FROM SYNTHESIS TO BEHAVIORAL ACTIVITY IN STREAMS: INVESTIGATIONS OF PUTATIVE SEA LAMPREY PHEROMONE COMPONENTS

By

Cory Olaf Brant

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ABSTRACT

FROM SYNTHESIS TO BEHAVIORAL ACTIVITY IN STREAMS: INVESTIGATIONS OF PUTATIVE SEA LAMPREY PHEROMONE COMPONENTS

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The sea lamprey (*Petromyzon marinus* L.) has become a model species in the study of bile acid production and release into the environment where these compounds function as intraspecific chemical signals. Throughout the later stages of their life history, sea lampreys have been shown to rely upon pheromone communication to mediate reproduction. Laboratory and stream behavioral bioassays have implicated 3-keto petromyzonol sulfate (3kPZS) as a lamprey mating pheromone, but the full function of this bile alcohol derivative remains to be elucidated. Further, the biosynthesis, regulation, and release of 3kPZS and other putative components of the pheromone remain only partially characterized. In Chapter 1 of this thesis, I observed the behaviors of migratory females to the presence of 3kPZS in streams across a typical migratory season. In Chapter 2, the synthesis, transport, and release of several steroid-derived compounds in adult male sea lampreys were further examined using analytical chemistry and molecular biology-based approaches in adult males. The data presented here further characterize the male mating pheromone in sea lamprey, contribute to the understanding of pheromone communication in vertebrates, and provide implications for controlling the invasive species in the Laurentian Great Lakes.

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INTRODUCTION TO THESIS

INTRODUCTION TO THESIS

Pheromone communication is often employed by animals to mediate and synchronize their reproductive activities. Many of the reproductive strategies enabled by pheromones have been described in aquatic vertebrate species (Hoar, 1965; Lambert and Resink, 1991; Kobayashi et al., 2002; Li et al., 2003; Stacy, 2003). However, there are very few aquatic vertebrate species with structurally-identified pheromone components, and fewer with identified functions of these components or blends (Stacy, 2003). The sea lamprey (*Petromyzon marinus* L.) has been shown to use pheromones to mediate migration (Teeter, 1980; Bjerselius et al. 2000), and reproduction (Teeter 1980; Siefkes et al., 2005), with over six bile acid and bile alcohol derivatives hypothesized to be pheromone components (Haslewood and Tokes, 1969; Li et al., 1995; 2002, Yun et al., 2003; Sorensen et al., 2005). As a result, sea lampreys have rapidly become a model species for studies of bile acid biosynthesis, transport pathways, and subsequent release into the environment (Siefkes et al., 2003; Sorensen et al., 2005). In addition, related studies pertaining to behavioral activities mediated by these compounds in aquatic environments have proliferated (Teeter, 1980; Li et al., 1995; 2002; Siefkes et al., 2005; Johnson et al., 2009; Wagner et al., 2006; 2009).

Once a male sea lamprey matures (spermiating), they begin to release a mating pheromone blend that attracts sexually mature (ovulatory) females to the male sender (Siefkes et al. 2005). The most abundant constituent of the mating pheromone has been described as 3-keto petromyzonol sulfate (3kPZS; Li et al., 2002, Yun et al., 2003). 3kPZS is released across gill epithelia of spermiating males (Siefkes et al. 2003), and has been shown to induce upstream movement of ovulatory females and attract them to within proximity of a nesting male (Siefkes

et al., 2005; Johnson et al., 2009). 3kPZS is therefore hypothesized to function as an indicator of male mate-readiness to the female receiver (Siefkes et al., 2005).

In-stream tests that examined immature adult (migratory) female sea lamprey indicated that 3kPZS was capable of influencing the movements of test subjects (Johnson, 2008 PhD. Dissertation), which was found to contradict previous studies that examined migratory female preference response to 3kPZS in a maze (Siefkes et al. 2005). The discrepancies in the data may be due to the fact that maze studies were conducted in the daytime when migratory sea lampreys are less active (Applegate, 1950; Vrieze et al. 2011). Environmental variables such as natural light/dark cycles and ranges of stream temperatures have both been shown to effect (in addition to migratory pheromone) activity patterns of migratory lamprey (Binder and McDonald, 2008; Wagner et al., 2009); therefore, nighttime stream testing may be more suitable when examining behavioral activity of migratory females in relation to a pheromone component such as 3kPZS, as it relates to natural environmental factors (Johnson and Li, 2010).

Additional evidence that 3kPZS may be a behaviorally active compound among migratory females sea lamprey is as follows: (1) higher proportions of males arrive at spawning grounds before females during the establishment of nesting sites, where they then release 3kPZS (Applegate, 1950; Manion and Hanson, 1980), (2) nesting males release 3kPZS at higher rates compared to other identified components (Siefkes et al., 2003) suggesting that 3kPZS functions at longer distances in streams, and (3) 3kPZS is a steroid-derived compound that is stable for over several weeks in stream water (Yun et al. 2003; K. Li, personal communication), and is therefore capable of reaching longer distances downstream. The behavioral activities of migratory females in relation to 3kPZS are yet to be fully characterized.

Therefore, in Chapter 1 of this thesis titled, "A male sea lamprey pheromone component modulates the effect of stream temperature on upstream migration of immature females," a working hypothesis that 3kPZS indicates the onset of the spawning season is further examined. I predicted that presence of 3kPZS in a stream will induce behavioral responses from migratory females that increase their probabilities of locating mates, similar to ovulatory females (Johnson et al., 2009). Using a proven in-stream experimental system (Siefkes et al., 2005; Johnson et al., 2009), I examined upstream, and directional, movements of migratory-phase female sea lamprey in relation to the presence of 3kPZS ($5x10^{-13}$ molar [M]) across a migratory season. Results show that 3kPZS induces upstream movement of migratory-phase female sea lamprey by over 40% during cold (12-15^{o} C) stream temperatures, when migratory sea lamprey are less likely to move upstream movement (Binder and McDonald, 2008), but does not induce finer-scale movement towards or around the source of 3kPZS. The result suggests an additional function of 3kPZS, dependent upon the life history phase of the sea lamprey. Presence of 3kPZS in streams may function to stimulate upstream movement of migratory-phase female sea lamprey during otherwise inactive periods by modulating the effects of cold stream temperatures, perhaps as an adaptive behavior that increases the likelihood of females locating spawning grounds and mating successfully. Chapter 1 is currently in preparation for its submittal to Animal Behaviour, and is therefore written in accordance with the guide for authors.

While the current understandings of the functions of pheromone components from sea lamprey have been advanced throughout the past decade through stream behavioral testing (Johnson and Li, 2010), the biochemical pathways, regulation, and release mechanisms of pheromone components have only begun to be elucidated (Siefkes et al., 2003; Venkatachalam et

al., 2004; Venkatachalam, 2005). Understanding these processes will further elucidate the function and regulation of these components of the sea lamprey pheromone.

In Chapter 2, "Production and selective release of bile acids by the adult male sea lamprey (*Petromyzon marinus* L.)," I hypothesized that the system for biosynthesis, transport, and release of multiple components of the mating pheromone, based on what has been described with release studies examining 3kPZS (Siefkes et al., 2003), is dramatically up-regulated during or after sexual maturation of male sea lamprey. I predicted that male sea lamprey produce steroid derived pheromone components from cholesterols in the liver, only after reaching sexual maturity. Additionally, I predicted that the final stages of biosynthesis and release of the novel component 3kPZS occurs in their gill epithelia cells before release. Using analytical techniques, I discovered that the production and transport of multiple bile acids and bile alcohols occurs specifically after sexual maturation in male sea lamprey, and several components, previously thought only to be released by larval sea lamprey, are released by sexually mature males from gills. Finally, I present evidence that the final conversion of petromyzonol sulfate (PZS) into 3kPZS likely occurs in the gills, and this final conversion is likely necessary for the selective release of 3kPZS into the environment once a male matures. Chapter 2 is written in accordance with the guide for authors for Journal of Chemical Ecology, and is currently in preparation for that journal.

The studies presented in this thesis support further characterization of the biosynthesis-through-biological functions of 3kPZS and other compounds unique to sea lamprey. Additionally, 3kPZS and other compounds continue to show potential in terms of designing pheromone-based control methods of the non-indigenous sea lamprey in the Laurentian Great

Lakes (Twohey et al., 2003; Li et al., 2007), a major goal of the Great Lakes Fishery Commission and partners.

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CHAPTER 1

A MALE SEA LAMPREY PHEROMONE COMPONENT MODULATES THE EFFECT OF STREAM TEMPERATURE ON UPSTREAM MIGRATION OF IMMATURE FEMALES

ABSTRACT

This study examines whether a male-released sea lamprey (*Petromyzon marinus* L.) mating pheromone modulates the effects of temperature on behaviours of immature female conspecifics during their spawning migration. Mature (spermiating) male sea lamprey secrete a sex pheromone, 3-keto petromyzonol sulfate (3kPZS), that indicates mate-readiness and attracts mature (ovulatory) females to the male sender. 3kPZS may influence behaviors of immature (migratory) females over long distances in streams, based on the high rate of release by males and stability of 3kPZS in stream water. Here, we determined: (1) whether upstream movement of migratory females changes in the presence or absence of 3kPZS over a wide range of temperatures, and (2) whether migratory females will change directions towards the source of 3kPZS once they arrive upstream. Using an in-stream bioassay, we showed that the proportion of migratory females that moved upstream increased by over 40% in cold stream temperatures (~14.5^{o}C) when 3kPZS ($5x10^{-13}$ M) was consistently administered into the stream (ANCOVA: $F_{1,12} = 7.02$, $P = 0.021$). The effects of 3kPZS and temperature on upstream movement were no longer distinguishable in warmer stream temperatures (>16^{o}C). Migratory females that moved upstream did not change their direction towards the odorant source when 3kPZS was administered, but did so when a migratory pheromone blend (extracted from larval sea lamprey) was administered, indicating a general upstream response induced by 3kPZS. 3kPZS appears to function in multiple ways, dependent upon the phase of life history of receiving females, to synchronize reproduction in the sea lamprey.

INTRODUCTION

Many aquatic animals utilize pheromones to relay and/or acquire information regarding mate readiness (Stacey 2003) or certain environmental qualities (Donahue 2006). The semelparous sea lamprey (*Petromyzon marinus* L.) relies largely upon both migratory (Teeter 1980; Bjerselius et al. 2000; Sorensen et al. 2003) and mating (Teeter 1980; Li et al. 2002) pheromones to coordinate their reproductive activities (Manion & Hanson 1980). This species is a model for characterizing pheromone communication in aquatic vertebrates (Li 2005). However, studies that have aimed to further characterize activity patterns of sea lamprey in relation to pheromone components have rarely considered the impact of environmental conditions; due to the limitations of laboratory testing (Johnson & Li 2010).

During migration, upstream movements of female sea lamprey are influenced by stream temperatures (Beamish & Potter 1975; Binder & McDonald 2008; Wagner et al. 2009), and a migratory pheromone (Teeter 1980; Bjerselius et al. 2000; Sorensen et al. 2005; Wagner et al. 2009). Sea lampreys do not home to natal streams (Bergstedt & Seelye 1995; Waldman et al. 2008), but instead have been shown to enter and ascend tributaries based upon the presence and intensity of larvae-released odours upstream (Teeter 1980; Wagner et al. 2009). In controlled laboratory settings, locomotor activity of immature adult (migratory, herein) female sea lamprey is reduced as temperatures decrease, until inactivity occurs below 10^{o} C (Binder and McDonald 2008). In streams, the frequency of upstream movement of migratory female sea lamprey in the presence of the migratory pheromone is reduced during cold temperatures (Wagner et al. 2009). Temperature-induced behaviours are proposed to represent an adaptive behaviour in sea lamprey that increases the probability of females reaching spawning grounds across a narrow thermal range for successful embryonic development and reproduction (Binder & McDonald 2008).

During the spawning season, a sulfated bile alcohol: 7α, 12α, 24-trihydroxy 5α-cholan-3-one 24-sulphate (3-keto petromyzonol sulphate or 3kPZS; Li et al. 2002; Yun et al. 2003) a major constituent of the mating pheromone blend released by mature (spermiating, herein) male sea lamprey (Siefkes et al. 2005; see Li et al. 2003; Li 2005 for review). Siefkes et al. (2005) and Johnson et al. (2006) used a stream behavioural bioassay to show preference responses of mature (ovulatory, herein) females to 3kPZS; including directional changes towards the odorant source and odorant plume tracking behaviours, respectively. Further, Johnson et al. (2009) showed that synthesized 3kPZS alone was capable of drawing ovulatory females past natural spermiating male odorants, and into baited riverine traps, confirming that greater concentrations of 3kPZS could be distinguished from the whole male pheromone blend by female conspecifics. Accordingly, the selectivity of olfactory receptors to 3kPZS was shown in female sea lamprey based on electro-olfactographic recordings by Siefkes & Li (2004).

Males often aggregate at upstream spawning grounds in streams where migration is still occurring (Applegate 1950; Manion & Hanson 1980). Once males reach maturity, they release 3kPZS and other compounds (Siefkes et al. 2003) to attract mates. Based on the high release rate of 3kPZS by spermiating males (~0.5 mg hr^{-1}) compared to other compounds (Li et al. 2002; Siefkes et al. 2003), it seems likely that 3kPZS functions as a long distance chemical cue, that my influence behaviours of migratory female conspecifics. Recent behavioral tests that examined migratory female sea lamprey indicated that 3kPZS was capable of influencing upstream movements of test subjects (Johnson 2008: PhD. Dissertation), which was a contradictory result to previous studies that examined migratory female preference responses to 3kPZS in a laboratory maze (Siefkes et al. 2005). The discrepancies in the data may be due to the fact that laboratory maze studies were conducted in the daytime when migratory sea lamprey are less

active (Applegate 1950; Vrieze et al. 2011), while stream tests were conducted at night where migration occurs naturally (Vrieze et al. 2011), and thermal ranges can be examined for a behavioural influence on migratory activity (Binder and McDonald 2008).

Here, we hypothesize that 3kPZS influences behaviours of migratory female sea lamprey in similar fashions (with slight modification, since migratory females are not ready to mate) to those documented in ovulatory females (Siefkes et al. 2005; Johnson et al. 2006: 2009). We examined two questions using stream bioassays: (1) Does 3kPZS increase the upstream migratory movement of the female sea lamprey at low stream temperatures (Binder and McDonald 2008); and (2) Do migratory female sea lamprey actively approach 3kPZS sources in the stream similar to ovulatory females (Siefkes et al. 2005; Johnson et al. 2009). Our results show that 3kPZS modulates the effects of cold stream temperatures on upstream movement of migratory female sea lamprey; whereby, test subjects moved upstream in the presence of 3kPZS during cold stream temperatures, but did not locate the 3kPZS source.

METHODS

Sea lamprey

All procedures involving sea lamprey were conducted in accordance with our Animal Use Form number 05-09-088-00, which was approved by the Michigan State University Institutional Animal Care and Use Committee prior to the start of the study. Migratory sea lamprey were captured by the United States Fish and Wildlife Service throughout tributaries to Lake Michigan, Lake Superior, and Lake Huron, U.S.A., in May-June 2009 and transported to the United States Geological Survey-Hammond Bay Biological Station (HBBS). Females were separated from males by applying gentle pressure to the abdomen and feeling for eggs. Their sex was later confirmed during surgical tagging procedures. Males were removed since only females were released for testing during this study. Females were held in 500-1000 litre-capacity flow through tanks at HBBS until use.

To confirm that test sea lamprey were pre-ovulatory, eight females were randomly selected on 17-May and 1-June, and 10 females were selected on 11-June-2009, and sacrificed for dissection. Samples of oocytes were collected from the posterior, medial, and anterior locations of the ovary. Oocytes were fixed in a 4% paraformaldehyde solution and placed in 4^{o}C until sectioning and hemotoxylin and eosin staining. Histological examination showed that all oocytes sampled were pre-ovulatory according to Applegate (1951), Lewis and McMillan (1965), and Yorke and McMillan (1980): see Figure 1-3S.

PIT tagging

A 23 mm-long half duplex passive integrated transponder (PIT) tag (Oregon RFID, Portland, Oregon, U.S.A.) was surgically implanted into each experimental animal through a 2-3 mm lateral incision in the mid-abdominal region. The incision was sealed with tissue adhesive (n-butyl cyanoacrylate, Vetbond; Minnesota Mining and Manufacturing, St. Paul, Minnesota, U.S.A.) immediately after PIT tag insertion. No anesthesia was used in this process to avoid the potential disruptive nature of common fish anesthetics to olfactory-mediated behaviours (Losey & Hugie 1994). The procedure typically took less than 30 seconds. Implanted animals were immediately transferred into aerated holding tanks with a constant flow of Lake Huron water for up to 24 hours, until they were stocked into stream acclimation cages. Tagged individuals were monitored throughout the day for signs of distress or mortality. Mortality was not observed during laboratory or stream acclimation.

Test odorants

Permission to administer 3kPZS into the stream was obtained from the United States Environmental Protection Agency via experimental use permit 75437-EUP-2. 3kPZS was custom synthesized by Bridge Organics (Vicksburg, Michigan, U.S.A.; purity >97%) in 2007, and stored at -80^{o}C. An analysis was conducted on 13-March-2009 to confirm 3kPZS purity was maintained before stream testing. The purity was determined by dissolving 0.538 mg of 3kPZS in a 1-ml mixture of 50:50 acetonitrile:water (v:v), and then subjecting 20 µl of the solution to high performance liquid chromatography (HPLC; Waters ACQUITY, Milford, Massachusetts, U.S.A) coupled with an evaporative light scattering detector (ELSD, Waters). Nuclear magnetic resonance (^{1}H NMR) was used to confirm the chemical structure of 3kPZS. From the analysis,

the purity was found to be greater than 95% prior to stream testing. A 10 mg ml^{-1} stock solution of synthesized 3kPZS (in 100% methanol) was prepared, vortexed (Vortex Genie 2, Daigger, Vernon Hills, Illinois, U.S.A.), and transferred into five vials of 10-ml aliquots, each. 3kPZS stock solution was stored at -80^{o}C until use.

Extracted larval odour, presumably containing all larval pheromone components, was used as a positive control to validate the experimental system. Larval extracts have been shown to induce strong swimming directional changes towards the odorant source from migratory female sea lamprey during past studies (Bjerselius et al. 2000; Wagner et al. 2009). Methods of extraction followed those by Fine et al. (2006), with slight modification. Over 20 000 larval sea lamprey were held in flowing 500 litre-capacity tanks at HBBS for collection of larval pheromone extracts from April-August 2008. Larvae were fed yeast on a weekly basis, and given a sand substrate for refuge. Tank flows were shut off at night allowing larval pheromone to accumulate. The larval-conditioned water was then passed through vertical columns containing 500 g of methanol-activated Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) using peristaltic pumps (Cole-Parmer, Vernon Hills, Illinois, U.S.A). Loading speed was ~300 ml min^{-1}. Three or more columns were loaded for up to 24 hours at a time. Each column was then eluted with 4-l of methanol and eluants were concentrated using a model R-210 roto-evaporator (Buchi Rotovapor, Flawil, Switzerland) and stored at -80^{o} C. All larval extracts were fully thawed, pooled, and thoroughly mixed before further analyses were conducted. Petromyzonamine disulfate (PADS), a component of larval extract with known chemical structure (Sorensen et al. 2005), was used as a benchmark compound when calculating the volume of extract to consistently apply to the stream. The concentration of PADS was determined using high performance liquid chromatography–tandem mass spectrometry (HPLC-

MS/MS). For reference, the PADS concentration was found to be 183.3 µg l^{-1} in the larval extract batch used (2008 batch).

Experimental site for behavioral tests

The experimental site consisted of a 250-m section of the Upper Ocqueoc River located in Millersburg, Michigan, U.S.A (T35N, R3E, Sec. 27). The upper reaches of the Ocqueoc River were historically infested with nesting sea lamprey (Applegate 1950). Wild populations of sea lamprey are now physically barred from entering the Upper Ocqueoc River due to the Ocqueoc Lake dam located ~20 km downstream. The first 45-m of the site was divided by a natural island, which separated two sub-channels of similar hydrologic and physical qualities (Figure 1-1). Any potential water leakage between the two sub-channels was prevented by the placement of sand bags. Test odorants were administered from a fixed point in the stream at the upstream end of each sub-channel. Two copper wire PIT antennas roughly 0.5 m-high x 6 m-long independently transected the mouth of each sub-channel at their downstream confluence. Each antenna was positioned to transect the mouth of each respective sub-channel, positioned perpendicular to the stream bed. Antennas were tuned to a sensitivity of roughly 0.3 m from upstream and downstream directions, and scan frequencies were roughly three scans $sec.^{-1}$. Downstream 205-m from the transecting antennas was an animal acclimation and release point at a fixed location in the centre of the stream.

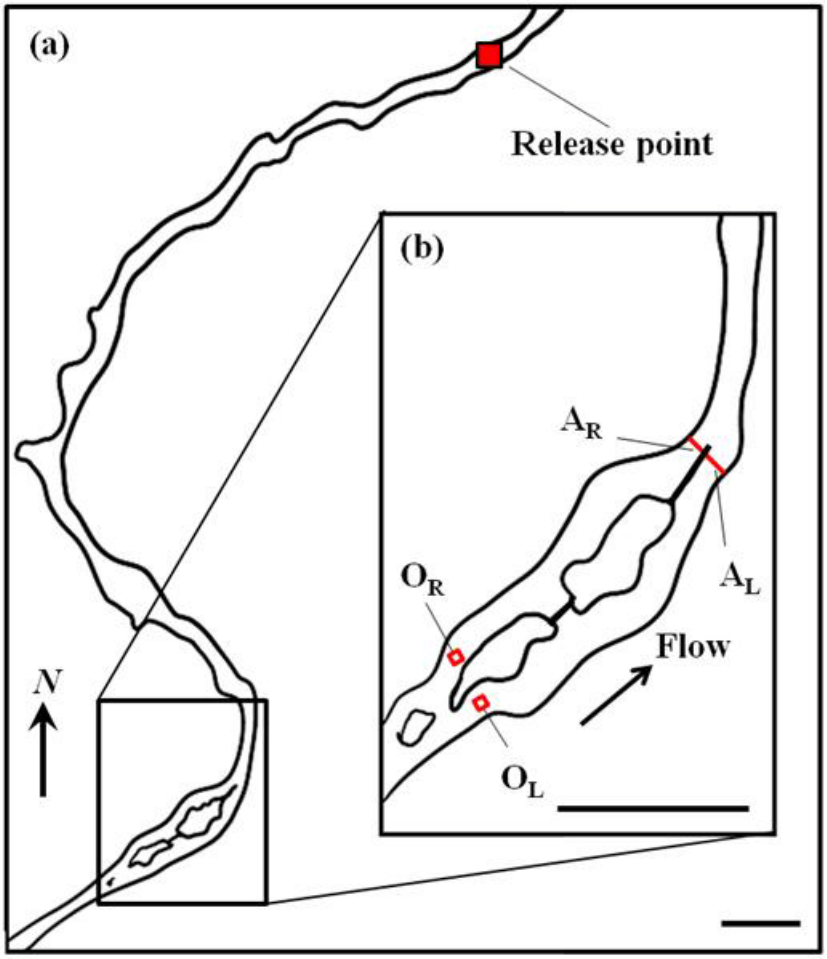

Figure 1-1. The section of the Upper Ocqueoc River, Millersburg, MI, U.S.A. (T35N, R3E, Sec. 27) used as the in-stream experimental system for observing upstream movement of migratory phase female sea lamprey in relation to male-released pheromone component 3kPZS. **(a)** Full 250 m-long section showing release point. **(b)** Close-up of system showing naturally bisected sub-channels with respective right channel (O_R) and left channel (O_L) odorant administration points (red boxes). Odorants were alternated in each sub-channel per trial. At the downstream confluence of the bisected channels, copper wire antennas recorded proportions of PIT-tagged animals moving into either the right ($A_{R)}$ or the left (A_L) sub-channels. Scale bars, 25 m.

Details of treatments and trials

A latex tube was fixed to the bottom of the stream bed at the upstream end of each sub-channel. Test odorants were diluted with 30 l of river water in large mixing bins. Bins were kept consistent for each test odorant to reduce the potential for contamination during dilution. Each solution was pumped into respective sub-channels through separate latex tubes at constant rates of 167 ml min^{-1} (±5 ml min^{-1}) over the span of three hours using peristaltic pumps (Masterflex 7553-70, Cole-Parmer, Vernon Hills, Illinois, U.S.A.). A test odorant was administered to one sub-channel (activated channel, herein) while an equal volume of methanol was administered into the adjacent sub-channel (control channel, herein), and the activated and control channels were alternated each trial to minimize channel bias. Test odorants included; (1) synthesized 3kPZS ($5x10^{-13}$ M) administered into one sub-channel (methanol into the adjacent sub-channel), (2) larval extract ($5x10^{-14}$ M PADS) administered into one sub-channel (methanol into the adjacent sub-channel), and (3) full negative control (methanol into both sub-channels). Stream velocity was taken every three days, or after every precipitation event, at a fixed location in the stream using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, Maryland, U.S.A.) to determine the volume of odorant to apply to the stream and maintain consistent concentrations across trials. Stream velocity ranged from 0.5 – 1.0 m sec^{-1} with an average of 0.78 m sec^{-1} ($X + SE = 0.78 + 0.018$ m sec^{-1}; $N = 50$).

Trials were conducted between 13-May and 11-June, 2009, at night when migrating sea lamprey are most active (Applegate 1950). A total of 21 full negative control trials, 16 synthesized 3kPZS trials, and four larval extract trials were conducted throughout the study. Up to two trials were conducted each night, depending upon animal availability. Twenty or 30 PIT-tagged sea lamprey were removed from holding tanks at HBBS and transported to their

respective stream acclimation cage between 0300-0500 hours (h) each night. Whether 20 or 30 animals were stocked and released per trial depended upon animal availability; however, the number of test animals stocked was consistent between early and late trials within each night. Early trial animals were held independently from late trial animals, in identical acclimation/release cages. Animals were then allowed an acclimation period in the stream of more than 15 hours, prior to a trial. No mortality occurred during acclimation. Acclimation cages were solid aluminum and stainless steel (~0.25 m^3), consisting of a sliding door that was removed manually upon release. Each trial was three hours long. The early trial was conducted from roughly 2020 h (starting at sundown) through 2320 h, and a late trial could then be run from roughly 0010 h through 0310 h.

At the start of each trial, the stream gauge height and stream temperature were recorded. Stream temperatures ranged from 12.4 – 23.0^{o}C, and average stream temperature during early trials was slightly greater than that of late trials ($X+SE$ = 17.2 + 0.5^{o}C and $X+SD$ = 15.6 + 0.6^{o}C, respectively). Stream gauge height ranged from 0.51 – 0.62 m with an average of 0.56 m ($X+SE$ = 0.56 + 0.005 m; N = 50). The average rate of stream temperature decrease from early to late trials was 2^{o}C $hour^{-1}$ ($X+SE$ = 1.99 + 0.13^{o}C $hour^{-1}$). The distribution of stream temperatures across dates can be seen in Figure 1-4S. In the first hour of each trial, the test odorant was administered to the stream allowing the current to carry the compound to the downstream acclimation cage prior to the release of test animals. At the start of the second hour, 20 – 30 PIT-tagged females were released. During the remaining two hours, animals were free to swim throughout the experimental system while odorants were administered.

The second trial started 30 minutes after the first. Test odorants were kept consistent for each night of trials (i.e. if 3kPZS was tested during the early trial, 3kPZS was also tested during

the late trial to prevent the possibility of any unwanted contamination from other test odorants). All equipment was thoroughly rinsed with stream water prior to a new trial. No animals were recovered from the stream after a trial. Movement data was consolidated and stored using a multiplexor (Oregon RFID, Portland, Oregon, U.S.A.). Data was uploaded each trial night using a hand-held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

Throughout the study season, all handling, transportation, lengths of stream acclimation periods of test subjects, and the concentration of test odorants applied to the stream remained consistent across all trials to minimize the chances of abnormal behaviours due to animal treatment.

Water sampling

Water samples were collected on 10-12 June 2009 during this study for analytical confirmation of in-stream 3kPZS concentrations during a trial, and to confirm that no natural 3kPZS was currently in the system. For each sampling event, triplicate 1-l samples were collected from a fixed location upstream of the odorant source point, and from downstream at the acclimation/release site, during 3kPZS trials. Analytical methods to determine 3kPZS concentrations from stream water were similar to those used by Fine and Sorenson (2005) for the quantification of closely related sea lamprey produced compound petromyzonol sulfate (PZS) in streams. Each sample was immediately spiked with 5 ng ml^{-1} (dissolved in 100 µl methanol) of 5-deuterated 3kPZS internal standard ($[^2H_5]$3kPZS; Bridge Organics, Vicksburg, MI, U.S.A.) and placed in -80^{o}C. 3kPZS was extracted from stream water with high recovery rates (> 90%) using solid phase extraction (single cation-exchange and reversed-phase mixed-mode cartridge;

Oasis MCX, Waters, Milford, Massachusetts, U.S.A.). Final quantification was accomplished using ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The limit of detection was acceptably small (< 0.1 ng l^{-1}). The experimental system was confirmed to be clear of any natural 3kPZS upstream of the field site and downstream of the odorant source prior to the start of a trial. 3kPZS was shown to reach the acclimation cage area of the site (250 m downstream) within one hour of active pumping, which confirmed that the acclimating animals were exposed to 3kPZS before their subsequent release. Further, in-stream concentrations of 3kPZS were shown to be acceptable ($X+SE$ = 5.76x10^{-13} + 7.65x10^{-14} M 3kPZS, N = 9), in relation to our target stream concentration of 5x10^{-13} M 3kPZS (Xi et al. *in review*).

Data analysis

Two main response variables from migratory female sea lamprey were examined in relation to the presence of test odorants: (1) the proportion of released animals that swam upstream (205 m) to the confluence of the two sub-channels, and (2) the proportion of upstream animals that entered the activated sub-channel. Since we administered methanol into both sub-channels for full negative control trials, we randomly assigned a sub-channel to be the "activated" channel for each of these trials during statistical analyses. When methanol was administered to both sub-channels during full negative control trials, the right-facing sub-channel (facing upstream) was randomly assigned as "activated" for the first trial conducted, and alternated between sub-channels for all trials thereafter.

An analysis of covariance (ANCOVA, α = 0.05) based on a generalized linear model was used to test whether certain factors had an influence on the response variables of interest in

relation to odorant treatments. The multiple mixed factors that were examined included: (1) stream gauge height, (2) date, (3) stream temperature, (4) rate of decreasing stream temperature per trial, (5) time of trial, and (6) the number of animals released per trial. Models incorporating mixed and fixed factor effects were constructed to compare residual log likelihood (LogLik) and AIC values using SAS statistical software (SAS Incorporated, Cary, North Carolina, U.S.A.; Table 1-1S). Differences of least squares means (unpaired t-test, $\alpha = 0.05$) of the proportion of animals moving upstream per trial were compared at the lower, median, and upper quartiles of covariate-temperature (14.5, 17.5, and 19.5^{o}C, respectively) to examine the interaction between the upstream movement of test subjects and stream temperatures. Slopes of regression lines for 3kPZS and negative control trials were compared to a slope of zero, and to each other, using an unpaired t-test ($\alpha = 0.05$).

RESULTS

Upstream movement of migratory females in the presence of odorants

The rate of stream temperature decrease per trial (^{o}C hr^{-1}), stream gauge height (m), time of trial (early or late), or the number of migratory females released per trial (20-30) did not influence the proportion of migrating females moving upstream across trials in our experimental system (ANCOVA: $F_{1,10}$ = 0.97, P = 0.348; $F_{1,12}$ = 0.42, P = 0.530; $F_{1,11}$= 0.01, P = 0.919; $F_{1,12}$ = 0.40, P = 0.540, respectively), and were therefore removed from the final model. The final model considered the effect of test odorant or stream temperature independently, and the interaction between the two, for best explaining the variability in the proportion of migratory sea lamprey moving upstream across trials. Statistical fitness values were lowest in this final model (*AIC* = -6.2, *logLik* = -12.2; Table 1-1S). Stream temperature across all trials can be seen in Figure 1-4S.

An average proportion of 0.61 (X+SE= 0.61+0.21, n = 233) of total released migratory females (N = 380) moved upstream to the confluence during 3kPZS trials. Full negative control trials showed an average proportion of 0.45 (X+SE= 0.45+0.13, n = 239) of released migratory females (N = 500) that moved upstream to the confluence of the sub-channels. When synthesized 3kPZS was administered to the stream, greater proportions of released migratory females moved upstream compared to full control trials (ANCOVA: $F_{1, 12}$ = 9.17, P = 0.012). Specifically during low stream temperatures (~14.5^{o} C), higher proportions of migratory females moved upstream during 3kPZS trials (ANCOVA: $F_{1,12}$ = 7.02, P = 0.021), compared to methanol-control trials (Figure 1-2, and herein). The slope of the regression line corresponding to 3kPZS trials (m = 0.03, R^2 = 0.14) was not different from zero, while that of full control trials (m = 0.1,

R^2 = 0.70) were different from zero (independent t-test: t_{10} = -1.87, P = 0.091; t_{10} = -2.88, P = 0.016, respectively). Further, the slopes of the 3kPZS and full control regression lines were different from one-another (independent t-test: t_{12} = -2.65, P = 0.021).

During larval extract trials, an average proportion of 0.53 (X+SE= 0.53+0.30, n = 63) of total released migratory females (N = 119) moved upstream 205 m to the confluence of the sub-channels. There was no difference between larval extract mean swim-up proportions across trials compared to full negative control or 3kPZS trials (ANCOVA: $F_{2,12}$ = 2.29, P = 0.144).

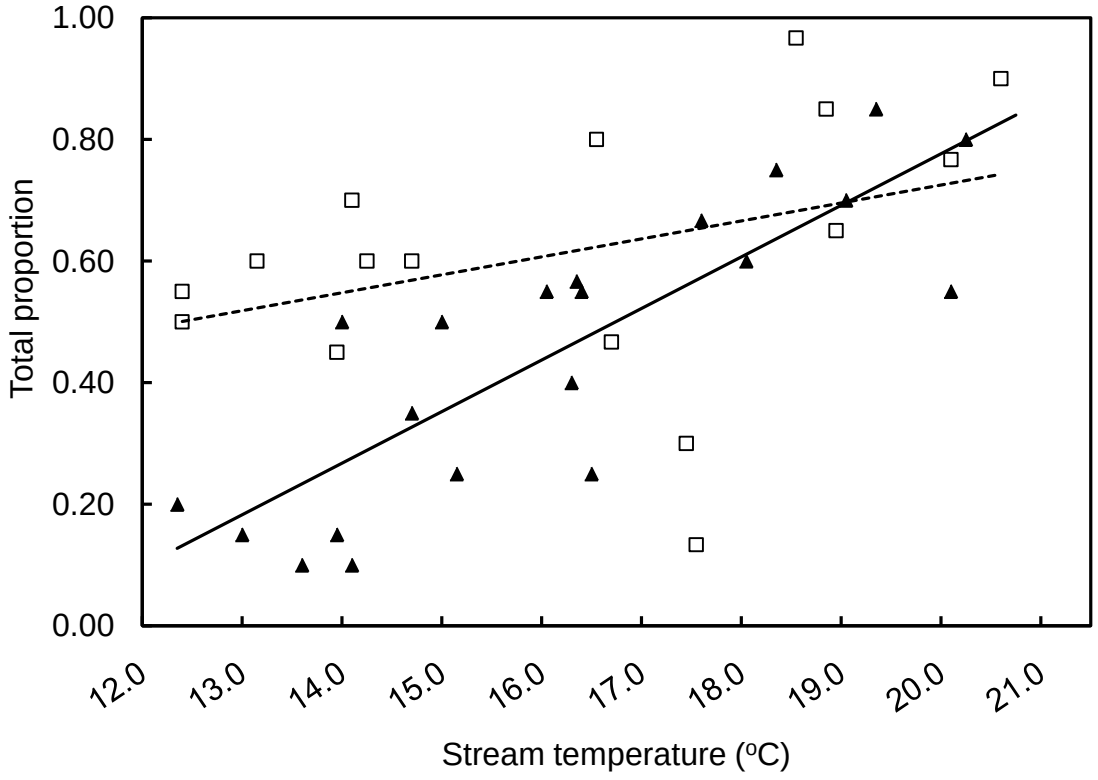

Figure 1-2. Effects of a pheromone component (3kPZS) on the proportion of migratory female sea lamprey that swam upstream across stream temperatures between 12.4 – 23.0°C. □: Trials where synthesized 3kPZS ($5x10^{-13}$ molar [M]) was administered to the stream. ▲: Trials where methanol (vehicle) was administered to the stream. The solid regression line represents methanol trials (R^2 = 0.70), and the dashed regression line represents 3kPZS trials (R^2 = 0.14). Trials were conducted at night in the Upper Ocqueoc River, Millersburg, Michigan, U.S.A.

Directional responses to odorant sources

Of the test animals that moved upstream during 3kPZS trials, an average proportion of 0.42 (X+SE= 0.42+0.24, n = 95) moved into the respective 3kPZS-activated sub-channel. During full negative control treatments, a proportion of 0.53 (X+SE= 0.53+0.29, n = 117) entered the randomly assigned 'activated' sub-channel. Full negative control and 3kPZS trials did not differ in terms of migratory female directional responses (ANCOVA: $F_{1,12}$ = 0.09, P = 0.774). Of the migratory females that moved upstream during larval extract trials, an average proportion of 0.86 (X+SE= 0.86+0.17, n = 55) entered the respective larval extract activated sub-channel; which in turn, was a much greater mean proportion when compared to 3kPZS and control trials (ANCOVA: $F_{2,15}$ = 18.99, P < 0.0001).

DISCUSSION

We showed that a male pheromone component 3kPZS increases the tendency of migratory female sea lampreys to move upstream at temperatures that typically are associated with low upstream movement. The results of this study demonstrate that 3kPZS in streams likely functions to induce upstream movement of migratory-phase female sea lamprey during otherwise inactive periods by modulating the effects of cold stream temperatures. The upstream response induced by the migratory pheromone is complimented by male released 3kPZS (Sorensen & Stacey 2004). This is likely an adaptive behavior, which increases the likelihood that females locate spawning grounds and mate successfully across a narrow thermal range. Our results confirm laboratory work by Binder and McDonald (2008) and a field experiment by Wagner et al. (2009) regarding temperature-induced effects on activity patterns of migratory sea lamprey. Furthermore, our discovery implicates that a single component of a pheromone (3kPZS) is capable of an aggregatory function in female sea lamprey (Sorensen & Stacey 2004) to spawning grounds during an immature phase, as well as a mating function to mates once maturation is reached (Li et al. 2002; Siefkes et al. 2005; Johnson et al. 2006; 2009).

The release of natural 3kPZS by male sea lamprey specifically after sexual maturation (Li et al. 2002; Siefkes et al. 2003), along with the robust attraction of ovulatory female sea lamprey to the male-sender (Siefkes et al. 2005; Johnson et al. 2006; 2009), indicates that 3kPZS signals the onset of mate-readiness to females. In our study, migratory females that were several days away from ovulation showed increased tendencies to move upstream, but did not change their direction towards 3kPZS odorant sources by entering the 3kPZS activated channel. It is not surprising that female sea lamprey with pre-ovulatory oocytes did not show behaviors typical of mate searching (see; Siefkes et al. 2005; Johnson et al. 2009), as females at this stage are not

ready to engage in spawning activities with a mate. An increase in activity of migratory female sea lamprey in the presence of 3kPZS is contradictory to previous maze studies described by Siefkes et al. (2005), which found no observable preference or response. The discrepancies between maze and stream data are likely due to the fact that maze studies were conducted in the daytime, when migratory female sea lampreys remain inactive, while stream studies were conducted at night where migration occurs naturally (Applegate 1950; Vrieze et al. 2011). Environmental variables such as natural light/dark cycles and ranges of stream temperatures have both been shown to effect (in addition to migratory pheromone) activity patterns of migratory lamprey (Binder and McDonald, 2008; Wagner et al., 2009); therefore, nighttime stream testing is more suitable when examining behavioral activity of migratory females in relation to a pheromone component such as 3kPZS, as it relates to natural environmental factors (Johnson and Li, 2010).

In this study, we provide an example of how a single component, 3kPZS, likely compliments the migratory pheromone to the advantage of the female receiver, both during the migratory and spawning phase of life history. 3kPZS likely functions as public information to migratory females regarding their downstream distance from spawning grounds, and serves as an aggregatory pheromone at this stage. Aggregatory pheromones are common among teleost fishes, and often this form of communication is learned or kin-related for the synchronization of reproduction (Stacey 2003; Sorensen & Stacey 2004). 3kPZS is likely inducing an adaptive behavior in migratory females to increase their tendency to move upstream during cold stream temperatures, continue direct upstream movement to aggregate around spawning grounds, and increase their probabilities of successfully reproducing once females mature.

Finally, many teleost fish species respond to odours from conspecifics by showing hormonal changes that promote maturation of gametes (Liley 1982; Stacey & Sorensen 2002; Sorensen & Stacey 2004). These changes are induced by primer pheromones, which we define in this manuscript as chemicals released by one species that induce slow physiological or developmental changes to the receiving individual (Wilson 1970). Endocrine responses to pheromones have been confirmed in goldfish (*Carassius auratus*) and in male sea lamprey (Stacey et al. 1989; Stacey 2003; Chung-Davidson et al. 2008). In goldfish, females release andostenedione (AD) and a 4-pregnen-17,20β-dihydroxy-3-one (17,20βP) almost exclusively across the gills (Sorensen & Stacey 2004). 17,20βP has been shown to induce hormonal changes in conspecific male goldfish and increase sperm and seminal fluid production (DeFraipont & Sorensen 1993; Sorensen & Stacey 2004). It is likely that 3kPZS acts as a primer pheromone in sea lamprey, similar to priming seen in goldfish. Migratory female sea lamprey would benefit to continue upstream migration, aggregate around areas of 3kPZS concentrations, and reach sexual maturity during a narrow thermal range before death. Once mature within areas of mate-ready males, females increase their probabilities of successful reproduction. It would be interesting to further test these theories using in-stream and molecular studies, and further characterize aggregation and priming functions of 3kPZS in migratory female sea lamprey (Stacey 2003; Sorensen & Stacey 2004; Li et al. 2004).

In summary, our data show that a lamprey-specific bile alcohol, 3kPZS, modulates the temperature-induced changes of upstream movement in migratory phase female sea lamprey. Evidently, 3kPZS exerts multiple functions depending on the life history phase of the female sea lamprey receiver. Migratory female sea lamprey that move upstream in the presence of 3kPZS during colder stream temperatures will likely aggregate in higher numbers to areas containing

3kPZS-baited traps. Once maturity is reached, trapping may be effective at removing high proportions of females from the system for control of the non-indigenous species in the Laurentian Great Lakes (Smith & Tibbles 1980; Twohey et al. 2003; Johnson et al. 2009).

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APPENDICES

APPENDIX A

SUPPLEMENTAL TABLES

Table 1-1S. Mixed and fixed effects examined during upstream movement of migratory sea lamprey in streams in relation to pheromones component 3kPZS.

Model	Fixed Effects	Interaction	logLik	AIC	
1	Period		-6.5	1.5	
2	Temp		-12.2	-4.2	
3	Gauge		-2.4	5.6	
4	$Rate_T$		0.6	8.6	
5	Odorant; Temp; Period	Odorant*Temp	-8.2	-2.2	
6	Odorant; Temp	Odorant*Temp*Period	3.2	9.2	
7	Odorant; Temp	Odorant*Temp	-12.2	-6.2	*
8	Odorant; Temp; Gauge	Odorant*Temp	-11.6	-3.6	
9	Odorant; Temp	Odorant*Temp*Gauge	-3.3	4.7	
10	Odorant; Temp; $Rate_T$	Odorant*Temp	-10.2	2.2	
11	Odorant; Temp	Odorant*Temp*$Rate_T$	-3.6	4.4	
12	Odorant; Temp; Released	Odorant*Temp	-2.9	5.1	
13	Odorant; Temp	Odorant*Temp*Released	11.9	19.9	
14	Odorant; Period	Odorant*Period	-3.8	4.2	
15	Odorant; Period	Odorant*Temp	-8.2	-2.2	
16	Odorant; Period	Odorant*Period*Temp	2.7	8.7	
17	Temp; Period	Temp*Period	-3.8	4.2	
18	Temp; Period	Odorant*Temp	-3.5	4.5	
19	Odorant; Temp; Period	Odorant*Temp	-8.2	-2.2	
20	Odorant; Temp; Period	Odorant*Temp; Odorant*Period	-7.2	-1.2	
21	Odorant; Temp; Period	Odorant*Period*Temp	2.7	8.7	
22	Odorant; Temp; Period	Temp*Period	-4.9	3.1	
23	Odorant; Temp; Period	Temp*Period; Odorant*Temp	-3.4	2.6	
24	Odorant; Temp; Period	Temp*Gauge; Odorant*Temp	-2.2	5.8	
25	Odorant; Temp	Odorant*Period	-7.7	0.3	
26	Odorant; Temp	Odorant*Period*Temp	3.2	9.2	
27	Odorant; Temp	Temp*Gauge; Odorant*Temp	-6.2	1.8	
28	Odorant; Temp; Released	Temp*Released; Odorant*Temp	6.7	14.7	

Odorant = 3kPZS or methanol (control) treatments, Period = early (2000-2300 h) or late (0000-0300 h) trial, Temp = stream temperature (^{o}C), $Rate_T$ = rate of stream temperature change during each trial (^{o}C hr $^{-1}$), Gauge = stream gauge height (m), Released = number of sea lamprey released during each trial (20-30). Fit statistics, including information criterion (AIC) and log-likelihood (logLik), are compared across models. *Model chosen using an ANCOVA in SAS Version 9.2 ($\alpha = 0.05$).

APPENDIX B

SUPPLEMENTAL FIGURES

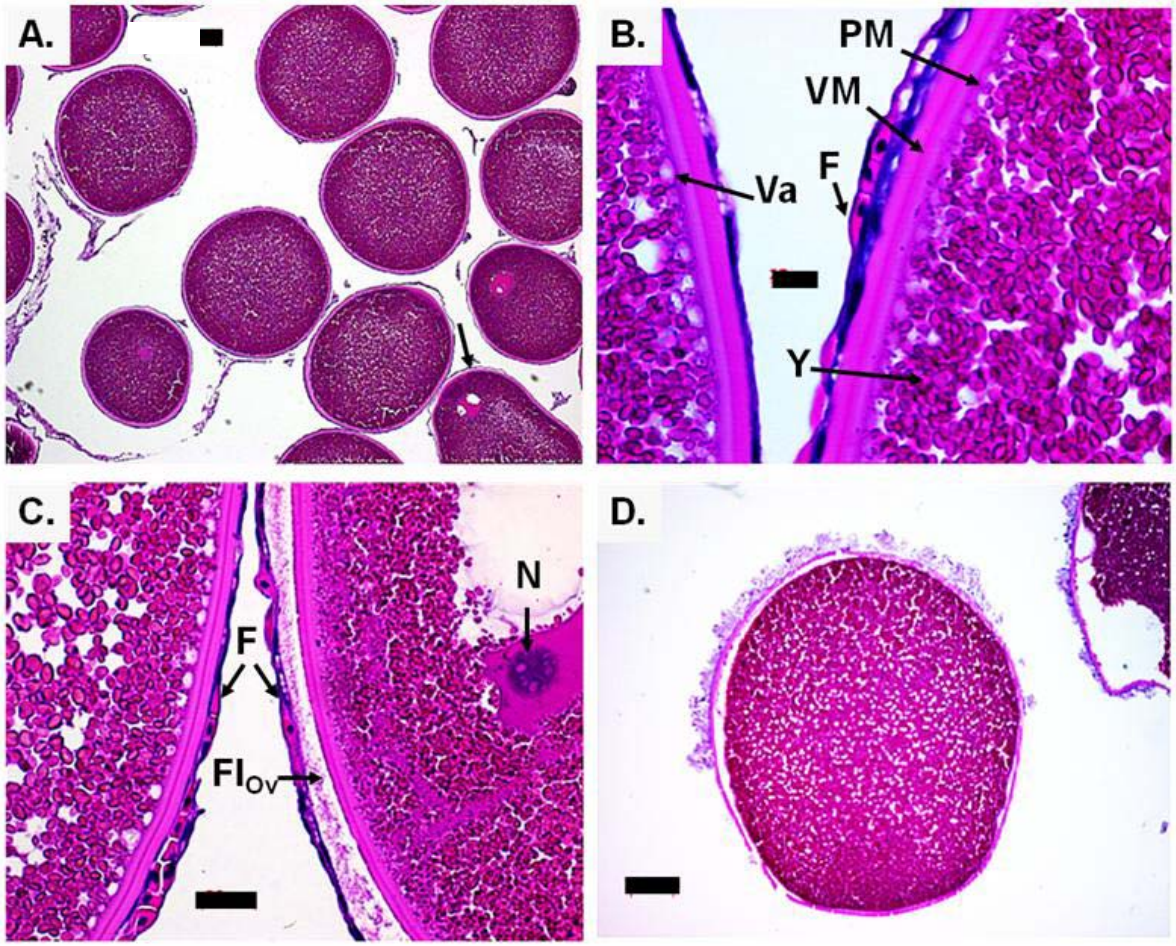

Figure 1-3S. Hemotoxylin and eosin stained sections of sea lamprey oocytes taken from a sub-sample of animals used in migratory field trials. **A)** Oocytes appearing pear-shaped (arrow) that were 5-10 days from ovulation (scale bar = 200 µm). **B)** Higher magnification showing location of the plasma membrane (PM) and yolk granules (Y; scale bar = 10 µm). A distinct vitelline membrane (VM) with peripheral striations, a follicular cell layer (F) intact, and vacuole build-up (V) under the vitelline membrane indicate a pre-ovulatory oocyte. **C)** The left oocyte was 5-10 days from ovulation, while the right shows a buildup of ovulatory fluid (Fl_{Ov}) between the vitelline membrane and follicular layer (F), 3-5 days from ovulation. Nucleus (N) is shown (scale bar = 20 µm). **D)** A mature (ovulatory) oocyte for reference that was taken from a female actively spawning in a nest (Cheboygan River dam, Michigan, U.S.A.) showing a degraded follicular layer (debris at top) and symmetrical shape (scale bar = 100 µm). No ovulatory oocytes were observed in all animals sampled for migratory trials, Ocqueoc River, Michigan, U.S.A. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

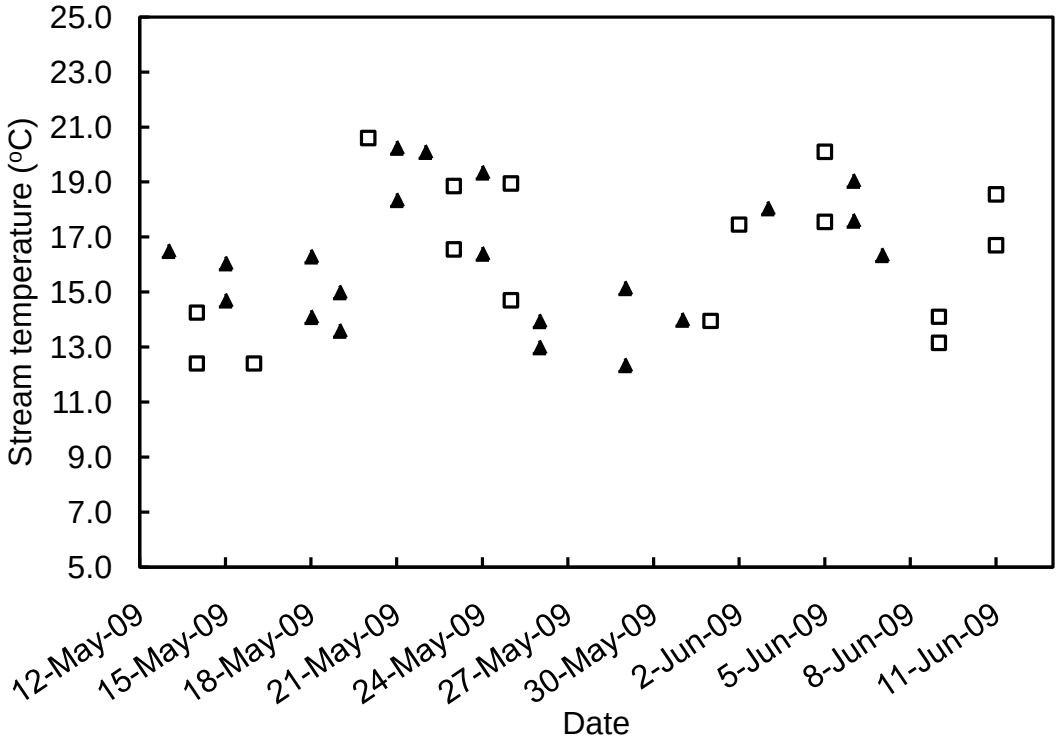

Figure 1-4S. Stream temperature taken during each nightly behavioural trial in the experimental system designed to observe upstream movements of migratory female sea lamprey in relation to synthesized 3kPZS, or methanol-control. □: Trials where synthesized 3kPZS ($5x10^{-13}$ molar [M]) was administered to the stream. ▲: Trials where methanol (vehicle) was administered to the stream. Day temperatures were not available. Ocqueoc River, Millersburg, MI.

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CHAPTER 2

PRODUCTION AND SELECTIVE RELEASE OF BILE ACIDS BY THE ADULT MALE SEA LAMPREY (*PETROMYZON MARINUS* L.)

ABSTRACT

Upon reaching sexual maturation, male sea lamprey (*Petromyzon marinus* L.) have been shown to release a bile alcohol-derived sex pheromone, 3-keto petromyzonol sulfate (3kPZS), across specialized gill epithelia cells. Details of the production, transport, and the release mechanism of 3kPZS and other steroidally-derived bile acids and bile alcohols from sea lamprey have not yet been elucidated. Here we further characterize the production and release of: (1) 3-keto allocholic acid (3kACA), (2) petromyzonol sulfate (PZS), (3) allocholic acid (ACA), (4) petromyzosterol disulfate (PSDS), and (5) petromyzonamine disulfate (PADS), in addition to 3kPZS. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) results show PZS is present in highest quantity in the liver, plasma, and gills (~100 times) of mature males compared to other compounds, whereas 3kPZS is present in highest quantity in washings from gills (~600 times) compared to other compounds. Bile acids and bile alcohols are transported from the liver through the cardiovascular system to the gills where certain derivatives are selectively released into the environment after males sexually mature. Quantitative Reverse Transcription PCR (QRT-PCR) results indicated that the conversion of bile acids and derivatives from cholesterols, as part of the biosynthetic pathway of these compounds, occurs in the liver of males after sexual maturation. We conclude that CYP7A1 transcripts and bile acid/bile alcohol production are both up-regulated in sexually mature male liver. The sea lamprey demonstrates a species with a direct and efficient mechanism for transport and subsequent release of bile acids and bile alcohols, and represents a model species for examining the biosynthesis of these biologically important compounds in vertebrates.

INTRODUCTION

The sea lamprey (*Petromyzon marinus* L.) represents a model species for comparative studies of bile acid synthesis and release through life history (Li, 2005; Vankatachalam, 2005). This species shows life stage-specific production of bile acids (bile acids will also universally refer to bile alcohols and derivatives, herein) that are hypothesized to function as chemical signals, or pheromones, for migration and reproduction (Li et al., 2003; Sorenson et al., 2005). Larval-phase sea lamprey release a lamprey-specific sulfated bile acid, petromyzonol sulfate (PZS), and an additional bile acid-derived compound hypothesized as a likely precursor in the biosynthesis of PZS known as allocholic acid (ACA; Haselwood and Tokes, 1969; Li et al., 1995). Both PZS and ACA are excreted with waste as metabolic by-products from larval sea lamprey. Sorenson et al. (2005) later identified two disulfated steroidal-derived compounds as components of the migratory pheromone: petromyzonamine disulfate (PADS), and petromyzosterol disulfate (PSDS; Table 2-4S), and demonstrated that these two compounds are released exclusively by larval sea lamprey as well.

Upon reaching sexual maturation (spermiating, herein), adult male sea lamprey no longer feed, and show a heavily atrophied intestine (Applegate, 1950), rendering the transport of bile acids and alcohols into the intestine useless in terms of lipid digestion. Spermiating male sea lampreys release at least two bile acids: 3-keto allocholic acid (3kACA) and 3-keto petromyzonol sulfate (3kPZS; Li et al., 2002; Yun et al., 2003; Table 2-4S). Both compounds are similar in structure to larval-released ACA and PZS, respectively, but instead contain a keto moiety in place of the hydroxyl at carbon-3 (C-3). 3kPZS has been confirmed to be secreted across the gill epithelia through specialized glandular cells (Siefkes et al. 2003); which were first

identified in sexually mature river lamprey, *Lampetra fluviatis* (Pickering, 1977). To date, the biosynthetic pathways of PZS have been regarded as specific to larval sea lamprey, and that of 3kPZS specific to spermiating males (Venkatachalam, 2005). Further, details of the onset of production, subsequent transport through the body, and mechanism of release of bile acids specific to sea lamprey have not been fully elucidated (Siefkes et al., 2003; Li, 2005). Finally, while PZS, ACA, PADS, and PSDS are confirmed to be released by larval sea lamprey, they have not yet been examined in regards to whether they are released by adults.

In this study, we compared production and release of five known bile acids in adult male sea lamprey before and after the onset of spermiation. In vertebrate animals, bile acids and bile alcohols are known to be derived cholesterols largely in the liver by a suite of enzymes which include Cholesterol 7α-hydroxilase, or cytochrome P450 7A1 (CYP7A1), as a rate limiting enzyme during synthesis (Chiang, 2009). We speculated that lamprey bile acids are also produced in the liver, and further hypothesized that all bile acids are transported directly to gills where they are selectively released into the environment from sexually mature males. We integrated biochemical and molecular techniques to examine the production and release of 3kPZS, PZS, 3kACA, ACA, PSDS, and PADS. Our data suggests that a dramatic up-regulation in CYP7A1 transcription leads to equally dramatic up-regulation in production of bile acids in mature males, and subsequently a substantial increase in the release of 3kPZS, a known mating pheromone component (Li et al., 2002).

METHODS

Sea lamprey collection and maintenance

All handling and dissections of sea lamprey were conducted in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee. Sea lampreys were captured by the United States Fish and Wildlife Service, or Fisheries and Oceans Canada, using traps placed near dams in tributaries to Lake Michigan, Lake Superior, and Lake Huron. All captured lamprey were brought to the United States Geological Survey - Hammond Bay Biological Station (HBBS) and held in 500-1000 l aerated tanks fed continuously with Lake Huron water. The tank temperatures were kept between 17-19^{o}C, which is similar to stream water temperatures during a typical sea lamprey spawning season (Applegate, 1950).

Immature male sea lamprey were visually identified and separated from females by carefully applying pressure to the lower abdomen to feel for eggs. Male sea lamprey were transferred to steel holding cages, ranging from approximately 0.25 – 1.0 m^3 in volume, in the lower Ocqueoc River (US23 bridge, Millersburg, MI, USA) to promote natural maturation in an actual spawning stream. Up to five acclimation cages, each containing roughly 10 – 15 male sea lampreys, were checked daily for signs of spermiation. Spermiating males were identified by secondary sexual characteristics (Manion and Hanson, 1980) including a pronounced rope-like ridge that develops dorsally along the length of the back to the proximal dorsal fin (Figure 2-1), and gamete release following gently pressure applied to the lower abdomen. Acclimation time typically lasted 5-10 days before animals became mature.

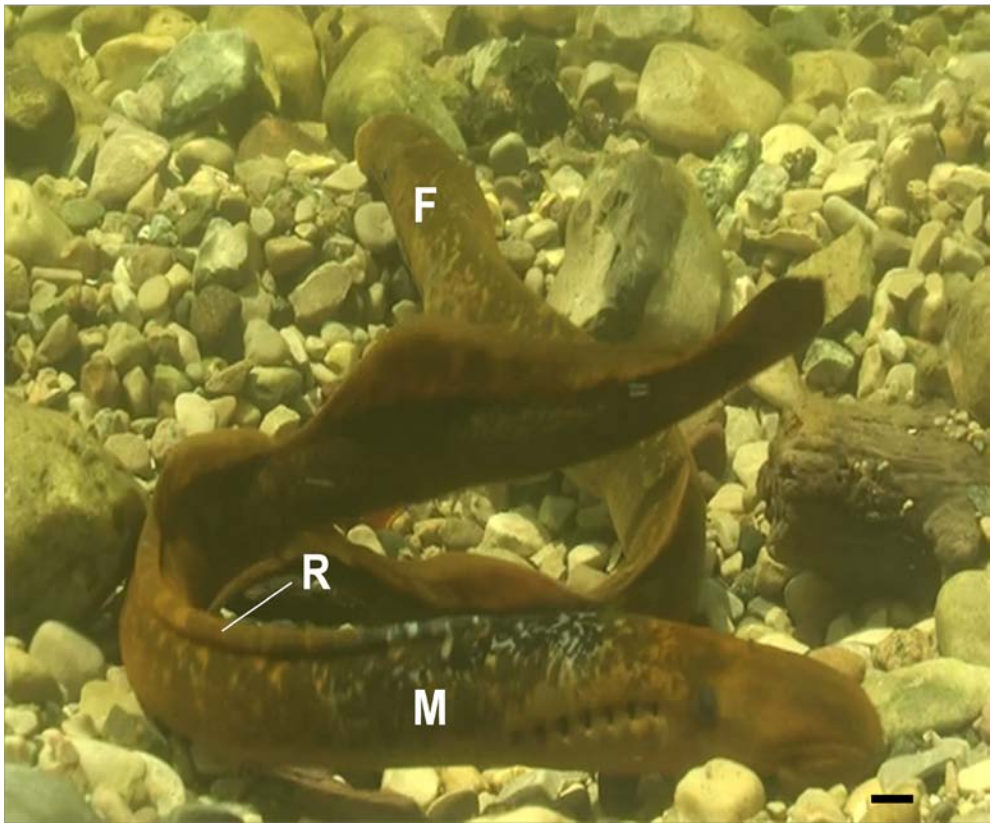

Figure 2-1. Nesting pair of sea lamprey showing a male (M) and female (F), as well as the male secondary sexual characteristic known as the rope (R). Scale bar = 20 mm, Photo by C. Brant.

Washings collection

Wash water samples from each male sea lamprey were collected for later quantification of known bile acids. The putative compound 3kPZS has been specifically shown to be released across the gill epithelia of spermiating male sea lamprey (Seifkes et al. 2003); therefore, a bisected chamber was constructed following Siefkes et al. (2003) with slight modification. The chamber was capable of collecting lamprey-conditioned water from the anterior and posterior region of an individual, independently (Figure 2-2A). Once an animal was secured into the aquarium, 3.5 l of deionized water (DI) was added to the posterior chamber, and the dividing air space was inspected for leaks. 3.5 l of DI water was then added to the anterior chamber. An additional non-bisected, free-swimming, washing chamber (Figure 2-2B) was used to confirm that chamber type did not severely alter release rates of compounds of interests compared to those of animals subjected to the bisected chamber. New individuals were transferred to a 20-l capacity container containing 5 l of temperature acclimated DI water. A portable aerator was used for a constant supply of oxygen. The temperature of the DI water used for washings was acclimated to that of the holding tanks from which the animals came (17-19^{o}C). Each chamber was aerated while an individual was washed for 1 hr.

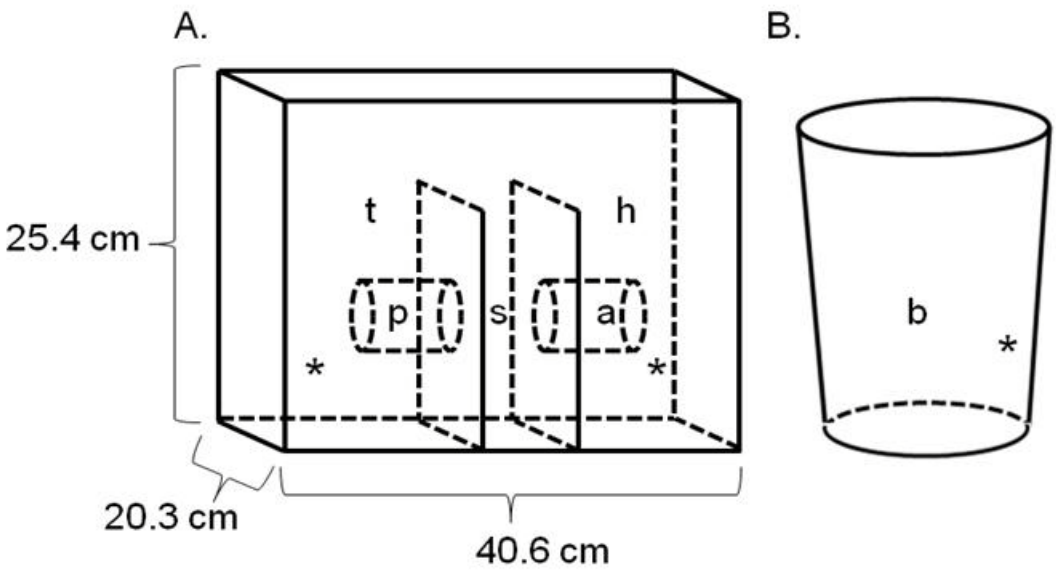

Figure 2-2. A) Schematic of a bisected chamber used to collect lamprey-conditioned water from the head (h) and tail (t) region of the animal following Siefkes et al. (2003), with slight modification. The anterior end of the animal was inserted into a perforated acrylic tube (a), securing the body with an adjustable gasket. The mid-region of the animal was suspended across an air space divider (s), assuring no possible leakage between h and t. The posterior was inserted into a perforated rubber tube (p), secured by an adjustable gasket. **B)** Schematic of basic 20 l washing chamber that collected lamprey-conditioned water from the entire body of the animal (b). *Air stone, cm: centimeter.

After washing, one 1-l and three 50-ml samples were taken from each chamber (i.e. head, tail, or whole body). All 1-l samples were immediately spiked with a 100 µl methanol 5-deuterated 3kPZS solution ([$^2H_5$]3kPZS; 5 ng/ml) that was custom synthesized by Bridge Organics Inc., Vicksburg, MI, as an internal standard (denoted as: 3kPZS-d_5 IS, herein). After thorough mixing, the samples were stored at -20^{o}C. All 50-ml samples were transferred directly to a -20^o C freezer. All samples were collected while wearing latex gloves, changing gloves between samples.

Plasma and tissue collection

Males removed from both washing chamber types were euthanized with an overdose of Tricaine methanesulfonate (MS222; Sigma-Aldrich, St. Louis, MO, USA). Blood was drawn directly from the heart using a 10 ml syringe (needle: 0.8 x 40 mm; BD SafetyGlide, Franklin Lakes, NJ, USA) until no more blood could be drawn. A 1.5 ml aliquot of blood from each male was transferred to a 1.5 ml Eppendorf Snap-Cap Microcentrifuge tube and centrifuged at 1500 rpm for 15 min at 4^{o}C. Supernatant plasma was carefully removed, transferred to a new vial, and frozen at -80^{o}C for later analyses.

Each male was dissected and the whole gill and liver were removed. Two sub-samples (20-50 mg, each) of each tissue type were transferred to separate 2 ml tubes, frozen in liquid nitrogen (N_2), and stored at -80^{o}C for later extraction of RNA (see: Quantitative real time PCR). Sub-samples were taken from consistent locations from each tissue. The remaining tissues were frozen at -20^{o}C for later extraction of bile acids.

Sample preparation and analysis by mass spectrometry

Each 1-l washing sample was thawed at room temperature and passed twice through a glass microfiber filter of 1.0 µm nominal pore size (Whatman, Piscataway, NJ, USA), followed by once through a metrical grid filter of 0.45 µm pore size (Poll Corporation, Ann Arbor, MI, USA). Solid phase extraction (SPE) was accomplished by passing each filtered sample through a methanol-activated Oasis MCX mixed-mode polymetric sorbent cartridge (6 cc/500 mg; Waters, Milford, MA, USA). Eluants in methanol (8 ml/sample) were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco, Kansas City, MO, USA). Residues were reconstituted with 100 µl of 50% high performance liquid chromatography (HPLC) grade methanol (Fisher Scientific, Fair Lawn, NJ, USA). Bile acid components were then identified and quantified using a Waters ACQUITY LC System coupled with the Waters Quattro Premier XE tandem quadrupole mass spectrometer (LC-MS/MS; Milford, MA, USA).

Liver and gill tissues were thawed on ice and weighed. Tissue sub-samples ranged from 15 – 60 mg. A 100 µl spike of 5 ng/ml 3kPZS-d_5 IS (in 75% ethanol) was added to pre-spermiating male tissue samples, and a 100 µl spike of 100 ng/ml 3kPZS-d_5 IS (in 75% ethanol) was added to spermiating male tissue samples. Ethanol (75%, in de-ionized water) was then added to each sample until the total volume reached 1 ml/100 mg of tissue. Tissues were homogenized and subjected to over 15 hr of shaking at room temperature (~100 rpm). Each sample was then centrifuged at 13000 rpm for 10 min at room temperature. Supernatants were transferred to new vials and evaporated (Labconco). Residues were re-constituted in 1 ml of deionized water for SPE. SPE procedures followed those of washing samples using Oasis MCX cartridges. Eluants (8-mL, each) were evaporated (Labconco) down to a dry powdered state. Residues were reconstituted with 100 µl of 1:1 methanol:DI water (*v*:*v*) before injection into LC-

MS/MS. Concentrations of each compound in each tissue was then standardized by the initial weight of each tissue sample (ng/g-tissue).

Plasma sample extraction was based on a published method by Scherer et al. (2009), with slight modification. Plasma samples were thawed on ice. 100 µl aliquots of plasma were transferred to 15 ml tubes. Each aliquot was spiked with 10 µl of 5 ng/ml 3kPZS-d_5 internal standard (in methanol). For protein precipitation, plasma was mixed with 1 ml acetonitrile, followed by 1 min of vortex-mixing. After 15 min of centrifugation (15000 rpm), the supernatant was filtered through a polyvinylidene fluoride (PVDF) syringe filter (0.22 µm pore size, 4 mm diameter; Membrane Solutions, Plano, TX, USA) and evaporated to a dry powered state. The samples were re-dissolved in 1 ml of 1:1 methanol:DI water (*v*:*v*). After an additional centrifugation (15000 rpm, 15 min), 10 µl of the methanolic supernatant was subjected to LC–MS/MS analyses. All statistical comparisons of mean concentrations in washings, tissues, and plasma, and pertaining to release rates, within spermiating and pre-spermiating males were accomplished using a Student's t-test ($\alpha = 0.05$).

Bile acids examined

Bile acids examined include: (1) 3-keto petromyzonol sulfate (3kPZS), (2) 3-keto allocholic acid (3kACA), (3) petromyzonol sulfate (PZS), (4) allocholic acid (ACA), petromyzosterol disulfate (PSDS), and (5) petromyzonamine disulfate (PADS). Refer to Table 2-4S for details of each compound and Figure 2-6S for details of structure.

Quantitative Reverse Transcription PCR

QRT-PCR followed the procedures described by Chung-Davidson et al. (2008a, b). Briefly, total RNA was extracted from gill and liver tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), treated with TURBO DNA-free kit (Applied Biosystems, Foster City, CA, USA), and diluted to 100 ng/µl. RNA samples were then reverse-transcribed into cDNA using Moloney Murine Leukemia reverse transcriptase (M-MLV RT; Invitrogen) and random hexamers (Promega, Madison, WI, USA). RTQ-PCR was performed using the TaqMan minor groove binder (MGB) system (Applied Biosystems). Each reaction consisted of 2 µl (5 ng/µl) cDNA, 8 µl TaqMan Universal PCR master mix, forward and reverse primers (900 nM of each), and 250 nM TaqMan MGB probe. Amplification plots were analyzed on an ABI-Applied Biosystems 7900 real-time PCR thermal cycler at the Michigan State University Research Technology Support Facility, East Lansing, Michigan, USA. Synthetic oligonucleotides were used as standards and ran on the sample plate.

Standards were PCR-amplified using the primers for RTQ-PCR seen in Figure 2-5S, purified with a MinElute PCR purification kit (Qiagen Inc., Valencia, CA, USA), and serially diluted (10-fold) into 10^{10} to 10^{3} molecules/2 µl solutions. Genes quantified in sea lamprey gill and liver included: (1) *CYP7A1* encoding for the Cholesterol 7α-hydroxilase or cytochrome P450 7A1 (CYP7A1) enzyme. (2) *SULT1C1* encoding for the sulfotransferase family cytosolic 1C1 (SULT1C1) general class of enzymes (standard), and (3) *40S* ribosomal protein (standard). The sequences for standards, primers and TaqMan MGB probe for each mRNA listed are shown in Figure 2-5S. Transcripts/ng total RNA of genes of interest were compared across gill and liver tissues from pre-spermiating and spermiating male sea lamprey using a generalized linear model

(ANOVA) and post hoc Fisher's protected least significant difference (PLSD) analyses ($\alpha = 0.05$).

RESULTS

Bile acids in washings, tissues, and plasma

From LC–MS/MS analyses, concentrations that were below 0.1 ng/ml were considered too low for reliable detection and, therefore, removed from the data set. 3kPZS was detected at the highest mean concentration ($X \pm SE$ = 122 ± 48 ng/ml, N = 6) in the head-washings of spermiating males, which was greater than all other compounds detected (Student's t-test: t = 2.1, $P < 0.001$). Minimal concentrations of compounds were detected in pre-spermiating male head, tail, or full body washings, or in spermiating male tail-washings (Table 2-1).

In liver and plasma samples of pre-spermiating males, PADS was detected at the highest mean concentrations ($X \pm SE$ = 593 ± 198 ng/g-liver, N = 6; and 269 ± 152 ng/l-plasma, N = 6), compared to all other compounds detected (Student's t-test: t = 2.06, P = 0.01 and $P < 0.001$, respectively). PZS was detected in slightly greater concentrations in gills compared to all compounds detected (Table 2-2). In spermiating males, mean concentration of PZS was found to be substantially greater in liver, plasma, and gill samples ($X \pm SE$ = 151674 ± 26321 ng/g-liver, N = 12; 11679 ± 2090 ng/l-plasma, N =11; and 15651 ± 4278 ng/g-gill, N = 12) when compared to mean concentrations of all other compounds detected (Student's t-test: t = 2.0, $P < 0.0001$; $P < 0.0001$; and $P < 0.0001$, respectively). All other compounds detected in the liver, gill, and plasma of spermiating males were lower (Figure 2-3b).

All detected bile acids, 3kPZS, PZS, 3kACA, ACA, and PADS, were released from the head-region of spermiating males. 3kPZS was released at the highest mean rate ($X \pm SE$ = 2273 ± 886 ng/hr/g-lamprey, N = 6), which was significantly higher compared to all other rates (Student's t-test: t = 2.05, P < 0.001). Release rates of PZS, 3kACA, ACA, and PADS were all

lower (3–80 ng/hr/g-lamprey) and not different from one-another (Student's t-test: $t = 2.05$, $P = 0.9$; Figure 2-3a).

The ratios of synthesized and released bile acids differ dramatically between pre-spermiating and spermiating males. In pre-spermiating males, ratios could not be calculated because these animals did not release any bile acids in detectible amounts. The ratios of 3kPZS:3kACA, 3kPZS:PZS, and 3kPZS:ACA stayed around 1:1 throughout the liver, plasma, and gills of pre-spermiating male sea lamprey. PADS was greater in concentration throughout liver, gill, and plasma, across all ratios. In spermiating males, the ratio of 3kPZS:3kACA shows 3kPZS to be in greater concentrations throughout the liver, plasma, and gills, and release. However, the ratio comparisons of 3kPZS:PZS show greater concentrations of PZS in the body, but much greater release rates of 3kPZS into the environment, similar to that seen across 3kPZS:PADS ratios (Table 2-3).

Transcripts of genes

The CYP7A1 gene was transcribed at 1000-fold higher levels in the liver tissue of spermiating males compared to spermiating male gills, and 1000-10000-fold higher levels compared to pre-spermiating male gills and liver, which was significant (ANOVA: $F_{3,56} = 10.98$, $P < 0.0001$; Figure 2-4a,b). Quantitative differences in transcripts of SULT1C1 (Figure 2-4c) and 40S ribosomal RNA (Figure 2-4d) using QRT-PCR analyses were not significant across liver and gill of pre-spermiating and spermiating males (ANOVA: $F_{3,56} = 1.55$, $P = 0.21$ and $F_{3,56} = 2.18$, $P = 0.10$, respectively). Standardization of CYP7A1 transcripts by 40S transcripts did not

change the relationships (Figure 2-4b). Standards and primers for RTQ-PCR of genes from liver and gill tissues can be seen in Figure 2-5S.

Table 2-1. Mean concentration (ng/ml) of bile acids from wash water collected from separate bodily regions of male sea lamprey.

Compound	PSM body	PSM head	PSM tail	SM body	SM head	SM tail
3kPZS	0	0	*ND*	101±14 ***a***	122±48 ***a***	0
3kACA	*ND*	0	*ND*	2±0.6 ***b***	1±0.4 ***b***	0
PZS	*ND*	0	*ND*	0.2±0.06 ***b***	0.2±0.07 ***b***	0
ACA	0	0	*ND*	3±1 ***b***	3±1 ***b***	0
PSDS	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
PADS	0	0	*ND*	0.6±0.2 ***b***	0.2±0.09 ***b***	0

Abbreviations: 3kPZS = 3-keto petromyzonol sulfate, 3kACA = 3-keto allocholic acid, PZS = petromyzonol sulfate, ACA = allocholic acid, PSDS = petromyzosterol disulfate, PADS = petromyzonamine disulfate, PSM = pre-spermiating male (N = 22), SM = spermiating male (N = 20), body = whole body washing, head = anterior region washing only, tail = posterior washing only, and *ND* = not detected. Bile acid concentrations were determined with LC-MS/MS and expressed as mean (ng/ml) ± 1 standard error. A value of 0 was given for values less than 0.1 ng/ml. Different lower-case letters across all rows and columns indicate statistically significant differences at $P < 0.05$ using Student's t-test.

Table 2-2. Mean concentration of bile acids detected in the body of pre-spermiating male sea lamprey.

Compound	L (ng/g)	P (ng/ml)	G (ng/g)
3kPZS	***A*** 27±7 ***b***	***A*** 6±1 ***b***	***A*** 30±14 ***ab***
3kACA	***B*** 39±18 ***b***	***B*** 6±1 ***b***	***A*** 41±11 ***a***
PZS	***A*** 167±38 ***b***	***B*** 14±2 ***b***	***B*** 37±3 ***a***
ACA	***A*** 7±3 ***b***	***A*** 2±0.3 ***b***	***A*** 7±2 ***b***
PSDS	*ND*	*ND*	*ND*
PADS	***A*** 593±198 ***a***	***A*** 269±152 ***a***	***B*** 30±15 ***ab***

Abbreviations: 3kPZS = 3-keto petromyzonol sulfate, 3kACA = 3-keto allocholic acid, PZS = petromyzonol sulfate, ACA = allocholic acid, PSDS = petromyzosterol disulfate, PADS = petromyzonamine disulfate, L = liver, P = plasma, G = gill, and *ND* = not detected. Tissues and plasma were collected from 6 pre-spermiating males. Concentrations were determined with LC-MS/MS and expressed as mean ± 1 standard error. Different upper-case letters indicate statistically significant differences across tissue types within each specific compound (i.e. row). Different lower-case letters indicate statistically significant differences across compounds within each specific tissue type (i.e. column). Significance was determined at $P < 0.05$ by Student's t-test.

Table 2-3. Ratios of bile acids detected in the body of, and subsequently released from, male sea lamprey.

Ratio	Liver (*c*:*c*)		Plasma (*c*:*c*)		Gill (*c*:*c*)		Release (*r*:*r*)	
	PSM	**SM**	**PSM**	**SM**	**PSM**	**SM**	**PSM**	**SM**
3kPZS:3kACA	1:1	46:1	1:1	53:1	1:1	22:1	*ND*	126:1
3kPZS:PZS	1:6	1:142	1:2	1:43	1:1	1:9	*ND*	553:1
3kPZS:ACA	4:1	5:1	3:1	3:1	4:1	5:1	*ND*	28:1
3kPZS:PADS	1:22	1:3	1:45	1:6	1:1	1:1	*ND*	737:1
3kACA:PZS	1:4	1:6595	1:2	1:2336	1:1	1:196	*ND*	4:1
3kACA:ACA	6:1	1:9	3:1	1:19	6:1	1:4	*ND*	1:4
3kACA:PADS	1:15	1:151	1:45	1:296	1:1	1:18	*ND*	6:1
ACA:PZS	1:24	1:705	1:7	1:121	1:5	1:47	*ND*	19:1
ACA:PADS	1:85	1:16	1:134	1:15	1:4	1:4	*ND*	26:1
PADS:PZS	4:1	1:44	19:1	1:8	1:1	1:10	*ND*	1:1

Abbreviations: 3kPZS = 3-keto petromyzonol sulfate, 3kACA = 3-keto allocholic acid, PZS = petromyzonol sulfate, ACA = allocholic acid, PSDS = petromyzosterol disulfate, PADS = petromyzonamine disulfate, PSM = pre-spermiating male (*N* = 6), SM = spermiating male (*N* = 12), *c*:*c* = Concentration ratios, *r*:*r* = release ratios, and *ND* = not detected.

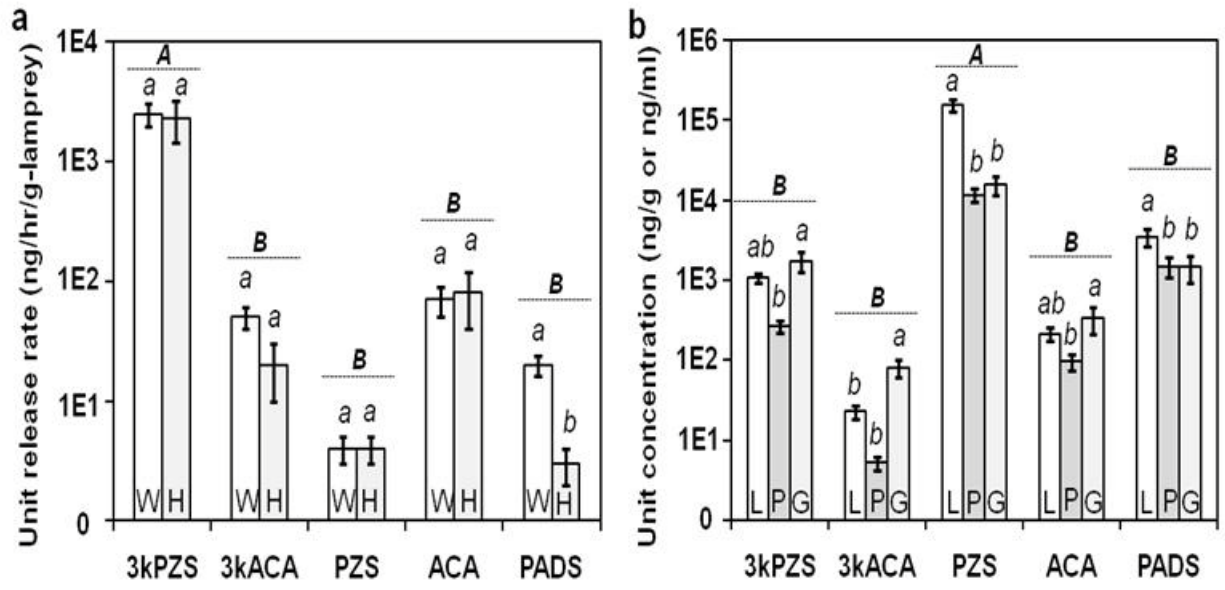

Figure 2-3. Quantification of bile acids: 3-keto petromyzonol sulfate (3kPZS), 3-keto allocholic acid (3kACA), petromyzonol sulfate (PZS), allocholic acid (ACA), and petromyzonamine disulfate (PADS) from spermiating male sea lamprey using LC-MS/MS. **a)** Release rate (ng/hr/g-sea lamprey) of compounds from wash water samples taken from the whole body (W; *N* = 14) of individuals and from just the head region (H; *N* = 6). **b)** Concentration of compounds from liver (L) and gill (G) tissues (ng/g-tissue), and plasma (P; ng/ml-plasma) from a sub-sample of **a** (*N* = 12). Different lower-case letters indicate statistically significant differences between vertical columns within each group (i.e. compound). A different upper-case letter indicates statistically significant differences between groups of columns across compounds. Columns indicate means ± 1 standard error (vertical bars), and were compared using Student's t-test (significantly different at $P < 0.05$). Petromyzosterol disulfate (PSDS) was not detected in spermiating males.

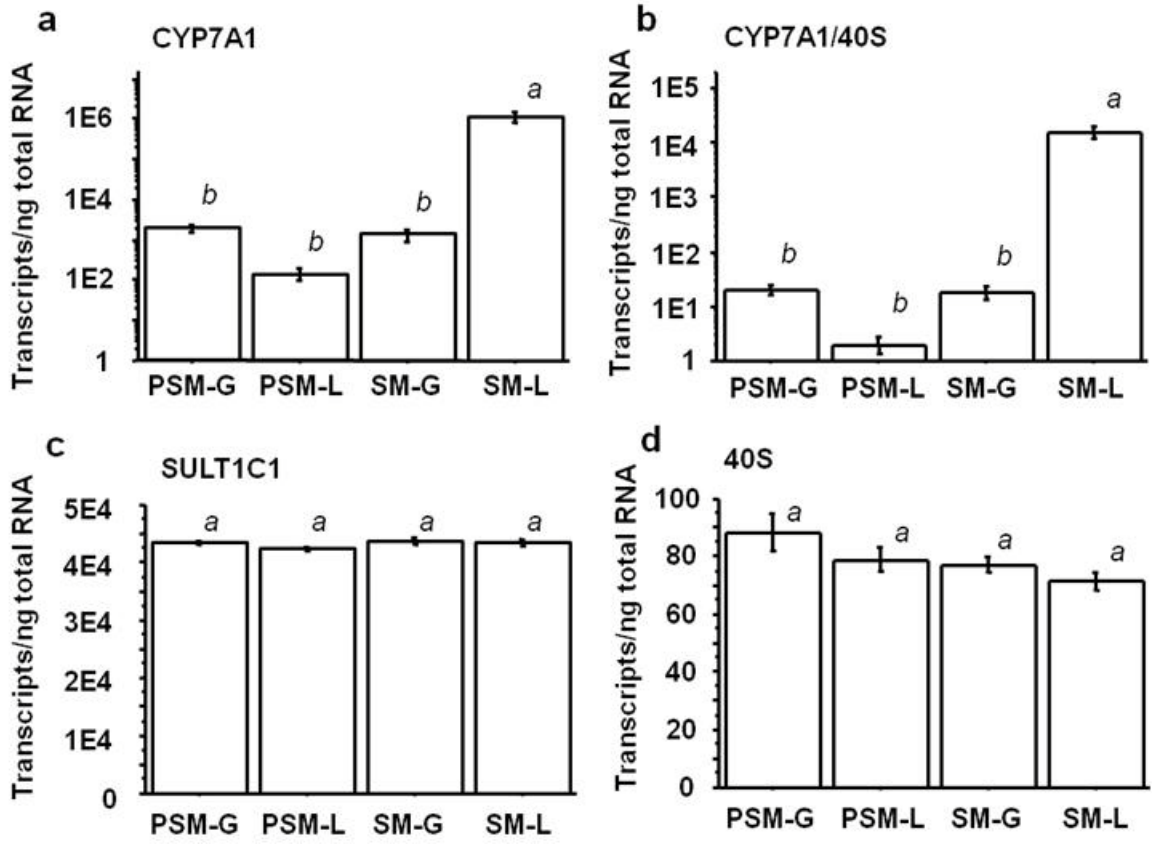

Figure 2-4. Quantitative Reverse Transcription PCR (QRT-PCR) analyses of gene transcripts in the gill (G) and liver (L) tissues from pre-spermiating (PSM, $N = 15$) and spermiating (SM, $N = 15$) males. **a)** Transcripts of cholesterol 7 alpha-hydroxylase (CYP7A1 gene). **b)** CYP7A1 expression standardized by 40S ribosomal DNA. **c)** Transcripts of sulfotransferase 1C1 (SULT1C1 gene). **d)** Transcripts of 40S ribosomal DNA (standard). Vertical columns represent mean transcripts ± 1 standard error. Different lower-case letters indicate statistically significant differences between vertical columns, which were compared using ANOVA and post-hoc Fisher's PLSD (significantly different at $P < 0.05$).

DISSCUSSION

This study confirms that a rate limiting CYP7A1 enzyme is up-regulated dramatically in the male sea lamprey liver after the animal reaches sexual maturation. We show that mature males release substantial amounts of 3kPZS from the gills compared to other bile acids that are up-regulated in gill tissue, indicating selective release of 3kPZS from the gills. Hepatic veins carry blood from organs such as the liver, directly to the heart, which in turn immediately pumps all blood through the gills (Augustinsson et al., 1956). The system comprises an effective pathway for bile acids to reach the gills. Our LC-MS/MS data confirm a pathway of multiple bile acids from the liver of males upon reaching sexual maturation, through the direct cardiovascular system, to the gills for release into the environment. Finally, bile acid quantification of washings collected from separate bodily regions of spermiating and pre-spermiating males confirm that 3kPZS, PZS, 3kACA, ACA, and PADS are released from the anterior end of spermiating males, likely across gill epithelia as previously shown with 3kPZS by Siefkes et al. (2003). Notably, these bile acids were detected in trace amounts, or not at all, in washings collected from the posterior end of spermiating males, and from all regions of pre-spermiating males.

Our LC-MS/MS and QRT-PCR results implicate a probable mechanism whereby 3kPZS is synthesized by the liver and the gills upon spermiation of a male sea lamprey, a process that has only been hypothesized in lamprey (Siefkes et al. 2003). Since PZS is present in high concentrations in the liver, plasma, and gill, yet 3kPZS is released across gill epithelia, it is highly likely that PZS is a precursor to 3kPZS and the conversion largely occurs in the gills. Venkatachalam (2005; and hereafter) suggested a pathway regarding the biosynthesis of PZS to 3kPZS from larvae to adult sea lamprey. The hypothesized pathway involves: (1) 3α, 7α, 12α,

trihydroxy 5α-cholan is hydroxilated at carbon-24 (C-24) forming 3α, 7α, 12α, 24 tetrahydroxy 5α-cholan (petromyzonol; PZ); (2) PZ-sulfotransferase (Venkatachalam et al. 2004) catalyzes the addition of a sulfate to C-24 of PZ, forming PZS; and (3) 5α-OH of PZS is speculated to be oxidized by 3α-dehydrogenase at C-3 to form 3kPZS, a male sex pheromone component (Li et al., 2002) selectively released across specialized glandular cells in the gills (Siefkes et al., 2003). It will be interesting and significant to confirm this biosynthetic pathway with further biochemical and gene expression studies, including a detailed study of hydroxysteroid dehydrogenase (3α-dehydrogenase, in this case) in spermiating male sea lamprey, and other direct evidence for the conversion of PZS to 3kPZS by gills.

From our data, we propose that sexually mature male sea lamprey produce and release more bile acids and bile alcohols than previously described. Until now, PZS, ACA, and PADS have been thought only to be released by larval-stage sea lamprey (Li et al., 1995; Polkinghorne et al., 2001; Yun et al., 2003; Sorenson et al., 2005). Our results clearly show the production of these bile acids in mature males. PSDS and PADS are two constituents of the larval pheromone with olfactory-stimulating capabilities based on electro-olfactographic evidence (Sorenson et al. 2005). PZS as ACA are also both potent olfactory stimuli in the adult sea lamprey (Li et al., 1995). Interestingly, PZS, ACA, and PADS are all hypothesized as components of the migratory pheromone released by larval sea lamprey upstream (Bjerselius et al. 2000; Sorenson et al., 2005).

Fine and Sorenson (2010) further showed that larval lamprey release identified larval compounds PZS, PSDS, and PADS at a rate of ~5-25 ng/hr/larva, where PSDS is released at twice the rate of the other two compounds. This release rate was determined after larvae had recently fed, through waste excretion. Assuming an average larval sea lamprey weighs ~1 g

(Applegate, 1950); we show an average release rate of 3kACA, PZS, ACA, and PADS of ~4-70 ng/hr/g-sea lamprey that is within the same range as the conspecific larvae, but from the gill region of males. It would be interesting to examine the release mechanisms of both adult and larvae in future studies to further characterize the biosynthesis and biological function of these bile acids among conspecifics during different phases of life history.

In summary, the sea lamprey dramatically up-regulates CYP7A1 and subsequent bile acids in the liver following maturation. Bile acids are transported through the direct cardiovascular system to the gills for release. The production of bile acids and their derivatives, including ACA, PZS, and PADS, are confirmed to be released from the head region of mature males. A clear discrepancy in the bile acids produced in the liver, compared to those released across gills, indicates that the mature male sea lamprey has adapted a mechanism for selective release of male mating pheromone component 3kPZS. Further studies that elucidate the biosynthetic pathway of bile acids, and the mechanism of release of these compounds from the gills, will accompany this research well, and likely be applied to other vertebrate species in the future. Knowledge of the synthesis and release of additional sea lamprey-specific compounds may be useful in the battle to control the invasive species within the Laurentian Great Lakes.

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APPENDICES

APPENDIX A

SUPPLEMENTAL TABLES

Table 2-4S. Bile acids, bile alcohols, and their derivatives produced and released by the sea lamprey (*Petromyzon marinus*).

Name	Abbr.	Chemical formula	Molecular weight	Sources
3-keto petromyzonol sulfate	3kPZS	$C_{24}H_{40}O_8S$	472	Li et al., 2002; Yun et al., 2003
3-keto allocholic acid	3kACA	$C_{24}H_{38}O_5$	406	Yun et al., 2002; Yun et al., 2003
allocholic acid	ACA	$C_{24}H_{40}O_5$	409	Li et al., 1995
petromyzonol sulfate	PZS	$C_{24}H_{38}O_5S$	474	Haselwood and Tokes, 1969; Li et al., 1995
petromyzosterol disulfate	PSDS	$C_{28}H_{46}O_9S_2$	509	Sorenson et al., 2005; Hoye et al., 2007
petromyzonamine disulfate	PADS	$C_{34}H_{60}N_2O_9S_2$	704	Sorenson et al., 2005; Hoye et al., 2007

APPENDIX B

SUPPLEMENTAL FIGURES

a

CYP7A1

CAACATGTCGGCGCTCATCGCCCTCCGAATACAACTCAATGACACGCTGTCTCGCATG

b

SULT1C1

CCGTGATGCGCGACAACCCCATGACGAATTACAGCACCTTGCCCACCGACTTCTTGGACCACTCCGTG

c

40S RIBOSOMAL PROTEIN

ACCTACGCAGGAACAGCTATGACCATCTCGAGCAGCTGAAGCTCCAATGTGGTGGAATTCGTCG

Figure 2-5S. Standards and primers for Quantitative Reverse Transcription PCR (RTQ-PCR) from liver and gill tissues of the sea lamprey (*Petromyzon marinus*). **a)** CYP7A1 primer design, **b)** SULT1C1 primer design, **c)** 40S ribosomal RNA primer design. Sequencing for 5' primer is shown in the yellow block, sequence for TaqMan MGB probe in green block, and sequence for 3' primer in blue block. Designed by Y-W. Chung-Davidson, Michigan State University, East Lansing, MI, USA.

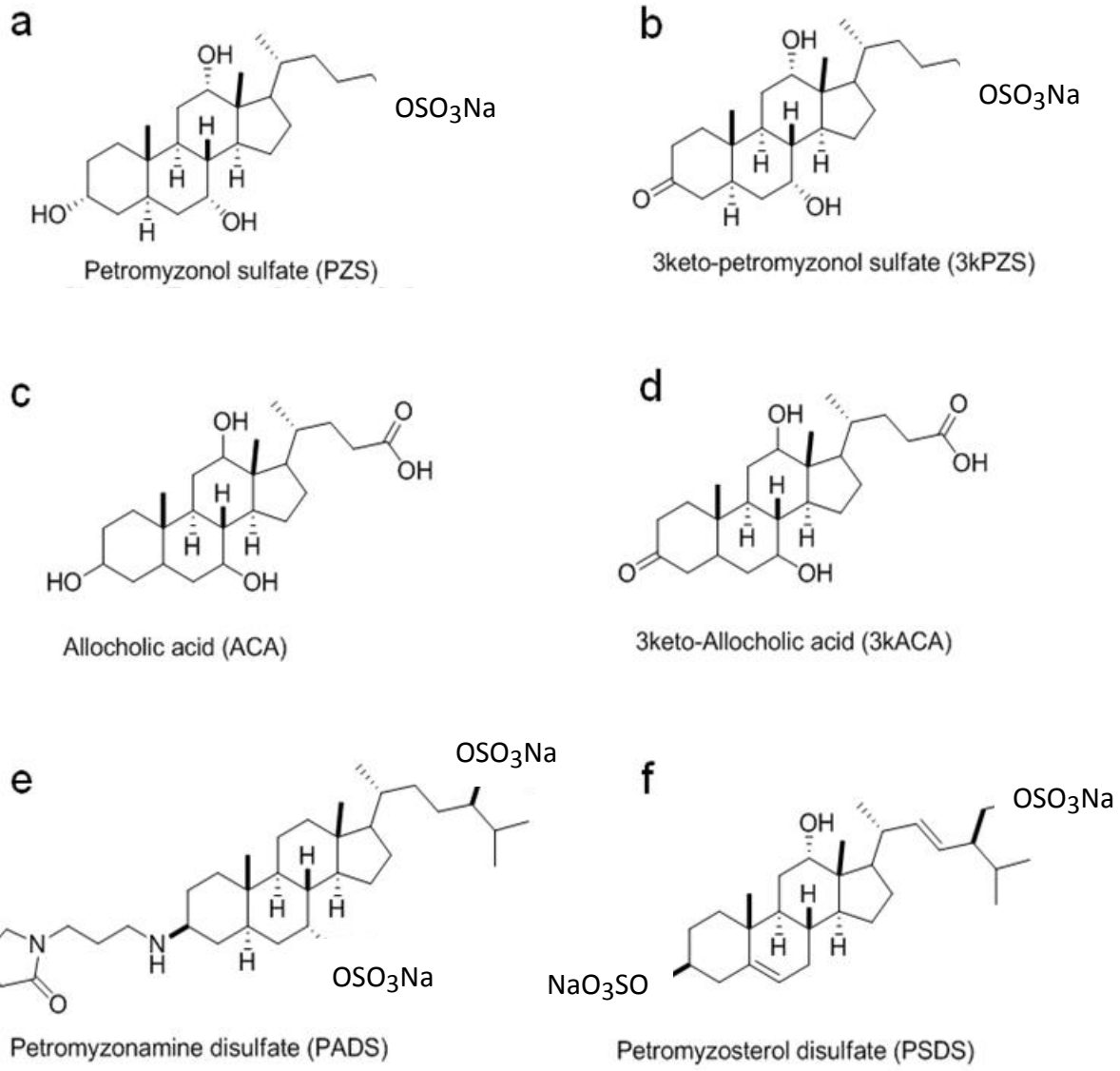

Figure 2-6S. Chemical structures of bile acids, alcohols, and steroidal derivatives of the sea lamprey. Structure illustrations courtesy of Dr. Ke Li, Michigan State University, USA. Refer to Table 2-4S for references regarding structure identification.

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