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Comparative Studies of Bile Acid Release in the Mature Male Lampreys

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Abstract

A comparative study of bile acid components from four lamprey species revealed that mature male chestnut lamprey *Ichthyomyzon castaneus* can produce 3 keto petromyzonol sulfate (3kPZS) while mature males of Pacific lamprey *Lampetra tridentata*, river lamprey *Lampetra fluviatilis* and American brook lamprey *Lethenteron appendix* produce petromyzonol sulfate (PZS). Identification of 3kPZS from a group of ancient lamprey species and of PZS from recently derived species led to a speculation that differentiation of bile acid biosynthetic systems has taken place during the course of evolution. Further studies on the biological functions of different bile acids in the adult lampreys are required to understand the evolution of chemical communication in lampreys.

Keywords: *Ichthyomyzon castaneus*, *Lampetra tridentata*, *Lampetra fluviatilis*, *Lethenteron appendix*, 3 Keto petromyzonol sulfate, Petromyzonol sulfate

Introduction

Lampreys are migratory, eel-shaped, jawless fishes. They have a multi-year stream-dwelling larval stage, metamorphose into ichthyo-parasitic juveniles that feed for 1-2 years in marine or lentic habitats, and mature into migratory adults that spawn in streams (Hardisty and Potter, 1971). Lampreys are extant representatives of the ancient vertebrates of which about 40 species remain (Janvier and Lund, 1983). All the northern hemisphere lampreys belong to a single family, Petromyzontidae (Porter, 1980).

Since a bile acid, petromyzonol sulfate (PZS), as presented in Fig. 1A, chemically identified from sea lamprey four decades ago (Haslewood and Tökés, 1969), there has been accumulating evidence that sea lamprey may use this bile acid as a chemical cue to guide the migrating lamprey to spawning streams (Li et al., 1995; Li and Sorensen, 1997; Bjerselius et al., 2000; Polkinghorne et al., 2001). In addition, another bile acid, allocholic acid (ACA), was implicated as a second compound that might play an important role as a migratory pheromone (Bjerselius et al., 2000; Polkinghorne et al., 2001).

Interestingly, sexually mature male sea lamprey can produce and release two bile acids, 3 keto petromyzonol sulfate (3kPZS), as presented in Fig. 1B, and 3 keto allocholic acid (3kACA), oxidized forms of the larval bile acids to attract conspecific for spawning (Li et al., 2002; Yun et al., 2003b). The concurrence of 3kPZS and 3kACA from mature lamprey, paralleled with the concurrence of PZS and ACA from larvae, led to speculation that sea lamprey have evolved a system to produce life stage specific chemical cues for their biological needs (Yun et al., 2003a).

It has been questioned whether other lamprey species use the same chemical cues for communication. The larval bile acid, PZS was found to exist in some larval lampreys, native to the Pacific coast, such as Pacific lamprey *Lampetra tridentata*, and western brook lamprey *L. richardsoni* although its biological roles are not yet known (Yun et al., 2003a). Based on these data, we have hypothesized that other lamprey species also can produce and release the oxidized form of this bile acid, 3kPZS, when they mature. To test this hypothesis,

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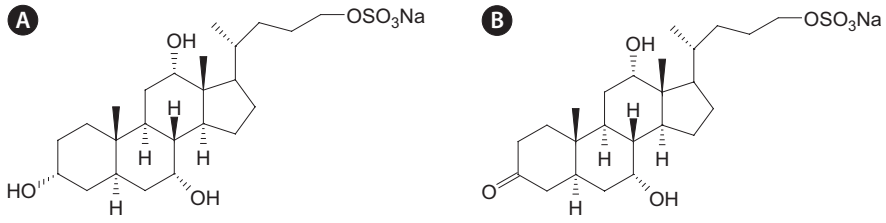

Petromyzonol sulfate (PZS) 3 Keto petromyzonol sulfate (3kPZS)

Fig. 1. Chemical structures of bile acids identified in lampreys, petromyzonol sulfate (A), and 3 keto petromyzonol sulfate (B).

extracts from the conditioned water with sexually mature male lampreys were analyzed with mass spectrometry (MS) and enzyme linked immunosorbent assays (ELISAs).

Here I report that chestnut lamprey *Ichthyomyzon castaneus,* produce and release 3kPZS while the two *Lampetra* species: Pacific lamprey, *L. tridentata*, and river lamprey *L. fluviatilis*; and American brook lamprey *Lethenteron appendix*, can produce PZS, a bile acid found in the sea lamprey larvae. Further, possible differentiation mechanisms are discussed in their evolutionary context.

Materials and Methods

Sample collection

Adult lampreys of the three species were collected from streams during their spring spawning migrations. Chestnut lamprey *I. castaneus* were collected 20 miles upstream from the mouth in the St. Joseph River, a southern Michigan tributary to Lake Michigan. Spawning individuals were collected in early April by the U.S. Fish and Wildlife Service and transported to Michigan State University where they were kept at 12°C in flow-through tanks. Animals were identified by their lateral circumoral dentition. Males were injected with 2, 100 μg/kg of body weight doses of lamprey gonadotropin releasing hormone I (Sower, 1990), 16 h apart. Three days after the injections, 8 males that were clearly spermiating were placed in a 10-L, aerated bucket. After 4 h, 2 L of water were collected and frozen at -80°C for later extraction.

Upstream migrating adult Pacific lamprey *L. tridentate* were collected from fish ladders at Willamette Falls on the Willamette River in Oregon and Bonneville Dam on the Columbia River in Washington in June 2001. Collected lampreys were transported to the Columbia River Research Laboratory (CRRL) and held in the onsite wet-lab facility. Lampreys were maintained on a simulated natural photoperiod with temperature-controlled, sand-filtered, flow-through river water from the Little White Salmon River. Water temperature was 15°C from June through October 2001, and near ambient Columbia River values, as measured at Bonneville Dam (5°C to 13°C), from November 2001 through June 2002. Conditioned water samples from the adult Pacific lamprey were collected in May 2002. Each individual lamprey was placed in 10 L of well water at 6°C (temperature of source) for 4 h with an air stone. After this time, lampreys were removed from their containers and anesthetized. Measurements of length, weight, and girths were made, and tissue samples were taken and stored. Concurrently, washing water was sampled and stored. Additionally, water from spermiating males, sampled on May 22, 2002, was pooled in equal amounts and extracted on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA).

River lamprey *L. fluviatilis* were collected from the River Ure, North Yorkshire, UK in April 2002 and held in a tank at the University of Durham aquarium facility at 7°C. Individual spermiating lamprey were held in a tank containing 5 L of water for 3 h. The conditioned water was collected.

Mature male and female American brook lamprey *L. appendix* were collected from the Carp River, south of Cecil, Michigan, USA. Females were gravid with eggs and males showed signs of spermiation. Individuals ranged in weight from 2.5 g to 7.5 g and in length from 87 mm to 119 mm. To collect the conditioned water samples, individual lampreys were placed in separate, aerated, Nalgene bottles containing 250 mL of Lake Huron water at 9.6ºC. The conditioned water was collected after 4 h.

Extraction of water conditioned with lampreys

The conditioned water samples from the four lamprey species were loaded onto Sep-Pak cartridges (Waters) where each Sep-Pak was eluted with 5 mL of methanol. The methanol eluates were pooled together, dried down, and subjected to MS and ELISA analyses.

Mass spectrometry

Mass spectra were obtained using a JEOL HX-110 double-focusing fast atom bombardment (FAB) mass spectrometer (JEOL, Peabody, MA, USA), operable in either the positive or negative ion mode. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV and the resolution was set at 3,000. Samples were prepared for MS by drying down the high-performance liquid

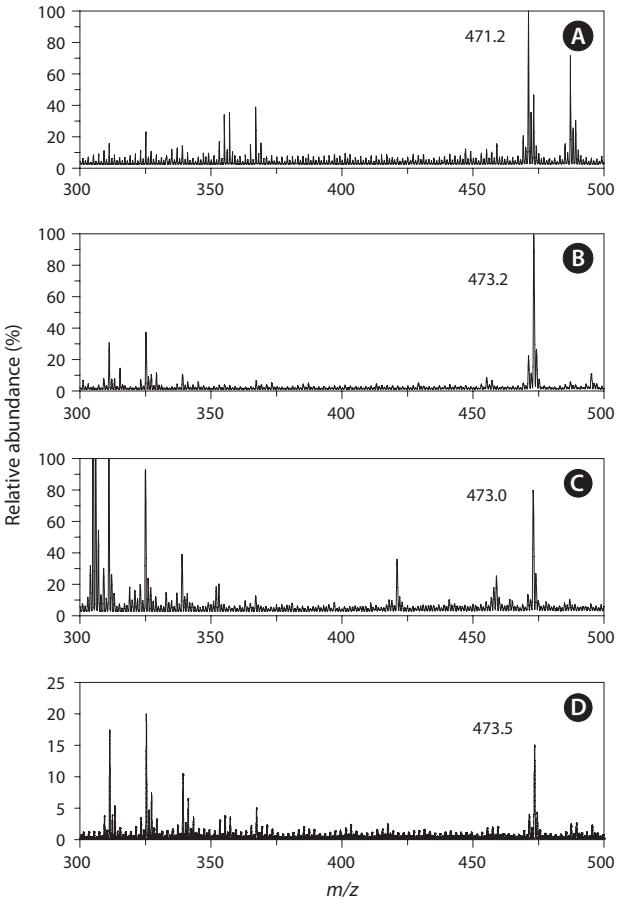

Fig. 2. Representative fast atom bombardment mass spectrometry analyses of water extracts conditioned with chestnut lamprey *Ichthyomyzon castaneus* (A), Pacific lamprey *Lampetra tridentata* (B), river lamprey *Lampetra fluviatilis* (C), and American brook lamprey *Lethenteron appendix* (D). An ionized peak at *m/z* 471.2 was observed from chestnut lamprey while ionized peaks at *m/z* 473.2, 473.0, and 473.5 were seen from Pacific, river and American brook lampreys.

chromatography fractions and re-dissolving them in methanol. High resolution MS was performed by peak matching with a resolution of 10,000. FAB MS was done at the NIH MS facility at Michigan State University.

ELISA analysis

The extracts of water samples conditioned with lampreys were analyzed using the ELISAs for 3kPZS and PZS as described previously (Yun et al., 2002; Yun et al., 2003a). Serial dilutions of the extracts were assayed along with both 3kPZS and PZS standards in a range of 20 pg-10 ng/well, to confirm the identities of bile acid compounds in the samples. The enzyme labels were generated by conjugating an enzyme, acetylcholine esterase, to 3kPZ-24-HS and PZ-24-HS. Labels were diluted 1,000-2,000 times while the antibodies were diluted 200,000 times.

Results

Identification of 3kPZS from silver lamprey by MS

MS analysis of the water extract from the male chestnut lamprey observed a major peak at *m/z* 471.2 in the negative mode (Fig. 2A). High resolution MS further confirmed the molecular mass of the major ionized peak to the 3.2 ppm level. No ionized peak for petromyzonol sulfate was found from this extract of conditioned water from chestnut lamprey.

Identification of PZS from Pacific, river, and American brook lampreys by MS

MS analyses of the water extracts from both the Pacific, river, and American brook lampreys revealed the major peak at *m/z* 473.2, 473.0, and 473.5, respectively and the molecular mass of the compound from the three extracts were further confirmed by high resolution MS at the 3.2 ppm, 1.8 ppm, and 0.3 ppm levels, respectively (Fig. 2B-2D). No ionized peak for 3kPZS was observed from these lamprey extracts.

Further verification of the bile acids in the lampreys (parallelism)

Close parallelism between the extract from the chestnut lamprey and 3kPZS standard was found (Fig. 3A). Similar close parallelism was also found between the extracts of the Pacific, river, and American brook lampreys and PZS standard (Fig. 3B).

Discussion

It has been well established that sea lamprey have developed an ability to produce life stage specific bile acid compounds to communicate between conspecifics (Li et al., 2003). I hypothesized that other lamprey species may have evolved the similar system because all the lamprey species have similar life histories. However, the present study revealed that at least the chemical identities of bile acids released by all other lamprey species are not parallel with those of sea lamprey. Water samples conditioned with four different lamprey species identified only 3kPZS from chestnut lamprey, and only PZS from Pacific, river, and American brook lampreys. It can be speculated that the two *Lampetra* species and one *Lethenteron* species have lost a system to convert 3α-OH steroids to 3 keto steroids during their life history, through the course of evolution.

It is interesting to observe that sexually mature males of evolutionary ancestral species (chestnut and sea lampreys) can produce 3kPZS while the three recently derived species (Pacific, river, and American brook lampreys) can produce PZS. Although controversy exists about the phylogeny and

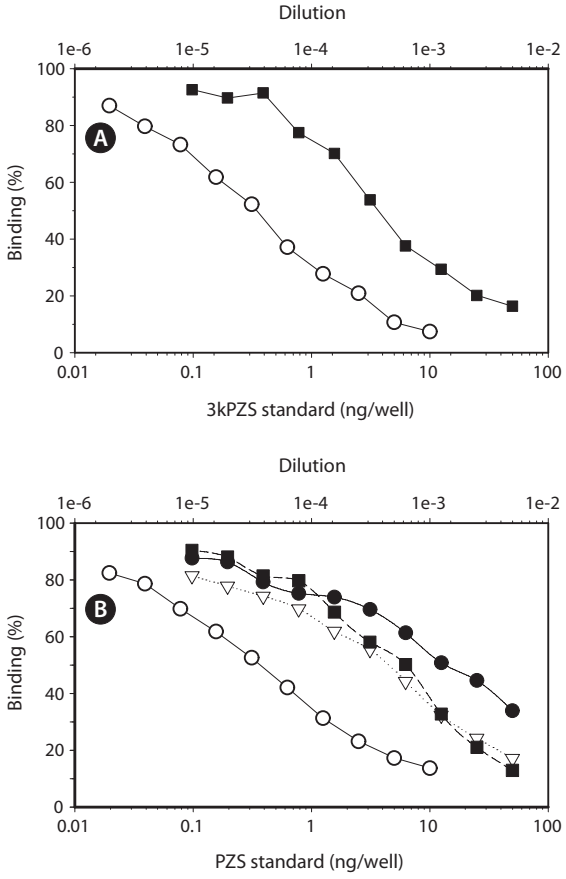

Fig. 3. Close parallelism between bile acid standards and water extracts conditioned with mature male lampreys. (A), close parallelism between serial dilution of water extract of chestnut lamprey *Ichthyomyzon castaneus* (■) and 3 keto petromyzonol sulfate (3kPZS) standard in a range of 20 pg-10 ng/well was observed (○). No displacement of 3kPZS standard by the extracts from Pacific lamprey *Lampetra tridentata*, river lamprey *Lampetra fluviatilis* and American brook lamprey *Lethenteron appendix* was found. (B), Serial dilution of water extracts from Pacific (●), river (■), and American brook (Δ) lampreys were assayed along with PZS standard (○) in a range of 20 pg-10 ng/well. No displacement of PZS standard by the extract from chestnut lamprey was found.

nomenclature of lamprey species (Bailey, 1980), phylogenies based on dentition (Hubbs and Potter, 1971; Porter 1980) have agreed that genus *Lampetra* is the most recently evolved of the extant lamprey genera. The genus *Lampetra* is further divided into three sub-genera, *Entosphenus*, *Lethenteron*, and *Lampetra,* with *Lampetra* being the most derived of the subgenera. A recent study used two mitochondrial genes to examine the phylogeny of *Lampetra*, and found evidence for *Entosphenus* to be a separate taxon, but that the division between *Lethenteron* and *Lampetra* does not exist and that this combined group is derived from *Entosphenus* (Docker et al., 1999). Although it has not been established whether all the lamprey species can produce PZS during their larval stage, it is likely that PZS is common among larval lampreys, based on a recent study that revealed that larvae of Pacific and western brook lampreys can produce and release PZS (Yun et al., 2003a). Differentiation of bile acids at adult stages seems to have taken place among the different group of lampreys during the course of evolution. However, present study cannot provide a further view as to when and how this differentiation has taken place because our sampling was limited to only ancient and recently derived groups. The findings in this study may raise more questions: 1) what is the biological function of these compounds; 2) why recently derived species have developed new biosynthetic pathways to produce PZS rather than using an old system that seemed to work efficiently in the ancient species; and 3) how they benefit from the switching the biosynthetic pathways to produce PZS in their adult stage. The answers to these questions should be addressed by studying chemical communication systems, bile acid biosynthetic pathways, and physiology of many ancestrally varied lamprey species.

Identification of life stage specific bile acids in sea lamprey led to a speculation that the enzyme 3α hydroxysteroid dehydrogenase (3α HSD) may have intervened in conversion of 3kPZS to PZS (Yun et al., 2003b). The lack of 3kPZS in the *Lampetra* and *Lethenteron* male adults suggests that the 3α HSD enzyme system has been lost or shut down in the recently derived lamprey species. However the possibility cannot be ruled out that other biosynthetic pathways have been involved in differentiation of bile acids in the newer species.

It makes ecological sense for some adult lampreys to release forms of bile acids that are different from larval bile acids, as observed in ancient lamprey species such as chestnut and sea lampreys. Otherwise these bile acids cannot be used as life-stage specific chemical cues. To the contrary, it seems very intriguing to find that some recently derived species release, even at their adult stage, the same bile acid as larval lamprey do considering they usually share the habitat with their larval stage conspecifics. There have been no studies that identify PZS as a putative pheromone in the *Lampetra* and *Lethenteron* species. However, it is possible that for these species to be able to use PZS as a chemical cue in their high background odor environment; more sophisticated olfactory system should have been developed.

In the Great Lakes, several strategies are used to control sea lamprey populations. The latest development is larval and sex pheromone research that uses pheromones as attractants to potentially enhance trapping efficiency (Li et al., 2003). Since some native species can produce both 3kPZS and PZS in their adult stage, application of bile acid pheromones to the natural environment poses possible ecological complications. Therefore, further systematic approaches are required to understand biological functions, geographical distributions, and phylogenetic relationships of bile acids in the native lamprey species of the Great Lakes region.

To summarize, we have identified 3kPZS from mature male chestnut lamprey and PZS from mature male Pacific, river, and American brook lampreys. Different bile acid identities between the ancient and recently derived lampreys suggest

that a modification of biosynthetic pathways for bile acid production has occurred during the course of evolution. Further systematic studies are required to better understand the evolution of chemical communications among lamprey species.

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