Sulfated Bile Acids as Putative Sex Pheromone Components in Pacific Lamprey

Sang-Seon Yun\textsuperscript{a}, Andras Szeitz\textsuperscript{b}, Andrew Wildbill\textsuperscript{c}, Michael Siefkes\textsuperscript{d} & David Close\textsuperscript{e}

\textsuperscript{a} Fisheries Centre, The University of British Columbia, 2202 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada
\textsuperscript{b} Mass Spectrometry Facility, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2405 Wesbrook Mall, Vancouver, British Columbia V6T 1Z3, Canada
\textsuperscript{c} Confederated Tribes of Warm Springs, Natural Resources Branch, The Dalles, Washington 97058, USA
\textsuperscript{d} Great Lakes Fishery Commission, Sea Lamprey Research Program, 2100 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA
\textsuperscript{e} Fisheries Centre and Zoology Department, The University of British Columbia, 2202 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

\textsuperscript{f} Present address: Faculty of Land and Food Systems, The University of British Columbia, 2357 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada.

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Sulfated Bile Acids as Putative Sex Pheromone Components in Pacific Lamprey

Sang-Seon Yun*1
Fisheries Centre, The University of British Columbia, 2202 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

Andras Szeitz
Mass Spectrometry Facility, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2405 Wesbrook Mall, Vancouver, British Columbia V6T 1Z3, Canada

Andrew Wildbill
Confederated Tribes of Warm Springs, Natural Resources Branch, The Dalles, Washington 97058, USA

Michael Siefkes
Great Lakes Fishery Commission, Sea Lamprey Research Program, 2100 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA

David Close*
Fisheries Centre and Zoology Department, The University of British Columbia, 2202 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

Abstract
Pacific Lamprey Entosphenus tridentatus, which is native to the Pacific coast of North America, is an important fisheries resource for some Native American communities and has been a conservation concern. Chemical analysis of water conditioned with mature male Pacific Lampreys and electrophysiological examination of the identified sulfated bile acids revealed that Pacific Lampreys may use the two bile acid compounds, 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS), as mating pheromones that can attract ovulatory females and stimulate them to nest. Liquid chromatography–mass spectrometry analysis on extracts of water conditioned with mature male Pacific Lampreys identified both 3kPZS, known as a major sex pheromone component, and PZS, known as a component of migratory pheromones in Sea Lamprey Petromyzon marinus. When combined with the previous electro-olfactogram (EOG) data demonstrating olfactory sensitivity of Pacific Lampreys to both compounds, the identification of the two bile acid compounds suggests that Pacific Lamprey evolved to have a chemical communication system for reproduction similar to that of Sea Lamprey. Further studies are required to confirm putative pheromonal functions of these two compounds in Pacific Lamprey. Comprehensive understanding of the reproductive behavior mediated by sex pheromones may provide a helpful tool in restoring the dwindling Pacific Lamprey populations along the North Pacific coast of North America.

The Pacific Lamprey Entosphenus tridentatus has declined throughout much of its range in the Columbia River basin of North America (Close et al. 2002). Concerns about the declining numbers of Pacific Lamprey led the state of Oregon to list them as a sensitive species in 1993, followed by protected status in 1996 (Kostow 2002) and reclassification as a species at risk in 2005 (ODFW 2006). Indigenous peoples from the Columbia River basin have experienced the loss of harvest opportunities (aboriginal rights) and culture associated with declining numbers of Pacific Lampreys (Close et al. 2002). The Pacific Lamprey is highly valued and considered one of

*Corresponding authors: s.yun@fisheries.ubc.ca; close@zoology.ubc.ca
1Present address: Faculty of Land and Food Systems, The University of British Columbia, 2357 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada.
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the important traditional foods of the indigenous peoples in the Columbia River basin (Close et al. 2002, 2004). Calls to restore this fish have become increasingly urgent.

Recent advancements in lamprey chemical communication systems using a Sea Lamprey Petromyzon marinus model demonstrated that chemical cues may play crucial roles in migratory and mating phases of their life history (Li et al. 2002; Sorensen et al. 2005). More specifically, a mixture of unique bile acid compounds released by river-resident larval lampreys can guide the migration of adult Sea Lampreys to the spawning ground (Fine et al. 2004; Sorensen et al. 2005; Fine and Sorensen 2008). A recent study by Yun et al. (2011) demonstrated that Pacific Lamprey larvae also release substances that can modify the migratory behavior of lampreys. Identification of the three bile acid compounds that are known to be components of a migratory pheromone in the Sea Lamprey strongly suggested there was a possible role for these three compounds to also be migratory pheromone components in Pacific Lamprey.

During the mating phase, sexually mature male Sea Lampreys release two bile acids, 3-keto petromyzonol sulfate (3kPZS) and 3-keto allocholic acid (3kACA) (Li et al. 2002; Yun et al. 2003b). Intensive electrophysiological and behavior studies demonstrated that 3kPZS is the major bile acid component that attracts ovulatory female Sea Lampreys on the spawning ground (Li et al. 2002; Johnson et al. 2005, 2009). In adult Pacific Lampreys, amino acids as well as lamprey bile acids can elicit olfactory responses when examined using the electro-olfactogram (EOG) (Robinson et al. 2009). That study revealed that adult Pacific Lampreys are sensitive to bile acid compounds such as petromyzonol sulfate (PZS) and 3kPZS, indicating possible roles of those compounds in chemical communication during the migratory and mating phases, if they are produced and released by male Pacific Lampreys.

Pacific Lampreys are known to have a similar life history to Sea Lampreys except for their longer migratory and spawning phases, which occur over a year (Keef er et al. 2009). However, it is well documented that Pacific Lampreys display an array of behaviors, including nest building, courtship, and spawning behavior, all of which are similar to those of Sea Lampreys. Recently, a close similarity in migratory cues was documented for Sea Lampreys and Pacific Lampreys (Yun et al. 2003b). Intensive electrophysiological and behavior studies demonstrated that 3kPZS is the major bile acid component that attracts ovulatory female Sea Lampreys on the spawning ground (Li et al. 2002; Johnson et al. 2005, 2009). In adult Pacific Lampreys, amino acids as well as lamprey bile acids can elicit olfactory responses when examined using the electro-olfactogram (EOG) (Robinson et al. 2009). That study revealed that adult Pacific Lampreys are sensitive to bile acid compounds such as petromyzonol sulfate (PZS) and 3kPZS, indicating possible roles of those compounds in chemical communication during the migratory and mating phases, if they are produced and released by male Pacific Lampreys.

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METHODS

Chemicals.—The synthetic bile acids, PZS, 3kPZS, and deuterated 3kPZS (3kPZS-5 d), were a gift from the Great Lakes Fishery Commission (Ann Arbor, Michigan). Acetonitrile and HPLC-grade methanol were purchased from Fisher Scientific (Ottawa, Ontario). All other chemicals were obtained from Sigma (St. Louis, Missouri) unless stated otherwise.

Collection of conditioned water and extraction.—Migrating Pacific Lampreys from the Columbia River were collected from the fishway (19–20°C) at the John Day Dam, Oregon, in June 2005 by the staff of the of the fisheries laboratory of the Confederated Tribes of the Umatilla Indian Reservation. The caught lampreys were transported to Michigan State University, East Lansing, Michigan, and held in 200-L flow-through tanks containing aerated well water at 12°C. In June 2006, male Pacific Lampreys were checked for maturation. Conditioned water, in which males showed signs of spermatiation, was collected. Spermatiation was confirmed by squeezing the fish’s abdomen to collect seminal fluid and then, under a microscope, checking for the presence of sperm in the seminal fluid. Fifteen spermatiating male lampreys were held in a static tank containing 200 L of well water for 24 h before water from the tank was collected. The conditioned water was then collected for filtration and the subsequent extraction process. The conditioned water was filtered with 0.45-μm glass-fiber filters (Whatman, Piscataway, New Jersey) and loaded onto a column (50 × 1,200 mm, Ace Glass, Vineland, New Jersey) packed with methanol-primed XAD 7HP resin (Sigma). After washing with deionized water, the column was eluted with 2 L of methanol. Methanol extracts were dried down under vacuum and stored at −80°C until analyzed.

LC–MS analysis of bile acids.—Samples of conditioned water extract from spermatiating lampreys were analyzed by LC–MS using a Scieix Qtrap 5500 mass spectrometer (Scieix, Framingham, Massachusetts) interfaced with an Agilent UPLC system (Santa Clara, California) at the Mass Spectrometry Facility, Faculty of Pharmaceutical Sciences, the University of British Columbia. Extract from water conditioned with spermatiating Pacific Lampreys was subjected to electrospray ionization mass spectrometry (ESI-MS) analysis in the negative mode by infusing 10 μL of the extract. The LC–MS analysis of the extract was performed using a C18 column (2.1 × 150 mm, Waters, Milford, Massachusetts) with a linear gradient. The mobile phase consisting of water with 0.1% formic acid and acetonitrile with 0.1% formic acid was applied at a flow rate of 200 μL/min, with a linear gradient of 0–100% acetonitrile with formic acid over 10 min. The effluent from the LC column was directed into the mass detector equipped with the ESI probe. A multiple reaction monitoring (MRM) scan was performed with transitions at a mass-to-charge ratio (m/z) of 473.2 > 97.2 for PZS, and m/z of 471.2 > 97.2 for 3kPZS, respectively. The collision energy used was −40 eV. Nitrogen and argon gas were used for desolvation and collision, respectively.

For quantification of PZS and 3kPZS concentrations in the extract of the conditioned water from mature male Pacific
Lampreys, an MRM scan was performed with transitions of 
\( m/z \) of 473.2 > 97.2 and \( m/z \) of 471.2 > 97 for PZS and 3kPZS, respectively. One nanogram of internal standard, 3kPZS-5 d, was added to each sample to calibrate. For both compounds, a standard curve was established in the range of 0.1–200 ng/mL. All MS data were collected and processed using Analyst software (Sciex).

RESULTS

Identification of the Sulfated Bile Acids: PZS and 3kPZS

Extraction of compounds from water conditioned with sexually mature male Pacific Lampreys was performed using XAD resin to obtain concentrated samples containing substances that were released by mature male Pacific Lampreys. Electrospray mass spectrometry scanning of the extract observed ionized peaks at \( m/z \) of 473.2 and \( m/z \) of 471.2 in the negative mode (Figure 1) along with some unknown peaks at \( m/z \) of 487.3 and \( m/z \) of 531.3. To confirm the identity of the compounds, MRM scanning using mass transitions of \( m/z \) of 473.2 > 97.2 and \( m/z \) of 472.2 > 97.2 for PZS and 3kPZS, respectively, was used, resulting in the identification of the two separate peaks that represented the two sulfated bile acid compounds (Figure 2A, B).

Estimation of the Ratio Between PZS and 3kPZS

The total concentrations of PZS and 3kPZS in the water extract were measured by LC–MS analysis using synthetic PZS and 3kPZS as standards. Peaks for each compound at retention time corresponding to that of synthetic copies of both compounds were identified and integrated. The PZS:3kPZS ratio was estimated based on the concentrations of both compounds in the extract and determined to be 0.015.

DISCUSSION

The decline of Pacific Lamprey populations in the Columbia River basin has raised concerns, prompting the urgent development of scientific tools to help restore their populations in the region. In the present study, we presented experimental data indicating that Pacific Lampreys may use the sulfated bile acid compounds, PZS and 3kPZS, as sex pheromone components. Comprehensive understanding of the physiological roles of Pacific Lamprey sex pheromones in the spawning grounds may lead to the development of a restoration tool by modifying their reproductive behavior in their natural environment.

In Sea Lamprey, 3kPZS was reported to be a main sex pheromone component with a possibility of another compound, 3kACA, being an auxiliary component that is involved in subtle spawning behavior (Yun et al. 2003b). Chemical, electrophysiological, and behavioral studies in Sea Lampreys clearly demonstrated that 3kPZS is a key sex pheromone component that can elicit unequivocal behavioral and electrophysiological responses in ovulatory females (Siefkes et al. 2005; Johnson...
et al. 2006, 2009). The chemical structures of both 3kPZS and PZS are illustrated in Figure 3. Surprisingly, the chemical profiling of the conditioned water extract of mature male Pacific Lampreys revealed that not only 3kPZS but also PZS was released into the water and that the concentration of PZS was approximately 70 times lower than that of 3kPZS. Further research is required to examine more specific roles of both PZS and 3kPZS in reproduction on the spawning grounds.

The EOG technique has been actively used to characterize olfactory mechanisms in fish (Scott and Scott-Johnson 2002). The EOG approaches have been successfully applied to examine the olfactory sensitivities of Pacific Lampreys to possible chemical cues, including amino acids and bile acids (Robinson et al. 2009; Yun et al. 2011). The two studies involving the EOG procedure with Pacific Lampreys demonstrated that the female Pacific Lamprey’s olfactory organs can detect 3kPZS and PZS at a concentration below $10^{-9}$ M. The Pacific Lamprey olfactory system responded more strongly to PZS and 3kPZS than to other sulfated compounds, such as petromyzonamine disulfate and petromyzosterol disulfate, and free bile acids, such as allocholic acid and 3kACA (Yun et al. 2011). In addition, cross-adaptation studies to characterize the specificity of each compound for olfactory receptors can further our understanding of olfactory mechanisms mediated by the bile acid compounds in Pacific Lampreys, as evidenced by an earlier study in Sea Lampreys (Sieckes et al. 2005; Li et al. 1995; Li and Sorensen 1997; Sieckes and Li 2004). Overall, results from the electrophysiological studies and chemical analyses strongly suggest that both PZS and 3kPZS act through olfaction to mediate critical physiological and behavioral functions in Pacific Lamprey.

Lampreys evolved 500 million years ago and are one of oldest extant vertebrates (Janvier 1996; Kumar and Hedges 1998). Phylogenetic analysis among lamprey species has indicated that the Sea Lamprey is a more ancient species than the Pacific Lamprey (Docke et al. 1999). Consequently, the use of chemical signals in Sea Lamprey suggests that this adaptation appeared early in lamprey evolution and was maintained in other species of lampreys. The production and detection of the same pheromones in Pacific Lamprey supports this conclusion. Moreover, our findings suggest a biological role for these pheromones in modifying migration behavior of Pacific Lamprey adults. This is particularly interesting in light of the fact that Pacific Lampreys must often execute spawning migrations over greater distances (hundreds of kilometers) and over longer time periods (1–2 years) than Sea Lampreys (Keefer et al. 2009). For this reason, other lamprey species may rely on the same chemosensory mechanisms found in the Pacific and Sea lampreys, possibly including those that do not even participate in anadromous migrations.

In summary, the identification of both 3kPZS and PZS from water conditioned with mature male Pacific Lampreys suggests that these sulfated bile acid compounds play a role as signaling molecules mediating sexual, attractive behavior in mature females on the spawning grounds. However, further designation of these compounds as chemical cues responsible for such behavior has yet to be validated by behavioral experiments that can demonstrate a positive attraction to the chemicals.

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