen is in the β -configuration, the B, C, and D rings are still in the same plane, but the A ring bends away and forms a kink which makes the nucleus L-shaped (Haslewood, 1967; Hay and Carey, 1990). Other bile acids listed in Table I differ from ACA or CA by the substituents attached to C-3, C-5, C-6, C-7, C-12, and C-24 positions. Cholesterol, the precursor of bile acids, has a double bond between C-5 and C-6, and a longer side chain.

10^{-10} M ACA, and 10^{-9} M TLS). All three odorants were tested both as adapting and test stimuli. TLS was chosen because it is the most stimulatory nonlarval bile acid.

Analysis of EOG Data

The magnitude of each EOG response was measured from the baseline to the peak of each phasic displacement and converted to millivolts. The methanol or ethanol control response, if present, was subtracted, and the duplicate responses averaged and calculated as percentages of the most recent average response to 10^{-5} M L-arginine. EOG data are generally standardized in this manner to reduce interindividual variability caused by differences in electrode size and placement. To analyze the structure-activity relationships, responses to ethanol control and bile

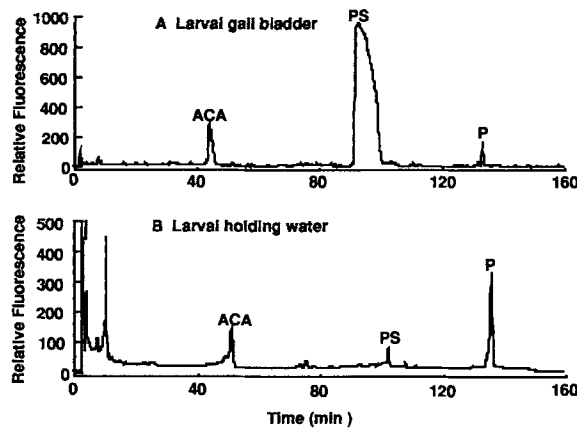


FIGURE 2. Digitized high performance liquid chromatography (HPLC) chromatograms of tissue and water samples. (A) Extracts of larval sea lamprey gall bladders. (B) Extracts of water in which larvae had been held. (ACA) Allocholic acid. (PS) Petromyzonol sulfate. (P) Petromyzonol.

acids at 10^{-10} M and 10^{-6} M were compared by a one way analysis of variance (ANOVA) using SYSTAT (SYSTAT, Inc., Evanston, IL). If the ANOVA indicated differences, all the bile acid responses were then compared to the control responses by Dunnet's test using JMP (SAS Institute Inc., Cary, NC). For cross-adaptation data, responses before and during adaptation were compared by Bonferroni paired *t* tests if ANOVA indicated differences among responses.

RESULTS

Bile Acid Production and Release

High performance liquid chromatography analysis indicated that gall bladders of larval sea lamprey contain three bile acids with retention times of ~44, 94, and 138 min (Fig. 2A). These retention times correspond to the retention times of synthetic ACA, PS, and P, respectively. Larval holding water also contained three bile acids whose retention times matched those of ACA, PS and P (Fig. 2B), demonstrating that

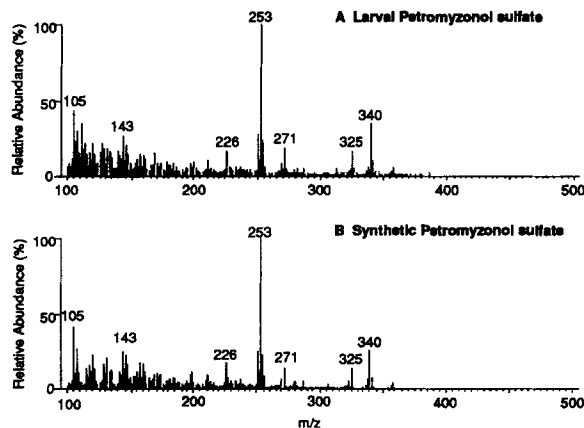


FIGURE 3. Electron impact (70 eV) mass spectra of (A) petromyzonol sulfate isolated from larval sea lamprey and (B) chemically synthesized petromyzonol sulfate.

these compounds are released into water. The ratio of P to PS in water extracts was larger than that for gall bladder extracts.

Mass spectrometry confirmed that the bile acids isolated from larval gall bladders were PS, ACA, and P. First, the EI mass spectra of larval PS and synthetic PS were essentially identical (Fig. 3). Larval PS and synthetic PS also had similar FAB spectra (Fig. 4, *A* and *B*): they had a MH^+ pseudomolecular ion at m/z 567, a glycerol adducted MH^+ at m/z 581, and a silver adducted M^+ at m/z 581, indicating a molecular mass at 474 as predicted from the formula ($C_{24}H_{42}O_7S$). Second, larval, and synthetic ACA showed nearly identical EI (not shown) and FAB mass spectra (Fig. 4, *C* and *D*): they had MH^+ , $[M + H + G]^+$ and $[M + Ag]^+$ at m/z 409, 501, and 515, respectively, indicating a molecular mass at 408, the same as predicted ($C_{24}H_{40}O_5$). Third, larval and synthetic P also had essentially identical EI (not shown) and FAB mass spectra (Fig. 4, *E* and *F*). The mass peaks at m/z 395, 487 and 501 for MH^+ , $[M + H + G]^+$ and $[M + G]^+$, respectively, indicated a molecule mass of 394, as predicted ($C_{24}H_{42}O_4$). Fourth, PS, ACA and P each showed at least three mass peaks corresponding to three neutral water losses during FAB ionization: PS had mass peaks at m/z 457, 439 and 421; ACA had 391, 373, and 355; and P had 377, 359 and 341. This is consistent with the original molecules having three or four hydroxyls. Last, the FAB spectrum of CA (Fig. 4 *G*) resembles that of ACA. However, CA had the base peak at m/z 355 whereas ACA had the base peak at m/z 373. Evidently, CA loses water more readily than ACA, suggesting a small difference in structure or configuration. Notably, the neutral water loss of both PS and P resembled that of ACA but contrasted with that of CA, suggesting that PS and P have three hydroxyls oriented the same as those of ACA but different from those of CA. FAB mass spectra with glycerol as the matrix showed similar results. It thus appears that larval bile acids have a similar structure around the steroid nucleus and that their different masses are due to differences in their side chains.

Olfactory Potency of Larval Bile Acids

EOG responses of lamprey to bile acids and L-arginine had distinct phasic and tonic components, typical of EOG responses of other vertebrates (Fig. 5). The average phasic EOG response elicited by 10^{-5} M L-arginine was 2.84 ± 0.24 mV ($n = 42$). The EOG response to ethanol at a 1:1,000 dilution (the concentration used in 10^{-6} M bile acid solutions) was 2.3 ± 0.7 percent ($n = 25$) of that elicited by standard, and the average EOG response to methanol at a 1:1,000 dilution was 0.7 ± 0.4 percent ($n = 10$) of the standard.

ACA and PS were the most potent bile acids tested. At concentrations of 10^{-10} M and 10^{-6} M (Fig. 6), synthetic ACA and PS elicited responses larger than the other bile acids tested ($P < 0.05$). ACA and PS had detection thresholds of 10^{-12} – 10^{-13} M (Fig. 7, *A* and *B*). Responses to PS plateaued at 10^{-6} M but responses to ACA did not. The concentration-response curves of synthetic and natural PS were very similar. The concentration-response curves of natural ACA showed a slightly different shape from that of synthetic ACA. However, their error bars overlapped and the difference was probably an artifact of the fact that we used different animals for these particular tests: the synthetic ACA was tested at the start of the spawning migration using much

more responsive animals from which EOG responses were more easily recorded. P was less potent than ACA and PS but more potent than the standard. The detection threshold of P was $\sim 10^{-8}$ M. The detection threshold of TLS was $\sim 10^{-12}$ M (Fig. 7 C).

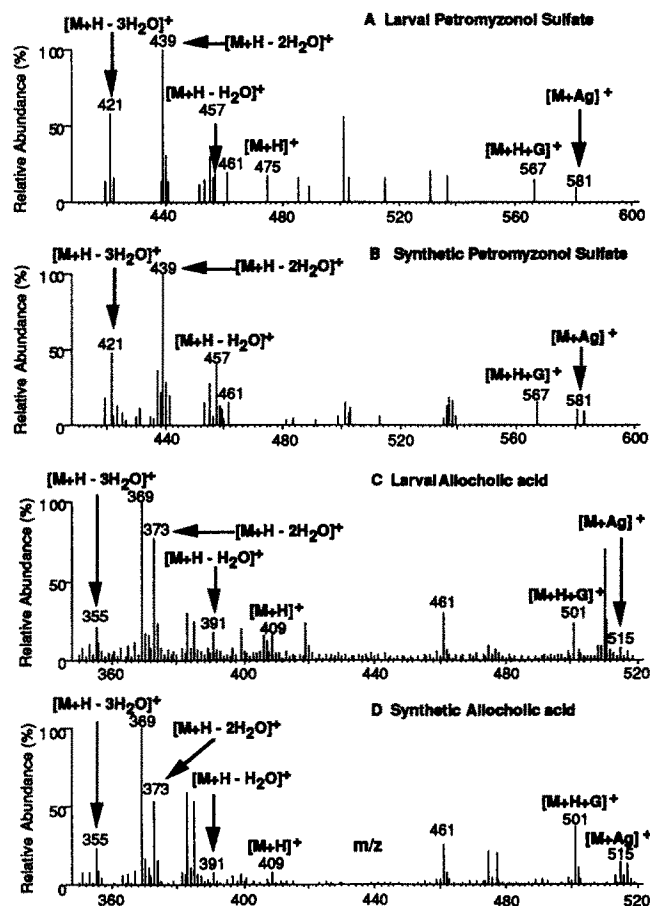


FIGURE 4. Fast atom bombardment (FAB) mass spectra of (A) petromyzonol sulfate isolated from larval sea lamprey; (B) chemically synthesized petromyzonol sulfate; (C) allocholic acid isolated from larval sea lamprey; (D) chemically synthesized allocholic acid; (E) petromyzonol isolated from larval sea lamprey; (F) chemically synthesized petromyzonol; and (G) cholic acid (Sigma Chemical Co.). Matrix: glycerol doped with silver nitrate. The glycerol peaks, at m/z 369 and 461, are not marked.

Structure-Activity Relationships of Bile Acids as Olfactory Stimulants

The olfactory organ of sea lamprey is highly specific in the manner with which it detects bile acids. At a concentration of 10^{-10} M, only ACA, PS, and TLS elicited larger responses than the control ($P < 0.05$, Fig. 6 B). Specificity was also evident at

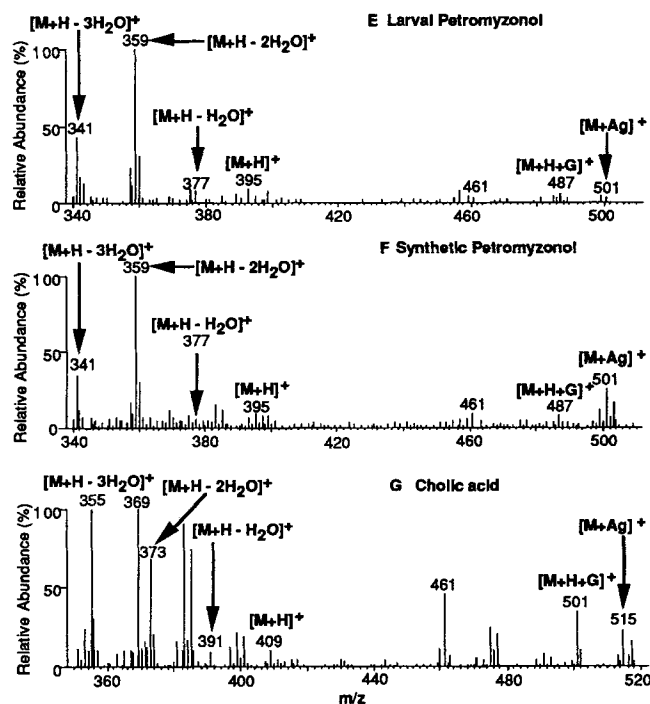


FIGURE 4. (Continued)

high concentrations; of 38 bile acids and related compounds tested at 10^{-6} M (Fig. 6A), only 15 elicited larger responses than the ethanol control ($P < 0.05$). The structure-activity relationship of bile acids suggests that four aspects of the molecule are critical for the potency of bile acids. First, a 5α -hydrogen (5α -H) appears to be important: 5α -bile acids ACA and P were much more potent than their 5β isomers, CA and 5β -P ($P < 0.01$). Second, in nonconjugated bile acids, compounds with axial hydroxyls are more potent than those with equatorial hydroxyls. For example, CDC (with a 7α -OH which is axial) was more potent than UDC (with a 7β -OH which is equatorial) ($P < 0.01$). Also, HCA has one axial hydroxyl, CA two, and ACA three; and their olfactory potencies were $HCA < CA < ACA$ ($P < 0.01$). Third, a 3-sulfate

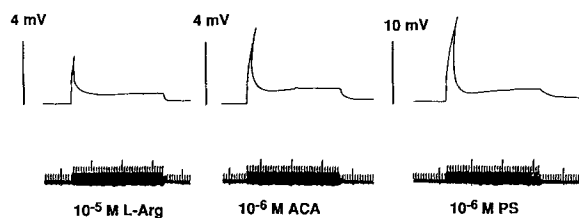


FIGURE 5. Electro-olfactogram (EOG) responses of a male upstream-migratory sea lamprey to 10^{-6} M petromyzonol sulfate (PS), 10^{-6} M allocholic acid (ACA) and 10^{-5} M L-arginine (L-Arg). A relatively long exposure time (3 min.) was used

to allow the phasic and tonic components of the EOG responses to be visualized. Responses were recorded on curvilinear graph paper. Note different scales for PS. Time signals, each division = 5 s.

ester is essential to the olfactory potency of bile acids with one hydroxyl. LCA, TLC and GLC, all bile acids with one hydroxyl, were more potent when sulfated at C-3 to become LCS, TLS and GLS ($P < 0.01$). The effect of sulfuric acid ester at the C-3 position was very specific and restricted to the aforementioned bile acids. Fourth, the nature of functional groups at C-24 has mixed effects on the olfactory potency of bile acids. Compared to a carboxyl, a sulfate ester at this position usually increased the potency of bile acid, whereas a hydroxyl decreased the potency of the bile acids (comparing PS to ACA and P, also CA to 5 β -P). Taurine conjugation increased the olfactory potency of LCA (with one hydroxyl) and decreased the potency of CA (with

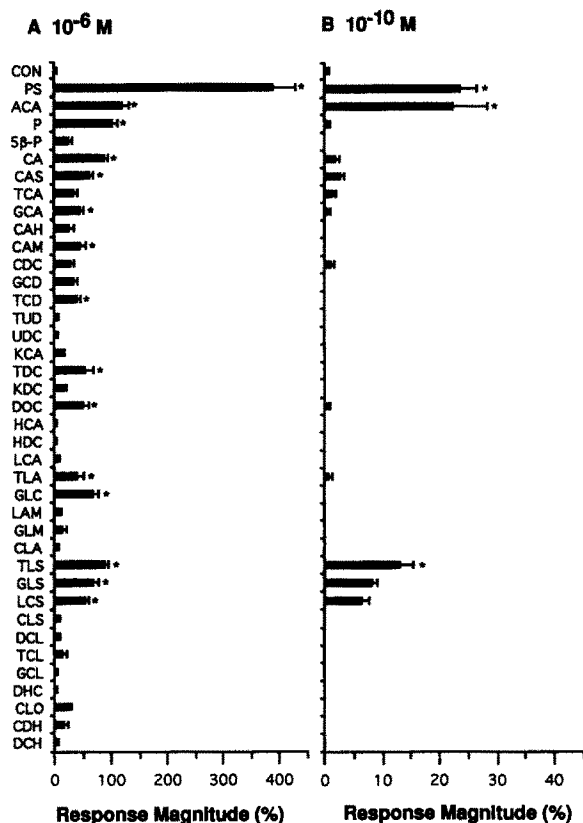


FIGURE 6. Electro-olfactogram (EOG) responses of migratory adult sea lamprey to bile acids at: (A) a concentration of 10^{-6} M ($n = 4-11$); (B) a concentration of 10^{-10} M ($n = 6-14$). Response magnitude relative to that elicited by the 10^{-5} M L-arginine standard. Notice the different scales in A and B. An empty space in A indicates that the compound elicited no response but an empty space in B indicates that the compound was not tested at 10^{-10} M. (Horizontal bars) Standard error. (Asterisks) Differences between the bile acid response and ethanol (A) or water control (B) ($P < 0.05$). Refer to Table I for abbreviations.

three hydroxyls), but did not change the potency of DOC (with two hydroxyls). Glycine conjugation had a similar effect to that of taurine.

The Specificity and Independence of Olfactory Receptor Sites

In each of the three cross-adaptation experiments, adapting the olfactory epithelium to one of the three bile acids suppressed responsiveness to itself ($P < 0.01$) but did not affect responsiveness to the other two bile acids ($P > 0.05$, Fig. 8).

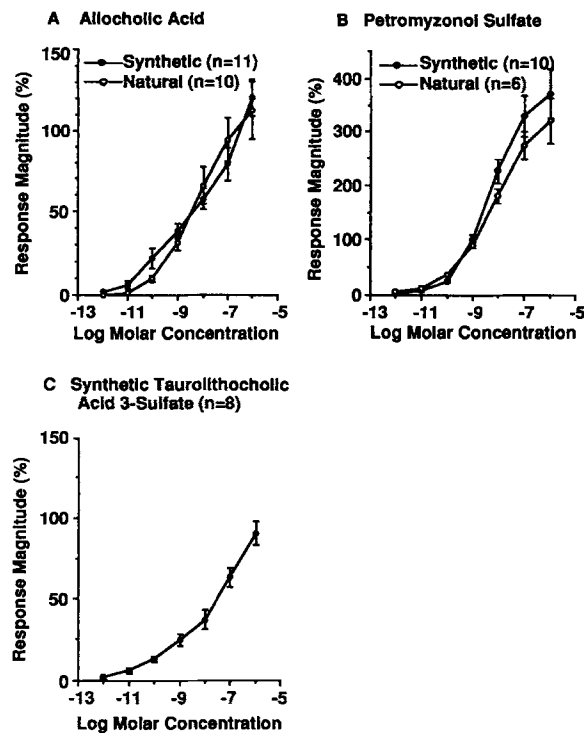


FIGURE 7. Semi-logarithmic plot of the concentration-response relationship of: (A) allocholic acid; (B) petromyzonol sulfate; and (C) taurolithocholic acid 3-sulfate in an ethanol-water or methanol-water solution for upstream-migratory sea lampreys. Synthetic indicates that the compounds were synthesized by chemical methods. Natural indicates that the compounds were purified from gallbladders of larval sea lamprey. Average response magnitude is represented as a percentage of that elicited by the standard stimulant, 10^{-5} M L-arginine. Vertical bars represent one standard error. Notice the different Y-axis scales.

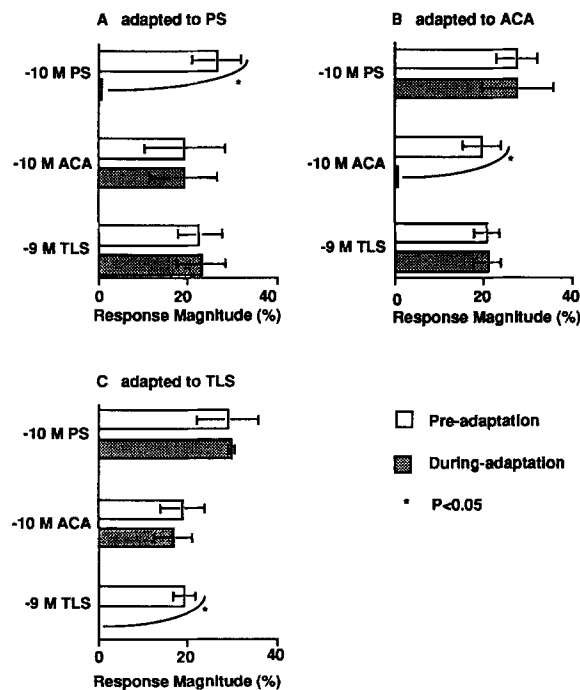


FIGURE 8. Cross-adaptation experiments. Electro-olfactogram responses elicited before (*open bars*) and during adaptation (*shaded bars*) to: (A) 10^{-10} M PS; (B) 10^{-10} M ACA; and (C) 10^{-9} M TLS. The response magnitude is represented as a percentage of that elicited by 10^{-5} M L-arginine. (*Horizontal bars*) One standard error. Odorant abbreviations are as defined in Table I. Concentrations are log molar. Pre and during responses were compared by Bonferroni paired *t* tests. Sample size, 3–6.

DISCUSSION

Our analyses of larval bile acids and their olfactory potency for adult sea lamprey strongly suggest that PS and ACA function as a conspecific olfactory signal for this species. Chemical analysis revealed that larval sea lamprey produce and release large quantities of ACA and PS. Petromyzonol sulfate is believed to be unique to lamprey (Haslewood et al., 1969) and our analyses demonstrated that, as proposed by Haslewood et al. (1969), it is the major bile acid produced by larvae. ACA was not identified by Haslewood et al. (1969) but this could have been due to the relative insensitivity of their technique (paper chromatography). The disproportionately large quantity of P in larval water could reflect bacterial degradation and warrants further study. Interestingly, larval bile acids are not found in either the liver or intestines of adult sea lamprey (Gallaher, Sorensen, and Olson, manuscript in preparation) which lose their gall bladders during metamorphosis (Yamamoto et al., 1986). Among other vertebrates studied, only the Australian lungfish, *Neoceratodus forsteri*, and the African lungfish, *Protopterus aethiopicus*, have been found to produce minor amounts of 5- β isomer of PS (Amos, Anderson, Haslewood, and Tokes, 1977; Haslewood, 1978). Allocholic acid has been found in a few other fish (Elliott, 1971; Hoshita, 1985) and whether these species also release unconjugated ACA is not known. Thus, larval lamprey are likely to be the only source of PS and P in lamprey habitats in North America. An important question yet to be addressed is whether other species of lamprey produce and release these bile acids and in what quantities.

The olfactory system of migratory adults is highly and specifically sensitive to ACA and PS. The detection thresholds for these two compounds were $\sim 10^{-12}$ M. This finding is particularly interesting because washings of larval sea lamprey, which we know now to contain ACA and PS, attract adults (Teeter, 1980). To our knowledge, these results are the first to demonstrate that larval fish release bile acids which are detectable by conspecific adults. It would be interesting to investigate this possibility for other species of fish. Similarly, it will be important to estimate the release rates of bile acids and their presence in natural river water.

Electro-olfactogram recording also demonstrates that the olfactory system of adult sea lamprey is specific enough to be capable of distinguishing larval bile acids from other bile acids. It is possible that sea lamprey use a mixture of ACA and PS as a signal. Two lines of evidence suggest that ACA, PS, and TLS are detected by three different types of receptor sites. First, the shapes of the concentration-response curves of ACA, PS and TLS are different. Second, adapting the olfactory epithelium to one compound does not suppress the EOG responsiveness of the epithelium to either of the other compounds. These results suggest that responses to these compounds are mediated by different receptor sites which also have independent signal transduction mechanisms. Because the difference between ACA and PS is at the C-24 position, it would appear that the C-24 sulfate ester is critical to the discrimination of these two bile acids. Similar effects of sulfate esters have been found in the olfactory detection of sex steroids by goldfish whose olfactory system is sensitive to both 17 α , 20 β -dihydroxy-4-pregn-3-one (17, 20 β P) and its metabolite, 17, 20 β P-20-sulfate (Sorensen, Goetz, Scott, and Stacey, 1992). It will be important to

fully elucidate the specificity of the lamprey olfactory system to a wide range of bile acids to determine exactly how these cues might be functioning.

The narrow sensory spectrum of the adult lamprey olfactory system to bile acids resembles that of the goldfish olfactory system to sex steroids. Of nearly 75 sex steroids tested on goldfish, only three are detectable at a concentration of 10^{-8} M (Sorensen, Hara, and Stacey, 1990; Sorensen et al., 1992; Sorensen and Scott, 1994; Sorensen, unpublished results). In contrast, the sensory spectra of teleost olfactory systems to the α -amino acids, a class of compounds postulated to function as food stimuli (Jones, 1992), are much broader. All teleosts tested to date are acutely sensitive to the majority of 20 L- α -amino acids (Hara, 1994). This may be advantageous because being sensitive to a large number of amino acids allows teleosts to learn to recognize a variety of food items (Atema, 1980). However, if ACA and PS function as a conspecific migratory signal for adult sea lampreys, then great sensitivity to these bile acids and poor sensitivity to other bile acids might be highly advantageous. The function of TLS as an odorant for lamprey is unknown.

The structure-activity relationship of bile acids suggests that the lamprey olfactory epithelium recognizes most, if not all, aspects of the PS and ACA molecules. For instance, a bile acid with a 5α -H evokes much larger EOG responses than a similar one with 5β -H. A 5α -H makes a bile acid molecule planar, while a 5β -H makes a bile acid L shaped, with the A-ring forming the short arm of the L (Haslewood, 1967; Hay and Carey, 1990). Furthermore, the axial orientation of hydroxyls makes bile acids more potent. However, a 5α -H and three axial hydroxyls does not ensure the olfactory potency of a bile acid as a negatively charged group at the 24-position is also critical to the potency of larval bile acids. Evidently, ACA and PS are the most potent bile acids because they have the combination of functional groups critical to the olfactory potency of bile acid molecules: three axial hydroxyls, a 5α -hydrogen and a negatively charged group at the 24 position.

An interesting exception to the latter situation is the fact that attachment of other negatively charged conjugating groups, taurine and glycine reduces the potency of taurocholic acid and other bile acids with three hydroxyls. This suggests an interesting similarity between sea lamprey and salmonids because in both cases the bile acids which they produce appear to be potent odorants for these species. In salmonids, taurine conjugation at the 24-position increases the olfactory potency of bile acids (Doving et al., 1980). Similarly, the major intestinal bile acids of the only salmonid examined to date, rainbow trout, are conjugated CA and CDC (Denton and Yousef, 1975; Sacquet, Lesel, Mejean, Riottot, and Leprince, 1979). Taurine conjugation is absent in lamprey bile acids. It thus appears that the olfactory epithelium of some fish may be tuned to conspecific bile acids.

Although our demonstration that the sea lamprey produce, release, and detect ACA and PS does not in itself prove that these bile acids function as a migratory pheromone, the evidence is compelling when considered in the context of several aspects of the life history of this species. First, although sea lamprey select only a small number of streams in which to spawn (Mormon, Cuddy, and Rugen, 1980), they do not always select natal streams (Applegate and Smith, 1951; Skidmore, 1959). In fact, adult sea lamprey appear to select streams for spawning that contain larvae as removing larvae from these streams frequently results in a decline in the

number of the adults entering the streams (Moore and Schleen, 1980). Second, when placed in a two choice maze, migratory adults stay longer in the side with larvae washings than in the control side (Teeter, 1980), suggesting that the migratory adults are attracted to chemical cues from larvae. Third, bile acids are stable, water soluble, and diverse enough to function as phylogenetic indicators (Haslewood, 1978, 1983; Hoshita, 1985). Fourth, the use of larval bile acids as a migratory cue makes ecological sense because the presence of larvae indicates habitat suitable for larvae survival. The critical test of our hypothesis will be determining if ACA and PS attract migratory adults to streams. This study is presently underway.

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