CHARACTERIZATION AND DISRUPTION OF SEA LAMPREY SEX PHEROMONE COMMUNICATION

By

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ABSTRACT

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Modulation of the sea lamprey (*Petromyzon marinus*) pheromone communication system may offer additional effective and environmentally benign approaches to manage populations in the Laurentian Great Lakes where they are invasive predators of large fishes. Previous studies showed that pheromones are indispensable cues that mediate sea lamprey migration and reproduction. Mature male sea lampreys release a multi-component sex pheromone that contains 3-keto petromyzonol sulfate (3kPZS) as a main component. 3kPZS is a potent odorant that induces upstream movement of ovulated females to the spawning grounds with nesting mature males. While the function and potential management implications of 3kPZS are well-studied, the identity of other putative sex pheromone components and the utility of pheromone antagonists that disrupt the sea lamprey pheromone communication system remain largely unknown. In this dissertation, I characterize novel pheromone components released by mature male sea lampreys and identify antagonists that disrupt the olfactory and behavioral responses to the sea lamprey sex pheromone components. In Chapter 1, I provide evidence that spermine is a sex pheromone released by mature male sea lampreys through the emission of milt (fish semen). Spermine is detected by two trace amine-associated receptors expressed in the olfactory epithelium, induces olfactory responses at a sub-femtomolar concentration, and attracts ovulated females but not mature males. These results reveal a new source and chemical template of sea lamprey pheromones in addition to identifying a secondary benefit of male gamete release; that is to recruit additional mates in sea lampreys. In Chapter 2, I provide evidence that PZS

treatment interferes with the responses to 3kPZS. Electro-olfactogram recordings from sea lampreys indicate PZS reduces the olfactory response to 3kPZS in a concentration-dependent manner. Behavioral results from maze assays and field experiments in-stream indicate the PZS disrupts 3kPZS-mediated behavioral responses in ovulated females as well. In Chapter 3, I provide evidence that petromyzonol-3,7,12,24-tetrasulfate (3sPZS), which was identified as a candidate 3kPZS antagonist through virtual screening in a previous study, selectively reduces the 3kPZS olfactory response. 3sPZS also reduces the behavioral preference of ovulated females for 3kPZS in the maze and reduces upstream movement, entry, and retention in artificial nest baited with 3kPZS in a natural spawning environment. The collective results of electrophysiology through field trials in a natural spawning environment provide a proof of concept that pheromone antagonists may be useful to disrupt vertebrate pheromone communication. In Chapter 4, I describe the methodology of bioassay-guided fractionation as an effective and interdisciplinary approach to isolate and characterize the structure, olfactory potency, and behavioral response of putative pheromones of sea lampreys. In Appendices A−C, I report the structures, olfactory potencies, and female behavioral preferences of eight novel bile salts or bile alcohols released from mature male sea lampreys. The results further elucidate the identity and bioactivity of the multi-component sex pheromone released by mature male sea lampreys. In addition, the identified naturally occurring and synthetic pheromone antagonists may offer new control tactics to manipulate behavioral responses or disrupt mating of sea lampreys. Taken together, the results on sea lamprey pheromones and pheromone antagonists can help guide an effective, integrated sea lamprey control program in the Laurentian Great Lakes.

This dissertation is dedicated to my mom, Susan Scott, who was a shining example of how to persevere in life with strength and determination.

ACKNOWLEDGMENTS

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INTRODUCTION TO DISSERTATION

SEMIOCHEMICALS MEDIATE INTERACTIONS BETWEEN ORGANISMS

Olfactory sensory inputs inform decisions throughout the animal kingdom (Wyatt 2014). Semiochemicals are chemicals emitted into the environment that mediate interactions between organisms (Nordlund and Lewis 1976). Semiochemicals are sub-divided into three categories: allelochemicals, pheromones, and signature mixtures. Allelochemicals are involved in the chemical interactions between a sender and receiver of different species, whereas pheromones and signature mixtures are within the same species.

In this dissertation, I refer to a pheromone as a chemical signal produced and released by an individual that elicits a specific stereotyped behavioral response (releaser effect) or physiological response (primer effect) in another individual of the same species (Karlson and Lüscher 1959). Pheromones evolve from cues, which are sources of public information (environmental, visual, audible, chemical, *etc.*) that only benefit the receiver (Symonds and Elgar 2008). Pheromones are specific chemical signals that benefit both the sender and receiver. Pheromones serve a range of functions and mediate interactions amongst conspecifics when searching for food or territory, avoiding risk, and evaluating competitors or mates (Wyatt 2014). Despite pheromones being a significant component of communication systems across many taxa, our knowledge of pheromone communication is largely based on studies of insect species (Carde and Minks 2012; El-Sayed 2009). Approximately 80% of the pheromone research in the past few decades is related to insect species, with members of the Lepidoptera order (butterflies and moths) alone comprising 38% (Symonds and Elgar 2008). Therefore, pheromones used by vertebrates are understudied when compared to that of insects. This creates a taxonomic gap of knowledge of pheromone communication across vertebrates. The research presented here

focuses on the pheromone communication of the sea lamprey (*Petromyzon marinus*) and helps fill this gap of knowledge on vertebrate pheromones. The sea lamprey is a unique model organism to study pheromone communication because it is a member of the most primitive group of vertebrates in the superclass Agnathan, or jawless fishes (Figure I-1). Understanding the function of pheromones in sea lampreys may provide insights into the evolution of vertebrate pheromone communication.

Figure I-1. Picture of a sexually mature male sea lamprey (*Petromyzon marinus*). All experiments described in this dissertation were conducted on sea lampreys.

SEA LAMPREY AS A MODEL ORGANISM TO STUDY PHEROMONE COMMUNICATION

The sea lamprey relies on pheromones to mediate its life cycle that consists of three distinct stages: larva, juvenile, and adult. The larvae burrow into the silt and sand sediment of freshwater streams for an average of 3-5 years and filter feed on organic material and microorganisms. Then the larvae undergo a drastic metamorphosis into parasites with a sucker-disc mouth, and the newly transformed parasites migrate downstream into the Laurentian Great Lakes or Atlantic Ocean to parasitize other fish (Hardisty and Potter 1971). After 1.5 years of feeding, immature adult sea lampreys cease feeding, detach from their host, and migrate back to spawning streams in the spring (Sorensen and Vrieze 2003; Wagner et al. 2009). Sea lampreys do not home to their natal streams like anadromous salmonids (Bergstedt and Seelye 1995) but instead detect the migratory cues released by stream-resident larvae. The migratory cue is comprised of multiple components that guide the adults into the spawning streams. The immature adults use the conspecific larval odors as a navigational cue to locate the general vicinity of a river with suitable spawning habitat and to initiate non-targeted upstream movement, but do not locate the specific source of release (Bjerselius et al. 2000; Sorensen et al. 2003; Sorensen et al. 2005; Teeter 1980). It was determined that immature adult sea lampreys rely on the olfactory detection of the migratory cue because immature adults rendered anosmic through naris plugging were unable to locate the river mouth (Vrieze et al. 2010; Vrieze et al. 2011).

Males precede females in ascending to the spawning grounds and construct nests from the gravel substrate using their oral discs (Applegate 1950). Migration of both males and females occurs primarily at night (Applegate 1950). At the onset of sexual maturation in May or

June when the water temperature reaches ~15 °C in tributaries of the Great Lakes basin, males release a multi-component sex pheromone that signals the males' reproductive status and nesting location to the females downstream (Johnson et al. 2015a). A main component of the sex pheromone is 3-keto petromyzonol sulfate (3kPZS), which induces the long distance upstream migration of females (Li et al. 2002). Females target the specific source of release and join the nesting males in the spawning grounds (Brant et al. 2015a; Johnson et al. 2009; Li et al. 2002; Siefkes and Li 2004; Siefkes et al. 2005). Females also detect minor bile salt components released by mature males including 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) and petromyzonamine-24-monosulfate (PAMS-24), which are presumed to induce preference behaviors when in close proximity and retention on the nest (Brant 2015; Brant et al. 2016; Li et al. 2013). The sea lamprey spawning season lasts for one to two weeks typically in June followed by death bceause sea lampreys are semelparous (Applegate 1950).

The pheromones are detected by the olfactory system and induce large magnitude electro-olfactogram (EOG) responses in adult sea lampreys (Li et al. 2013; Siefkes and Li 2004). The highly specialized adult sea lamprey olfactory system consists of a peripheral olfactory organ containing both the main olfactory epithelium and accessory olfactory organ in a cartilaginous capsule with a single nostril located on the dorsal surface of the head. Water is pumped in and out of the naris in a bi-directional manner. The well-developed olfactory organ has many longitudinal folds called lamellae that combine to form a flower-like structure called the olfactory rosette (Kleerekoper 1972). The olfactory organ has a midline raphe from which bilateral lamellae radiate. The pseudostratified layers of the olfactory epithelium consist of supporting cells, ciliated non-sensory cells, basal cells, and ciliated olfactory sensory neurons (VanDenbossche et al. 1995). Olfactory receptors on the dendrites of the three distinct sea

lamprey morphotypes of olfactory sensory neurons bind the odorant and trigger a signal cascade ultimately resulting in a physiological or behavioral response (Laframboise et al. 2007). Adult sea lampreys also have large olfactory bulbs relative to the rest of the brain (Stoddart 1990) to receive and integrate the olfactory signals of conspecifics (Kleerekoper 1972).

INVASIVE SEA LAMPREY CONTROL

The sea lamprey invaded the Laurentian Great Lakes in the 1800s and early 1900s. The sea lamprey invasion resulted in a series of deleterious changes in the aquatic ecosystem that precipitated the collapse of the Great Lakes fishery (Smith and Tibbles 1980). In addition, sea lamprey extirpated several native fish populations (Siefkes et al. 2013). Sea lamprey parasitism in combination with overfishing nearly eliminated the lake trout (*Salvelinus namaycush*) population. The Great Lakes Fishery Commission (GLFC) was jointly established by the United States and Canada in 1955 to control sea lampreys to enhance survival and reproduction of desirable fishes (Commission 2011). Management of invasive sea lampreys is integral to rehabilitate the native biodiversity, maintain a healthy ecosystem, and sustain viable Great Lakes fisheries valued to be worth at least $7 billion annually (Commission 2011). The current sea lamprey control program suppresses sea lamprey abundance; however, controlling sea lampreys is an ongoing battle. Selective pesticides (lampricides) are applied to tributaries to kill stream-resident larvae. Barriers and traps minimize the spawning migrations and remove adults before spawning (Siefkes et al. 2013). Despite these efforts, sea lampreys continue to be a significant source of mortality for large and medium sized fish in the Great Lakes. Ongoing research seeks

to improve the effectiveness, efficiency, and selectivity of sea lamprey control tactics (Siefkes 2017).

Given chemical cues and pheromones are indispensible for successful migration and reproduction, they are therefore aspects of sea lamprey biology that may be exploited to control invasive populations in the Laurentian Great Lakes. Previous research indicates that application of synthesized pheromone can increase the efficacy of sea lamprey traps (Johnson et al. 2013; Johnson et al. 2015b), but other options that modulate sea lamprey pheromone biology remain unexplored. Insect chemical ecologists modulate species-specific pheromone responses in an effort to manage nuisance pest and suppress their detrimental impacts (Witzgall et al. 2010). However, similar techniques have not been applied to vertebrate pests.

OVERVIEW OF DISSERTATION CHAPTERS

The goal of my dissertation is to further characterize the novel putative pheromone components released by mature male sea lampreys and identify antagonists that disrupt the olfactory and behavioral responses to the pheromone components. Strategies that disrupt the pheromone olfactory and behavioral responses may provide additional effective and environmentally benign approaches for sea lamprey management. Each chapter was formatted as a draft manuscript for a specific journal listed below. All manuscripts generated from the chapters will have multiple authors and I will be the first author when submitted.

Pheromones are known to mediate reproduction in sea lampreys, but it is unclear their role in the last stage of life involving mate choice. The previously characterized spermiated male sex pheromone components are sulfated bile acids released from the gills (Buchinger et al. 2015;

Siefkes et al. 2003); however, other sources of pheromones have not been examined. Chapter 1 titled "Spermine as a male sex pheromone detected by two TAAR-like receptors" characterizes the function of spermine as a male sea lamprey sex pheromone from semen, referred to as milt in fish species. This chapter was written in the format of *Current Biology*. Zhe Zhang, Liang Jia, Ke Li, Qinghua Zhang, Thomas Dexheimer, Edmund Ellsworth, Jianfeng Ren, Yu-Wen Chung-Davidson, Yao Zu, Richard Neubig, and Weiming Li are listed as co-authors on the submitted manuscript which constitutes Chapter 1. Previous studies found that spermine is more abundant in semen than any other tissue or fluid (Mann 1974; Rosenthal and Tabor 1956). I reasoned that spermine emitted via milt indicates sperm availability, and thus would serve as a signal that benefits both sexes seeking multiple mates in a polygynandrous mating system. I hypothesized that if spermine functions as a male sex pheromone, milt should be the main source of waterborne spermine that stimulates the lamprey olfactory system and induces preference behaviors in females. Spermine was confirmed to be present in sea lamprey milt. Using an integrative approach, the activation of two trace amine-associated receptors located in the olfactory epithelium was linked to the olfactory detection of spermine and the subsequent behavioral attraction of females but not males. The results indicate that spermine is a sex pheromone transmitted via the novel substrate of milt.

Previous studies found female sea lampreys rendered anosmic fail to locate spawning habitats (Vrieze et al. 2010) and interact with males (Johnson et al. 2006), indicating that application of pheromone inhibitors that block the olfactory detection of pheromones might effectively halt reproduction. Given pheromones are detected via the olfactory system, the search for inhibitors focused on compounds that disrupt olfactory responses to the sea lamprey sex pheromone components. Petromyzonol sulfate (PZS), which is released by larval sea

lamprey, reduced the olfactory detection of 3kPZS (Siefkes and Li 2004). Virtual screening by collaborators Dr. Leslie Kuhn and Dr. Sebastian Raschka (Michigan State University) also suggested PZS may be a candidate inhibitor of 3kPZS (Raschka et al. 2018). Chapter 2 titled "A conspecific chemical cue disrupts responses to a male pheromone in the invasive sea lamprey" further explores these findings to determine the potential of PZS to disrupt the 3kPZS chemical communication of sea lampreys. Chapter 2 was written in the format of the *Journal of Applied Ecology*. Based on the known disruption of the 3kPZS olfactory response by PZS, I hypothesized that PZS disrupts the attraction of ovulated females to 3kPZS in sea lampreys. I combined electrophysiology and behavioral experiments to demonstrate PZS is an effective 3kPZS olfactory and behavioral inhibitor across a range of concentrations. Detailed tracking of sexually mature females in a spawning stream when 3kPZS was applied with and without PZS further support the conclusion that PZS alters 3kPZS-mediated behaviors in the field. At the end of the chapter, I provide a discussion of how these results may be translated into sea lamprey management tactics.

Chapter 3 titled "A synthetic antagonist reduces olfactory and alters female behavioral responses to a male sex pheromone in sea lamprey" integrated virtual screening results with electrophysiology and behavioral assays to identify an additional potent and selective agent that disrupts the olfactory and behavioral responses to 3kPZS in sea lampreys. Chapter 3 was also written in the format of the *Journal of Applied Ecology*. In this chapter, I assessed if the eight polysulfated petromyzonol candidate antagonists could reduce the 3kPZS olfactory or behavioral response of ovulated females. I provide evidence that petromyzonol-3,7,12,24-tetrasulfate (3sPZS), a candidate 3kPZS antagonist identified through virtual screening by collaborators Dr. Leslie Kuhn and Dr. Sebastian Raschka (Michigan State University) (Raschka et al. 2018),

reduces the 3kPZS olfactory response. 3sPZS also reduces the behavioral preference of females for 3kPZS in the maze and reduces upstream movement, entry, and retention in artificial nest baited with 3kPZS in a natural spawning environment. I conclude the chapter with a highlight of the potential management implications of sea lamprey pheromone antagonists.

Sea lamprey behavioral studies indicated the full suite of sex pheromone components found in water conditioned with spermiated male sea lampreys resulted in greater attraction, prolonged retention, and increased display of nesting behaviors compared to the mixture of the known sex pheromones including 3kPZS, DkPES, and PAMS-24 (Fissette, unpublished). These results suggest males release additional sex pheromone components. In Chapter 4 titled "Identification of sea lamprey pheromones using bioassay-guided fractionation," I describe a methodological approach used to identify the unknown active sex pheromone components that elicit these additional responses. Ke Li and Weiming Li are co-authors on the submitted manuscript which constitutes Chapter 4 that is under review at the *Journal of Visualized Experiments*. This chapter focuses on bioassay-guided fractionation as an effective and interdisciplinary approach to isolate and characterize the structures, olfactory potencies, and behavioral responses of putative pheromones of sea lampreys. The chapter provides representative results, troubleshooting tips, potential modifications of the protocol, and applications of this technique to other taxa.

OVERVIEW OF DISSERTATION APPENDICES

Using bioassay-guided fractionation, eight novel bile salts or bile alcohols released from spermiated male sea lampreys were isolated and characterized. The results are reported in three

appendices of the dissertation. The Appendices were adapted from manuscripts that were written for specific target journals. The Appendices are included to reflect the research that I conducted in collaboration with Dr. Ke Li (Michigan State University). Bioassay-guided fractionation is an integrated approach that spans the two fields of chemistry and biology. While my interests primarily focus on understanding the biology of pheromone communication, my research has benefitted from the collaboration with chemists including Dr. Ke Li. Dr. Ke Li isolated and elucidated the structures of the eight putative pheromones or cues and is the lead author on published or drafted manuscripts that constitute Appendices A−C. I am the second author on these manuscripts. I characterized the olfactory potency and behavioral activity of the putative pheromones, analyzed the respective data, and contributed substantially to the writing of the manuscripts.

Appendix A titled "Bile salt-like dienones having a novel skeleton or a rare substitution pattern function as chemical cues in adult sea lamprey" is adapted from a publication in *Organic Letters* (Li et al. 2017a). Ke Li, Cory Brant, Skye Fissette, Joseph Riedy, Thomas Hoye, and Weiming Li are co-authors on the manuscript that was adapted for Appendix A. Appendix A reports the structure, olfactory potency, and female behavioral preferences of two novel sulfated bile salt-like dienones (petromyzene A–B) from water conditioned with mature male sea lampreys.

Appendix B titled "Three novel bile alcohols of mature male sea lamprey (*Petromyzon marinus*) act as chemical cues for conspecifics" is adapted from a publication in the *Journal of Chemical Ecology* (Li et al. 2017b). Ke Li, Joseph Riedy, Skye Fissette, Zoe Middleton, and Weiming Li are co-authors on the manuscript that was adapted for Appendix B. Appendix B reports the structure, olfactory potency, and female behavioral preferences of the three novel

sulfated bile alcohols (petromyzone A–C) from water conditioned with mature male sea lampreys.

Appendix C titled “Bile salts petromylidene A−C isolated from male sea lamprey (*Petromyzon marinus*) act as putative pheromones” is formatted for the *Journal of Natural Products*. Ke Li, Skye Fissette, Tyler Buchinger, Joseph Riedy, and Weiming Li contributed to a draft manuscript which has been adapted for Appendix C. Appendix C reports the structure, olfactory potency, and female behavioral preferences of the three novel bile acid derivatives (petromylidene A−C) from water conditioned with mature male sea lampreys.

CONCLUSION

In summary, these studies presented in my dissertation characterize novel pheromone components released by mature male sea lampreys and identify pheromone antagonists that disrupt the olfactory and behavioral responses to a male sea lamprey pheromone. The results further elucidate the identity and bioactivity of the multi-component sex pheromone released by mature male sea lampreys. The sex pheromone contains numerous potent odorants that mediate behavioral responses in conspecifics. In collaboration with other researchers, I identified spermine as a new pheromone component present in milt, two pheromone antagonists, and eight sex pheromone components or cues from water conditioned with mature male sea lampreys. Understanding sea lamprey pheromone communication and the factors that modulate pheromone behavioral responses are especially important in order to successfully implement pheromones as a component of integrated sea lamprey management (Li et al. 2007). Naturally occurring or synthetic pheromone antagonists may offer new control tactics to manipulate sea lamprey behavioral responses or to disrupt sea lamprey mating. At the end of the dissertation, I provide concluding remarks that highlight the implications and future directions of this research. In conclusion, sea lamprey pheromones and pheromone antagonists can help guide an effective sea lamprey control program in the Laurentian Great Lakes, where the sea lamprey is a destructive invader.

CHAPTER 1

SPERMINE AS A MALE SEX PHEROMONE DETECTED BY TWO TAAR-LIKE RECEPTORS

Adapted from Scott, A.M., Zhang, Z., Liang, J., Zhang, Q., Dexheimer, T., Ellsworth, E., Ren, J., Chung-Davidson, Y.W., Zu, Y., Neubig, R.R., & Li, W. (2018). Spermine as a male sex pheromone detected by two TAAR-like receptors. Manuscript submitted.

SUMMARY

Sexual selection was initially inferred from elaborate traits that increase male competitiveness for access to mates. Male competition continues after copulation; semen is also selected to contain anti-aphrodisiacs that deter promiscuity in copulated females (Gilbert 1976; Kukuk 1985; Mann and Lutwak-Mann 1981). We posited that the same selective pressure should have resulted in semen containing compounds that attract additional female mates. Female animals across taxa mate with multiple partners (Taylor et al. 2014) and promiscuity drastically increases their offspring's lifetime fitness (Gerlach et al. 2011). We chose to test the hypothesis that sex pheromones are present in semen of sea lamprey (*Petromyzon marinus*), who spawn in a lek-like system. In their spawning season, sexually mature lampreys aggregate in gravel patches and spawn intermittently for approximately one week before death (Johnson et al. 2015a). During that period, each male with expressible semen, referred to as milt in fish species, defends a nest, while ovulated females move from nest to nest and mate with numerous nesting males. Here we report that spermine (Tabor and Tabor 1984) is a male sex pheromone that attracts mature females. Spermine is released by mature males through the emission of milt, induces olfactory responses at a sub-femtomolar concentration, and attracts ovulated females but not mature males. Spermine activates two trace amine-associated receptors (TAARs) expressed in HEK293T cells. An antagonist to the receptors reduces olfactory responses and abolishes female attraction to spermine. Our results demonstrate an unexpected function of semen in recruiting mates and implicate a molecular mechanism for the olfactory detection of semen.

RESULTS AND DISCUSSION

If spermine functions as a male sex pheromone, emitted milt should be the main source of waterborne spermine that stimulates the sea lamprey olfactory system and induces preference behaviors in ovulated females because previous studies found spermine is more abundant in semen than any other tissue or fluid (Mann 1974; Rosenthal and Tabor 1956). To measure emitted spermine accurately, Dr. Ke Li optimized an ultra-high performance liquid chromatography-tandem mass spectrometry assay and found that sea lamprey milt contained 172 ± 21 ng g^{-1} of spermine (mean ± S.E.M., n = 6), of which 69.8% of the spermine was contained in seminal plasma (Figure 1-1A). In comparison, ovarian fluid from ovulated females did not contain detectable spermine (limit of detection: 1.0 ng mL^{-1}), nor did water conditioned with mature males that expressed milt (spermiated males) or ovulated females (Table 1-1). Although spermine is a ubiquitous polyamine with wide ranging cellular functions, our results indicate that only milt, in particular the seminal plasma, is the main source of waterborne spermine from mature sea lampreys.

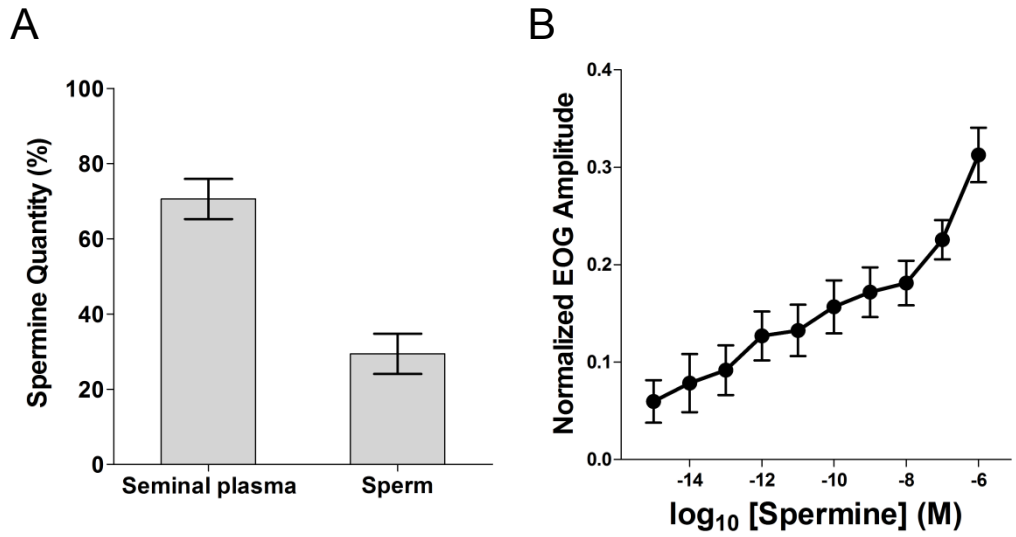

Figure 1-1. The seminal content and olfactory potency of spermine. (A) Mean percentage of spermine by weight in spermiated male sea lamprey milt components (seminal plasma and precipitated sperm) determined with ultra-performance liquid chromatography connected to a mass spectrometer (UHPLC–MS/MS) (mean ± S.E.M., n = 6). (B) Semi-logarithmic plot of electro-olfactogram (EOG) concentration-response relationship shows that spermine is a potent odorant for the adult sea lampreys. Data are presented as the mean EOG amplitude normalized by the response to 10^{-5} M $_{L}$-arginine (n = 12). Error bars represent one S.E.M.

Table 1-1. Concentration of spermine in water conditioned with spermiated male and ovulated female sea lampreys determined with UHPLC–MS/MS. SM, Spermiated male; OF, Ovulated female. N.D., Not detected. [a]The handling of sea lampreys most likely resulted in the incidental release of expressible milt into the water samples. Adult lamprey skin is slippery. To transfer lampreys in and out of water sampling buckets, one needs to grip both head and tail regions of the lamprey, and accidently pressure on the abdomen is difficult to avoid.

Sample ID	Concentration (ng ml^{-1})
SM-1	N.D.
SM-2	N.D.
SM-3	5.9[a]
SM-4	N.D.
SM-5	N.D.
SM-6	182.3[a]
SM-7	N.D.
SM-8	N.D.
SM-9	N.D.
SM-10	N.D.
OF-1	N.D.
OF-2	N.D.
OF-3	N.D.
OF-4	N.D.
OF-5	N.D.
OF-6	N.D.
OF-7	N.D.
OF-8	N.D.
OF-9	N.D.
OF-10	N.D.

Our electro-olfactogram (EOG) recordings showed 10^{-15} M to 10^{-6} M spermine evoked concentration-dependent responses from adult sea lamprey olfactory epithelia (Figure 1-1B), with the threshold of detection lower than 10^{-15} M (paired t-test, $t = -2.58$, $df = 11$, $p = 0.013$). Because spermine potentiates the action of NMDA receptors (Rock and Macdonald 1992), we assessed if NMDA receptors mediated the olfactory response of spermine. We subjected the sea lamprey naris to prolonged treatment of an NMDA receptor antagonist (10^{-5} M kynurenic acid) and observed no changes in EOG responses to spermine or other odorants tested. We inferred that NMDA receptors did not contribute to the EOG response of spermine. Previous studies have shown that spermine is responsible for the characteristic odor of human semen (Keel and Webster 1990), and evokes olfactory responses at concentrations as low as 10^{-8} M in goldfish and 10^{-6} M in zebrafish (Michel et al. 2003; Rolen et al. 2003). Olfactory detection of spermine by sea lampreys at sub-femtomolar concentrations is likely adaptive. Unlike goldfish and zebrafish that spawn in stagnant water, sea lamprey spawn in rapids with water velocities ranging between 0.5-1.5 m s^{-1} (Johnson et al. 2015a), where spermine released along with milt emission could become diluted rapidly.

As a direct test of the pheromone function of spermine, we examined the effects of milt and spermine on the behavior of sexually mature sea lampreys in a two-choice maze. All tested ovulated females preferred the channel conditioned with milt (estimated to result in 2.2×10^{-14} M spermine in the maze) compared to the channel conditioned with the vehicle (Figure 1-2). Likewise, ovulated females preferred 10^{-12} M and 10^{-10} M spermine compared to the vehicle. The behavioral preferences of ovulated females to milt, 10^{-12} M spermine, and 10^{-10} M spermine were not different (ANOVA, $F = 0.679$, $df = 2, 20$, $p = 0.518$). Spermiated males also preferred milt compared to the vehicle; however, they showed no preference to 10^{-12} M or 10^{-10} M

spermine (Figure 1-2). The dichotomy in male and female behavioral responses to spermine supports the hypothesis that spermiated males residing on nests emit spermine that attracts ovulated females. Other males may detect milt odors to cue in on spawning habitat and spawning activities, as mature male odorants are known to attract males en route to spawning grounds in sea lamprey (Brant et al. 2015b). If, however, a male sea lamprey intrudes an occupied nest, an agonistic fight begins immediately with the victorious male retaining the nest (Manion and Hanson 1980), thus maintaining the one male per nest in this polygynandry system. The milt odorants that attract males remain to be identified, but do not include spermine. Based on the chemical, electrophysiological, and behavioral evidence, we concluded that spermine is a sex pheromone that attracts ovulated females.

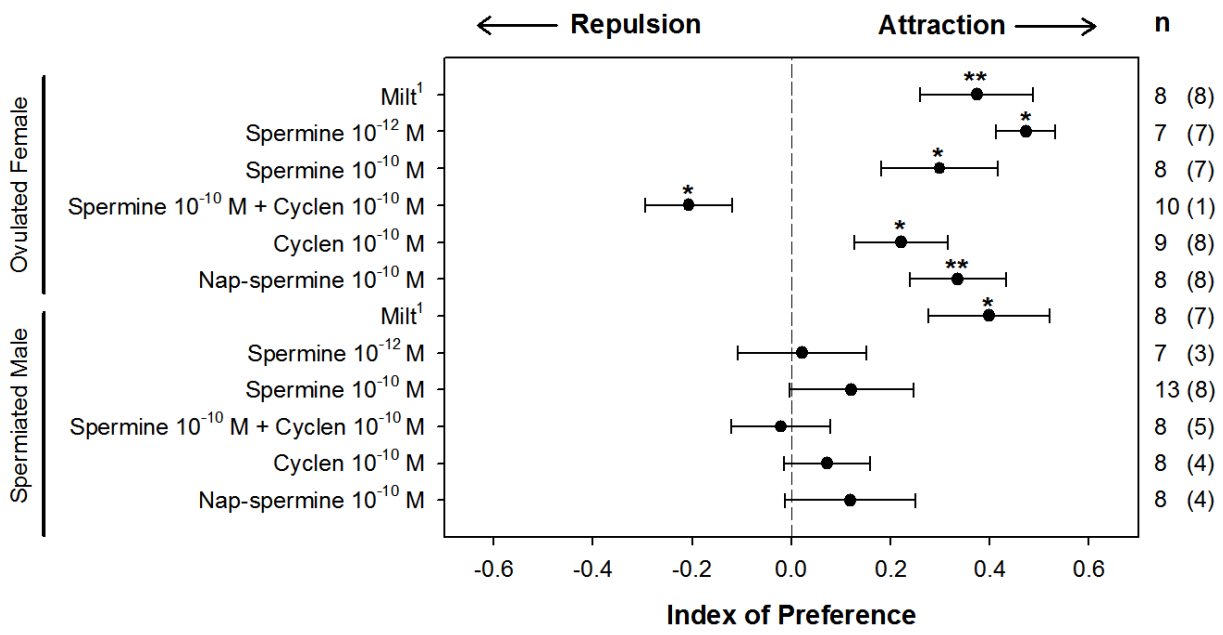

Figure 1-2. Spermine attracts ovulated females but not spermiated males. Adult sea lamprey behavioral responses were measured in a two-choice maze. The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined by Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The index of preference is presented as the mean ± S.E.M. (horizontal bar). The index value was evaluated using a Wilcoxon signed-rank test. n, sample size, with the number in the parentheses indicating the number of test subjects spending more time in the treatment side. [1]Milt was estimated to result in 2.2×10^{-14} M spermine assuming complete dilution in the maze. *, $p < 0.05$. **, $p < 0.01$.

We then set out to identify the olfactory chemoreceptors that detect spermine and focused our search on trace amine-associated receptors (TAARs), a family of receptors known to mediate olfactory responses to monoamines and diamines in vertebrates (Hussain et al. 2009; Liberles and Buck 2006). Collaborators Zhe Zhang, Liang Jia, Qinghua Zhang, Thomas Dexheimer, Yao Zu, and Richard Neubig designed and performed receptor assays. In HEK293T cells, collaborators expressed 26 sea lamprey TAAR-like genes mined and cloned from the sea lamprey genome (Smith et al. 2013), of which 21 targeted to the cell membrane. We found that only TAAR-like 348 and TAAR-like 365 (TAAR348 and TAAR365, respectively, hereafter) showed robust responses to 10 μM spermine (Figure 1-3A and Figure 1-4). We also screened spermine against 35 odorant receptor and V1R receptor genes mined and cloned from sea lamprey genome and expressed in HEK293T cells. Of the 25 odorant and V1R receptors that targeted to the cell membrane, none showed responses to spermine (data not shown), suggesting that other types of olfactory chemoreceptors do not respond to spermine.

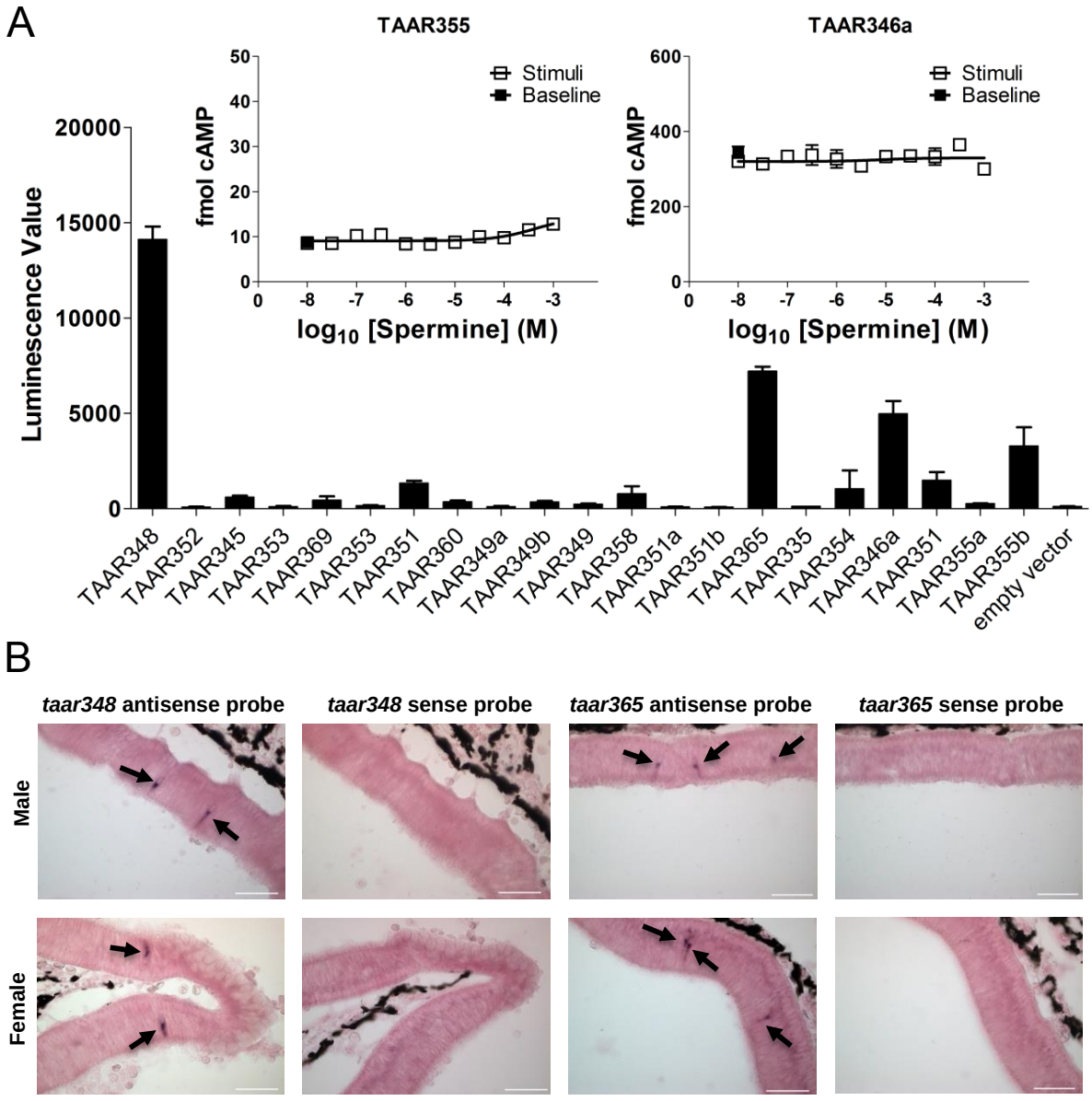

Figure 1-3. TAAR348 and TAAR365 showed robust responses to spermine and are located in the olfactory epithelium of adult sea lampreys. (A) HEK293T cells were reverse transfected with the 21 membrane expressed TAAR plasmids or an empty vector along with a CRE-luciferase reporter plasmid and then incubated for 48 h. Transfected cells were stimulated with 10 μM spermine and incubated for 4 h. Luciferase activity was measured by detecting luminescence value, mean ± S.E.M. (n = 2). Insets: Follow-up cAMP assays on TAAR346a and TAAR355b indicated these two receptors were not cognate receptors for spermine; rather they caused a high baseline activation with minimal response to spermine, mean ± S.E.M. (one representative experiment performed in triplicate).

Figure 1-3 (cont'd). cAMP production in HEK293T cells expressing TAAR346a or TAAR355b was measured with a TR-FRET assay. Black squares represent the baseline cAMP level induced by the stimulation buffer. Cells were stimulated with increasing concentrations of spermine shown as the white squares. (B) *In situ* hybridization using *taar348*- and *taar365*-specific antisense probe and sense probe (negative control), displayed on cross-sectional views of the main olfactory epithelium. Both *taar348* and *taar365* mRNAs were localized to the odorant receptor neurons of adult male and female sea lampreys. *taar348* and *taar365* mRNA positive cells are denoted with blue stain (NBT/BCIP) with black arrows. Sections were counterstained with Nuclear Fast Red. Black melanophores in the lamina propria are characteristic of sea lamprey olfactory epithelia. Scale bars represent 50 µm.

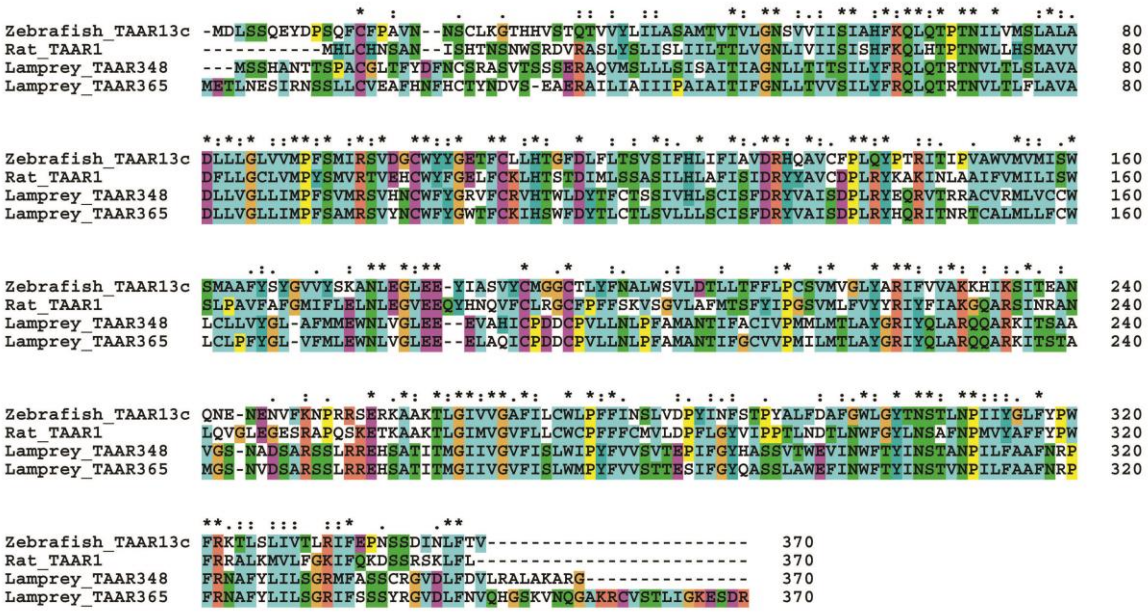

Figure 1-4. Comparisons of primary structures of sea lamprey TAAR348 and TAAR365 with those of zebrafish TAAR13c and rat TAAR1. This figure was provided by Jianfeng Ren who performed the bioinformatics analyses. Zebrafish TAAR13c (NP_001076509, 341 amino acid residues) and rat TAAR1 (NP_599155, 332 amino acid residues) were aligned with sea lamprey TAAR348 and TAAR365 (348 and 365 amino acid residues, respectively) using CLUSTAL 2.1 with default parameters. Marks for highly conserved substitutions: "*", residues which have a single, fully conserved residue; ":", residues with one of the following 'strong' groups fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; and ".", residues with one of the following 'weak' groups fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. We used positive scores from the Gonnet Pam250 matrix to define strong (score > 0.5) and weak groups (score ≤ 0.5). Color assignment was based on the amino acid residue profile specified in http://www.jalview.org/help/html/colourSchemes/clustal.html.

TAAR348 was highly specific in its responses to spermine (Figure 1-5B, Figure 1-5C), while TAAR365 responded to many amines (data not shown). In HEK293T cells, both TAAR348 and TAAR365 induced cAMP production in a concentration-dependent manner in response to spermine (Figure 1-6A). TAAR348 responded strongly to spermine (EC_{50} = 34 ± 1 μM). TAAR365 showed a similar level of potency (EC_{50} = 16 ± 2 μM) but a lower level of efficacy. Transcripts of *taar348* and *taar365* were found in the olfactory sensory neurons of adult male and female sea lampreys (Figure 1-3B). These data demonstrate that TAAR348 and TAAR365 are cognate receptors of spermine with TAAR348 showing remarkable specificity. These results expand the known ligands of TAARs to include not only mono- and di-amines (Hussain et al. 2013; Liberles 2015), but also other biogenic polyamines. Notably, the gene family encoding TAAR-like receptors first appeared in lampreys and evolved independently from the TAAR gene family of jawed vertebrates (Hashiguchi and Nishida 2007). It would be interesting to de-orphanize the olfactory chemoreceptors that detect spermine in jawed vertebrate animals, hence to infer if the olfactory sensitivity to spermine in jawless and jawed vertebrates is due to a convergent evolution of certain members in the TAAR gene families (Hashiguchi and Nishida 2007; Hussain et al. 2009; Keel and Webster 1990; Liberles and Buck 2006; Michel et al. 2003; Rolen et al. 2003).

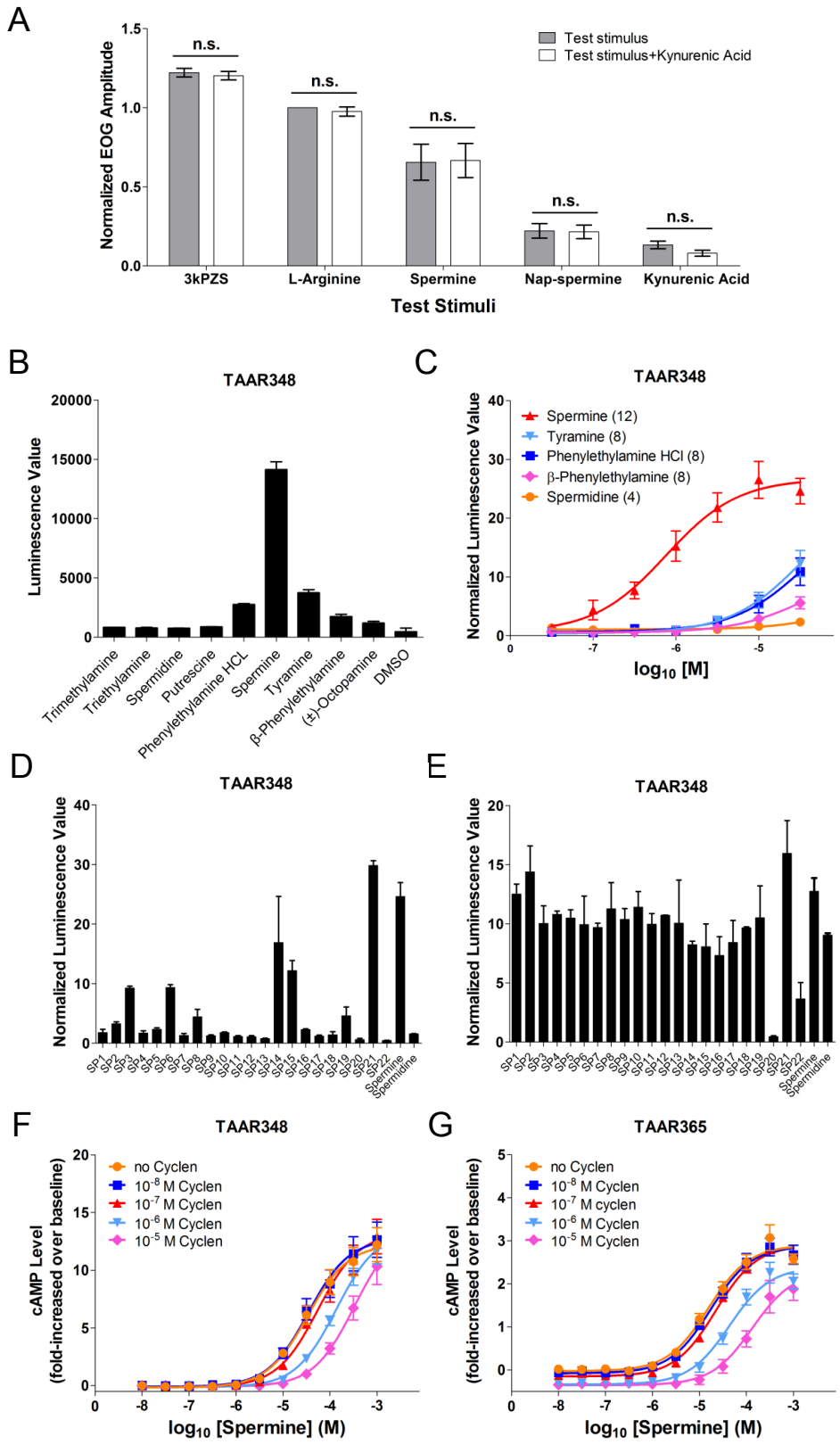

Figure 1-5. Characterization of spermine receptors as well as their agonist and antagonist.

Figure 1-5 (cont'd). (A) Kynurenic acid (10^{-5} M) treatment did not reduce the response magnitude to 3kPZS (10^{-7} M), $_{L}$-arginine (10^{-5} M), spermine (10^{-5} M), nap-spermine (10^{-6} M), or itself in the olfactory epithelia. Data are presented as the mean EOG amplitude in adult sea lampreys normalized to the response of 10^{-5} M $_{L}$-arginine and blank-corrected (n = 5). Error bars represent one S.E.M. Difference in responses for each stimulus before (gray) and during (white) continuous exposure of the naris to kynurenic acid was evaluated with a paired t test. n.s., not significant. Related to Figure 1-1B. (B) Spermine induced higher luciferase activity than other tested amines in TAAR348. HEK293T cells were reverse transfected with TAAR348 plasmid and luciferase reporter vector, and stimulated with 10 μM spermine or one of the other eight amines for 4 h before measuring luciferase activity. Luciferase activity was measured by detecting luminescence value, mean ± S.E.M. (n = 2). (C) Spermine induced a full sigmoidal dose-response curve in TAAR348. HEK293T cells were reverse transfected with TAAR348 plasmid and luciferase reporter vector and stimulated with increasing concentrations of spermine. Luciferase activity was normalized by DMSO stimulated luminescence value [Luc (Ligand)/Luc (DMSO)], mean ± S.E.M. Sample size is indicated by the number in the parentheses. (D) HEK293T reverse transfected cells with TAAR348 plasmid containing a luciferase reporter gene were incubated for 48 h, stimulated with 10 μM of one of the 22 spermine analog (Table 1-2) or DMSO (negative control), and incubated for 4 h. SP21 (Nap-spermine) was identified as a full TAAR348 agonist. Luciferase activity was measured by detecting luminescence value and normalized by DMSO stimulated luminescence value, mean ± S.E.M. (n = 2). (E) HEK293T reverse transfected cells with TAAR348 plasmid containing a luciferase reporter gene were incubated for 48 h, stimulated simultaneously application of 10 μM of one of the 22 spermine analog with 10 μM spermine and incubated for 4 h. SP20 (Cyclen) inhibited the 10 μM spermine induced luciferase activity. Luciferase activity was measured by detecting luminescence value and normalized by DMSO stimulated luminescence value, mean ± S.E.M. (n = 2). Increasing concentrations of cyclen induced increasing inhibition of the concentration-dependent cAMP production induced by spermine by (F) TAAR348 or (G) TAAR365, respectively, as measured with a TR-FRET assay. cAMP level was normalized with the cAMP baseline of buffer treated cells. Data are presented as mean. Error bars indicate one S.E.M. (n = 5).

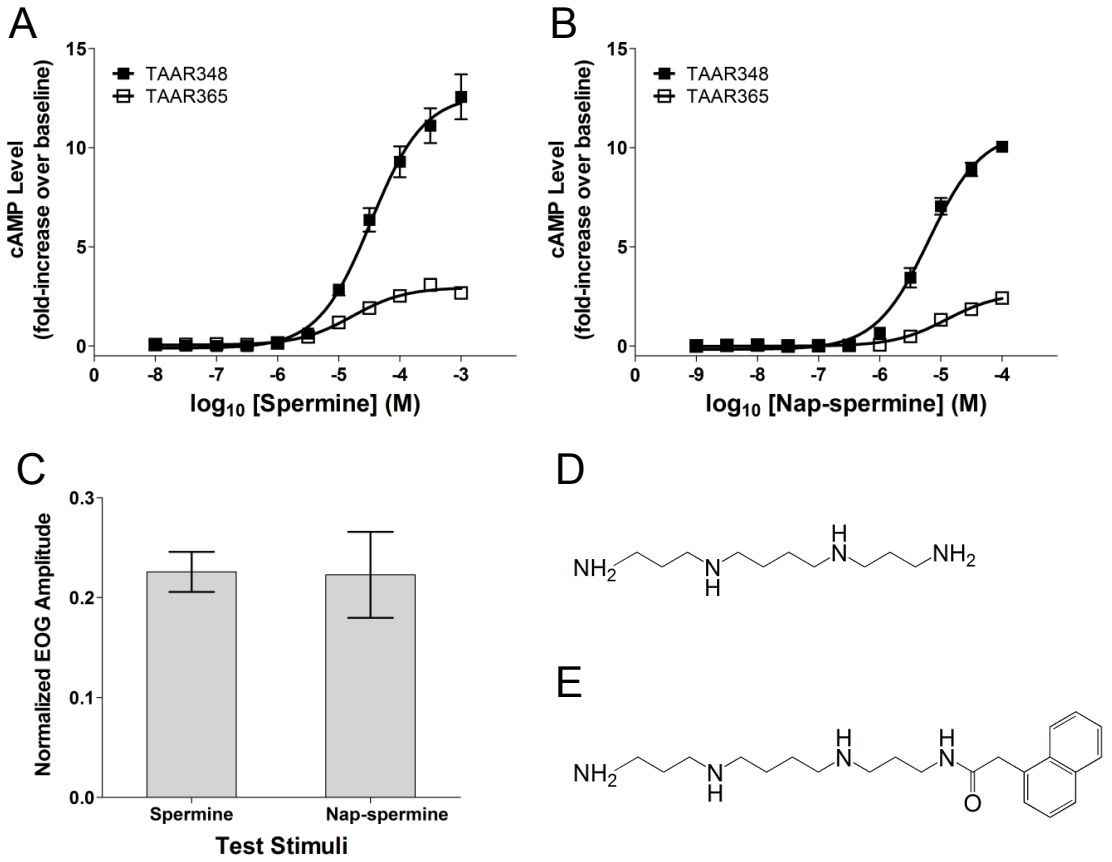

Figure 1-6. Characterization of spermine receptors and their agonist, nap-spermine. cAMP production in HEK293T cells expressing TAAR348 or TAAR365 was measured with a TR-FRET assay. cAMP level was normalized with the cAMP baseline of buffer treated cells. (A) Spermine induced cAMP production in a concentration-dependent manner by activating TAAR348 or TAAR365. Data are presented as mean. Error bars indicate one S.E.M. (n = 5). (B) Nap-spermine induced cAMP production in a concentration-dependent manner in TAAR348 and TAAR365. Data are presented as mean. Error bars indicate one S.E.M. (n = 5). (C) 10^{-7} M spermine (n = 12) and 10^{-7} M nap-spermine induced similar EOG response magnitudes (n = 6). Data are presented as the mean EOG amplitude in adult sea lampreys normalized to the response of 10^{-5} M $_{L}$-arginine and blank-corrected. Error bars represent one S.E.M. (D) Chemical structure of spermine. (E) Chemical structure of 1-naphthylacetyl spermine (nap-spermine).

In our final steps to elucidate a molecular mechanism of spermine detection, we sought to determine if TAAR348 and TAAR365 mediated the observed olfactory and behavioral responses to spermine in sea lampreys. Because gene knockout in adult lampreys is not technologically feasible (Zu et al. 2016), we screened for ligands that could block TAAR348 and TAAR365 pharmacologically. We identified cyclen (1,4,7,10-tetraazacyclododecane; Inset of Figure 1-7B) as an antagonist and nap-spermine (1-naphthylacetyl spermine; Figure 1-6E) as an agonist of TAAR348 (Figure 1-5D, Figure 1-5E, respectively) after testing 22 structural analogs of spermine (Table 1-2). Cyclen did not induce cAMP production in cells with TAAR348 or TAAR365 (Figure 1-7B), but was highly effective in inhibiting spermine-induced cAMP production, with an IC_{50} of 0.6 ± 0.1 μM for TAAR348 and an IC_{50} of 1.3 ± 0.2 μM for TAAR365 (Figure 1-7A). Cyclen treatment resulted in a sigmoidal, concentration-dependent reduction in cAMP production induced by spermine in TAAR348 and TAAR365 (Figure 1-5F, Figure 1-5G, respectively). The action is not due to non-specific interference with the assay system, as cyclen treatment did not inhibit cAMP production induced in the β1-Adrenergic receptor by 100 nM isoprenaline, an agonist of the β1-Adrenergic receptor (Figure 1-7D). The incomplete inhibition of TAAR348 and TAAR365 activity by cyclen (Figure 1-7A, Figure 1-5F, Figure 1-5G) suggests that cyclen may be a negative allosteric modulator (Christopoulos and Kenakin 2002).

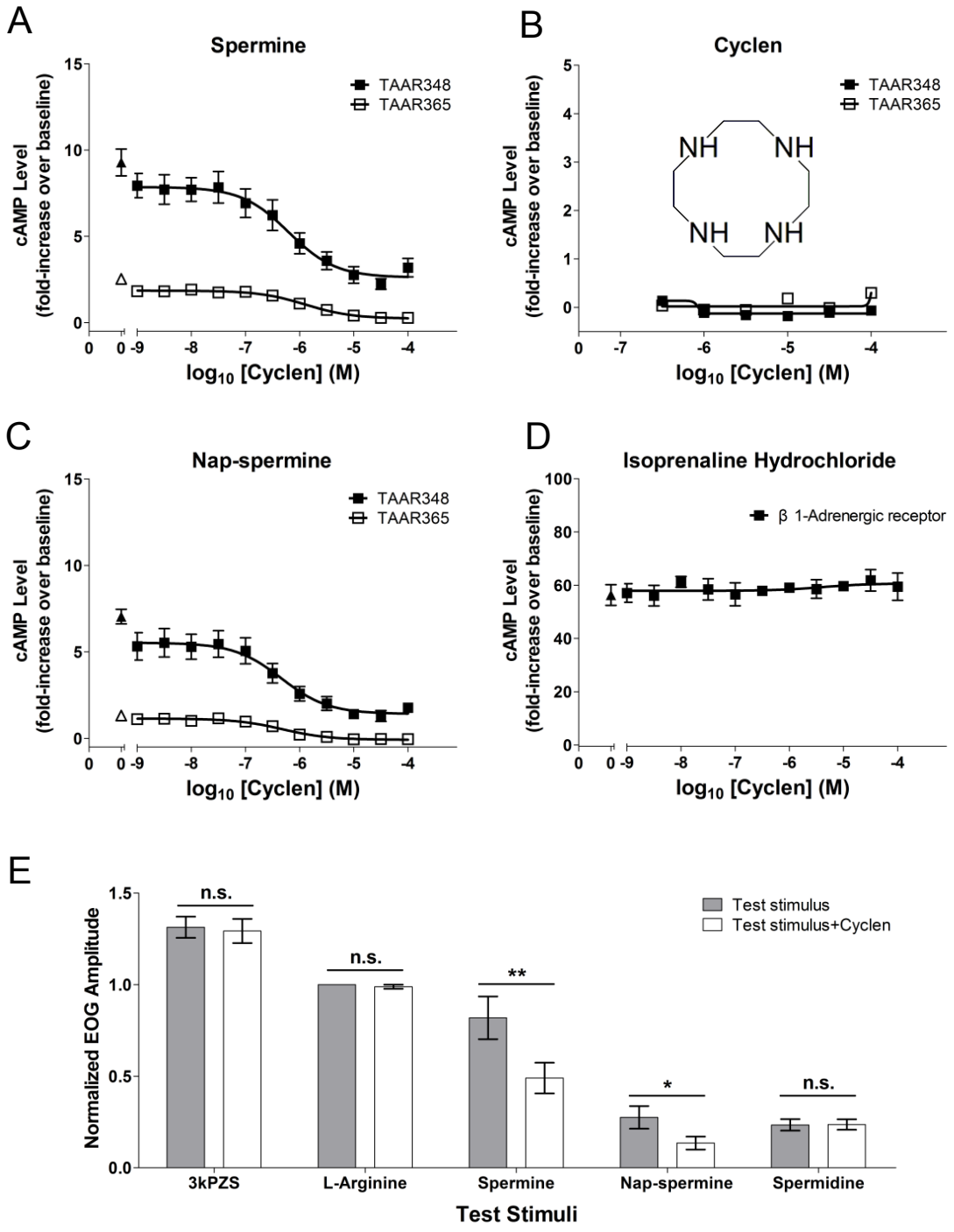

Figure 1-7. Characterization of an antagonist of the spermine receptors, cyclen. cAMP production in HEK293T cells expressing TAAR348 or TAAR365 was measured with a TR-FRET assay. cAMP level was normalized with the cAMP baseline of buffer treated cells.

Figure 1-7 (cont'd). (A) Cyclen inhibited cAMP production induced by exposure of TAAR348 or TAAR365 to 10^{-4} M spermine. Filled triangles and open triangles represent the cAMP production induced by TAAR348 or TAAR365 exposed to only 10^{-4} M spermine, respectively. Data are presented as mean. Error bars indicate one S.E.M. (n = 5). (B) Cyclen did not induce cAMP production in HEK293T cells expressing TAAR348 or TAAR365. Data represented reads from triplicate wells. Error bars represent one S.E.M. Inset: Structure of cyclen (1,4,7,10-tetraazacyclododecane). (C) Cyclen inhibited cAMP production induced by exposure of TAAR348 and TAAR365 to 10^{-5} M nap-spermine. Filled triangle and open triangle represent the cAMP production induced by TAAR348 or TAAR365 to only 10^{-5} M nap-spermine, respectively. Data are presented as mean. Error bars indicate one S.E.M. (n = 5). (D) Cyclen did not inhibit the cAMP production induced by exposure of β1-Adrenergic receptor to 100 nM isoprenaline hydrochloride. Filled triangle represents the cAMP production induced by β1-Adrenergic receptor to only 100 nM isoprenaline hydrochloride. Data are presented as mean. Error bars indicate one S.E.M. (n = 3). (E) Cyclen treatment (10^{-5} M) reduced the response magnitude to spermine (10^{-5} M) and nap-spermine (10^{-6} M), but not to spermidine (10^{-5} M), $_{L}$-arginine (10^{-5} M), or 3kPZS (10^{-7} M) in the olfactory epithelia. Data are presented as the mean EOG amplitude in adult sea lampreys normalized to the response of 10^{-5} M $_{L}$-arginine and blank-corrected (n = 5). Error bars represent one S.E.M. Difference in responses for each stimulus before (gray) and during (white) continuous exposure of the naris to cyclen (adaptation) was evaluated with a paired t test. ∗, $p < 0.05$. ∗∗, $p < 0.01$. n.s., not significant.

Table 1-2. Spermine structural analogs screened for spermine receptor agonists and antagonists. CAS, Chemical abstract service.

Analog number	CAS number	Common name
SP1	109-76-2	1,3-Diaminopropane
SP2	110-60-1	1,4-Diaminobutane
SP3	462-94-2	1,5-Diaminopentane
SP4	646-19-5	1,7-Diaminoheptane
SP5	373-44-4	1,8-Diaminooctane
SP6	646-25-3	1,10-Diaminodecane
SP7	124-09-4	Hexamethylenediamine
SP8	4605-14-5	N,N′-Bis(3-aminopropyl)-1,3-propanediamine
SP9	4741-99-5	N,N′-Bis(2-aminoethyl)-1,3-propanediamine
SP10	10563-26-5	1,2-Bis(3-aminopropylamino)ethane
SP11	7209-38-3	1,4-Bis(3-aminopropyl)piperazine
SP12	295-37-4	1,4,8,11-Tetraazacyclotetradecane
SP13	15439-16-4	1,4,8,12-Tetraazacyclopentadecane
SP14	113812-15-0	N1,N12-Diethylspermine tetrahydrochloride
SP15	77928-70-2	N1-Acetylspermine trihydrochloride
SP16	177213-61-5	N4,N9-di-Boc-spermine
SP17	112-24-3	Triethylenetetramine
SP18	112-57-2	Tetraethylenepentamine
SP19	1310544-60-5	N,N'-Bis(2-pyridylmethyl)-1,2-ethylenediamine tetrahydrochloride
SP20	294-90-6	Cyclen (1,4,7,10-Tetraazacyclododecane)
SP21	122306-11-0	Nap-spermine (1-Naphthylacetyl spermine)
SP22	295-14-7	1,4,7,10-Tetraazacyclotridecane

Subsequently, we determined if cyclen treatment resulted in a loss or modification of olfactory and behavioral responses to spermine in ovulated females. In the EOG experiments, pre-incubation of the olfactory epithelium with cyclen reduced the response magnitude to spermine, but not to other potent sea lamprey odorants including $_{L}$-arginine (Li and Sorensen 1997), 3kPZS, a known sea lamprey pheromone (Li et al. 2002), or spermidine, a biogenic polyamine that is a precursor in the biosynthesis of spermine (Figure 1-7E). Cyclen treatment reversed the female preference behavior induced by spermine; an equal molar mixture of 10^{-10} M spermine and cyclen repulsed ovulated females in the maze (Figure 1-2). As expected, the same mixture did not alter the behavior of spermiated males in the maze (Figure 1-2). The modification of the electrophysiological and behavioral responses to spermine in animals exposed to cyclen is consistent with the cyclen-mediated inhibition of cAMP responses to spermine in HEK293T cells with TAAR348 and TAAR365. The effect of cyclen in altering the female, but not the male, behavioral response to spermine lends further support that spermine is a specific male sex pheromone.

We further reasoned that the identified agonist, nap-spermine, should replicate the effects of spermine at the levels of the receptor, olfactory epithelium, and whole organism behavior. As predicted, nap-spermine activated both TAAR348 (EC_{50} = 5 ± 1 μM) and TAAR365 (EC_{50} = 11 ± 3 μM; Figure 1-6B), and induced EOG responses with magnitudes comparable to those induced by spermine (Figure 1-6C). As was the case for spermine, nap-spermine also attracted ovulated females but not spermiated males in the maze (Figure 1-2). Moreover, the cAMP production induced by nap-spermine in cells with TAAR348 or TAAR365 was reduced by cyclen, with an IC_{50} of 0.5 ± 0.3 μM and 0.5 ± 0.06 μM, respectively (Figure 1-7C). Likewise, cyclen treatment reduced the EOG response magnitude to nap-spermine (Figure 1-7E). In

summary, cyclen consistently nullified the receptor, olfactory, and female behavioral responses induced by spermine, whereas nap-spermine consistently induced responses similar to those induced by spermine. These results, coupled with the lack of spermine-induced responses for all other examined sea lamprey olfactory chemoreceptors, provide strong evidence that TAAR348 and possibly TAAR365 play a role in mediating the olfactory and behavioral responses to spermine.

It is not surprising that spermine has been co-opted as a sex pheromone. This type of pheromone often evolves from compounds once originally intended for other functions that are also linked to mate choice (Johansson and Jones 2007). We speculate that spermine is critical to male fertility and sperm mobility in sea lampreys, as has been shown in mammals (Stanger and Quinn 1982), and thus provides a reliable indicator for sperm quality and quantity. An ovulated female lamprey should be keenly attuned to such a signal to maximize the fertilization rate of her roughly 100,000 eggs (Johnson et al. 2015a) while optimizing the genetic benefit and lifelong fitness of her offspring (Taylor et al. 2014).

Our results reveal an unexpected and unknown pheromone function of semen in recruiting additional mates, which constitutes another aspect of post-copulatory sexual selection. It is well established that sexual competition between males continues after copulation; however, previous studies on this topic regarding semen have focused on the anti-aphrodisiac effects that deter promiscuity in copulated females. Our results suggest the same selective pressure may have selected for spermine, and possibly other semen compounds, to attract additional female mates. Where studied, all vertebrate animals smell spermine (Michel et al. 2003; Rolen et al. 2003) and express TAARs in their olfactory epithelia (Liberles 2015). Whether other taxonomic

groups have evolved a semen-borne pheromone, in particular the use of spermine as a pheromone that relies on TAARs, remains an open question.

METHODS

Experimental model and subject details

All procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (IACUC) (AUF# 03/14-054-00 and 02/17-031-00). Adult sea lampreys were captured in tributaries of the Laurentian Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada with scientific collection permits, transported to the U.S. Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, USA, and held in 500–1000 L aerated flow through tanks maintained at 15–19 °C. Immature adult sea lampreys used for electro-olfactogram recordings (237.9 g ± 36.8, 489.9 mm ± 18.3; mean ± S.E.M.) were transported to University Research Containment Facility at Michigan State University, East Lansing, Michigan, USA, held in flow through tanks (250 L) supplied with aerated, chilled well water maintained at 7–9 °C, and were used in April and May 2016 and 2017. Sexually mature adults (male and females) were used for behavioral assays conducted in June and July 2016 and 2017 and for sample collection for spermine quantification. To produce sexually mature adults, immature adults were held in cages constructed on polyurethane mesh and plastic pipe (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan, USA to allow natural maturation. These animals were monitored daily for signs of sexual maturation.

HEK293T cells were maintained at 37 °C with 5% CO_2 and grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) with no antibiotic.

Quantification of spermine

Ovulated female and spermiated male sea lampreys were separated by sex and held for 14 h in two 500 L tanks supplied with continuous flow-through aerated Lake Huron water at 17.7 °C before collecting samples for quantification of spermine. Each lamprey was transferred to a 20 L bucket supplied with continuous flow-through aerated Lake Huron water and acclimated for 1 h. All water was drained from the bucket and 3 L of deionized water with an air stone was added. After 1 h, the lamprey was removed from the bucket, a conditioned water sample was collected, and the water sample was stored at −20 °C until further analysis. Milt (sperm with seminal plasma) and eggs with ovarian fluid were collected from sexually mature lampreys by applying gentle pressure to their abdomen, resulting in expression of eggs or milt from the cloacal opening. The samples were centrifuged (10 mins, 1020 x*g* at 4 °C) to separate the fluid from the gametes. The supernatant fluid was transferred to a new tube and the gametes were kept in the same tube. The samples were stored at −80 °C until further analysis.

Dr. Ke Li designed and performed the quantification analysis. All chemicals for chromatography were purchased from Sigma-Aldrich (MO, USA). To prepare the stock solutions of spermine (≥ 99%) and [$^{2}H_{8}$] -spermine (Sp-d_8, 95%), each compound was dissolved in water/methanol (7:3, v/v) at a concentration of 1 mg ml^{-1}. The stock solution of internal standard (Sp-d_8; IS herein) was further diluted with water/methanol (7:3, v/v) to 500 ng ml^{-1}. 10 µl IS was added to each sample before extraction as described (Magnes et al. 2014) with modification. Briefly, 1 mL sample spiked with IS was treated with trichloroacetic acid (TCA, 4%, 100 µL) (Glaubitz et al. 2015), vortexed (5 min), and centrifuged (10 min, 10,000 x*g*), resulting in 500 µL supernatant that was mixed with 500 µL deionized water. Subsequently, sodium carbonate buffer (0.1 M, pH = 9, 125 µL) and isobutyl chloroformate (25 µL) were added

and incubated at 35 °C for 15 min. The isobutyl chloroformate residue was cleaned (Byun et al. 2008) and reconstituted to 100 µL with the initial mobile phase.

A Waters ACQUITY H-Class ultra-performance liquid chromatography (UHPLC) system connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer was used to detect spermine in the conditioned water and gamete samples (Waters Corp., MA, USA). The mobile phase consisted of water (containing 0.1% formic acid) as (A) and acetonitrile (containing 0.1% formic acid) as (B). A Waters BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) coupled with an Acquity UHPLC™ column in-line filter kit (0.2 µm filter) was used. Samples were separated with a gradient program at a flow rate of 250 µL min^{-1} for 12 min at 35 ^{o}C: 70 % A for 1 min, decreased to 0% A from 1 to 7 min, and then maintained at 0 % A from 7.01 to 9.0 min, increased to 70 % A from 9.0 to 9.01 min, and then maintained at 70% A to 12 min for column equilibrium. The sample injection volume was 10 µL. Spermine was detected by Multiple Reaction Monitoring (MRM) mode and processed using Masslynx 4.1 software. The UHPLC–MS/MS parameters were optimized for the transition of the spermine analyte as follow: $[M + H]^{+}$*m/z* 603.4, multiple reaction monitoring *m/z* 603.4 > 154.9, cone voltage 49 V, collision energy 40 eV, and retention time 6.92 min; and for the spermine-d_8 analyte as follow: $[M + H]^{+}$*m/z* 611.4, multiple reaction monitoring *m/z* 611.4 > 163.1, cone voltage 36 V, collision energy 40 eV, and retention time 6.92 min.

The UHPLC effluent was introduced into the mass spectrometer with electrospray ionization in the negative mode. The ESI-MS/MS parameters were set as follows: capillary voltage, 2.60 kV; extractor voltage, 5 V; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 800 L h^{-1} (N_2, 99.9% purity). Argon (99.9999% purity) was introduced as the collision gas into the collision cell at a flow rate of 0.15 mL min^{-1}. Data were

collected in centroid mode with a scan range of 50–1000 *m/z*. The dwell time established for each transition was 0.2 s and the interscan delay was set at 20 ms. Data acquisition was carried out using Masslynx 4.1 software and processed using TargetLynx (Waters Corp.). The detection limit was 1.0 ng ml^{-1}.

Two-choice maze behavioral assay

The preference of ovulated female and spermiated male sea lampreys to the odorants was evaluated using a two-choice maze that was described in a previous study (Li et al. 2017b). Briefly, a single lamprey was introduced to the acclimation cage at the downstream end of the maze for 5 min. The lamprey was released and the cumulative amount of time the lamprey spent in each channel was recorded for 10 min. The test stimulus was introduced to a randomly chosen channel and vehicle (water when milt was test stimulus and methanol for other test stimuli) to the other channel at constant rates of 200 ± 5 mL min^{-1}. Both test stimulus and vehicle were pumped into the maze for 5 min, and the cumulative amount of time the lamprey spent in each channel was recorded for 10 min. The maze was flushed with water for 10 min before the start of the next experiment to remove any remaining odorant. The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined by Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$) to determine if the index of preference was significantly different from zero. A significant positive value of the index of preference indicated attraction. A significant negative value of the index of preference indicated repulsion. A non-significant value of the index of preference indicated neutral. The trial was discarded if

the sea lamprey failed to enter the control and experimental channel for at least 10 seconds during the 10 min period before the odorant was applied, as this is an indication of strong side bias or inactivity.

Electro-olfactogram recordings

Electro-olfactogram (EOG) setup and recordings were conducted following established procedures (Li et al. 2017a) to determine if the adult sea lamprey olfactory organ was sensitive to spermine. Sea lampreys were anesthetized with 3-aminobenzoic acid ethyl ester (MS222; 100 mg L^{-1}), immobilized with an intra-muscular injection of gallamine triethiodide (3 mg kg^{-1} of body weight, in 0.9% saline), and placed in a V-shaped plastic stand. Gills were continuously irrigated with aerated water containing 50 mg L^{-1} MS222. The olfactory lamellae were surgically exposed by removing the skin on the surface of the olfactory capsule. The differential EOG response to each test stimulus was recorded using borosilicate electrodes filled with 0.04% agar in 0.9% saline connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, CT, USA) in 3M KCl. The recording electrode was placed between two olfactory lamellae and adjusted to maximize the response to $_{L}$-arginine standard while minimizing the response to the blank control (charcoal-filtered water), and the reference electrode was placed on the external skin near the naris. Electrical signals were amplified by a NeuroLog system (model NL102, Digitimer Ltd., England, UK), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized by Digidata 1440A (Molecular Devices LLC, CA, USA), and recorded on a PC running AxoScope 10.4 software (Molecular Devices LLC).

For concentration-response curves, the olfactory epithelia of sea lampreys were exposed to 10^{-15} M to 10^{-6} M solutions of spermine. A 10^{-3} M stock solution of spermine in

water/methanol (1:1, v:v) was prepared, stored at -20 ^{o}C, and then serially diluted with filtered water to yield 10^{-15} M to 10^{-6} M solutions. A 10^{-2} M stock solution of $_{L}$-arginine in deionized water was prepared, stored at 4 ^{o}C, and diluted with filtered water to yield a 10^{-5} M solution. A 10^{-5} M $_{L}$-arginine solution was introduced to the olfactory epithelium for 4 s and the response was recorded as a reference. The olfactory epithelium was flushed with filtered water for 2 min and then the blank control (charcoal-filtered water) was introduced and recorded to confirm the absence of a response in the charcoal filtered water supply. Next, the test stimulus starting at 10^{-15} M to 10^{-6} M was applied, recorded, and flushed. Blank control and 10^{-5} M $_{L}$-arginine standard was measured after every two concentrations. The EOG response magnitudes were measured in mV, normalized relative to 10^{-5} M $_{L}$-arginine (2.854 ± 0.081; absolute raw mean ± S.E.M.), and corrected for blank response (0.166 ± 0.008; absolute raw mean ± S.E.M.) as defined in Normalized EOG Amplitude = (Rt-Rb)/ (Ra- Rb), where Rt is the response magnitude to the test stimulus, Rb is the response magnitude to the blank, and Ra is the response magnitude to 10^{-5} M $_{L}$-arginine. The $_{L}$-arginine standard and blank control responses were comparable to previous studies (Li et al. 2013; Li et al. 2017a; Li et al. 2017b). The detection threshold was defined as the lowest concentration where the test stimulus elicited a larger response than the blank control (paired t-test, one tailed).

For continuous naris exposure experiment, the EOG responses to the test stimuli (10^{-5} M spermine, 10^{-5} M $_{L}$-arginine, 10^{-7} M 3-keto petromyzonol sulfate (3kPZS), 10^{-5} M spermidine, and 10^{-6} M nap-spermine) were first recorded in the similar manner as the concentration response curve. The naris was continuously exposed to either 10^{-5} M kynurenic acid or 10^{-5} M cyclen as the adapting solution for 5 min. Next, the EOG responses to mixtures of the test stimuli with the adapting solutions were recorded. The naris was rinsed with charcoal filtered water for 2 min

followed by recording the responses to the test stimuli to ensure recovery of the olfactory system. The normalized EOG response of each test stimuli before and during adaptation was evaluated with a two-tailed paired t-test.

High throughput ligand screening of TAARs

Zhe Zhang, Liang Jia, Qinghua Zhang, Thomas Dexheimer, Yao Zu, and Richard Neubig designed and/or performed all receptor experiments. The open reading frames of *TAARs* were mined from the sea lamprey genome assembly (Pmarinus_7.0) (Smith et al. 2013). 26 *TAARs* were annotated, cloned from genomic DNA, and introduced into Rho-pCMV modified from pCMV-Tag-2B (Agilent Technologies 211172) by introducing a Rho-tag (first 21 amino acids of bovine rhodopsin) at N-terminal to replace the intrinsic flag-tag (Zhuang and Matsunami 2008).

HEK293T cells were seeded at 5,500 cells per well on a 384-well plate and co-transfected with 5 µL DNA-transfection mixture that contained 5 ng of a TAAR plasmid, pCI-mRTPs (provided by Dr. H. Matsunami, Duke University), and pEGFP-N1 (6085-1, Clontech, CA, USA). Empty plasmid, pCI-mRTPs, and pEGFP-N1 were co-transfected as a negative control. The plate was incubated for 48 h at 37 °C with 5% CO_2, fixed with 3 µL 37% formaldehyde per cell for 15 min at room temperature (RT), washed with 50 µL PBS three times, and incubated with 25 µL blocking buffer (1 × PBS with 5% BSA) for 1 h at RT. Subsequently, 25 µL mouse monoclonal anti-rhodopsin antibody (1:500, MABN15, Millipore, MA, USA) was added to each well and incubated at 4 °C overnight. The antibody solution was aspirated, washed with 50 µL PBS three times, and incubated with Alexa Fluor 594 goat anti-mouse IgG (1:500, A11005, Thermo Scientific, MA, USA) for 1 h at RT. Cells were counterstained with DAPI (1:1000, D1306, Thermo Scientific, MA, USA). Images were acquired at 200x magnification

under Cytation 3 Cell Imaging Multi-Mode Reader (BioTek) with DAPI, GFP, and Texas Red filters. Images were sequentially acquired in single XY-plane and merged with ImageJ software (NIH). Results indicated that 21 TAARs targeted to the plasma membrane, which were used for further screening.

The initial high throughput screening (HTS) was performed in 384-well plates as described (Zhuang and Matsunami 2008) with the following modifications. For the reverse transfection, each well contained 5 µL DNA-transfection mixture (25 ng CRE-Luciferase vector pGL4.29 (E8471, Promega, WI, USA), 5 ng TAAR plasmid, 5 ng pCI-mRTPs, 5 ng pCI-$G_{\alpha olf}$ (provided by Dr. H. Matsunami, Duke University), and 5,500 HEK293T cells in 25 µL 0.5% FBS DMEM medium. The plates were incubated for 48 h at 37 °C with 5% CO_2. Each stimulus solution (20 mM in DMSO; 150 nL) was dispensed into a designated well using Biomek FXP liquid handling automation workstation (Beckman Coulter, CA, USA). The negative control stimulus was 150 nL DMSO. Plates were then incubated for 4 h at 37 °C with 5% CO_2. Luciferase activity was measured using Steady Glo Luciferase Assay System (Promega E2520), and luminescence was read on a Synergy Neo multi-mode microplate reader (BioTek, VT, USA). Luciferase activity was normalized by DMSO stimulated luminescence value with following formula [Luc (Ligand)/Luc (DMSO)].

Assay for cAMP production

To characterize TAAR348 and TAAR36, the cAMP production assay was performed in 384-well plates as described in LANCE Ultra cAMP Kit manual (TRF0263, PerkinElmer, MA, USA). Briefly, HEK293T cells were seeded in a 100 mm dish with 3×10^6 cells in 10 mL complete culture medium (DMEM medium with 10% FBS and 1x Anti-Anti) and incubated for

24 h at 37 °C with 5% CO_2. Cells were then transfected with 5 µg pGL4.29, 1 µg pCI-mRTPs, 1 µg pCI-$G_{\alpha olf}$, and 1 µg TAAR plasmid and incubated at 37 °C with 5% CO_2 for 24 h. Transfected cells were detached with Versene (15040066, Thermo Scientific, MA, USA) and transferred to 384 well plates at 5 µL (2,000 cells) per well. 5 µL of the 2x spermine serial solutions were added to each well and incubated for 30 min at RT. Afterwards, 5 µL 4x Eu-cAMP tracer working solution and 5 µL 4x ULight-anti-cAMP working solution were added to each well and incubated for 1 h at RT. Plates were read with the Synerg Neo multi-mode microplate reader for TR-FRET emissions at 620 nm (as internal reference) and 665 nm (as biological response). The ratio of 665/620 allows normalization for the well-to-well variability and interference due to assay components.

Screening of agonists and antagonists of spermine receptor

Twenty-two spermine structural analogs (Table 1-2) were screened to identify agonists for TAAR348 using a strategy similar to the initial high throughput screening with the following modifications. Briefly, 3×10^6 HEK293T cells were seeded in 10 mL complete culture medium and incubated for 24 h on day 1. On day 2, the cells were co-transfected with 5 µg pGL4.29, 1 µg pCI-mRTPs, 1 µg pCI- $G_{\alpha olf}$, and 1 µg TAAR plasmid, and then incubated for 24 h. On day 3, the transfected cells were harvested and then re-seeded in 384 well plates at a density of 9,000 cells per well in 30 µL 0.5% FBS DMEM medium, and incubated for 24 h. On day 4, 150 nL of each analog solution (2 mM in DMSO), or 150 nL DMSO as a negative control, was dispensed into a designated well and subsequently incubated for 4 h. Luciferase activity was measured as the indicator for receptor activity.

To identify antagonists of the TAAR348 spermine-induced responses, the screening was performed on the same 22 spermine structural analogs using the agonist screening procedure from day 1 through day 3. On day 4, each well was dispensed with 150 nL of an analog solution (2 mM), or 150 nL DMSO as a negative control, and incubated for 30 min at 37 °C with 5% CO_2. Afterwards 5 µL 6×10^{-5} M spermine was added to each well and incubated for 4 h at 37 °C with 5% CO_2 before the plates were read for luciferase activity. To assay for spermine-cyclen competition, 2.5 µL 4x cyclen solutions and 2.5 µL 4x spermine solutions were added to each well.

To examine the specificity of cyclen, the effect of cyclen on the β1-Adrenergic receptor induced cAMP production was assessed. The cAMP production assay was performed as described for characterization of TAAR348 and TAAR365. Briefly, HEK293T cells were transfected with 5 µg pGL4.29, 1 µg pCI-mRTPs, and 1 µg mouse β1-Adrenergic receptor plasmid (provided by Dr. B. Kobilka, Stanford University), and stimulated with cyclen serial solutions and 100 nM isoprenaline hydrochloride (β1-Adrenergic receptor agonist; Sigma I5627). Subsequently, Eu-cAMP tracer and ULight-anti-cAMP were added, incubated, and read for TR-FRET emissions at 620 nm (as internal reference) and 665 nm (as biological response).

In situ hybridizations of *taar348* and *taar365*

Dr. Yu-Wen Chung-Davidson and Anne Scott assisted Liang Jia with *in situ* hybridizations of *taar348* and *taar365*. Probes (~350-400bp) were designed based on the coding regions of *taar348* and *taar365*. The amplified DNA fragments were cloned into a pGEM-T vector (Promega A3610) and the sequences verified. Each plasmid was linearized using restriction enzyme Nco (anti-sense probe) or Spe (sense probe) and used for synthesis of

digoxigenin-labeled RNA probes with DIG RNA labeling kit (SP6/T7) (11175025910, Roche, Switzerland). *In situ* hybridization was conducted following previously described methods (Chung-Davidson et al. 2004). Briefly, 20 μm frozen sections of olfactory epithelium were hybridized with RNA probes (3 ng μL^{-1}) overnight at 65 °C in the hybridization solution (50% deionized formamide, 1x Denhart's solution, 5% dextran sulfate, 750 mM sodium chloride, 25 mM ethylenediaminetetraacetic acid, 25 mM piperazine-N,N′-bis-2-ethanesulfonic acid, 0.25 mg mL^{-1} fish sperm DNA, 0.25 mg mL^{-1} poly A acid, and 0.2% sodium dodecyl sulfate). After hybridization, sections were washed three times for 5 min each in 4x saline-sodium citrate (SSC). Subsequently, sections were washed sequentially in 2x SSC containing 0.3% Tween-20 and 0.2x SSC containing 0.3% Tween-20 three times each for 15 min each at 68 °C. Sections were washed in 0.1x SSC containing 0.3% Tween-20 for 15 min followed by three washes of 5 min each in 0.1M PBS containing 0.3% Tween-20 at room temperature (RT). The sections were then incubated with blocking solution (1x PBS, 2 mg/mL bovine serum albumin, 0.3% Tween-20, and 10% normal sheep serum) for 1 hour at RT, followed by incubation with alkaline phosphatase-conjugated sheep-anti-digoxigenin Fab fragments (1:1000 diluted in blocking solution, Roche 11093274910) overnight at 4 °C. Hybridization signals were detected by incubating the sections in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Thermo Scientific 34042) for 2 hours at RT and then counterstained with Nuclear Fast Red (Vector Laboratories H3403) for 5 min at RT. All images were observed and photographed with a Zeiss Axioskop2 mot plus microscope equipped with a 40x Plan-Neofluar objective. Control experiments (sense probe) were conducted simultaneously with the identical procedure and conditions.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (Ver. 5) and Microsoft Excel. All statistical tests were two-tailed, unless otherwise noted. Nonparametric tests were used when the data did not meet the assumptions of the parametric test. The detection threshold for the spermine EOG concentration-response relationship was defined as the lowest concentration where the test stimulus elicited a larger response than the blank control and was evaluated with a one tailed paired t-test. For the adaptation EOG experiments with cyclen or kynurenic acid as the adapting stimuli, the normalized EOG response of each test stimuli before and during adaptation was evaluated with a two-tailed paired t-test. The indices of preference calculated for the behavioral maze trials were evaluated using a Wilcoxon signed-rank test to determine if the preference was significantly different from zero. Differences were considered significant at $p < 0.05$. Spermine quantification, EOG responses, behavior index, and receptor assay data are presented as mean ± S.E.M with the sample sizes listed in the corresponding figure legends.

Data availability

The mRNA sequences of *taar348* and *taar365* are available in GenBank (Accession numbers MH037334 and MH037351, respectively). The antisense probe sequence of *taar348* and *taar365* are available in GenBank (Accession numbers MH037360 and MH037361, respectively).

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CHAPTER 2

A CONSPECIFIC CHEMICAL CUE DISRUPTS RESPONSES TO A MALE PHEROMONE IN THE INVASIVE SEA LAMPREY

ABSTRACT

Invasion of aquatic species are a growing global ecological threat. The sea lamprey (*Petromyzon marinus*) is a successful invasive jawless fish that has substantially altered the Laurentian Great Lakes ecosystem. Integrated sea lamprey management is necessary to sustain viable fisheries, rehabilitate the biodiversity, and maintain a healthy ecosystem in the Great Lakes. Olfaction is known to play a key role in the sea lamprey life cycle. Ovulated female sea lampreys detect and are attracted to a sex pheromone, 3-keto petromyzonol sulfate (3kPZS), released by mature males. Modulation of the olfactory and behavioral responses to 3kPZS may offer novel methods for sea lamprey control. Previous research suggests petromyzonol sulfate (PZS), which is released by larval sea lamprey, reduces the olfactory detection of 3kPZS by the adults. We integrated an electro-olfactogram (EOG) approach with behavioral assays in a maze and spawning stream to characterize the disruption of PZS on the 3kPZS olfactory detection and attraction of ovulated female sea lampreys. Exposing the adult sea lamprey naris to a range of concentrations of PZS (10^{-10} M to 10^{-6} M) reduced the 3kPZS olfactory response in a concentration-dependent manner. At equal concentrations (10^{-6} M), PZS reduced the 3kPZS olfactory response by 92%. In a two-choice maze, 10^{-12} M or 10^{-11} M PZS mixed with 10^{-12} M 3kPZS neutralized the preference of ovulated females for 3kPZS. 10^{-10} M PZS mixed with 10^{-12} M 3kPZS averted females in a maze. In a natural field experiment, 5×10^{-13} M PZS reduced the upstream movement, entry, and retention of ovulated females to an artificial nest baited with 5×10^{-13} M 3kPZS. We identified a naturally occurring antagonist of the pheromone induced responses in sea lampreys. PZS effectively inhibits olfactory and behavioral responses to 3kPZS in sea lampreys and can likely be implemented to 1) disrupt mating, 2) re-direct lampreys into

specific locations, and 3) enhance selective fish passages. Our results represent a step forward in utilizing pheromone antagonists as an environmentally benign control agent to reduce the negative impacts of invasive sea lampreys in the Great Lakes.

INTRODUCTION

Invasions of aquatic species have had devastating effects on the ecosystem and are a growing global threat facilitated by increased international trade networks (Mack et al. 2000; Simberloff 2013). The Laurentian Great Lakes region is highly vulnerable to aquatic invasive species because of its interconnected waterways and role in international trade. At least 184 nonindigenous species have been reported in the Great Lakes region and many have successfully established populations (Escobar et al. 2018). The sea lamprey *(Petromyzon marinus)* is a destructive jawless fish that invaded the upper Great Lakes in the early 1900s. The sea lamprey invasion resulted in the extirpation of several native fish populations and precipitated the collapse of the Great Lakes fishery (Smith and Tibbles 1980). Management of sea lamprey is integral to sustain viable fisheries, rehabilitate the native biodiversity, and maintain a healthy ecosystem. Many aspects of the sea lamprey life cycle have been studied and the knowledge gained through such experiments have informed the development of control tactics including the application of selective pesticides (lampricide) to kill stream-resident larvae and the use of barriers and traps to minimize spawning migrations and to remove adults before spawning (Siefkes 2017; Siefkes et al. 2013). An emerging area of interest for control tactics is to disrupt olfaction, or the sense of smell, in this species (Buchinger et al. 2015; Li et al. 2007; Siefkes 2017).

Olfaction is known to play a key role in the sea lamprey life cycle (Buchinger et al. 2015). After detaching from their host fishes in the Great Lakes or Atlantic Ocean, immature adults migrate into a river with suitable spawning habitat, guided by the odors released by conspecific stream resident larvae (Bjerselius et al. 2000; Sorensen et al. 2003; Sorensen et al.

2005; Teeter 1980). Sexually mature males ascend to spawning grounds first, build nests, and release a multi-component sex pheromone that attracts females to a nesting location (Johnson et al. 2015a). As females enter the river and move upstream, they rely on olfaction to locate mates to spawn. Specifically, ovulated females detect 3-keto petromyzonol sulfate (3kPZS; 7α,12α, 24-trihydroxy-3-one-5α-cholan-24-sulfate), a main component of the sex pheromone released by males, which induces long distance upstream movement and targeted attraction (Brant et al. 2015a; Johnson et al. 2009; Li et al. 2002; Siefkes and Li 2004; Siefkes et al. 2005). After arriving at the spawning grounds, females respond to minor components presumed to induce preference behaviors and retention (Brant et al. 2016; Li et al. 2013).

We speculate that interfering with sea lamprey responses to pheromones may provide an additional approach for sea lamprey management. Insect chemical ecologists pioneered the concept of modulation of species-specific pheromone responses in an effort to manage nuisance pests (Witzgall et al. 2010). Pheromone inhibitors or antagonists, which are often structurally similar compounds to the major pheromone component, selectively interfere with the detection and behavioral responses to pheromones (Evenden et al. 1999; Giner et al. 2009; McDonough et al. 1996; Millar and Rice 1996; Solé et al. 2008). Application of pheromone inhibitors to manage invasive species in aquatic ecosystems has not been tested, though pheromones have been implicated in many nuisance or invasive fishes (Sorensen and Wisenden 2015). Previous electrophysiology cross-adaptation results suggest petromyzonol sulfate (PZS; 3α, 7α,12α, 24-tetrahydroxy-5α-cholan-24-sulfate), which is known to be released by larval sea lamprey (Haslewood and Tökés 1969; Polkinghorne et al. 2001; Sorensen et al. 2005), reduces with the olfactory response of 3kPZS (Siefkes and Li 2004). We speculate that PZS, which shares a high degree of structural similarity with 3kPZS, may function as an inhibitor. Virtual screening of

more than 12 million organic molecules by collaborators Dr. Leslie Kuhn and Dr. Sebastian Raschka also identified PZS as a candidate inhibitor of 3kPZS based on the high degree volumetric and functional group similarity of PZS with 3kPZS (Raschka et al. 2018).

We hypothesize that PZS disrupts the olfactory detection and attraction of ovulated females to 3kPZS in sea lampreys. First, we studied the olfactory inhibition of 3kPZS by PZS over a wide range of concentrations using electro-olfactogram (EOG). Second, we determined the behavioral responses of ovulated females in a two-choice maze and a natural field experimental site exposed to 3kPZS in the presence of PZS. Our results indicate that PZS disrupts female responses to 3kPZS and may offer new sea lamprey control tactics.

MATERIALS AND METHODS

Experimental animals

All procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (Animal usage form numbers: 03/12-063-00 and 12/14-223-00). Adult sea lampreys were captured in tributaries of the Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada, transported to the U.S. Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station (Millersburg, MI, USA), and held in 1000 L capacity aerated flow through tanks supplied with Lake Huron water maintained at 15–19 °C. Adult sea lampreys used for electro-olfactogram recordings were transported to the University Research Containment Facility at Michigan State University (East Lansing, MI) and held in 250 L flow through tanks supplied with aerated, chilled well water maintained at 7–9 °C. Ovulated females used for behavioral assays were held in cages located in the lower Ocqueoc River (Presque Isle County, MI) to allow for natural maturation. These animals were monitored daily for signs of sexual maturation by assessing defined secondary sex characteristics (Johnson et al. 2015a).

Electro-olfactogram (EOG) setup

The EOG setup followed previously described procedures (Li et al. 2017a) with modification. In brief, sea lampreys were anesthetized via immersion in a 3-aminobenzoic acid ethyl ester bath (100 mg L^{-1}; MS222, Sigma-Aldrich, St. Louis, MO, USA), immobilized with an intra-muscular injection of gallamine triethiodide (3 mg kg^{-1} of body weight, in 0.9% saline; Sigma-Aldrich), and placed in a V-shaped plastic stand. Gills were continuously irrigated with

aerated water containing 50 mg L^{-1} MS222 throughout the experiment. The olfactory lamellae were surgically exposed by removing the skin on the surface of the olfactory capsule. A small capillary tube delivered the stimuli and charcoal filtered water to the olfactory epithelium by gravity flow to prevent desiccation. The differential EOG response to each test stimulus was recorded using thin wall glass capillaries pulled to a tip to fabricate the borosilicate electrodes (filled with 0.04% agar in 0.9% saline) and connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, Hamden, CT, USA) in 3 M KCl. The recording electrode was placed between two olfactory lamellae in the olfactory epithelium and adjusted to maximize the response to $_{L}$-arginine (a known odorant highly stimulatory to the sea lamprey olfactory epithelium) (Li and Sorensen 1997), while minimizing the response to the blank control (charcoal filtered water). The reference electrode was placed on the external skin near the naris. Electrical signals were amplified by a NeuroLog system (model NL102, Digitimer Ltd., Hertfordshire, England, United Kingdom), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized (Digidata 1440A Molecular Devices LLC, Sunnyvale, CA, USA), and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC).

Electro-olfactogram (EOG) recordings

Electro-olfactogram (EOG) recordings are used to measures the *in vivo* olfactory response to stimuli. EOGs measure the summated generator potential of the olfactory sensory neurons in the epithelia after exposure to an odorant (Ottoson 1955; Scott and Scott-Johnson 2002). Since the EOG results of 3kPZS were consistent with behavioral results when the pheromone 3kPZS was initially identified (Li et al. 2002), we used this method to measure how

PZS disrupts 3kPZS olfaction as an initial experiment before conducting behavior experiments. To record the olfactory responses to a range of concentrations of 3kPZS and PZS, 10^{-5} M $_{L}$-arginine standard was first introduced to the olfactory epithelium for 4 s and the response was recorded as a reference of the electrical activity. EOG response magnitudes were measured in millivolts (mV) as the amplitude of the negative deflection in response to odorant stimulation. Then, the olfactory epithelium was flushed with charcoal filtered water for 2 min, and the response to a blank control (charcoal filtered water) was recorded to confirm the absence of a response in the charcoal filtered water supply. A 10^{-3} M stock solution of 3kPZS (Bridge Organics Co., Vicksburg, MI, USA; methanol: deionized water, 1:1) was prepared, stored at -20^{o}C, and serially diluted with charcoal filtered water to yield 10^{-12} M to 10^{-6} M working solutions. Increasing concentrations of 3kPZS starting at 10^{-12} M to 10^{-6} M were applied, recorded, flushed, and then repeated with the next higher concentration. Blank control and 10^{-5} M $_{L}$-arginine responses were measured after every two concentrations. The EOG response magnitudes were normalized relative to 10^{-5} M $_{L}$-arginine and corrected for blank response as defined in Equation 1: Normalized EOG Amplitude = $(R_t - R_b)/(R_a - R_b)$, where R_t is the response magnitude to the test stimulus, R_b is the response magnitude to the blank, and R_a is the response magnitude to 10^{-5} M $_{L}$-arginine. The solution preparation and EOG recordings for PZS were conducted in the same manner as 3kPZS. The $_{L}$-arginine standard and blank control responses were comparable to previous studies (Li et al. 2013; Li et al. 2017a; Li et al. 2017b).

To determine whether PZS reduced the olfactory potency of the 3kPZS, the EOG response to the test stimulus (3kPZS or $_{L}$-arginine) before and during exposure to PZS were recorded and the reduction of the olfactory response due to PZS treatment was calculated. It was presumed that if PZS and the stimulus (3kPZS or $_{L}$-arginine) share the same receptors, olfactory

sensory neurons activated by an adapting solution of PZS will have less binding sites available to respond to a test stimulus, resulting in a decreased total EOG response to the stimuli (Caprio and Byrd 1984). The responses to the blank control, 10^{-5} M $_{L}$-arginine, 10^{-6} M 3kPZS, and 10^{-6} M PZS were recorded. The olfactory epithelium was exposed to 10^{-6} M PZS for 2 min and then the responses to a mixture of 10^{-6} M PZS and 10^{-5} M $_{L}$-arginine followed by a mixture of 10^{-6} M PZS and 10^{-6} M 3kPZS were recorded while exposing the epithelium to 10^{-6} M PZS between recordings. The olfactory epithelium was flushed with charcoal filtered water for 2 min, and responses to the blank control, 10^{-5} M $_{L}$-arginine, 10^{-6} M 3kPZS, and 10^{-6} M PZS were recorded to ensure recovery of the olfactory epithelium.

The change in the olfactory response before and during exposure to PZS for either $_{L}$-arginine or 3kPZS was calculated as the ratio between the blank corrected adapted response (during exposure) and the non-adapted response (before exposure), as defined in Equation 2: Ratio of EOG amplitudes = $(R_{adapt} - \text{blank}) / (R_{non\text{-}adapt} - \text{blank})$. For $_{L}$-arginine, R_{adapt} was the response of the mixture of 10^{-6} M PZS and 10^{-5} M $_{L}$-arginine relative to $R_{non\text{-}adapt}$, the response of 10^{-5} M $_{L}$-arginine. For 3kPZS, R_{adapt} was the response of the mixture of 10^{-6} M PZS and 10^{-6} M 3kPZS relative to $R_{non\text{-}adapt}$, the response of 10^{-6} M 3kPZS. A ratio of EOG amplitudes of 1.0 for the mixture of PZS and 3kPZS indicates the olfactory response of 3kPZS is the same magnitude before and during exposure of PZS, suggesting PZS does not influence the 3kPZS olfactory response. A value of 0.5 indicates a 50% reduction of the 3kPZS olfactory response during exposure to PZS.

This experiment was repeated and expanded across a range of concentrations for adapting solution of PZS or 3kPZS (10^{-10} M to 10^{-6} M) with the test stimulus concentration held constant

(10^{-6} M for 3kPZS or PZS and 10^{-5} M $_{L}$-arginine). The ratio of the EOG amplitudes was calculated in a similar manner as defined in Equation 2.

Two-choice maze behavioral assay

Since previous experiments have shown the male sex pheromone biases the preference of female lampreys in a maze (Siefkes et al. 2005), the preference of females to mixtures of 3kPZS and PZS was measured in a two-choice maze to gauge the potential of PZS to disrupt this type of behavior. The preferences of ovulated female sea lampreys to the odorants were evaluated in June and July 2013 – 2014 following established procedures (Li et al. 2017b; Li et al. 2002). To begin, a single lamprey was introduced to the furthest point downstream in the maze in an acclimation cage. After 5 min, the lamprey was released and the cumulative amount of time the lamprey spent in each channel was recorded for 10 min to assess side bias before odorant application. The test odorant (3kPZS, PZS, or 3kPZS mixed with PZS) was randomly applied to one channel and vehicle (methanol) to the other at constant rates of 200 ± 5 mL min^{-1} for 15 min. No behavior was recorded for the first 5 min of odorant application to allow sufficient time for the odorant to flow through the length maze, but the cumulative time spent in each channel was recorded for the final 10 min. The maze was flushed with water for 10 min before the start of the next experiment to remove any remaining odorant. A 10-minute flushing period was deemed to be sufficient time in previous experiments (Li et al. 2002) and confirmed with a rhodamine dye test. The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined in Equation 3: Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The indices of preference were evaluated

using a Wilcoxon signed-rank test ($\alpha = 0.05$) to determine if the index of preference was different from zero. A significant positive value of the index of preference indicated attraction, a significant negative value indicated repulsion, and a value not different from zero indicated neutral. The trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10 s during the 10 min period before odorant application, as this is an indication of strong side bias or inactivity.

Field behavioral assay

In stream behavioral assays provided a more realistic assessment on the influence of PZS on the 3kPZS-induced long distance upstream movement, entry into an artificial nest, and retention of ovulated females (Johnson et al. 2009). In stream behavioral trials were conducted in July 2014 in a 45 m long stretch of the upper Ocqueoc River (Presque Isle County, MI). The stream trials characterize the influence of PZS on the behavioral movements of ovulated female sea lampreys to 3kPZS in a natural spawning environment. A barrier prevents sea lampreys from infesting the upper river, allowing the number of lamprey in the system and background pheromone concentrations to be controlled. The in stream assays followed previously described procedures with modification (Brant et al. 2016). A 23mm long half duplex passive integrated transponder (PIT) tag each with a unique identification number (Oregon RFID, Portland, OR, USA) was inserted into a latex sleeve and sutured to the mid-dorsal region of each ovulated female. Two unique colored floy tags were attached to the mid-dorsal region to aid in visual identification of each individual. Tagged animals were held in aerated holding tanks with flow-through Lake Huron water for up to 12 h, transferred to release cages in stream at the field site, and allowed to acclimate for approximately 8 h.

The PIT antennas were arrayed at four locations to monitor the movement of each female equipped with a PIT tag: across the stream channel 3 m below the release cage to track downstream movement, across the stream channel 3 m above the release cage to track upstream movement, and square antennae (1 m^2) around each of the two odorant sources designed to mimic artificial nests (side-by-side, 1.5 m apart) to track nest entry. Antennae were wired to a multiplexor and data logger (Oregon RFID, Portland, OR, USA) to store the data during the trial that was subsequently uploaded to a personal digital assistant after each trial (Meazura model MEZ1000, Aceeca International Limited, Christchurch, New Zealand). Two release cages (0.25 m^3) were positioned in the center of the stream 45 m downstream from the odorant sources.

Stream temperature was recorded at the start and end of each trial with a digital thermometer. Test odorants were diluted with 15 L of river water in separate mixing bins on shore. Odorants were pumped (Masterflex 7553-70, Cole-Parmer, Vernon Hills, IL, USA) for 2 h into the center of each 1 m^2 odorant sources at constant rates of 167 ± 5 mL min^{-1}. After 30 min of pumping, females were released from cages and their behavior was observed for the remaining 90 min. The PIT antenna system tracked the movement of the fish and swim tracks were drawn based on visual observations. In control trials, 3kPZS (5 x 10^{-13} M) was applied to both odorant sources, replicating the concentration and plume of 3kPZS released by spermiated males on two nests (Xi et al. 2011). In experimental trials, the mixture of 3kPZS (5 x 10^{-13} M) and PZS (5 x 10^{-13} M) was applied to the treatment odorant source, while 3kPZS (5 x 10^{-13} M) was applied at the control odorant source. Experimental trials represented the introduction of a candidate pheromone antagonist (PZS) over part of the background 3kPZS plume. Treatments applied to each odorant sources were alternated each trial. To assure the correct molar concentration of the odorants, stream discharge was taken every three days or after precipitation

events using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, MD, USA).

The stream was sectioned with fixed transects to serve as a visual reference as the movement of each fish was tracked. The first 10 transects from the odorant source towards downstream were 1 m apart, and the remaining were spaced every 5 m thereafter until the release cages (Figure 2-4). Fish were visually observed and the swim track of each individual was recorded by hand based on the unique color combination of floy tags from the release cage upstream onto scaled maps. Swim tracks were later compiled onto a digital map. When the female entered the odorant source, the observer recorded the amount of time spent in the 1 m^2 area (retention in seconds). The cumulative amount of time each female spent in the treatment versus control odorant source was evaluated with a paired t test or Wilcoxon signed-rank test for the control and experimental trials.

To characterize the odorant distribution at the field site, rhodamine (Cole-Parmer Rhodamine Red, Vernon Hills, IL, USA) dye tests were conducted using previously validated methods (Johnson et al. 2009). In brief, rhodamine dye was applied to the center of one of the odorant sources at a constant rate of 167 ± 5 mL min^{-1} using a peristaltic pump for 30 min prior to sampling. The rhodamine concentration was sampled at the 10 points along each of the transects during active administration of dye for 90 min using Cyclops 7 (Model number 2100-000, Serial number 2101754, Turner Designs, Sunnyvale, CA, USA) affixed with Data Bank (Model number 2900-005, Serial number 2900211, Turner Designs). The river was thoroughly flushed with water for 1 h, the absence of dye was confirmed with rhodamine sampling, and then the dye test was repeated from the other odorant source. The values were compiled to determine the relative concentration of the odorant plume from the odorant sources to the release cages.

Statistical analysis of PIT data

The response variables (downstream movement, upstream movement, odorant source entry) across treatments were analyzed in R (R version 2.15.1, Vienna, Austria) using a logistic regression with a binomial distribution following established methods (Brant et al. 2016; Johnson et al. 2009). Briefly, the binary response variables examined were 1) the distribution of released females that swam 3 m or more downstream from the release cage and stayed downstream of the release cages for the duration of the trial, 2) the distribution of released females that swam 3 m or more upstream from release cages and stayed upstream of the release cages for the duration of the trial, 3) of the females that moved upstream entered an odorant source, the distribution that initially entered the treatment odorant source.

RESULTS

Olfactory responses

3kPZS and PZS were potent odorants that stimulated the adult sea lamprey olfactory epithelium in a concentration-dependent manner as measured by electro-olfactogram (EOG) assays (Figure 2-1). 3kPZS and PZS were detected at low concentrations (10^{-10} M).

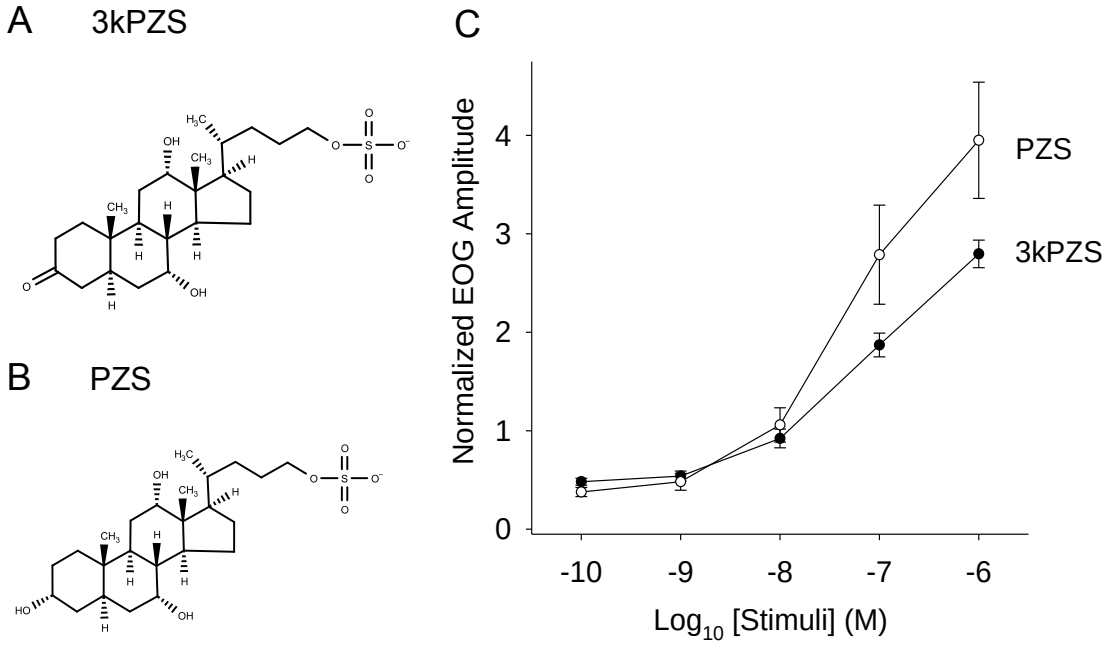

Figure 2-1. The chemical structures of the stimuli and the semi-logarithmic plot of the 3kPZS and PZS electro-olfactogram (EOG) concentration response curves. Chemical structures of (A) 3kPZS (3-keto petromyzonol sulfate; 7α,12α, 24-trihydroxy-3-one-5α-cholan-24-sulfate) and (B) PZS (petromyzonol sulfate; 3α, 7α,12α, 24-tetrahydroxy-5α-cholan-24-sulfate). (C) Semi-logarithmic plot of EOG concentration-response relationship shows 3kPZS (•) and PZS (○) were stimulatory to the adult sea lamprey olfactory epithelium and were detected at low concentrations (10^{-10} M). Data are presented as the mean normalized EOG amplitude ± SEM (n = 5).

Exposing the adult sea lamprey olfactory epithelium to 10^{-6} M PZS reduced the olfactory response to 10^{-6} M 3kPZS more than 10^{-6} M PZS reduced the olfactory response to 10^{-5} M $_{L}$-arginine (paired t-test, $p = 0.036$, $t = 5.158$, $df = 2$). The normalized EOG response of 10^{-6} M PZS mixed with 10^{-6} M 3kPZS after exposing the olfactory epithelium for 2 min with 10^{-6} M PZS was 0.08 ± 0.03 (mean ± SEM, $n = 4$), indicating a 92% reduction of the 3kPZS olfactory response. In contrast, the normalized EOG response of 10^{-6} M PZS mixed with 10^{-5} M $_{L}$-arginine was 0.45 ± 0.09 ($n = 4$).

Exposing the adult sea lamprey olfactory epithelium to 10^{-10} M to 10^{-6} M PZS reduced the EOG responses of 10^{-6} M 3kPZS of adult sea lampreys in a concentration-dependent manner, but not 10^{-5} M $_{L}$-arginine (Figure 2-2A). Exposure to 10^{-10} M to 10^{-6} M 3kPZS reduced the EOG responses of 10^{-6} PZS when 3kPZS was applied at higher concentrations (10^{-7} M and 10^{-6} M) (Figure 2-2B).

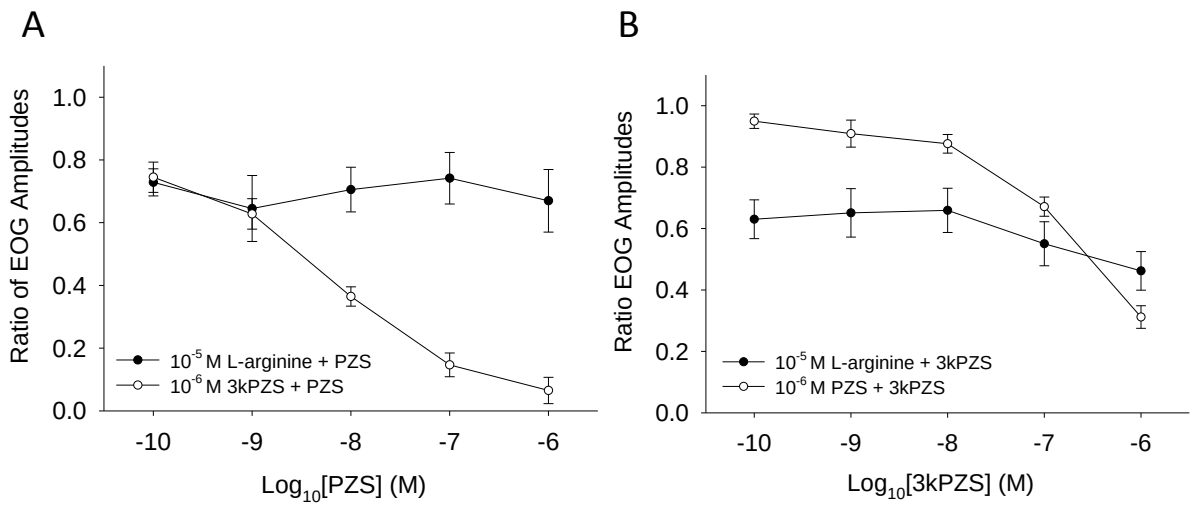

Figure 2-2. Electro-olfactogram (EOG) responses of (A) 3kPZS after exposure to PZS and (B) PZS after exposure to 3kPZS. Exposure to PZS (10^{-10} M to 10^{-6} M) reduced the EOG responses of 10^{-6} M 3kPZS of adult sea lamprey in a concentration-dependent manner, but not 10^{-5} M $_{L}$-arginine. Exposure to 3kPZS (10^{-10} M to 10^{-6} M) reduced the EOG responses of 10^{-6} M PZS at high concentrations. Mean EOG response ± SEM is the ratio of the EOG amplitude of $_{L}$-arginine 10^{-5} M, 3kPZS 10^{-6} M, or PZS 10^{-6} M during versus before exposure to increasing concentrations of (A) PZS or (B) 3kPZS (n = 6).

Disruption of behavioral preference response for 3kPZS

In the two-choice maze behavioral assays, ovulated female sea lampreys were attracted to 10^{-12} M 3kPZS (0.369 ± 0.059, mean index of preference ± SEM, n = 41, Wilcoxon signed-rank test, $p < 0.001$; Figure 2-3). The preference for 3kPZS was nullified with the addition of PZS. The mixture of 10^{-12} M 3kPZS and 10^{-12} M PZS or 10^{-11} M PZS was behaviorally neutral (−0.046 ± 0.128, n = 15, p = 0.934 and 0.034 ± 0.104, n = 15, p = 0.934, respectively; Figure 2-3). The mixture of 10^{-12} M 3kPZS with 10^{-10} M PZS was repulsive (−0.491 ± 0.086, n = 15, $p < 0.001$). Females were repulsed from 10^{-12} M to 10^{-10} M PZS (−0.271 ± 0.101, n = 17, p = 0.020, −0.626 ± 0.053, n = 15, $p < 0.001$, and −0.432 ± 0.066, n = 15, $p < 0.001$, respectively; Figure 2-3).

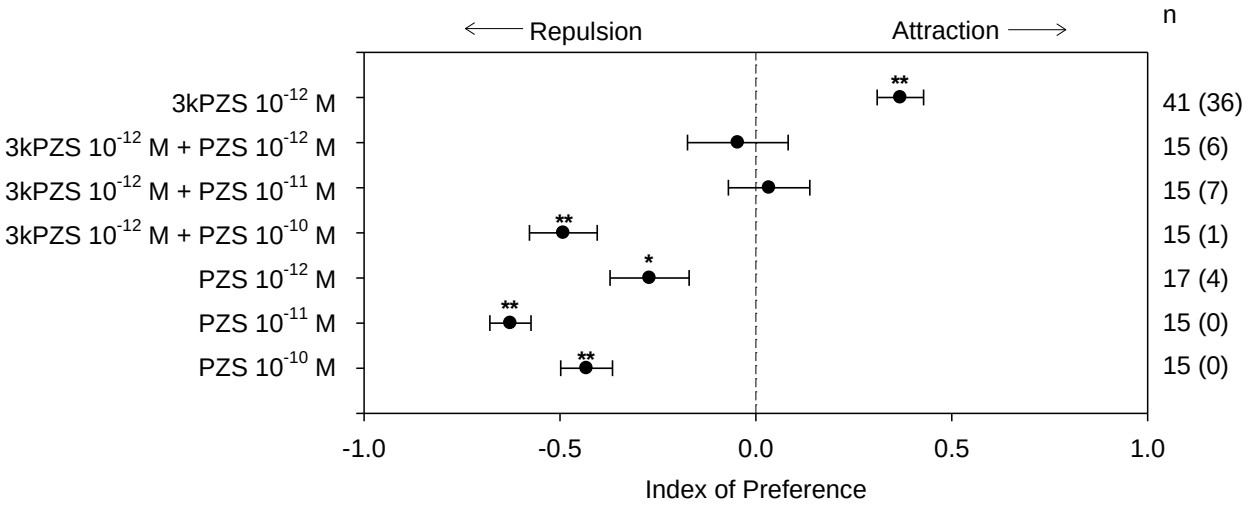

Figure 2-3. Ovulated female sea lamprey behavioral responses in a two-choice maze indicating females had a preference of 3kPZS that was disrupted with the addition of PZS. The time the lamprey spent in the control (Bc) and experimental (Be) channel of the maze before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined by Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. Data are presented as the mean index of preference ± SEM (horizontal bar). A positive index value indicates attraction and a negative index value indicates repulsion. The index value was evaluated using a Wilcoxon signed-rank test. The sample size, n, is the number outside of parentheses with the number in the parentheses indicating the number of test subjects spending more time in the treatment side. ∗, $p < 0.05$. ∗∗, $p < 0.001$.

Disruption of 3kPZS-induced upstream movement, nest entry, and nest retention in a field experiment

PZS treatment affected the proportion of ovulated females that swam upstream (logistic regression: $\chi^2 = 4.54$, $df = 1$, $p = 0.03$) and entered the odorant sources ($\chi^2 = 6.20$, $df = 1$, $p = 0.01$) based on the PIT telemetry data form the field experiment (Table 2-1). The experimental trials with a mixture of 3kPZS and PZS (1:1, 5×10^{-13} M) versus the adjacent nest of 3kPZS (5×10^{-13} M) alone reduced upstream movement compared to the control trials with 3kPZS (5×10^{-13} M) applied to both odorant sources. Of the females that swam upstream and entered an odorant source in the control trials, approximately equal proportions of ovulated females entered each of the odorant sources (52% and 48%, respectively). In the experimental trials, a reduced proportion of ovulated females entered the treatment odorant source containing the mixture of 3kPZS and PZS. Of the females that swam upstream and entered an odorant source in the experimental trials, only 19% entered the source with the mixture of 3kPZS and PZS.

Application of PZS also reduced retention of each female in the 1 m^2 odorant source compared to the adjacent odorant source of 3kPZS alone in the experimental trials (Wilcoxon signed-rank test, $p = 0.002$, $Z = 3.039$, $n = 28$) (retention in treatment odorant source 3kPZS & PZS in seconds: 61.9 s ± 25.9, mean ± SEM; retention in control odorant source 3kPZS: 148.5 s ± 36.3). In the control trials, retention in the odorant source did not differ between the two sources (paired t test, $p = 0.968$, $t = 0.041$, $df = 18$) (retention treatment odorant source 3kPZS: 115.4 s ± 47.5; retention in control odorant source 3kPZS: 114.3 s ± 54.6).

Table 2-1. Behavioral responses of ovulated female sea lampreys exposed to 3kPZS or 3kPZS mixed with PZS in a 45 m section of the upper Ocqueoc River (Presque Isle County, MI). In control trials (first row), 3kPZS was applied to both odorant sources (5×10^{-13} M, final in stream concentration) and in experimental trials (second row), 3kPZS (5×10^{-13} M) mixed with PZS (5×10^{-13} M) was applied to the treatment odorant source and 3kPZS (5×10^{-13} M) was applied to the control odorant source. Downstream movement: the percentage (the count) of the released ovulated females that moved at least 3 m downstream of the release cages. Upstream movement: the percentage (the count) of the released females that moved at least 3 m upstream of the release cages. Enter Odorant Source: the percentage (the count) of the females that moved upstream that then entered the 1 m^2 odorant source. Movement responses were evaluated with a logistic regression with a binomial distribution. Responses within a column that share a letter are not significantly different ($\alpha = 0.05$).

Odorant						Enter Odorant Source	
Treatment	**Control**	**Trials**	**Released**	**Down**	**Up**	**Treatment**	**Control**
3kPZS	3kPZS	6	54	7% (4) A	56% (30) A	52% (11) A	48% (10) A
3kPZS & PZS	3kPZS	20	149	6% (10) A	39% (70) B	19% (6) B	81% (25) B
			χ^2	0.23	4.54	6.20	
			df	1	1	1	
			P-value	0.63	0.03	0.01	

Swim tracks overlain onto the odorant plume map illustrate that application of PZS reduced the preference of ovulated females for 3kPZS (Figure 2-4 and Figure 2-5). PZS reduced the entry into the odorant source. Stream temperatures in the upper Ocqueoc River showed minimal variation throughout the field experiments from 25 June to 29 July 2014 (20.03 ± 0.75 °C, overall mean ± SEM, n = 26). The mean temperature for the control (3kPZS vs. 3kPZS) and experimental trials (3kPZS and PZS vs. 3kPZS) were not significantly different from each other (control: 19.14 ± 0.95 °C, n = 6, and experimental: 20.30 ± 0.36 °C, n = 20; t test, p = 0.180, t = -1.381, *df* = 24).

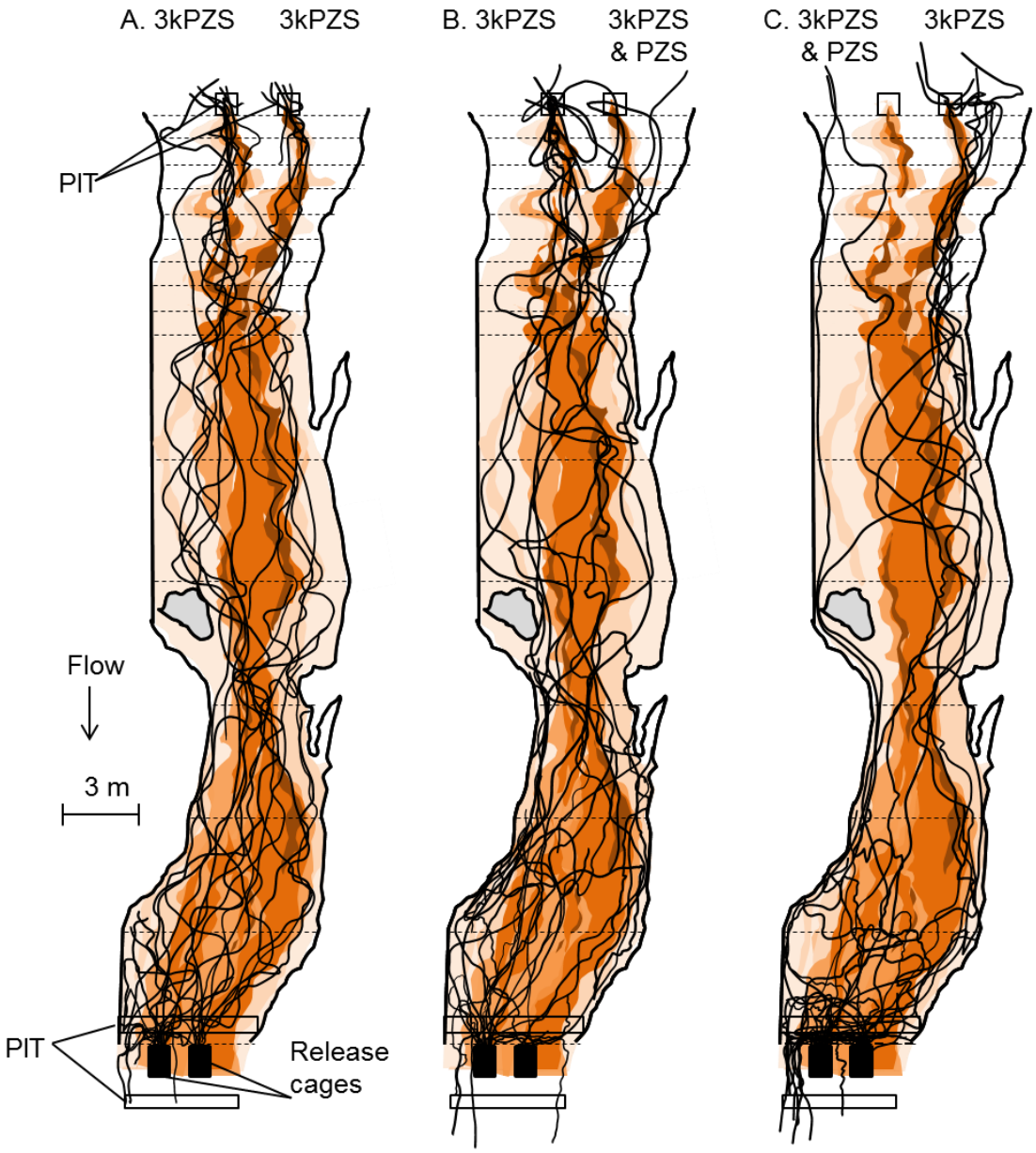

Figure 2-4. Representative swim track movements of ovulated females in a 45 m section of upper Ocqueoc River (Presque Isle County, MI, USA). Females were exposed to (A) 3kPZS (5×10^{-13} M, final in stream concentration) in both odorant sources (control, n = 54) or to (B, C) 3kPZS (5×10^{-13} M) versus a mixture of 3kPZS (5×10^{-13} M) and PZS (5×10^{-13} M) in the adjacent odorant source (experimental, n = 149). The movement of females from the release cages (solid black boxes downstream) was monitored with 4 passive integrated transponder (PIT) antennas (locations shown) and visual observations. Each line indicates the swim track of an individual female mapped. Rhodamine dye was administered in the center of the odorant source and sampled at ten points along each transect (transecting dashed lines) to map the odorant plume. The shading of the odorant plume corresponds to the relative molar concentration of the odorant. Darker orange/brown indicates a higher relative odorant concentration.

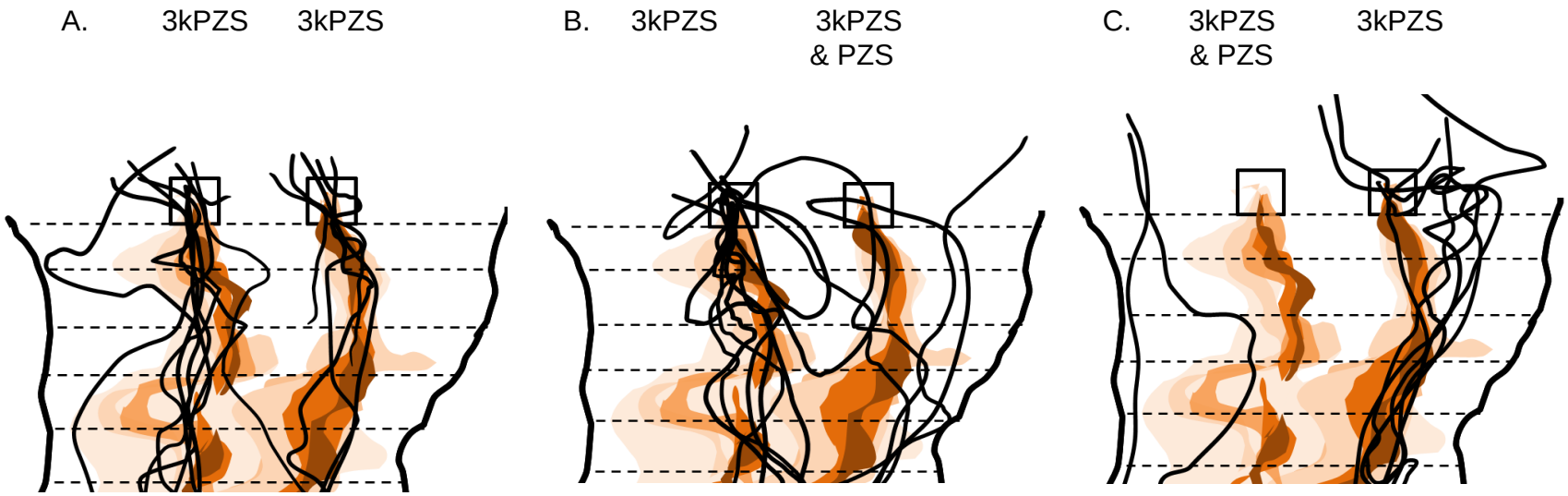

Figure 2-5. Representative swim track movements of ovulated females within 5 m of the odorant sources in upper Ocqueoc River (Presque Isle County, MI). This figure zooms in on Figure 2-4 to highlight the movements of females approaching the odorant sources mimicking artificial nests from 5 m below the odorant sources. Females were exposed to (A) 3kPZS (5×10^{-13} M, final in stream concentration) in both odorant sources (control, n = 54) or to (B, C) 3kPZS (5×10^{-13} M) versus a mixture of 3kPZS (5×10^{-13} M) and PZS (5×10^{-13} M) in the adjacent odorant source (experimental, n = 149). The movement of females was monitored with passive integrated transponder (PIT) antennas around each of the odorant sources (1 m^2) and visual observations. Each line indicates the swim track of an individual female mapped. Rhodamine dye was administered in the center of the odorant source and sampled at ten points along each transect (transecting dashed lines) to map the odorant plume. The shading of the odorant plume corresponds to the relative molar concentration of the odorant. Darker orange/brown indicates a higher relative odorant concentration.

DISCUSSION

Ovulated females rely on the detection of 3kPZS to locate a mature male (Li et al. 2002). The electrophysiology and behavioral data presented here replicate the previous studies of the pheromonal function of 3kPZS (Johnson et al. 2009; Li et al. 2002; Siefkes and Li 2004; Siefkes et al. 2005), and establish that PZS disrupts the olfactory detection to 3kPZS and consequently reduces the attraction of ovulated females to 3kPZS. Data from EOG recordings indicate PZS is an effective 3kPZS olfactory inhibitor. Data from maze and field behavioral assays indicate PZS is also a behavioral antagonist of 3kPZS. This study supports our hypothesis that disrupting the olfactory responses to 3kPZS with PZS alters 3kPZS-mediated behaviors in the field, and indicates that the use PZS might offer a new sea lamprey control tactic.

Based on electrophysiology recordings, we found PZS over a wide range of concentrations is a potent inhibitor of the 3kPZS olfactory responses. The adult sea lamprey olfactory epithelium exhibited reduced responses to 3kPZS after exposure to PZS. The influence of PZS on the olfactory detection of 3kPZS was dose-dependent and non-reciprocal. A lower concentration of PZS (10^{-10} M) reduced the olfactory response of 3kPZS by 25%. Notably, when the naris was exposed to 10^{-6} M PZS, the 3kPZS olfactory response was reduced by 92% compared to the 3kPZS response before PZS exposure. Exposing the olfactory epithelium to increasing concentrations of PZS reduced the olfactory responses to 3kPZS more than exposure to increasing concentrations of 3kPZS reduced the olfactory responses to PZS. We speculate the pattern of non-reciprocal influence of 3kPZS and PZS suggests there are at least two different receptor types for PZS and 3kPZS, or PZS has a higher affinity than 3kPZS at the olfactory receptor level; however, this warrants further investigation. Our findings are supported by

Siefkes and Li (2004) who found PZS reduced the olfactory response of 3kPZS more than 3kPZS reduced PZS in cross-adaptation experiments. 3kPZS as the adapting stimulus partially adapted PZS by approximately 40%, whereas PZS as the adapting stimulus partially adapted 3kPZS by about 60% (Siefkes and Li 2004). Since PZS diminished the olfactory response of 3kPZS, the relative concentration of PZS that interacted with 3kPZS EOG responses guided the design of the behavioral assays.

Behavioral observations in the maze and field experiment provide further evidence that PZS not only reduces the 3kPZS olfactory response but also disrupts the attraction to 3kPZS. Maze results indicate that PZS can bias the preferences of females, which could limit their ability to locate a nesting male and successfully spawn. PZS reduced the behavioral preference for 3kPZS in the maze in a concentration-dependent manner, which is similar to the results observed in the EOG responses. Ovulated females preferred the channel scented with 3kPZS. Application of PZS at the same concentration of 3kPZS (10^{-12} M) neutralized the preference of ovulated females for 3kPZS. This is consistent with the EOG results where PZS at an equal molar concentration reduced the response to 3kPZS close to baseline. When the concentration of PZS was increased relative to 3kPZS (100:1, PZS: 3kPZS) in the maze, ovulated females avoided the mixture. Experiments in stream conditions under the physical contexts associated with a natural environment provide a more realistic understanding of how PZS disrupts female upstream movement over a longer distance (45 m) in addition to the attraction to nests (Johnson and Li 2010). When only 3kPZS was applied at the odorant source in a spawning stream, ovulated females swam upstream and located the odorant source. Our findings show when PZS is applied with 3kPZS, PZS disrupts the 3kPZS-induced upstream movement, entry, and retention in a nest baited with 3kPZS. Likewise, some insect pheromone antagonists can suppress the ability of

insects to track pheromone plumes, resulting in frequent lateral casting and in some instances regression downward (Bau et al. 1999; Riba et al. 2005).

We suspect that applying sea lamprey pheromone antagonists will be more socially acceptable than other sea lamprey control efforts (*i.e.*, lampricide) because the pheromone antagonist described here targets sea lamprey olfactory detection and behavioral response to 3kPZS. When 3kPZS was applied at a management scale level, no adverse non-target effects were reported (Johnson et al. 2013) and we predict a similar outcome with application of a 3kPZS antagonist. In addition, PZS is already present in the spawning rivers as a natural product released by larval and adult sea lampreys.

Larval sea lamprey synthesize PZS in the liver, store it in the gall bladder, and then excrete it through the intestine with feces (Haslewood and Tökés 1969). Larvae release a 3kPZS: PZS ratio of approximately 0.1: 1 (Brant et al. 2015a). Adult male sea lampreys also produce PZS in the liver, transport it to gills where PZS is converted to 3kPZS, and then release 3kPZS from the gills (Brant et al. 2013). Mature males release a 3kPZS: PZS ratio of 1000: 1 (Brant et al. 2013). Inside the body, mature males have 10 to 100-fold more PZS relative to 3kPZS depending on the specific tissue (Brant et al. 2013). We speculate as semelparous sea lampreys approach the end of their reproductive life, the body of mature males begins to break down and metabolites found within the body cavity could be passively released into the water. These metabolites could serve as a cue to influence the movement of conspecific females. Indeed, the odors produced by the putrefying carcasses of sea lampreys repulse other sea lamprey (Wagner et al. 2011). Ovulated females may use PZS as an indicator of absence of suitable mates. We speculate if high concentrations of PZS relative to 3kPZS signal the presence of larvae or poor quality males approaching death, the discovery of PZS as an antagonist of

3kPZS may be an exploitation of a natural reaction of ovulated females and hence may be highly effective when implemented. However, this needs to be further tested in the future.

Implications for Management

The discovery that sea lamprey behavior can be exploited with a naturally occurring pheromone antagonist opens up new possibilities for targeted control. We envision the deployment of pheromone antagonists to strategic locations above spawning grounds to disrupt mating as one component of the integrated sea lamprey control tool kit. Pheromone behavioral antagonists should be easy to apply and versatile because they are not limited by the presence of infrastructure like other control efforts (*i.e.*, traps and barriers). Mass trapping requires traps to be built, deployed, baited, and checked, whereas mating disruption requires only the application of the disrupting agent (El-Sayed et al. 2006). Application of sea lamprey pheromone behavioral antagonists to streams would likely be less technical than lampricide. The concentration range in which the lampricide kills lampreys but not other fishes is narrow (Hubert 2003). We found PZS over a wide range of concentrations reduces the olfactory and behavioral response to a single concentration of 3kPZS with different levels of effectiveness. Given that the PZS works over a broad concentration range and the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is effective only at a narrow range, we suspect pheromone antagonist application would be less technical than lampricide treatment. PZS can also be embedded in dissolvable polymers designed to release an antagonist when stream temperatures are suitable for sea lamprey spawning (15 – 22 °C) (Applegate 1950) to quickly render the spawning ground unsuitable for sea lamprey mating, thus alleviating the need for mechanical pumps to administer PZS (Hanson and Wagner 2012). A cost-effective, environmentally benign polymer-based emitter has already been developed for

deployment of the sea lamprey pheromone 3kPZS and its utility confirmed (Hanson and Wagner 2012).

Aside of mating disruption, pheromone antagonists may be used to re-distribute sea lamprey. Pheromone antagonists may offer a cost effective control tool to 1) Re-direct adult lampreys away from large rivers where lampricide application are prohibitively expensive (Siefkes et al. 2013); 2) Re-direct adult lampreys into areas with poor habitat that would reduce fitness; and 3) Enhance selective fish passages to block sea lamprey upstream migration while allowing mobility valued species currently obstructed by sea lamprey barriers (McLaughlin et al. 2013). The continued coordination of numerous tactics within an integrated management plan, with behavioral antagonists as one component, will likely enhance the efficacy and selectivity of invasive sea lamprey control.

Given most fish rely extensively on chemical signals that mediate behaviors (Sorensen and Wisenden 2015), research on manipulating these signals is broadly relevant to species management. In the present study, we discuss the use of a pheromone behavioral antagonist targeted at the invasive sea lamprey. Identification and application of pheromone behavioral antagonists for Asian carp (bighead carp *Hypophthalmichthys nobilis*, black carp *Mylopharyngodon piceus*, grass carp *Ctenopharyngodon idelle*, and silver carp *Hypophthalmichthys molitrix*) and round goby (*Neogobius melanostomus*), which are highly successful invaders that use pheromones, may provide additional management tactics (Sorensen and Stacey 2004; Sorensen and Wisenden 2015). Applying conspecific chemical cues may provide tools for the restoration of imperiled species as well. For example, the American eel (*Anguilla rostrata*) has declined to less than 1% of its peak abundance and applying conspecific chemical cues may be used to guide migrations into desirable habitats (Galbraith et al. 2017).

In summary, we identified the first vertebrate pheromone antagonist that nullifies behavioral responses to a male sex pheromone in a natural environment. PZS treatment effectively reduces the 3kPZS olfactory responses and disrupts the 3kPZS behavioral responses of female sea lampreys in a maze and field assay. Our results represent a step forward in utilizing pheromone antagonists as tools for controlling the invasive sea lampreys in the Great Lakes.

ACKNOWLEDGMENTS

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CHAPTER 3

A SYNTHETIC ANTAGONIST REDUCES OLFACTORY AND ALTERS FEMALE BEHAVIORAL RESPONSES TO A MALE SEX PHEROMONE IN SEA LAMPREY

ABSTRACT

The sea lamprey is a parasitic jawless fish that invaded the Laurentian Great Lakes and inflicted catastrophic ecological and economic damage. The mature males release a sex pheromone, 3-keto petromyzonol sulfate (3kPZS), that is indispensable for sea lamprey reproduction. Blocking sex pheromone detection in naris-plugged ovulated female sea lampreys effectively halts spawning. Targeting sex pheromones may offer potential tools to enhance the effectiveness and selectivity of the sea lamprey control program. Our goal is to find a way to disrupt the reproductive behaviors of sea lamprey mediated by 3kPZS. We reason a potent and selective sea lamprey pheromone antagonist that renders females anosmic to 3kPZS should reduce the 3kPZS olfactory and behavioral responses. In this study, we integrated electro-olfactogram (EOG) recordings and two-choice maze assays to assess if any of the eight polysulfated petromyzonol candidate antagonists reduce the 3kPZS olfactory or behavioral responses of ovulated females. We then conducted field behavioral assays to determine if the most effective antagonist from the maze assays altered the upstream movement and reproductive behaviors of ovulated females induced by 3kPZS in a natural spawning environment. Exposing the adult sea lamprey naris to 10^{-6} M petromyzonol-3,7,12,24-tetrasulfate (3sPZS) reduced the olfactory response of 10^{-6} M 3kPZS by 59%. In a two-choice maze, ovulated females preferred the channel with 10^{-12} M 3kPZS and avoided the channel with a mixture 3kPZS and 3sPZS (1:1, each at 10^{-12} M). Spermiated males showed no behavioral preference for the mixture of 3kPZS and 3sPZS. In a field experiment, 3sPZS when applied with 3kPZS (1:1, each at 5×10^{-13} M) at an artificial nest reduced upstream movement, entry, and retention of ovulated females compared to application of 3kPZS alone. We conclude that 3sPZS is an effective antagonist that alters the

olfactory and behavioral response to 3kPZS. 3sPZS shows the potential to suppress sea lamprey reproduction to a level that will result in reduced reproductive output, especially when implemented together with other adult control initiatives.

INTRODUCTION

Aquatic invasive species can have profound negative impacts on the ecosystem and require control efforts to mitigate their impacts (Mack et al. 2000). The establishment of invasive species combined with anthropogenic habitat degradation and exploitation of fishes has substantially and irreversibly altered the Laurentian Great Lakes. The sea lamprey is a parasitic jawless fish native to the Atlantic Ocean that invaded the upper Great Lakes in the 20th century, inflicted catastrophic ecological and economic damage, and contributed to the collapse of native fish communities and the Great Lakes fishery (Siefkes et al. 2013; Smith and Tibbles 1980). An effective, integrated sea lamprey control program currently in place suppresses sea lamprey abundance. Application of selective pesticides (lampricides) kill stream resident larvae, and barriers with associated traps minimize the spawning migrations and remove adults before spawning (Siefkes et al. 2013). While lampricide treatments are an effective control method, lampricide can have negative impacts on non-target amphibians and fishes (Boogaard et al. 2003; Dahl and McDonald 1980) and barriers can block the migration and dispersal of recreational and economically important fish (McLaughlin et al. 2013). Therefore, the existing sea lamprey control efforts come with an associated ecological cost and face increased scrutiny because of high implementation costs and waning social acceptance of chemical lampricides and barriers to fish migration. To stop or reduce the current sea lamprey control methods, however, would likely decimate the sustainable fishery and biodiversity in the Great Lakes (Christie and Goddard 2003; McLaughlin et al. 2013). Ongoing research seeks to improve the effectiveness, efficiency, and selectivity of sea lamprey control tactics (Siefkes 2017).

Pheromones are indispensable for sea lamprey migration and reproduction and may offer potential tools to enhance the effectiveness and selectivity of sea lamprey control (Buchinger et al. 2015). Sea lampreys rely on their well-developed olfactory organs to detect conspecific odors that mediate behavioral responses. Stream resident larvae release chemical cues that guide adults migrating from the lakes or oceans back into the spawning streams (Bjerselius et al. 2000; Teeter 1980). Once in the spawning streams, sexually mature males ascend to spawning grounds and release a multi-component sex pheromone that elicits long-distance upstream movement and spawning behaviors in females (Johnson et al. 2015a). The olfactory system detects the sex pheromone that includes 3-keto petromyzonol sulfate (3kPZS) as a main component, which lures females long distances upstream (Johnson et al. 2009; Li et al. 2002; Siefkes et al. 2005), and additional minor components (Brant et al. 2016; Johnson et al. 2014b; Li et al. 2017a; Li et al. 2017b). The sexually mature lampreys intermittently spawn for approximately one week and then die. This is a key window of time to focus on for targeted adult sea lamprey control because adults congregate in known spawning habitat. A new strategy targeting the adults' reliance on pheromones might provide an additional tactic to control invasive sea lamprey populations.

A series of studies have shown blocking the sex pheromone detection in ovulated female sea lampreys effectively halts reproduction. When 3kPZS is absent or the olfactory organ is physically occluded, ovulated females halt upstream movement (Johnson et al. 2006) and fail to interact and spawn with nesting mature males (Johnson 2005). In insects, pheromone antagonists that share a high degree of structural similarity to the major pheromone component can block pheromone detection and behavioral responses (Bau et al. 1999; Evenden et al. 1999; Giner et al. 2009; McDonough et al. 1996; Millar and Rice 1996; Riba et al. 2005; Solé et al. 2008). We reasoned that a sea lamprey pheromone antagonist that renders females anosmic to

3kPZS should have similar disruptive effects. Indeed, petromyzonol sulfate (PZS), a compound known to be released by larval sea lamprey (Haslewood and Tökés 1969; Polkinghorne et al. 2001; Sorensen et al. 2005) that is structurally very similar to 3kPZS, functions as an inhibitor of 3kPZS. PZS disrupts the 3kPZS-induced olfactory responses (Siefkes and Li 2004), reduces the behavioral preference of females for 3kPZS in a maze, and disrupts an array of 3kPZS-induced behavioral responses of females in a field experiment (Chapter 2).

In a previous study (Raschka et al. 2018), more than 12 million organic molecules were virtual screened and prioritized based on the degree of volumetric and chemical similarity of the candidate 3kPZS antagonists with 3kPZS. Candidate antagonists were further prioritized based on the degree of overlapping spatial arrangement of key 3kPZS functional groups. Using this approach, collaborators Dr. Leslie Kuhn and Dr. Sebastian Raschka (Michigan State University) identified petromyzonol-3,7,12,24-tetrasulfate (3sPZS) as a candidate 3kPZS antagonist. Given the structural similarity amongst 3kPZS, 3sPZS, and PZS, we adopted an explorative approach and varied the functional groups at three positions (C3, C7, and C12) with common functional groups of sea lamprey pheromones (sulfate, hydroxyl, and keto groups) to yield eight synthetic polysulfated petromyzonol compounds to be tested as candidate 3kPZS antagonists (Figure 3-1). We integrated electro-olfactogram (EOG) recordings, two-choice maze assays, and field behavioral assays to identify an effective candidate antagonist that reduced the olfactory responses and behavioral attraction to 3kPZS.

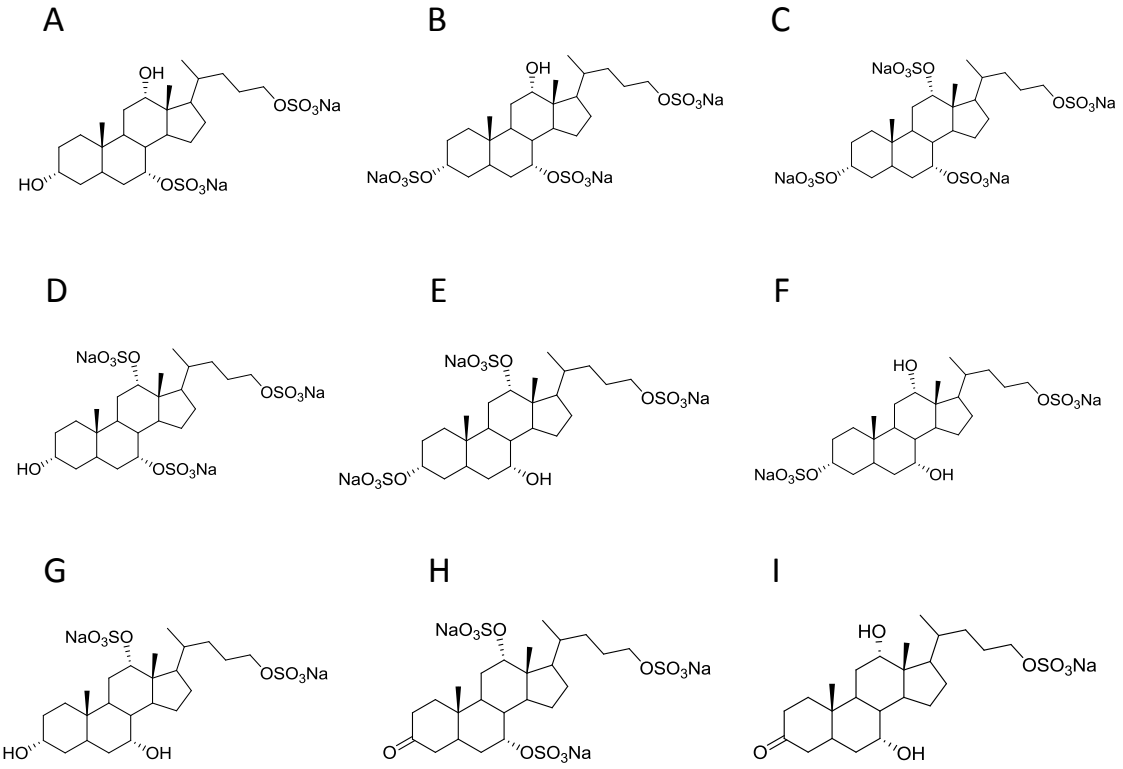

Figure 3-1. The chemical structures of the eight synthetic polysulfated petromyzonol compounds and a main component of the mature male sea lamprey sex pheromone. (A) Petromyzonol-7,24-disulfate disodium salt; (B) Petromyzonol-3,7,24-trisulfate trisodium salt; (C) Petromyzonol-3,7,12,24-tetrasulfate tetrasodium salt (3sPZS); (D) Petromyzonol-7,12,24-trisulfate trisodium salt; (E) Petromyzonol-3,12,24-trisulfate trisodium salt; (F) Petromyzonol-3,24-disulfate disodium salt; (G) Petromyzonol-12,24-disulfate disodium salt; (H) 3-keto-petromyzonol-7,12,24-trisulfate trisodium salt; (I) 3-keto-petromyzonol-24-sulfate sodium salt (3kPZS).

MATERIALS AND METHODS

Experimental animals

All procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (Animal usage form numbers: 03/12-063-00 and 12/14-223-00). Adult sea lampreys were captured in tributaries of the Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada, transported to the U.S. Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station (Millersburg, MI, USA), and held in 1000 L capacity aerated flow through tanks supplied with Lake Huron water maintained at 15–19 °C. Adult sea lampreys used for electro-olfactogram recordings were transported to the University Research Containment Facility at Michigan State University (East Lansing, MI) and held in 250 L flow through tanks supplied with aerated, chilled well water maintained at 7–9 °C. Mature adults used for behavioral assays were held in cages constructed with PVC and mesh located in the lower Ocqueoc River (Presque Isle County, MI) to allow for natural maturation and assessed daily for defined secondary sex characteristics (Johnson et al. 2015a).

Test stimuli

Eight candidate 3kPZS antagonists and 3kPZS (Figure 3-1) were purchased from Bridge Organics Co. (Vicksburg, MI, USA). Each compound (~10 mg each) was dissolved in the initial mobile phase of liquid chromatography, subjected to semi-preparative high performance liquid chromatography (HPLC), and further purified with successive size exclusion chromatography. The purity of all compounds was confirmed to be ≥ 95%. The purity of 3kPZS was determined

by comparison of HPLC results with that of standards. The purity of the candidate 3kPZS antagonists was evaluated by HPLC purity normalization procedures since standards are not available. Stock solutions at 10^{-3} M (methanol: deionized water, 1:1) were prepared for each compound and stored at -20 ^{o}C. All polysulfated petromyzonol compounds and 3kPZS were tested as sodium salts of the corresponding sulfate.

Electro-olfactogram (EOG) setup

Electro-olfactograms (EOGs), which measure the sum of the field potential generated upon activation of olfactory sensory neurons in the epithelia after odorant exposure (Ottoson 1955; Scott and Scott-Johnson 2002), were recorded to determine if the polysulfated petromyzonol compounds reduced the 3kPZS olfactory response in adult sea lampreys. The EOG setup followed previously described procedures (Li et al. 2017a) with modification. Sea lamprey were anesthetized via immersion in a 3-aminobenzoic acid ethyl ester bath (100 mg L^{-1}; MS222, Sigma-Aldrich, St. Louis, MO, USA), immobilized with an intra-muscular injection of gallamine triethiodide (3 mg kg^{-1} of body weight, in 0.9% saline; Sigma-Aldrich), and placed in a V-shaped plastic stand. Gills were continuously irrigated with aerated water containing 50 mg L^{-1} MS222 throughout the experiment. The olfactory lamellae were surgically exposed by removing the skin from the surface of the olfactory capsule. A small capillary tube delivered the stimuli and charcoal filtered water to the olfactory epithelium by gravity flow to prevent desiccation. The differential EOG response to each test stimulus was recorded using thin wall glass capillaries pulled to a tip to fabricate the borosilicate electrodes (filled with 0.04% agar in 0.9% saline) and connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, Hamden, CT, USA) in 3 M KCl. The recording electrode was placed

between two olfactory lamellae and adjusted to maximize the response to $_{L}$-arginine (a known odorant highly stimulatory to the sea lamprey olfactory epithelium) (Li and Sorensen 1997), while minimizing the response to the blank control (charcoal filtered water). The reference electrode was placed on the external skin near the naris. Electrical signals were amplified by a NeuroLog system (model NL102, Digitimer Ltd., Hertfordshire, England, United Kingdom), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized (Digidata 1440A Molecular Devices LLC, Sunnyvale, CA, USA), and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC).

Recording the change in 3kPZS EOG responses before and during exposure to candidate antagonists

The EOG response to the test stimulus (3kPZS or $_{L}$-arginine) was measured before and during exposure to one of the candidate antagonist polysulfated petromyzonol compounds and calculated the reduction of the 3kPZS olfactory response due to the candidate antagonist treatment. It was presumed that if the candidate antagonist and the stimulus (3kPZS or $_{L}$-arginine) share the same receptors, olfactory sensory neurons activated by an adapting solution of the antagonist will have less binding sites available to respond to a test stimulus, resulting in a decreased total EOG response to the stimuli (Caprio and Byrd 1984). The 10^{-5} M $_{L}$-arginine standard was first introduced to the olfactory epithelium for 4 s and the response was recorded as a reference of the electrical activity. EOG response magnitudes were measured in millivolts (mV) as the amplitude of the negative deflection in response to odorant stimulation. The olfactory epithelium was flushed with charcoal filtered water for 2 min, and the response to a blank control (charcoal filtered water) was recorded to confirm the absence of a response in the

charcoal filtered water supply. Then the responses to 10^{-6} M 3kPZS followed by 10^{-6} M candidate antagonist were recorded. The olfactory epithelium was exposed to 10^{-6} M candidate antagonist for 2 min and the responses to a mixture of 10^{-6} M candidate antagonist and 10^{-5} M $_{L}$-arginine followed by a mixture of 10^{-6} M candidate antagonist and 10^{-6} M 3kPZS were recorded while exposing the epithelium to 10^{-6} M candidate antagonist between recordings. The olfactory epithelium was flushed with charcoal filtered water for 2 min, and responses to the blank control, 10^{-5} M $_{L}$-arginine, 10^{-6} M 3kPZS, and 10^{-6} M candidate antagonist were recorded to ensure recovery of the olfactory epithelium.

The change in the olfactory response before and during exposure to the candidate antagonist for either $_{L}$-arginine or 3kPZS was calculated as the ratio between the blank corrected adapted response (during exposure) and the non-adapted response (before exposure), as defined in Equation 1: Ratio of EOG amplitudes = $(R_{adapt} - \text{blank}) / (R_{non\text{-}adapt} - \text{blank})$. For $_{L}$-arginine, R_{adapt} was the response of the mixture of 10^{-6} M candidate antagonist and 10^{-5} M $_{L}$-arginine relative to $R_{non\text{-}adapt}$, the response of 10^{-5} M $_{L}$-arginine. For 3kPZS, R_{adapt} was the response of the mixture of 10^{-6} M candidate antagonist and 10^{-6} M 3kPZS relative to $R_{non\text{-}adapt}$, the response of 10^{-6} M 3kPZS. A ratio of 1.0 for the mixture of a candidate antagonist and 3kPZS indicates the 3kPZS olfactory response is the same magnitude before and during exposure of the candidate antagonist, suggesting the candidate antagonist does not influence the 3kPZS olfactory response. A value of 0.5 indicates a 50% reduction of the 3kPZS olfactory response. Differences in the ratio of the EOG amplitudes for the mixture of the candidate antagonist and 3kPZS compared to the candidate antagonist and $_{L}$-arginine were evaluated with a two-tailed paired t-test.

EOG concentration response curves of top candidate 3kPZS antagonists

The olfactory concentration response curves were recorded for candidate antagonists that reduced the olfactory response to 10^{-6} M 3kPZS significantly more than 10^{-5} M $_{L}$-arginine. The 10^{-3} M stock solution of the candidate antagonist was serially diluted with charcoal filtered water to yield 10^{-12} M to 10^{-6} M solutions. The response to 10^{-5} M $_{L}$-arginine was recorded. Then increasing concentrations of the candidate antagonists starting at 10^{-12} M to 10^{-6} M were applied, recorded, flushed, and then repeated with the next higher concentration. Blank control and 10^{-5} M $_{L}$-arginine responses were measured after every two concentrations. The EOG response magnitudes were normalized relative to 10^{-5} M $_{L}$-arginine and corrected for blank response as defined in Equation 2: Normalized EOG Amplitude = $(R_t - R_b)/ (R_a - R_b)$, where R_t is the response magnitude to the test stimulus, R_b is the response magnitude to the blank, and R_a is the response magnitude to 10^{-5} M $_{L}$-arginine. The $_{L}$-arginine standard and blank control responses were comparable to previous studies (Li et al. 2017a; Li et al. 2017b). The detection threshold was defined as the lowest concentration where the candidate antagonist elicited a larger response than the blank control (paired t-test, one tailed).

Two-choice maze behavioral assay

A two-choice maze was used to determine if the candidate antagonists disrupted the behavioral preference to 3kPZS. The preferences of ovulated female and spermiated male sea lampreys to odorants were evaluated in June and July 2015 – 2017 following established procedures (Li et al. 2017b; Li et al. 2002). A single lamprey was introduced to the furthest point downstream in the maze in an acclimation cage. After 5 min, the lamprey was released and the cumulative amount of time the lamprey spent in each channel was recorded for 10 min to

assess side bias before odorant application. The test odorant (3kPZS 10^{-12} M, 3kPZS 10^{-12} M and the candidate antagonist 10^{-12} M, or candidate antagonist 10^{-12} M) was randomly applied to one channel and vehicle (methanol) to the other at constant rates of 200 ± 5 mL min^{-1} for 15 min. No behavior was recorded for the first 5 min of odorant application to allow sufficient time for the odorant to flow through the length maze, but the cumulative time spent in each channel was recorded for the final 10 min. The maze was flushed with water for 10 min before the start of the next experiment to remove any remaining odorant. The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined in Equation 3: Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$) to determine if the index of preference was different from zero. A significant positive value of the index of preference indicated attraction, a significant negative value indicated repulsion, and a value not different from zero indicated neutral. The trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10 s during the 10 min before odorant period, as this is an indication of strong side bias or inactivity.

Field behavioral assay

In stream behavioral assays characterized the influence of an odorant on the long distance upstream movement, attraction into the artificial nests baited with odorants, and retention at the nest (Johnson et al. 2009). In stream behavioral trials were conducted in July 2016 in a 45 m long stretch of the upper Ocqueoc River (Presque Isle County, MI) to characterize the influence 3sPZS, the most effective 3kPZS behavioral antagonist from the maze, on the 3kPZS-induced

behaviors of ovulated females in a natural spawning environment. A barrier prevents sea lampreys from infesting the upper river, allowing the number of lampreys in the system and background pheromone concentrations to be controlled. In stream assays followed previously described procedures with modification (Brant et al. 2016). A 23mm long half duplex passive integrated transponder (PIT) tag each with a unique identification number (Oregon RFID, Portland, OR, USA) was inserted into a latex sleeve and sutured to the mid-dorsal region of each ovulated female. Two unique colored floy tags were attached to the mid-dorsal region to aid in visual identification of each individual. Tagged animals were held in aerated holding tanks with flow-through Lake Huron water for up to 12 h, transferred to release cages in stream at the field site, and allowed to acclimate for approximately 8 h.

The PIT antennas were arrayed at four locations to monitor the movement of each female equipped with a PIT tag: across the stream channel 3 m below the release cage to track downstream movement, across the stream channel 3 m above the release cage to track upstream movement, and square antennae (1 m^2) around each of the two odorant sources designed to mimic artificial nests (side-by-side, 1.5m apart) to track nest entry (Figure 3-5 and Figure 3-6). Antennae were wired to a multiplexor and data logger (Oregon RFID, Portland, OR, USA) to store the data during the trial that was subsequently uploaded to a personal digital assistant after each trial (Meazura model MEZ1000, Aceeca International Limited, Christchurch, New Zealand). Four response variables measured with the PIT array downstream movement, upstream movement, entry into the treatment odorant source, and entry into the control odorant source. The two release cages (0.25 m^3) were positioned in the center of the stream 45 m downstream from the odorant sources.

Stream temperature was recorded at the start and end of each trial with a digital

thermometer. Test odorants were diluted with 15 L of river water in separate mixing bins on shore. Odorants were pumped (Masterflex 7553-70, Cole-Parmer, Vernon Hills, IL, USA) for 90 min into the center of each 1 m^2 odorant sources at constant rates of 167 ± 5 mL min^{-1}. After 15 min of pumping, females were released from cages and their behavior was observed for the remaining 75 min. The PIT antenna system tracked the movement of the fish and swim tracks were drawn based on visual observations. In control trials, 3kPZS (5 x 10^{-13} M) was applied to both odorant sources, replicating the concentration and plume of 3kPZS released by spermiated males on two nests (Xi et al. 2011). In experimental trials, the mixture of 3kPZS (5 x 10^{-13} M) and 3sPZS (5 x 10^{-13} M) was applied to the treatment odorant source, while 3kPZS (5 x 10^{-13} M) was applied at the control odorant source. Experimental trials represented the introduction of a candidate pheromone antagonist (3sPZS) over approximately half of the background 3kPZS plume. Treatments applied to each odorant sources were alternated each trial. To assure the correct molar concentration of the odorants, stream discharge was taken every three days or after precipitation events using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, MD, USA).

The stream was sectioned with fixed transects to serve as a visual reference as the movement of each fish was tracked. The first 10 transects from the odorant source towards downstream were 1 m apart, and the remaining were spaced every 5 m thereafter until the release cages (Figure 3-5). Fish were visually observed and the swim track of each individual was recorded by hand the based on the color combination of floy tags, which were later compiled onto a digital map. When the female entered the odorant source, the observer recorded the amount of time spent in the 1 m^2 area (retention in seconds). The cumulative amount of time

each female spent in the treatment versus control odorant source was evaluated with a paired t-test or Wilcoxon signed-rank test for the control and experimental trials.

To characterize the odorant distribution at the field site, rhodamine (Cole-Parmer Rhodamine Red, Vernon Hills, IL, USA) dye tests were conducted using previously established methods (Johnson et al. 2009). Rhodamine dye was applied to the center of one of the odorant sources at a constant rate of 167 ± 5 mL min^{-1} using a peristaltic pump for 15 min prior to sampling. The rhodamine concentration was sampled at the 10 points along each of the transects during active administration of dye for 75 min using Cyclops 7 (Model number 2100-000, Serial number 2101754, Turner Designs, Sunnyvale, CA, USA) affixed with Data Bank (Model number 2900-005, Serial number 2900211, Turner Designs). The river was thoroughly flushed with water for 1 h, the absence of dye was confirmed with rhodamine sampling, and then the dye test was repeated from the other odorant source. The values were compiled to determine the relative concentration of the odorant plume from the odorant sources to the release cages.

The response variables (downstream movement, upstream movement, odorant source entry) across treatments were analyzed in R (R version 2.15.1, Vienna, Austria) using a logistic regression with a binomial distribution following established methods (Brant et al. 2016; Johnson et al. 2009).

RESULTS

Selective reduction of 3kPZS olfactory response during exposure to polysulfated petromyzonol compounds

Exposing the adult sea lamprey naris to 10^{-6} M petromyzonol-3,24-disulfate or 10^{-6} M 3sPZS reduced the olfactory response of 10^{-6} M 3kPZS significantly more than the olfactory response to 10^{-5} M $_{L}$-arginine (paired t-test, p = 0.015, t = 4.093, *df* = 4; p = 0.014, t = 5.113, *df* = 3, respectively; Figure 3-2). Petomyzonol-3,24-disulfate reduced the olfactory response to 3kPZS 56% more than the response to $_{L}$-arginine. The normalized EOG response of 10^{-6} M petromyzonol-3,24-disulfate mixed with 10^{-6} M 3kPZS was 0.335 ± 0.048 (mean ± SEM, n = 5), indicating a 66% reduction of the 3kPZS olfactory response. The normalized EOG response of 10^{-6} M petromyzonol-3,24-disulfate mixed with 10^{-5} M $_{L}$-arginine was 0.891 ± 0.089 (n = 5). 3sPZS reduced the olfactory response to 3kPZS 18% more than the response to $_{L}$-arginine. The normalized EOG response of 10^{-6} M 3sPZS mixed with 10^{-6} M 3kPZS was 0.411 ± 0.052 (n = 4), indicating a 59% reduction of the 3kPZS olfactory response. In contrast, the normalized EOG response of 10^{-6} M 3sPZS mixed with 10^{-5} M $_{L}$-arginine was 0.586 ± 0.086 (n = 4). The remaining six polysulfated petromyzonol compounds did not reduce the 3kPZS olfactory response significantly more than the $_{L}$-arginine olfactory response (p > 0.05; Figure 3-2).

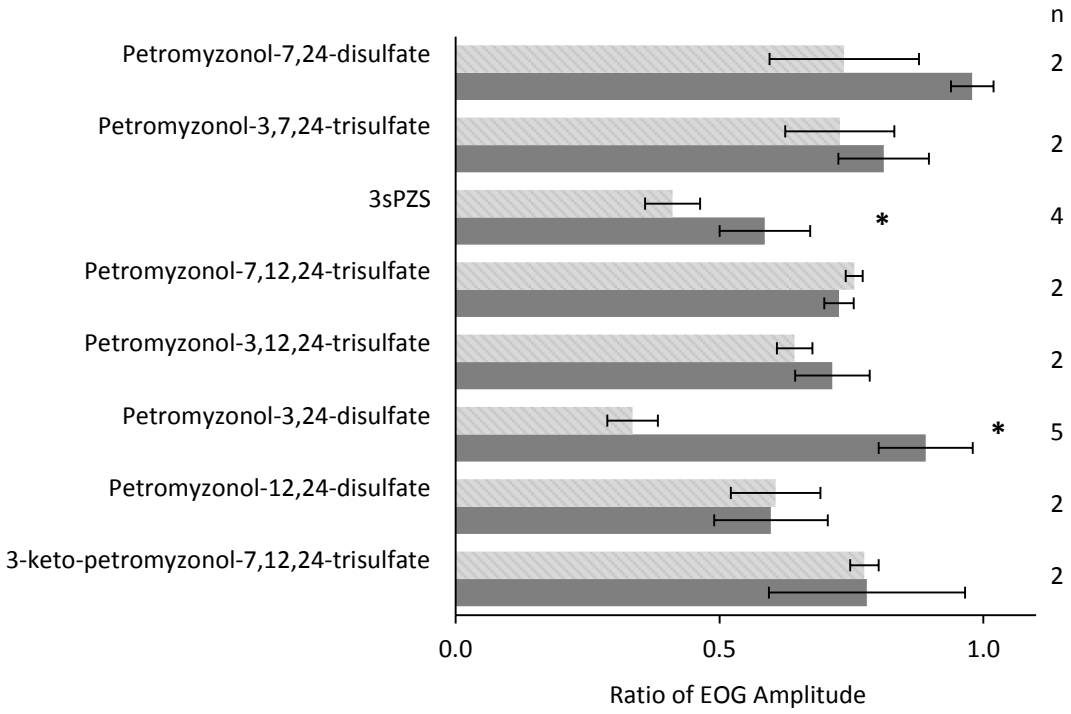

Figure 3-2. Ratio of the electro-olfactogram amplitude during and before exposure to 10^{-6} M of each of the eight polysulfated petromyzonol compounds of 10^{-6} M 3kPZS (light bars) and 10^{-5} M $_{L}$-arginine (dark bars). Adaptation of the olfactory epithelium with 3sPZS and petromyzonol-3,24-disulfate reduced the response magnitude to 10^{-6} M 3kPZS significantly more than 10^{-5} M $_{L}$-arginine. Data are presented as the mean ratio of EOG amplitude in adult sea lampreys normalized to the response of 10^{-6} M 3kPZS or 10^{-5} M $_{L}$-arginine. Error bars represent one SEM. Differences in the ratio of the EOG amplitudes of 3kPZS and $_{L}$-arginine for the indicated polysulfated petromyzonol compound were evaluated with a two-tailed paired t-test. $* p < 0.05$. The sample size is indicated ($n = 2-5$).

Olfactory potency of two 3kPZS candidate olfactory antagonists

3sPZS and petromyzonol-3,24-disulfate stimulated the adult sea lamprey olfactory epithelium in a concentration-dependent manner (Figure 3-3). The threshold of detection, which is the lowest concentration that elicited an olfactory response greater than the blank water control, for 3sPZS was 10^{-10} M (paired t-test, p = 0.027, t = -2.72, *df* = 4) and for petromyzonol-3,24-disulfate was less than 10^{-12} M (paired t-test, p = 0.017, t = -3.16, *df* = 4).

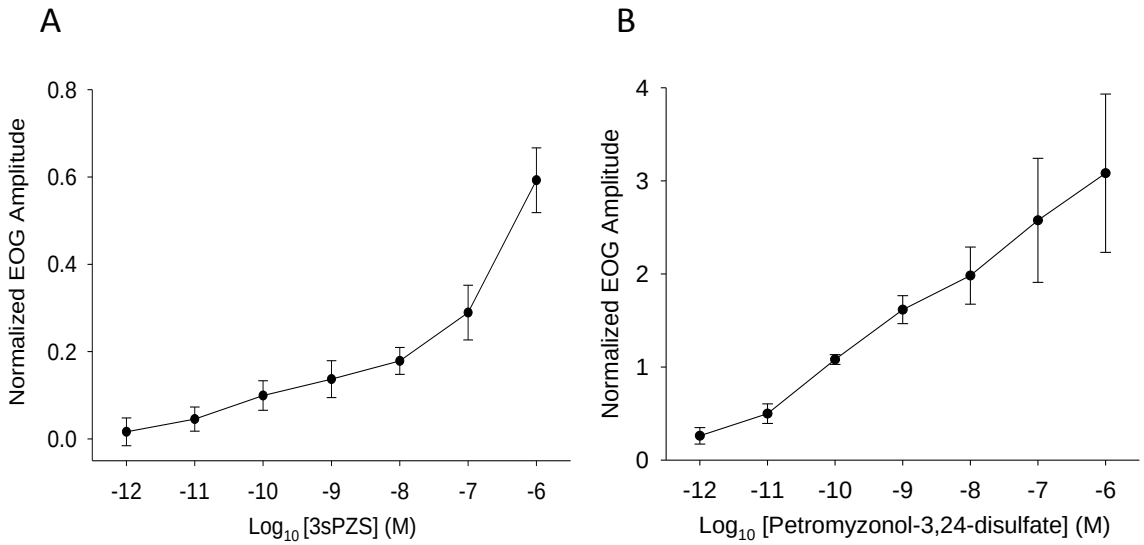

Figure 3-3. Semi-logarithmic plot of electro-olfactogram concentration response curve of two candidate 3kPZS antagonists. The plot shows that (A) 3sPZS and (B) petromyzonol-3,24-disulfate stimulated the adult sea lamprey olfactory epithelium in a concentration-dependent manner and have low detection thresholds. Data are presented as the mean normalized EOG amplitude ± SEM (n = 5) (See Equation 2 in Materials and Methods).

3sPZS reversed behavioral preference of females for 3kPZS in two-choice maze

In the two-choice maze behavioral assay used to assess preferences, ovulated female sea lampreys were attracted to 10^{-12} M 3kPZS (0.509 ± 0.054, mean index of preference ± SEM, n = 28,Wilcoxon signed-rank test, $p < 0.001$, Figure 3-4). Two polysulfated petromyzonol compounds when mixed with 3kPZS were significantly repulsive to ovulated females. The mixture of 10^{-12} M 3kPZS and 10^{-12} M petromyzonol-7,12,24-trisulfate repulsed ovulated females (−0.246 ± 0.095, n = 8, p = 0.039, Figure 3-4), as did the mixture of 10^{-12} M 3kPZS and 10^{-12} M 3sPZS (−0.457 ± 0.063, n = 22, $p < 0.001$, Figure 3-4). Similarly, 10^{-12} M 3sPZS alone repulsed ovulated females (−0.365 ± 0.064, n = 27, $p < 0.001$, Figure 3-4). Four of the polysulfated petromyzonol compounds when mixed with 10^{-12} M 3kPZS neutralized the attraction of ovulated females to 3kPZS: petromyzonol-3,7,24-trisulfate (−0.104 ± 0.136, n = 7, p = 0.813); petromyzonol-3,12,24-trisulfate (−0.036 ± 0.112, n = 8, p = 0.742); petromyzonol-12,24-disulfate (−0.062 ± 0.132, n = 7, p = 0.813); and 3-keto-petromyzonol-7,12,24-trisulfate (0.193 ± 0.189, n = 8, p = 0.383) (Figure 3-4).

Spermiated males were attracted to 10^{-12} M 3kPZS (0.309 ± 0.079, n = 10, p = 0.002). However, the mixture of 10^{-12} M 3kPZS and 10^{-12} M 3sPZS or 10^{-12} M 3sPZS alone did not induce a preference (−0.057 ± 0.146, n = 7, p = 0.813, and 0.151 ± 0.106, n = 7, p = 0.297, respectively, Figure 3-4).

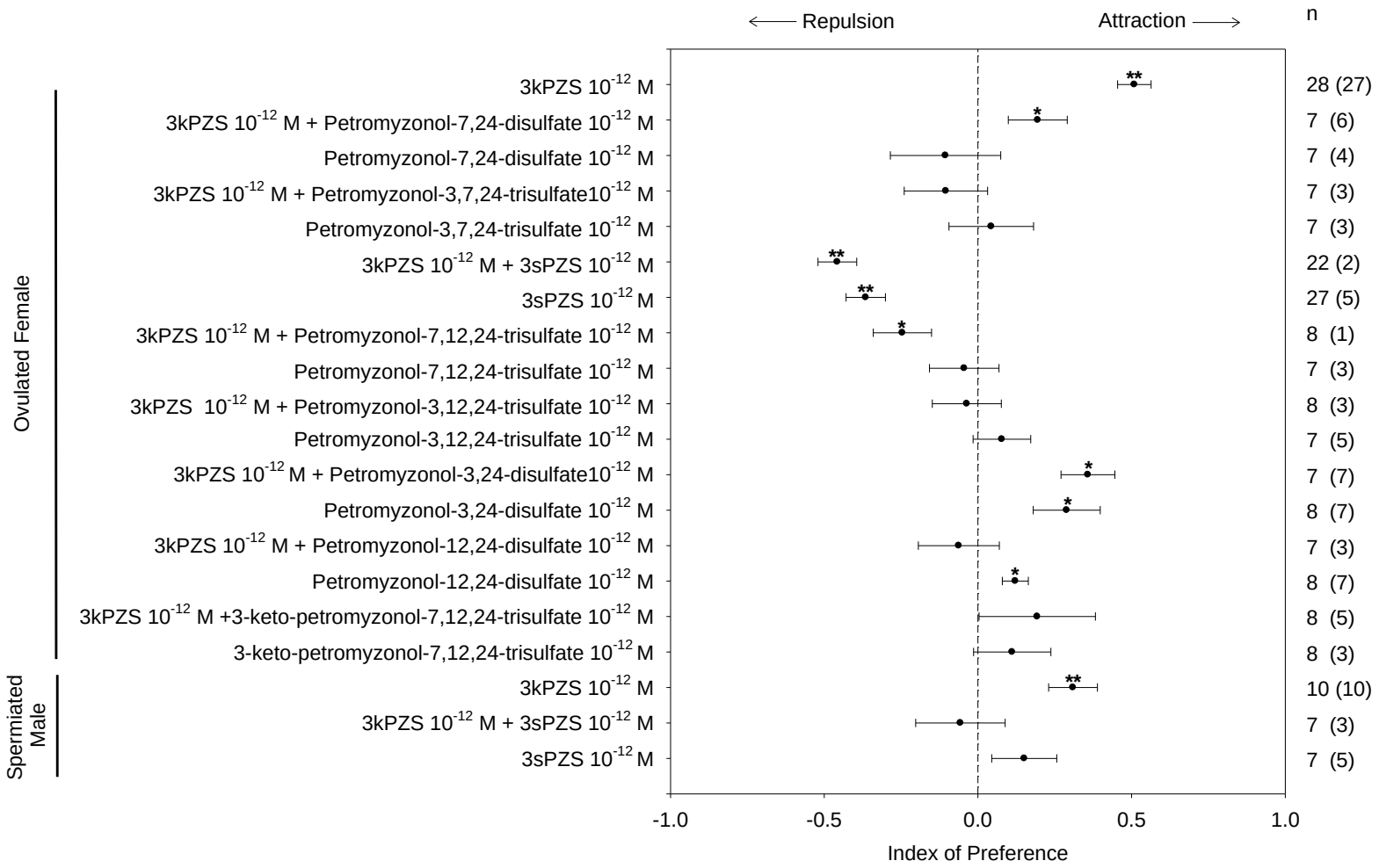

Figure 3-4. Adult sea lamprey behavioral responses in a two-choice maze to 3kPZS, the eight synthetic polysulfated petromyzonol compounds, and the mixtures 3kPZS with each of the polysulfated petromyzonol compounds. The results indicated both ovulated females and spermiated males were attracted to 3kPZS, however, only females were repulsed from the mixture of 3kPZS with 3sPZS.

Figure 3-4 (cont'd). The time the lamprey spent in the control (Bc) and experimental (Be) channel of the maze before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate an index of preference for each trial as defined by Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. Data are presented as the mean index of preference ± SEM (horizontal bar). A positive index value indicates attraction and a negative index value indicates repulsion. The index value was evaluated using a Wilcoxon signed-rank test. The sample size, n, is the number outside of parentheses with the number in the parentheses indicating the number of test subjects that spent more time in the treatment side. * $p < 0.05$. ** $p < 0.01$.

3sPZS reduced upstream movement, entry, and retention of ovulated females in artificial nest baited with 3kPZS in a natural spawning environment

Given 3sPZS was the most effective 3kPZS antagonist from the maze assay, we proceeded with testing the influence of 3sPZS on 3kPZS-induced responses of ovulated females in a natural spawning stream environment. 3sPZS treatment affected the proportion of ovulated females that swam downstream (logistic regression; $\chi^2 = 4.69$, $df = 1$, $p = 0.030$), swam upstream ($\chi^2 = 4.89$, $df = 1$, $p = 0.027$), and entered the odorant sources ($\chi^2 = 6.20$, $df = 1$, $p = 0.013$) (Table 3-1). The experimental trials (mixture of 3kPZS and 3sPZS versus 3kPZS) resulted in increased downstream movement and reduced upstream movement compared to the control trials (3kPZS applied to both odorant sources). Of the females that swam upstream and entered an odorant source in the control trials, approximately equal proportions entered the treatment and control odorant sources (49% and 51%, respectively). Therefore, no evidence of side bias in the stream was observed. In the experimental trials, 3sPZS application reduced entry into the treatment odorant source compared to the control trials.

In the experimental trials, application of 3kPZS with 3sPZS reduced retention of each female in the 1 m^2 treatment odorant source compared to 3kPZS alone (retention in treatment odorant source 3kPZS & 3sPZS in seconds: 17.4 s ± 6.7, mean ± SEM; retention in control odorant source 3kPZS: 115.8 s ± 36.9) (Wilcoxon signed-rank test, $p < 0.001$, $Z = 4.330$, $n = 65$). In the control trials, retention in the odorant source did not differ between the two sources (retention treatment odorant source 3kPZS: 51.9 s ± 15.6; retention in control odorant source 3kPZS: 40.5 s ± 11.7) (Wilcoxon signed-rank test, $p = 0.496$, $Z = -0.688$, $n = 42$).

Table 3-1. Behavioral responses of ovulated female sea lampreys exposed to 3kPZS or a mixture of 3kPZS and 3sPZS in a 45 m section of the upper Ocqueoc River (Presque Isle County, MI). In control trials (first row), 3kPZS was applied to both odorant sources (5×10^{-13} M, final in stream concentration) and in experimental trials (second row), 3kPZS (5×10^{-13} M) mixed with 3sPZS (5×10^{-13} M) was applied to the treatment odorant source and 3kPZS (5×10^{-13} M) was applied to the control odorant source. Downstream movement: the percentage (the count) of the released ovulated females that moved at least 3 m downstream of the release cages. Upstream movement: the percentage (the count) of the released females that moved at least 3 m upstream of the release cages. Enter Odorant Source: the percentage (the count) of the females that moved upstream and entered the 1 m^2 odorant source. Movement responses were evaluated with a logistic regression with a binomial distribution. Responses within a column that share a letter are not significantly different ($\alpha = 0.05$).

Odorant						**Enter Odorant Source**	
Treatment	**Control**	**Trials**	**Released**	**Down**	**Up**	**Treatment**	**Control**
3kPZS	3kPZS	8	75	4% (3) A	86% (64) A	49% (26) A	51% (27) A
3kPZS & 3sPZS	3kPZS	14	153	12% (19) B	73% (111) B	27% (20) B	73% (53) B
			χ^2	4.69	4.89	6.20	
			df	1	1	1	
			P value	0.03	0.03	0.01	

Swim tracks overlaid onto the odorant plume map show 3sPZS application reduced the preference of ovulated females for 3kPZS (Figure 3-5 and Figure 3-6). 3sPZS application reduced nest entry. Stream temperature, a factor known to influence sea lamprey upstream movement, showed minimal variation in the upper Ocqueoc River throughout the field experiments from 06 July to 17 July 2016 (20.34 ± 0.46 °C, overall mean ± SEM, n = 21) (Brant et al. 2015b). The mean temperature for the control (3kPZS vs. 3kPZS) and experimental trials (3kPZS and 3sPZS vs. 3kPZS) were not significantly different from each other (control: 20.81 ± 0.59 °C, n = 7, and experimental: 20.10 ± 0.63 °C, n = 14; t test, p = 0.477, t = 0.726, *df* = 19).

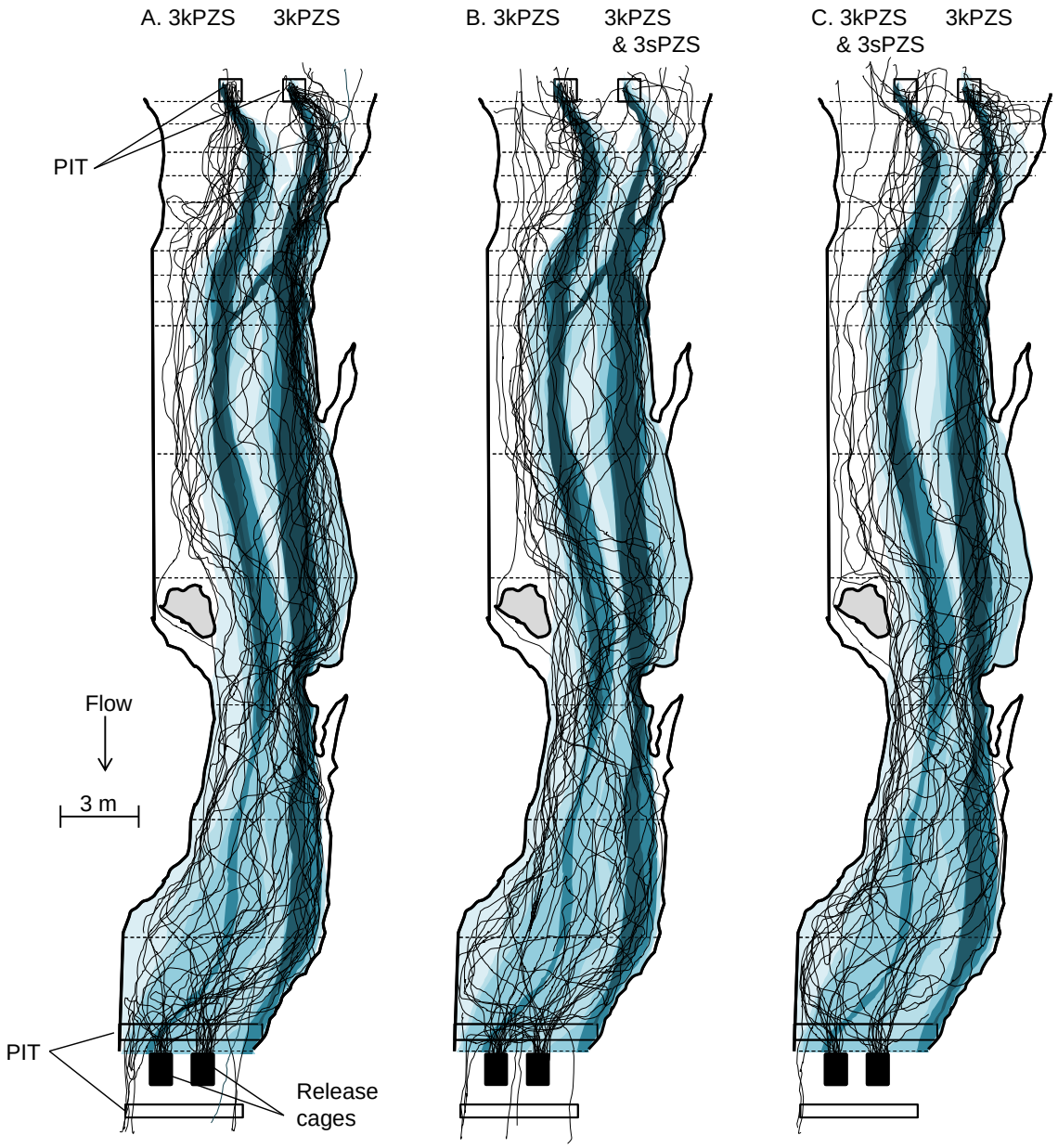

Figure 3-5. Representative swim track movements of ovulated females in a 45 m section of upper Ocqueoc River (Presque Isle County, MI). Females were exposed to (A) 3kPZS (5 x 10^{-13} M, final in stream concentration) in both odorant sources (control, n = 75) or to (B, C) 3kPZS (5 x 10^{-13} M) versus a mixture of 3kPZS (5 x 10^{-13} M) and 3sPZS (5 x 10^{-13} M) in the adjacent odorant source (experimental, n = 153). The movement of females from the release cages (solid black boxes downstream) was monitored with four passive integrated transponder (PIT) antennas (locations shown) and visual observations. Each line indicates the swim track of an individual female. Rhodamine dye was administered in the center of the odorant source and sampled at ten points along each transect (transecting dashed lines) to map the odorant plume. The shading of the odorant plume corresponds to the relative molar concentration of the odorant. Darker blue indicates a higher relative odorant concentration.

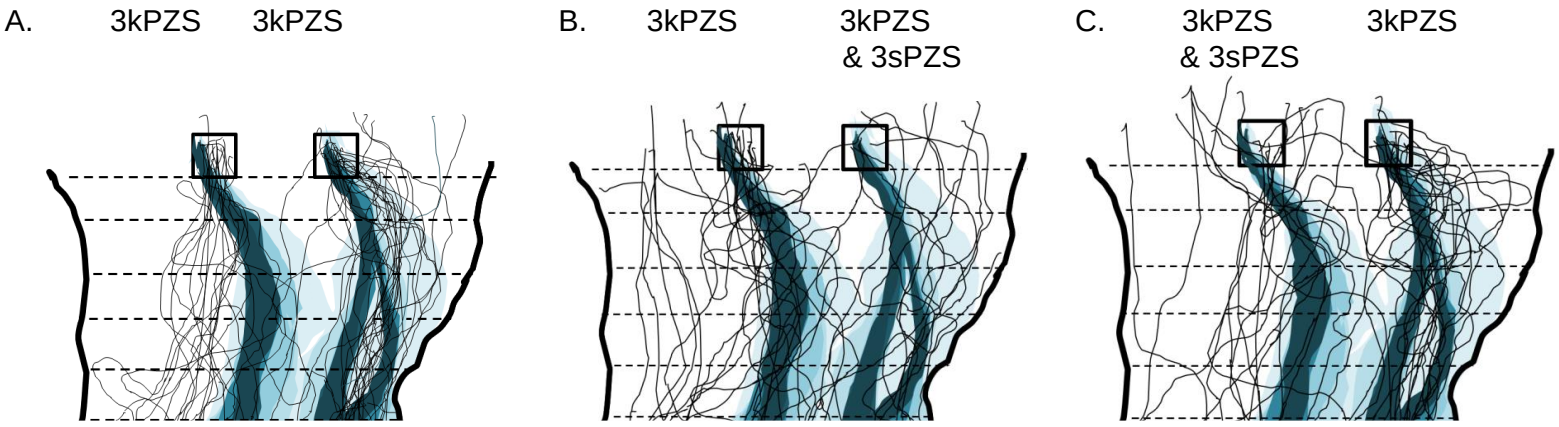

Figure 3-6. Representative swim track movements of ovulated females within 5 m of the odorant sources in upper Ocqueoc River (Presque Isle County, MI). This figure zooms in on Figure 3-5 to highlight the movement of females approaching the odorant sources mimicking artificial nests from 5 m below the odorant sources. Females were exposed to (A) 3kPZS (5×10^{-13} M, final in stream concentration) in both odorant sources (control, n = 75) or to (B, C) 3kPZS (5×10^{-13} M) versus a mixture of 3kPZS (5×10^{-13} M) and 3sPZS (5×10^{-13} M) in the adjacent odorant source (experimental, n = 153). The movement of females was monitored with passive integrated transponder (PIT) antennas around each odorant source (1 m^2) and visual observations. Each line indicates the swim track of an individual female. Rhodamine dye was administered in the center of the odorant source and sampled at ten points along each transect (transecting dashed lines) to map the odorant plume. The shading of the odorant plume corresponds to the relative molar concentration of the odorant. Darker blue indicates a higher relative odorant concentration.

DISCUSSION

Our results demonstrate 3sPZS is a 3kPZS antagonist that reduces the olfactory and behavioral responses of adult sea lampreys to 3kPZS. In this study, we found that 3sPZS inhibited the behavioral preferences for 3kPZS in a maze, reduced upstream movement, and reduced entry and retention in an artificial nest baited with 3kPZS in a natural spawning stream environment. 3sPZS, as an effective sea lamprey 3kPZS pheromone antagonist, has a similar overall shape and spatial arrangement of select key functional groups as 3kPZS, which is consistent with the results of Raschka *et al.* (2018).

The data from electrophysiology and behavioral assays (maze and field) show 3sPZS is a potent inhibitor of 3kPZS-induced responses in sea lamprey. The specific concentration of 3sPZS that influences the 3kPZS response varies with the sensitivity of the assay. The concentration response curve for 3sPZS showed a steep increase in the olfactory response with increasing concentrations and a low detection threshold at 10^{-10} M (Figure 3-3A), which are typical dynamics when an odorant is interacting with a specific receptor (Li et al. 2013). Pre-exposure to 3sPZS also reduced the 3kPZS EOG response. These data support 3sPZS is likely acting through an odorant receptor. In a similar manner, pre-exposure of codling moths (Giner et al. 2009) and European corn borers (Riba et al. 2005) to their respective pheromone antagonists resulted in reduced electrophysiological responses to their pheromone and disruptive behavioral responses to a source baited with pheromone relative to untreated insects. 3sPZS inhibited preferences of females for 3kPZS in a two-choice maze at 10^{-12} M (Figure 3-4) as well as upstream movement, entry, and retention in an artificial nest baited with 3kPZS in a natural spawning environment when applied at 5×10^{-13} M (Figure 3-5, Figure 3-6, Table 3-1). A low

effective concentration of 3sPZS is desirable to keep costs for potential management application minimal because adult sea lamprey live in fast-flowing riffle habitat that can reach 1 m s^{-1} (Applegate 1950), which is capable of quickly diluting the 3sPZS from its point of application.

A decrease in the 3kPZS EOG response after adaptation to a polysulfated petromyzonol compound in the EOG recordings indicates the polysulfated petromyzonol compound likely shares one or several receptors with 3kPZS. However, these results do not indicate the mode of action of the polysulfated petromyzonol compound. Based on the electrophysiology results, both 3sPZS and petromyzonol-3,24-disulfate reduced the 3kPZS olfactory response. Then in the maze, these two compounds when mixed with 3kPZS induced different behavioral responses in ovulated females. Females avoided the mixture of 3sPZS and 3kPZS in the maze, whereas females preferred petromyzonol-3,24-disulfate when tested alone or when mixed with 3kPZS (Figure 3-4). Petromyzonol-3,24-disulfate could be preventing 3kPZS from binding to its cognate receptor and functioning as an antagonist or petromyzonol-3,24-disulfate could be acting as an agonist and activating the 3kPZS receptor. If petromyzonol-3,24-disulfate is an agonist of a 3kPZS receptor, it should induce a similar EOG concentration response curve and behavioral response as 3kPZS. Indeed, petromyzonol-3,24-disulfate induces an EOG concentration response curve similar to 3kPZS (Figure 3-3B) (Li et al. 2013) and elicits behavioral attraction of ovulated females in the maze like 3kPZS (Figure 3-4). With the goal of our study to identify a 3kPZS behavioral antagonist, we proceeded with 3sPZS as the 3kPZS candidate antagonist of choice for the in stream experiment.

The results from the natural spawning stream experiment with 3sPZS are consistent with the results seen when females were rendered anosmic through naris-plugging. 3kPZS has been demonstrated to be a main component of the male sex pheromone (Li et al. 2002), and when

3kPZS is absent, ovulated females not only halt upstream movement but actually turn around and move downstream (Johnson et al. 2006; Siefkes et al. 2005). Likewise, Johnson et al. (2005) reported that fewer anosmic females moved upstream when released in a spawning stream compared to sham-treated females, and those that did move upstream, failed to interact or spawn with the mature males presumably releasing pheromones. These results are consistent with our observations from the field experiment that suggest that 3sPZS disrupts the behavioral response to 3kPZS and show that 3sPZS increases downstream movement, reduces upstream movement, and deters entry and retention in 3kPZS-baited artificial nest (Table 3-1).

Based on the electrophysiology and behavioral results, we speculate 3sPZS is likely a sea lamprey 3kPZS antagonist. 3sPZS reduces the olfactory response to 3kPZS disproportionally more than $_{L}$-arginine (Figure 3-2). These results suggest that 3sPZS influences 3kPZS at the olfactory level and is not a general olfactory suppressant. Follow-up receptor pharmacology experiments to determine the specific mechanism of action of 3sPZS should be conducted to provide further support of its selectivity. Specifically blocking olfactory and subsequent behavioral responses to 3kPZS, a sea lamprey sex pheromone component, is unlikely to influence other aquatic organisms that live in sympatry with spawning adult lampreys (Buchinger et al. 2013). In addition, we speculate 3sPZS is unlikely to have non-target effects and will be stable in part due to the high degree of similarity with 3kPZS, the first vertebrate pheromone biopesticide approved by the U.S. Environmental Protection Agency (Gaden and Lubeck 2016). Studies on potential non-target effects of 3sPZS should be conducted to confirm its impact. Furthermore, 3sPZS exerts sex specific behavioral effects at low concentrations on sea lamprey. Females avoided the mixture of 3kPZS and 3sPZS and spent more time in the control channel in the maze despite females having a preference for the channel with 3kPZS

relative to the vehicle. However, in the same experimental setup, males showed no significant behavioral preferences to the mixture of 3kPZS and 3sPZS or 3sPZS alone. The sex-specific behavioral responses suggest 3sPZS is not a general repellent. We reason applying 3sPZS would re-direct the movement of females but would not displace males from spawning grounds. This would likely result in the separation of females and males and reduce spawning.

Implications for Management

The application of a pheromone antagonist may suppress sea lamprey reproduction to a level that will result in reduced reproductive output. Over time, continued reduced reproductive output should result in a decrease in the sea lamprey population. Previous sea lamprey recruitment modeling has specified an adult control strategy should aim to reduce ovulated female abundance to 0.2 females/100 m^2 (Dawson and Jones 2009). Dawson and Jones (2009) suggest that in order to achieve the desired target, an 80% or greater reduction of reproduction within a stream is necessary. In the maze, we observed 96% of females preferred 3kPZS (27 of 28) in comparison to only 9% of females preferred the mixture of 3sPZS and 3kPZS (2 of 22) (Figure 3-4). Therefore, we find that when 3sPZS is added to the sex pheromone 3kPZS, it reduces the preference of ovulated females for 3kPZS substantially. These values are comparable with insect wind tunnel experiments that reported that the addition of the pheromone behavioral antagonist to the pheromone reduced the proportion of pest European corn borers from reaching the odorant source by 83% (Gemeno et al. 2006) and 88% (Linn et al. 2007). In a natural stream habitat, 3sPZS deterred nest entry and biased the movement of the females moving upstream, resulting in 73% entering the nest baited with 3kPZS alone (Table 3-1). A higher proportion of females failed to enter an artificial nest during experimental in stream trials

when 3sPZS was applied compared to controls of 3kPZS alone, suggesting that the addition of 3sPZS disrupts pheromone-mediated swim orientation with a reduced ability to reliably track a pheromone plume and locate the odorant source. In a large scale pheromone antagonist treatment of maize fields, an antagonist of the major pheromone component of Mediterranean corn borers was dispensed and reduced the larvae per plant by 67-98% and crop damage by 86-90% (Solé et al. 2008). Solé *et al*. (2008) suggested the lower density of Mediterranean corn borer larvae in treated fields resulted from the antagonist influencing female oviposition behaviors. We aim to achieve similar or greater effects with a sea lamprey pheromone antagonist.

Some of the same control strategies discussed for the application of PZS as a naturally occurring 3kPZS antagonist are also relevant for 3sPZS as a synthetic 3kPZS antagonist. 3sPZS can be implemented to likely 1) disrupt sea lamprey mating at the spawning grounds, 2) re-direct sea lamprey into specific locations, and 3) enhance selective fish passages by selectively blocking sea lamprey upstream movement and allowing other fish to pass (Chapter 2). Further optimization of the sea lamprey pheromone antagonist by identifying the most effective concentration and formula will increase its management effectiveness. We determined 3sPZS applied at the same concentration as the pheromone influences the behavioral response of ovulated females, but other concentrations of 3sPZS relative to 3kPZS should be tested to determine the optimal concentration. Based on our observations, 3sPZS or PZS (Chapter 2) prevented most but not all ovulated females from entering the artificial baited nest from in stream assays. Whether these two 3kPZS antagonists combined may function in an additive or synergistic manner to reduce attraction and retention should be investigated. The male sex pheromone also contains minor components predicted to mediate spawning behaviors in close

proximity to the nest (Brant et al. 2016; Johnson et al. 2014b; Li et al. 2017a; Li et al. 2017b). Identifying behavioral antagonists to these minor components may result in a greater reduction of spawning success, thereby enhancing the utility of sea lamprey behavioral antagonists. Together with other adult sea lamprey control initiatives, such as pheromone baited-trapping (Johnson et al. 2013), electrical guidance (Johnson et al. 2014a), or alarm substance application (Hume et al. 2015), we predict that it is achievable to reduce spawner abundance below the recommended threshold.

We conclude 3sPZS is a potent and effective synthetic antagonist that alters the physiological and behavioral responses of female sea lampreys to 3kPZS, a sex pheromone component known to be indispensable for sea lampreys to successfully mate. We illustrated an effective approach to identify pheromone antagonists that can be adapted for other pest species. This approach may lead to the identification of pheromone antagonists for the management of other nuisance or invasive taxa known to use pheromones (Sorensen and Wisenden 2015). These results strongly implicate that the development and implementation of a novel and benign pheromone antagonist, such as 3sPZS, should be considered in future strategies to control the invasive sea lampreys in the Great Lakes.

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CHAPTER 4

IDENTIFICATION OF SEA LAMPREY PHEROMONES USING BIOASSAY-GUIDED FRACTIONATION

Adapted from Scott, A. M., Li, K., & Li, W. (2018). Identification of sea lamprey pheromones using bioassay-guided fractionation. *Journal of Visualized Experiments*. Manuscript under review.

ABSTRACT

Bioassay-guided fractionation is an iterative approach that uses the results of physiological and behavioral bioassays to guide the isolation and identification of an active pheromone compound. This method has resulted in the successful characterization of chemical signals that function as pheromones in a wide range of animal species. Sea lampreys rely on olfaction to detect pheromones that mediate behavioral or physiological responses. We use this knowledge of fish biology to posit functions of putative pheromones and to guide the isolation and identification of active pheromone components. Chromatography is used to extract, concentrate, and separate compounds from the conditioned water. Electro-olfactogram (EOG) recordings are conducted to determine which compounds elicit olfactory responses. Two-choice maze behavioral assays are then used to determine if any of the odorous compounds are also behaviorally active and induce a preference. Spectrometric and spectroscopic methods provide the molecular weight and structural information to assist with the structure elucidation. The behavioral responses observed in the maze should ultimately be validated in a field setting to confirm their function in a natural stream setting. These bioassays play a dual role to 1) guide the fractionation process and 2) confirm and further define the bioactivity of isolated components. Here, we report representative results of sea lamprey pheromone identification that exemplify the utility of the bioassay-guided fractionation approach. The identification of sea lamprey pheromones is particularly important because modulation of its pheromone communication system is among the options considered to control the invasive population in the Laurentian Great Lakes. This method can be readily adapted to characterize the chemical communication in a broad array of taxa and shed light on waterborne chemical ecology.

INTRODUCTION

Pheromones are specific chemical signals released by individuals that aid in locating food sources, detecting predators, and mediating social interactions amongst conspecifics (Wyatt 2014). Pheromone communication in insects has been well studied (El-Sayed 2009; Symonds and Elgar 2008); however, the chemical identification and biological function of aquatic vertebrate pheromones have not been studied as extensively. Knowledge of the identity and function of the pheromones released can be applied to facilitate the recovery of threatened species (Leal 2017; Zhu et al. 2017) or control pest species (Witzgall et al. 2010). Application of these techniques necessitates the isolation and characterization of the bioactive pheromone components.

Pheromone identification is a branch of natural product chemistry. Progress in pheromone research has been partially limited due to the nature of pheromone molecules themselves. Pheromones are often unstable and released in small quantities, and few sampling techniques exist to detect minute amounts of volatile (Cheng et al. 2017; Howse et al. 2013) or water-soluble compounds (Pizzolon et al. 2010). Approaches to identify pheromones include 1) targeted screening of known compounds, 2) metabolomics, and 3) bioassay-guided fractionation. Targeted screening of known compounds tests commercially available metabolic by-products of physiological processes hypothesized to function as pheromones. This approach is limiting because researchers can only test known and available compounds. However, it has resulted in the successful identification of sex hormones in goldfish that function as pheromones (Kobayashi et al. 2002; Stacey 2015; Stacey and Sorensen 2011). Metabolomics is a second pheromone identification approach that distinguishes potential small molecule metabolic products within a

biological system (Kuhlisch and Pohnert 2015). A comparison of the metabolic profiles of two groups (*i.e.,* an active versus inactive extract) enables the identification of a potential metabolic profile from which the metabolite is purified, the structure is identified, and the bioactivity is confirmed (Prince and Pohnert 2010). Additive or synergistic effects of complex formulations of specific mixtures are more likely to be detected with metabolomics because metabolites are considered together rather than a series of fractions (Kuhlisch and Pohnert 2015). Yet, the implementation of metabolomics relies on the availability of synthetic references because the resulting data do not facilitate the elucidation of novel structures.

Bioassay-guided fractionation is an integrated, iterative approach that spans two fields: chemistry and biology. This approach uses the results of physiological and behavioral bioassays to guide the isolation and identification of an active pheromone compound. A crude extract is fractionated by a chemical property (*i.e.*, molecular size, polarity, *etc.*) and tested with electro-olfactogram (EOG) recordings and/or in a bioassay. The bioactive components are screened out by repeating these steps of fractionation and EOGs and/or bioassays. The structures of pure active compounds are elucidated by spectrometric and spectroscopic methods, which provide the molecular weight and structural information to produce a template of the compound to be synthesized. Bioassay-guided fractionation can yield diverse metabolites and potentially novel pheromones with unique chemical skeletons that are unlikely to be predicted from the biosynthetic pathways.

Here, we describe the bioassay-guided fractionation protocol used to isolate and characterize the bioactivity of male sea lamprey sex pheromone compounds. The sea lamprey (*Petromyzon marinus*) is an ideal vertebrate model to study pheromone communication because these fish rely heavily on the olfactory detection of chemical cues to mediate their anadromous

life history comprised of three distinct stages: larva, juvenile, and adult. Sea lamprey larvae burrow into the sediment of freshwater streams, undergo a drastic metamorphosis, and transform into juveniles that migrate to a lake or ocean where they parasitize large host fish. After detaching from the host fish, the adults migrate back into spawning streams, guided by the migratory pheromones released by resident larvae (Moore and Schleen 1980; Teeter 1980; Vrieze et al. 2011; Wagner et al. 2006; Wagner et al. 2009). Mature males ascend to spawning grounds, release a multi-component sex pheromone to attract mates, intermittently spawn for approximately a week, and then die (Siefkes et al. 2005; Teeter 1980). The identification of sea lamprey pheromones is important because modulation of the pheromone communication system is among the options considered to control the invasive sea lampreys in the Laurentian Great Lakes (Siefkes et al. 2013).

PROTOCOL

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University (AUF# 03/14-054-00 and 02/17-031-00).

Phase 1. Collection and extraction of sea lamprey conditioned water

1.1. Place sexually mature male sea lampreys (15–30 animals) in a tank supplied with 250 L of aerated Lake Huron water maintained at 16–18 ^{o}C.

1.2. Collect the male conditioned water throughout each night from June to July. Sea lampreys naturally die after spawning. If a fish is nearing this point in its life, it is replaced with a fresh mature male.

1.3. Extract the conditioned water by solid phase extraction. Pass the conditioned water through a bed of 2 kg of Amberlite XAD 7 HP resin, contained in a series of four 2.5 L-capacity glass columns. Maintain load speed at 800 mL/min.

1.4. CAUTION: This step uses methanol and acetone. Both are flammable and poisonous. Elute the metabolites with 10 L of methanol followed immediately by 5 L of acetone.

Note: The pooled residual can be stored at -80 ^{o}C until further processed.

1.5. Remove the organic solvent and harvest the extract by rotoevaporating under reduced pressure at 40 ^{o}C for 48 h and concentrate the water residue by lyophilization at -20 ^{o}C until dry.

Phase 2. Isolation of fraction pools with chromatography

2.1. CAUTION: The chloroform used in this step is a poisonous reagent. Subject the extract to liquid chromatography over silica gel (gradient elution from 95% $CHCl_3$ (chloroform)/MeOH

(methanol) to 100% MeOH, 2.5 L total volume). Perform column chromatography on silica gel (70-230 and 230-400 mesh).

2.2. Guide the pooling of the eluents into 20 fractions by thin layer chromatography (TLC) analysis. Use a gradient of methanol in water from 30 to 100% over 30 min with pre-coated silica gel plates. Visualize spots first under UV light at 254 nm and then stain by spraying with an acidic methanol solution with 5% anisaldehyde.

2.3. Concentrate the fraction to a residue with size exclusion chromatography using Sephadex LH-20. First use a $CHCl_3$-MeOH (1:1) column and then a MeOH (100%) column to yield the compounds.

Phase 3. Electro-olfactogram (EOG) recordings to identify odorous fractions/compounds

3.1. Pull borosilicate glass capillaries with a micropipette puller.

3.2. Score and cut the opening at the tip of the capillary with a diamond-tipped glass cutter and fill with molten 0.4% agar in 0.9% saline.

3.3. Fill the solid state electrodes holders with Ag/AgCl pellets and pulled electrodes with 3 M KCl using a micropipette.

Note: Dislodge any air bubbles in the pulled electrode or electrode holder.

3.4. Insert the pulled electrodes in the electrode holders.

3.5. Prepare 100 mL of 10^{-5} M $_L$-arginine in charcoal-filtered water from a 10^{-2} M $_L$-arginine stock solution in deionized water (stored at 4 °C) in a volumetric flask. Transfer 20 mL of 10^{-5} M $_L$-arginine to a glass vial. For the concentration-response curve, prepare 10 mL of 10-fold dilutions of the pheromone in glass vials (*i.e.*, 10^{-6} M to 10^{-13} M from a 10^{-3} M pheromone stock

solution in 50% methanol/water stored at -20 °C). Prepare fresh dilutions daily prior to experiments and use within a day.

3.6. Put the glass vials of working solutions of $_{L}$-arginine and putative pheromones in a re-circulating water bath to allow the temperature to equilibrate.

3.7. Anesthetize the lamprey with 3-aminobenzoic acid ethyl ester (MS222; 100 mg/L) and immobilize with an intra-muscular injection of gallamine triethiodide (3 mg/kg of body weight, in 0.9% saline).

3.8. Orient the lamprey in a V-shaped stand and wrap with wet paper towel to prevent desiccation.

Note: Do not obstruct the opercula.

3.9. Insert tube of re-circulating aerated water containing 50 mg/L MS222 into the buccal cavity, adjust flow rate, and ensure water is coming out via the opercula to continuously irrigate the gills.

3.10. Remove 5 mm^2 section of skin on the surface of the olfactory capsule to expose the olfactory epithelium using a scalpel and forceps under the microscope.

3.11. Flush odorant delivery tubing with filtered water and connect to the valve mounted on micromanipulator. Place the odorant delivery capillary tube into the olfactory epithelium cavity using the micromanipulator to deliver filtered water to the olfactory epithelium to prevent desiccation when not administering odorants.

3.12. Mount the recording and reference electrodes on the micromanipulators. Lower the reference electrode onto the external skin near the naris. Using the microscope, lower the recording electrode to barely touch the surface of the olfactory epithelium.

3.13. Transfer the uptake of the odorant delivery tube from the background filtered water to the 10^{-5} M $_{L}$-arginine solution.

3.14. Turn on the computer, amplifier (set to DC mode), filter, and digitizer. Using the software, program the procedure to administer a 4 s single pulse of odorant and initiate a stimulation protocol. Trigger the odorant pulse in the software and record the differential EOG response amplitude in the data acquisition software.

Note: Use the micromanipulator to maneuver the positions of the recording electrode, reference electrode, or odorant delivery tube to increase the signal-to-noise ratio with a maximum response to $_{L}$-arginine standard and minimal response to the blank control (filtered water). Ground the amplifier while moving the electrodes.

3.15. Start recording the blank control, $_{L}$-arginine, and then the odorants from low to high concentrations with a 2 min flush of filtered water between applications.

3.16. After recording all desired responses, ground the amplifier and carefully retract the electrodes and odorant delivery capillary tube.

3.17. Analyze and plot the data using analysis software.

Phase 4. Two-choice maze bioassay to identify behaviorally active fractions/compounds

4.1. Acclimate the sea lamprey in the release cage in the maze for 5 min.

4.2. Release the sea lamprey and record the cumulative amount of time the lamprey spends in the experimental and control channel for 10 min (before odorant application period).

Note: If the sea lamprey fails to enter the experimental and control channel for at least 10 s during this 10 min period, end the trial as this is an indication of inactivity or strong side bias.

4.3. Apply the test stimulus (*i.e.*, putative pheromone at 10^{-12} M) to the randomly assigned experimental channel and vehicle (50% methanol/deionized water) to the control channel using a peristaltic pump at constant rates of 200 mL/min for 5 min.

4.4. Apply the test stimulus and vehicle for 10 additional min and record the cumulative amount of time the lamprey spends in the experimental and control channel (after odorant application period).

4.5. Flush the maze with water for 10 min before the start of the next trial. Repeat 4.1- 4.5 with at least seven lampreys if sufficient test stimulus is available.

4.6. Calculate an index of preference (Li et al. 2017b) for each trial and evaluate the significance using a Wilcoxon signed-rank test. Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)], where Bc is the time spent by the lamprey in the control channel before odorant application, Be is the time spent in the experimental channel before odorant application, Ac is the time spent in the control channel after odorant application, and Ae is the time spent in the experimental channel after odorant application.

Phase 5. Repeat Phase 2 (Chromatography) to isolate pure compounds from active fractions

Phase 6. Structure elucidation of pure compound with mass spectrometry (MS) and nuclear magnetic resonance (NMR)

6.1. Dissolve the purified compound in the initial mobile phase of the high performance liquid chromatography (HPLC) (methanol: water, 1:1, v:v) to form a solution approximately 1 μg/ml. Measure molecular weight by electrospray ionization mass spectrometry (ESIMS) and high-resolution ESIMS (HRESIMS) spectra using a TQ-S TOF LC mass spectrometer.

6.2. Predict the molecular formula with the database on Masslynx 4.1 software.

6.2.1. Open the chromatogram of the injected sample and obtain its mass spectrum by selecting the chromatographic peak.

6.2.2. Input the measured mass to ion (*m/z*) value (4 decimals) in "Elemental composition" under the "Tool" module.

6.2.3. Set the tolerance parameter to PPM lower than 5.

6.2.4. Adjust the symbol parameters to fit the element composition of the measured molecule. The software produces a prediction of molecular formula based on the single mass analysis.

6.3. Record the 1D (^{1}H, ^{13}C) and 2D NMR (^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC)) spectra on an Agilent 900 MHz spectrometer following the Bruker user guide.

6.3.1. Place sample inside the spinner turbine. Use the depth gauge to ensure the sample height is in the middle of the measuring window.

6.3.2. Open the TOPSPIN software. Click the "Lift" button to change the sample in the magnet.

Note: You should be able to feel (holding your hand over the top of the magnet) and hear the whistling sound of gas coming from the top of the magnet.

6.3.3. Gently place the sample on the air cushion on top of the magnet and push the "Lift" button again to descend the sample into the NMR magnet.

Note: Listen for a click noise to indicate the sample is in right position.

6.3.4. Create a new data set and load the standard parameters.

6.3.5. Type "lock" to invoke the automatic locking procedure and select the solvent at the prompt page. For fine tuning, at the manipulate panel, adjust the button at "unlock" model, adjust the cursor on manipulate panel, and lock the magnet again.

6.3.6. At the prompt “atma” page, adjust the parameter in the manipulate panel to the tuning procedure which may takes a few minutes. Wait until the tuning is completed to proceed.

6.3.7. Start the automatic shimming routine by typing “topshim” in the prompt. Wait until the shimming is completed to proceed.

6.3.8. Type “rga” to set automatic receiving gain adjustments, then type “d1” to set the delay between pulses, then type “zg” to start the acquisition, and wait until the acquisition finishes.

6.3.9. Type “ef” or “efp” to process the data and then type “apk” for automatic phasing. Process the data on the MestReNova software.

6.4. Elucidate the chemical structure by interpretation of the MS and NMR data analysis.

6.4.1. Count the carbon signals in ^{13}C NMR spectrum. Select the molecular formula that matches the predicted result in HRESIMS.

6.4.2. Integrate the proton signals in ^{1}H NMR spectrum and assign the connectivity of carbon and proton based on the signals on HSQC spectrum.

6.4.3. Assign the connectivity of carbon skeleton based on the correlations in ^{1}H-^{1}H COSY and HMBC spectrum.

6.4.4. Tentatively assign the chemical structure based on structure rationale (Breitmaier and Sinnema 1993). Search the tentative structure in the Reaxys and Scifinder databases. Compare the tentative structure with the analogs in references.

6.4.5. Assign the relative configuration with the NOESY spectrum and assign absolute configuration by a derivative reaction if needed.

Phase 7. Repeat Phase 3 (EOG) and Phase 4 (Bioassay) to confirm pure compounds are odorous and behaviorally active

REPRESENTATIVE RESULTS

A diagram summarizing the steps described in the protocol of bioassay-guided fractionation is shown in Figure 4-1. The protocol involves the steps to isolate and characterize the structure, olfactory potency, and behavioral activity of five putative sea lamprey pheromones or cues (Figure 4-2). Using the mass spectrometric and NMR data (Figure 4-3 to Figure 4-8), the structures of petromyzene A-B and petromyzone A-C were elucidated from water conditioned with mature male sea lampreys (Li et al. 2017a; Li et al. 2017b).

Our representative data from the EOG responses (Figure 4-9) demonstrate petromyzene A-B and petromyzone A-C were all potent odorants that stimulated the adult sea lamprey olfactory epithelium and had low thresholds of detection (Figure 4-10). Of particular note, the EOG recordings require proper placement of the recording electrode on the surface of the olfactory epithelium relative to the stimulus delivery tube to result in a good signal-to-noise ratio for reliable odorant response recordings (Figure 4-9B). If this critical step is not followed, it will be difficult to discern the signal induced by the odorant amongst the high noise. The downward deflection of the EOG trace after odorant exposure is a negative potential. A good EOG signal of a potent odorant should show odorant administration resulting in a quick and sharp response followed by a recovery to the baseline. The olfactory responses to the putative pheromones were also normalized to the responses of 10^{-5} M $_{L}$-arginine, a positive control odorant in sea lamprey, tested throughout the experiment to ensure the integrity of the recordings was maintained.

In the two-choice maze behavioral assays (Figure 4-11), ovulated female sea lampreys were attracted to petromyzone A, petromyzene A, and petromyzene B and repulsed from petromyzone C. Ovulated females appeared to be repulsed from petromyzone B; however, the

behavioral response was not significant (Figure 4-12). A larger sample size was not possible due to the limited quantity of petromyzone B. To properly assess the behavioral response to an odorant, it is critical to record the pre-treatment bias for the control and experimental channel of each lamprey.

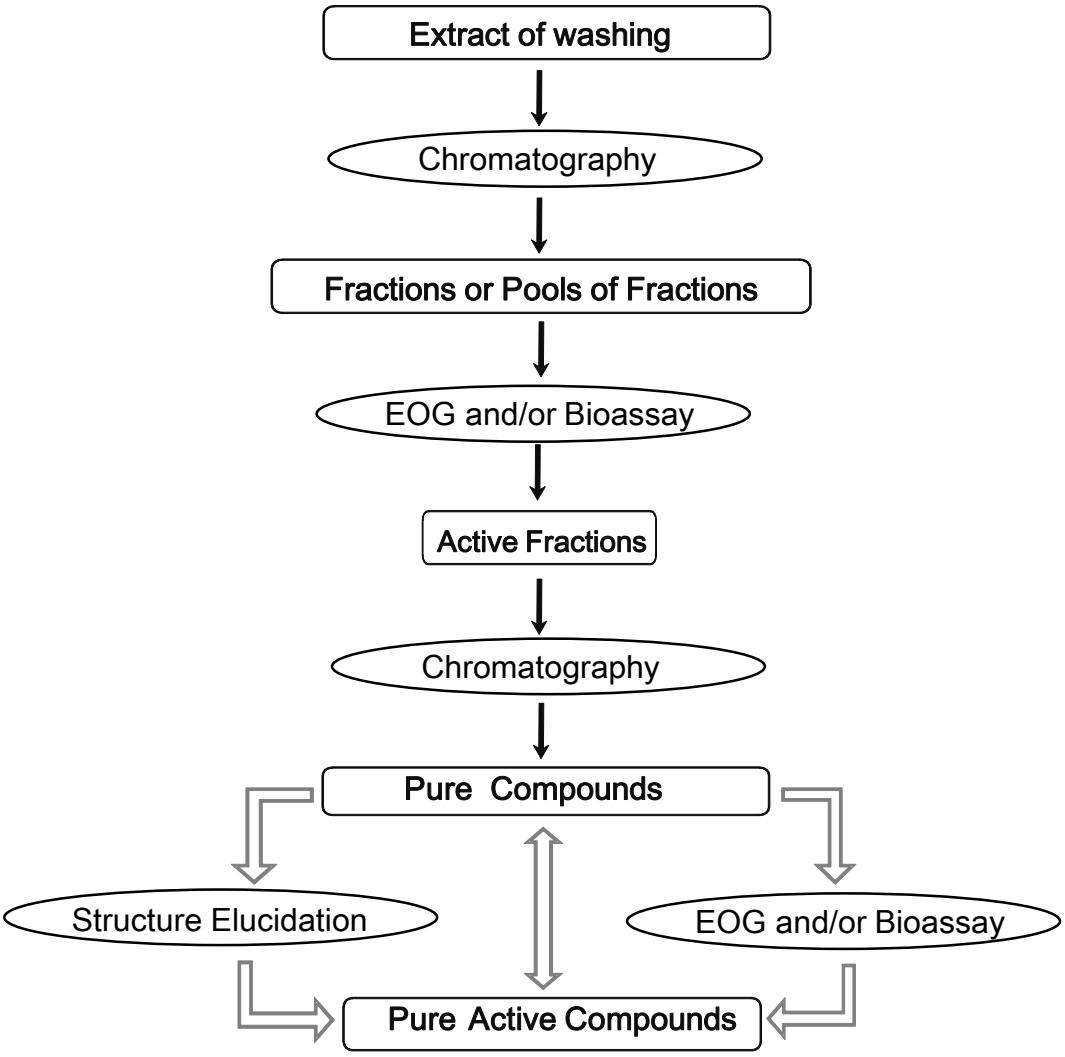

Figure 4-1. Flowchart of the bioassay-guided fractionation of pheromones. The boxes indicate the resulting chemical product from the technique indicated in the oval. Sexually mature male sea lampreys are held in a tank for collection of conditioned water. The conditioned water (washings) is extracted. The extract is subjected to chromatography to separate into fractions or pools. Fractions or compounds are tested with electro-olfactogram (EOG) recordings to identify those that are odorous. Odorous fractions or compounds are tested in a bioassay to evaluate the behavioral activity. Active fractions are further separated with chromatograph to isolate the pure compounds. The structure of the pure compound is elucidated with mass spectrometry (MS) and nuclear magnetic resonance (NMR). The bioactivity of the pure compound is confirmed with EOG and a behavior bioassay. The spectra of the pure compound and the pure active compound are compared.

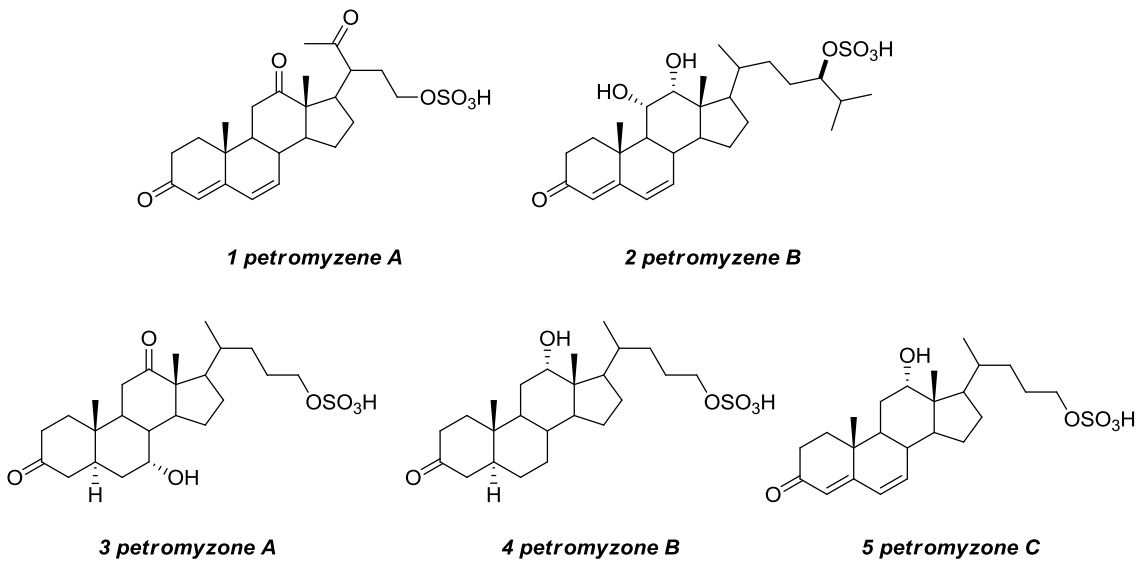

Figure 4-2. Chemical structures of petromyzene A-B and petromyzone A-C. These compounds were identified with bioassay-guided fractionation. This figure was modified from Figures 1 in the references (Li et al. 2017a; Li et al. 2017b).

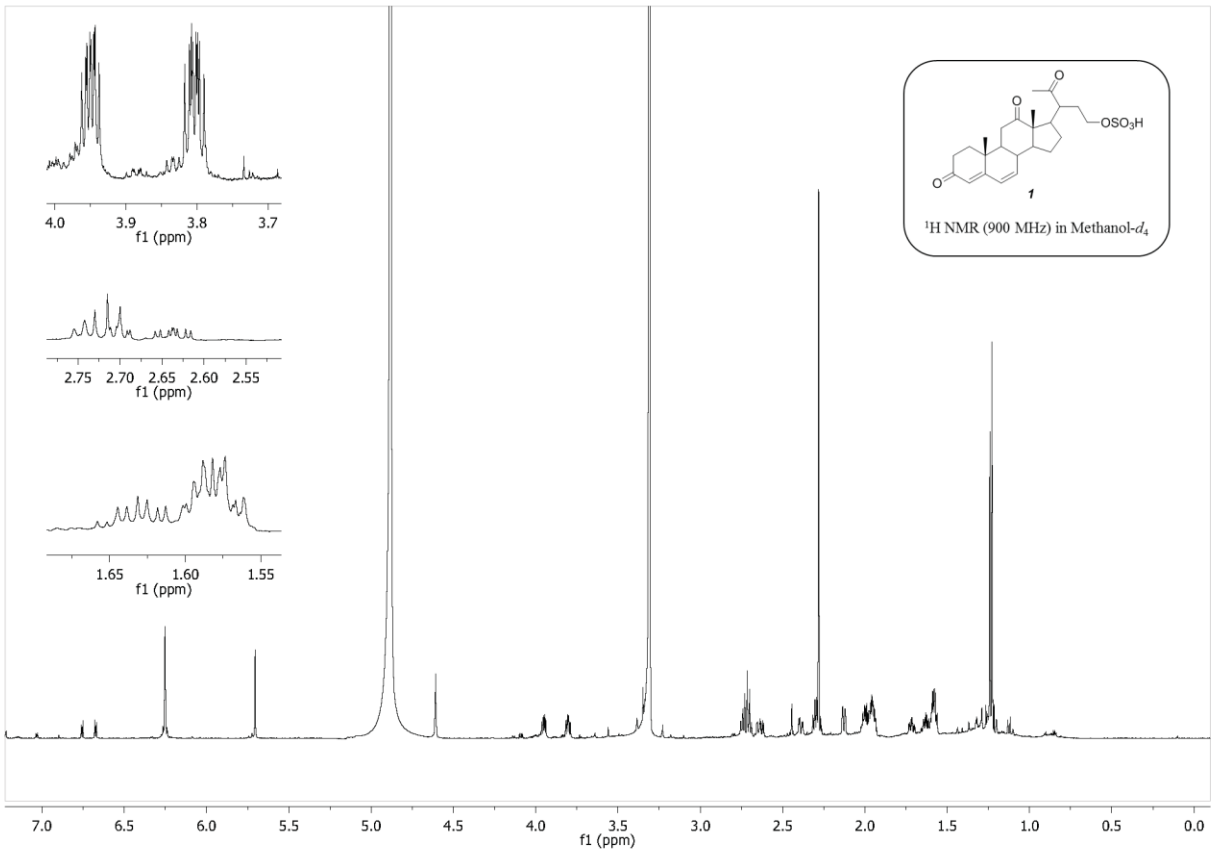

Figure 4-3. ^{1}H NMR (900 MHz) spectrum of petromyzene A. The ^{1}H NMR spectrum identifies the hybridization of the H-atoms based on the chemical shift, the number of equivalent H-atoms based on the integral of the resonance, and the number of neighboring H-atoms based on the coupling constants and splitting patterns of the signal. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

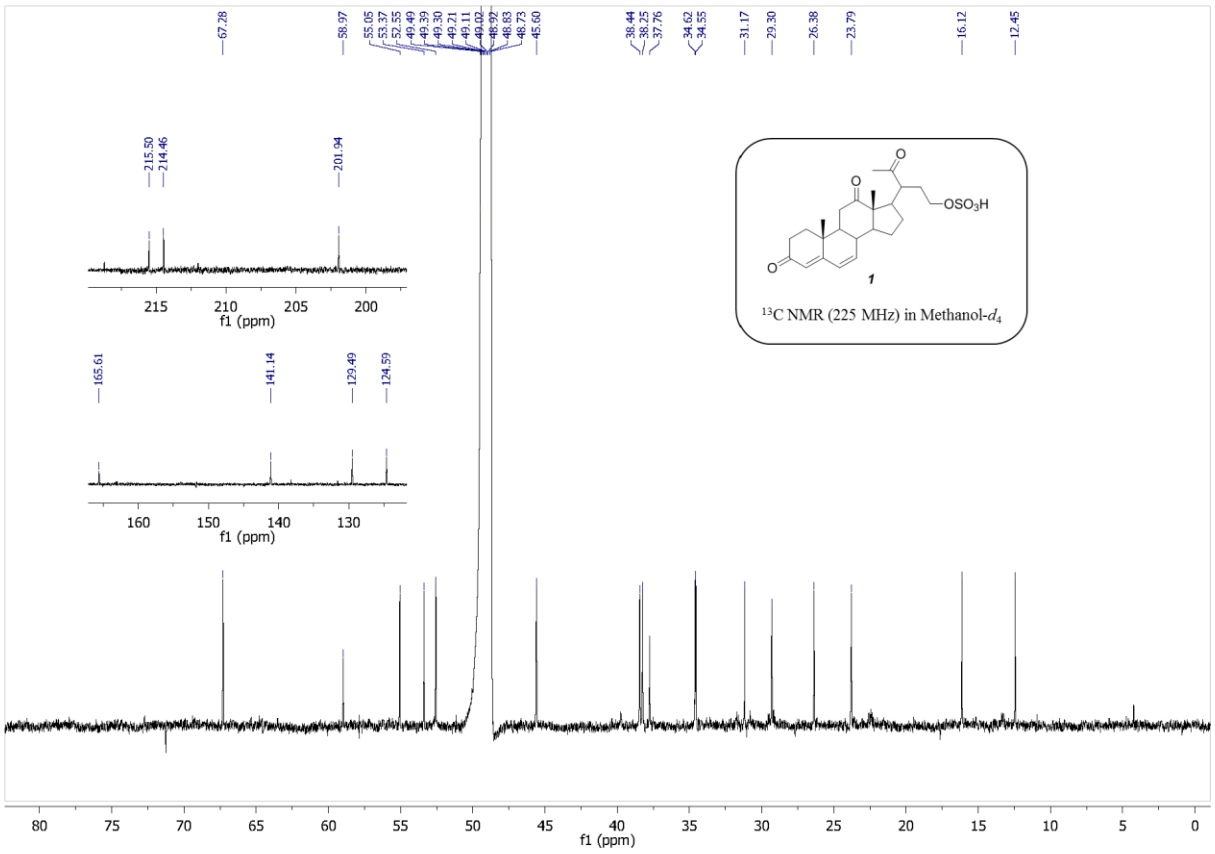

Figure 4-4. ^{13}C NMR (225 MHz) spectrum petromyzene A. The ^{13}C NMR spectrum identifies the substituent pattern and number of carbons based on the number of signals and the hybridization state of each carbon based on the chemical shift of the structure of the measured compound. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

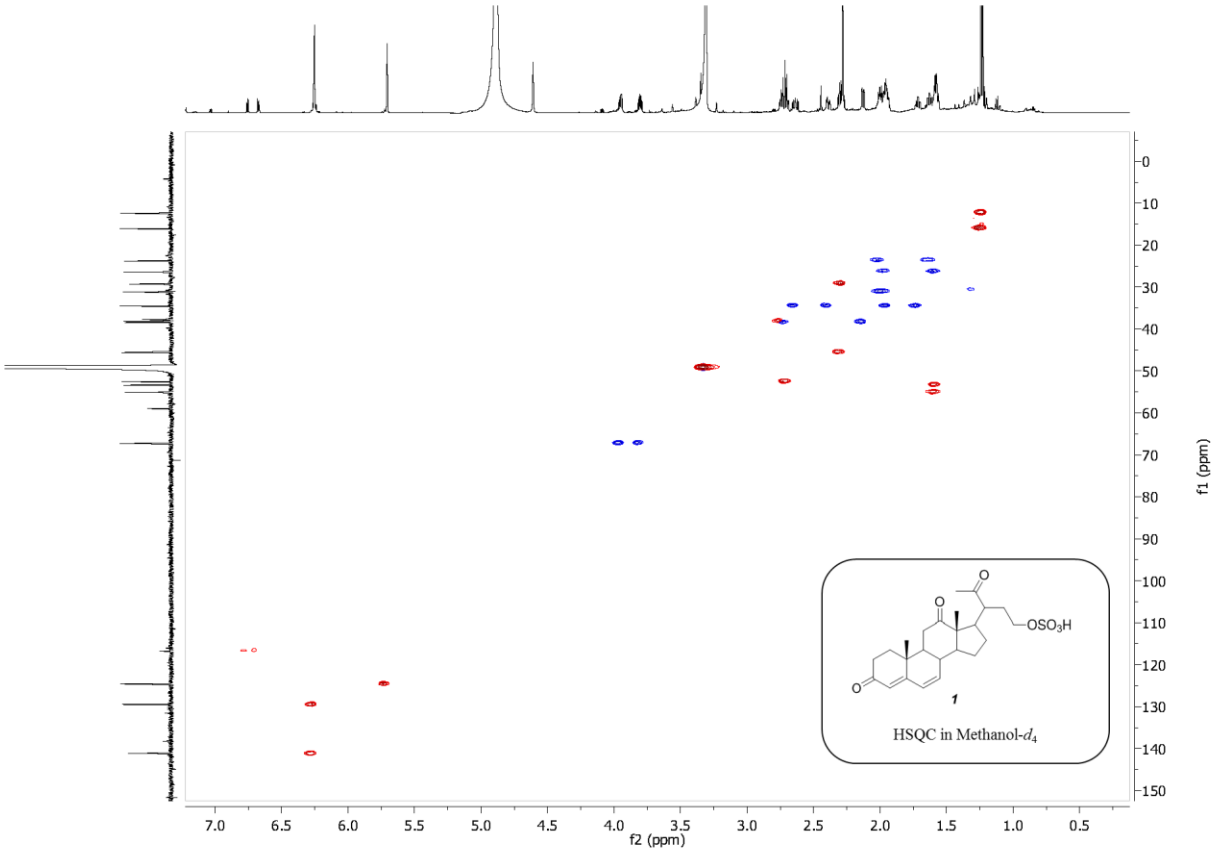

Figure 4-5. HSQC spectrum of petromyzene A. The HSQC (heteronuclear single quantum coherence) spectrum assists in assigning the connectivity of the carbons and protons of the structure of the measured compound, indicating the hybridization of the carbons. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

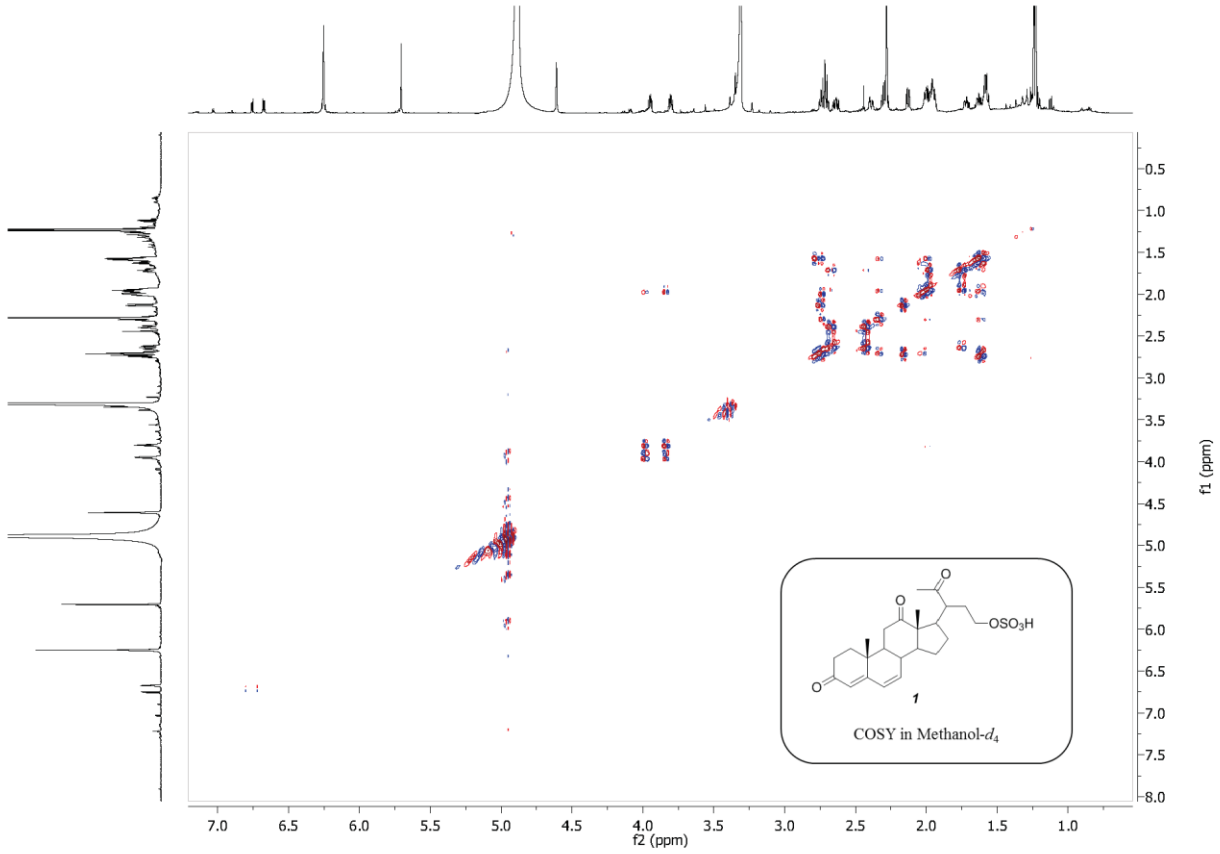

Figure 4-6. ^{1}H-^{1}H COSY spectrum of petromyzene A. The ^{1}H-^{1}H COSY (correlation spectroscopy) spectrum assists in assigning the connectivity between the carbons of the structure of the measured compound. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

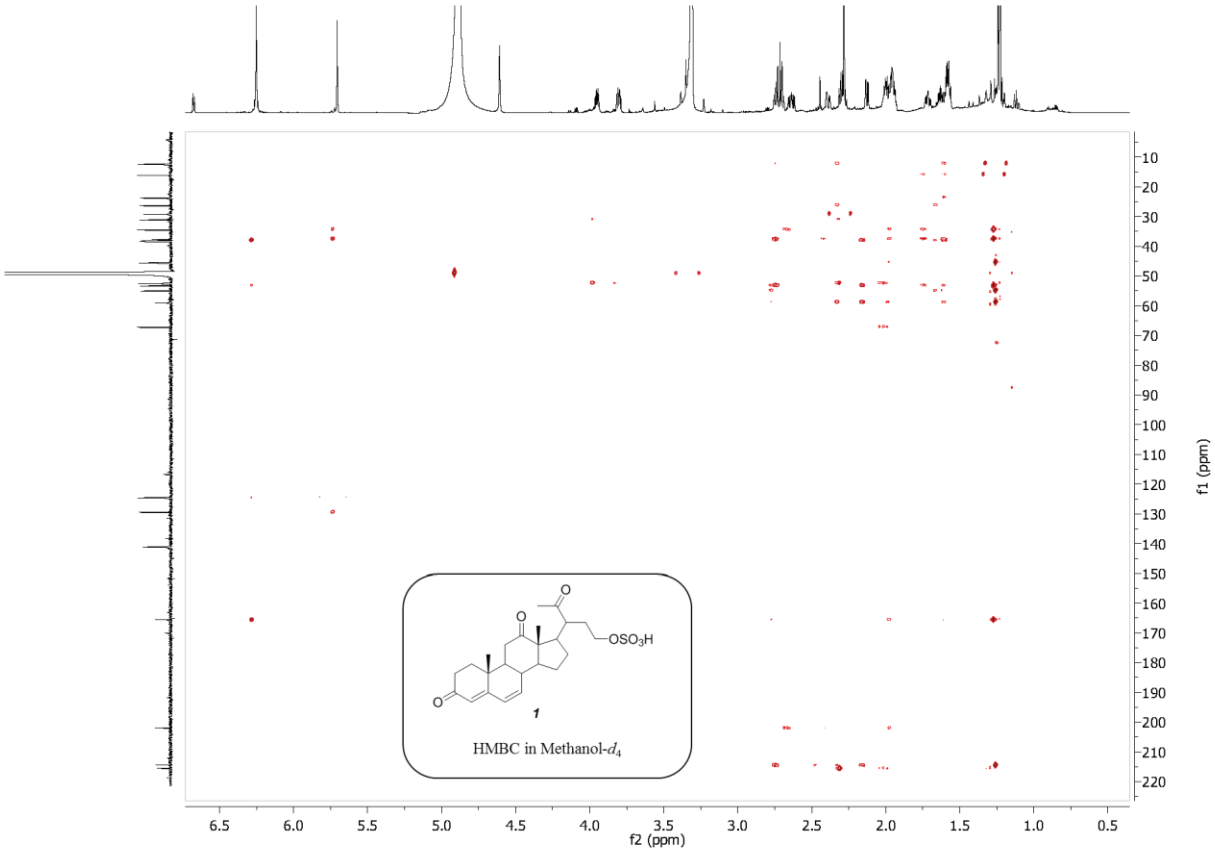

Figure 4-7. HMBC spectrum of petromyzene A. The HMBC (heteronuclear multiple bond correlation) spectrum assists in assigning the connectivity of the carbons and protons of the structure of the measured compound and further confirm the tentative structural assignment. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

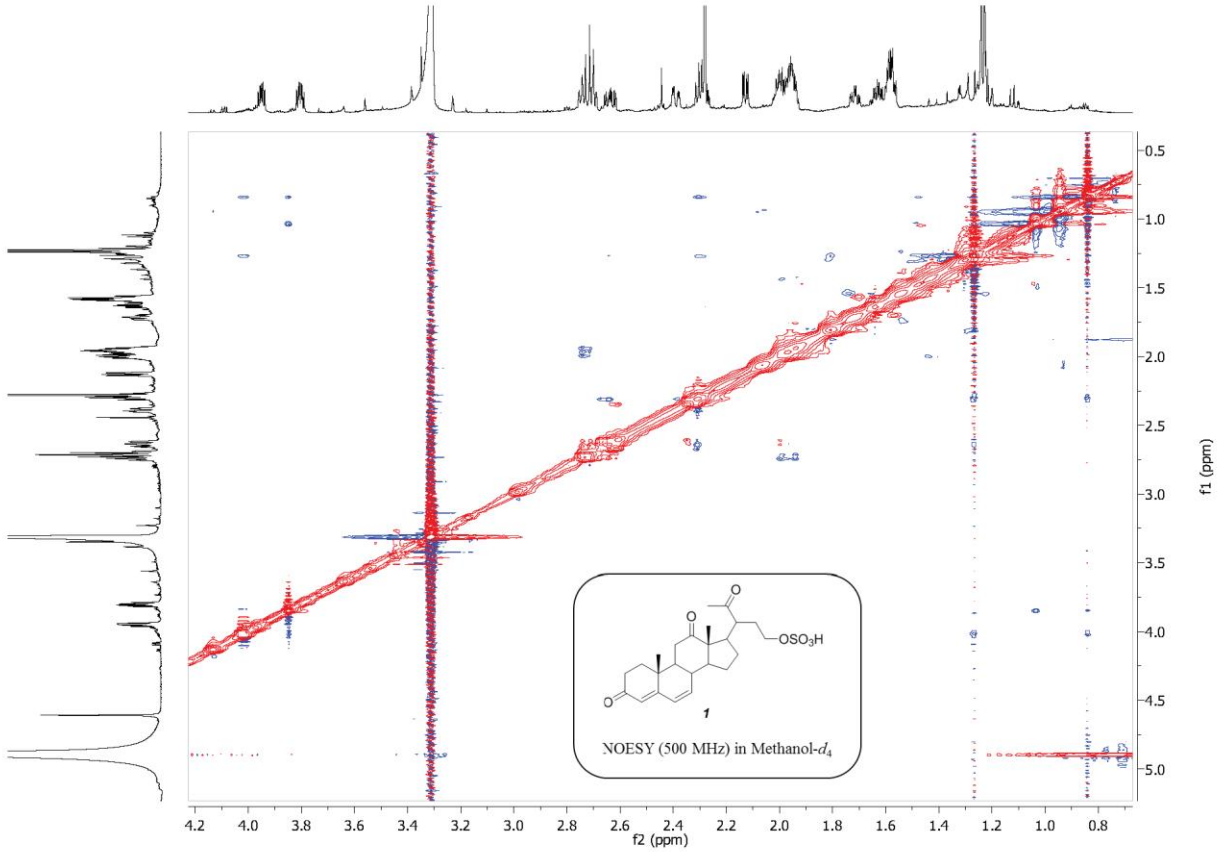

Figure 4-8. NOESY spectrum of petromyzene A. The NOSEY (nuclear Overhauser effect spectroscopy) spectrum assists in assigning the relative configuration of the structure of the measured compound. This figure was provided by Dr. Ke Li and is from the supporting information of the reference (Li et al. 2017a).

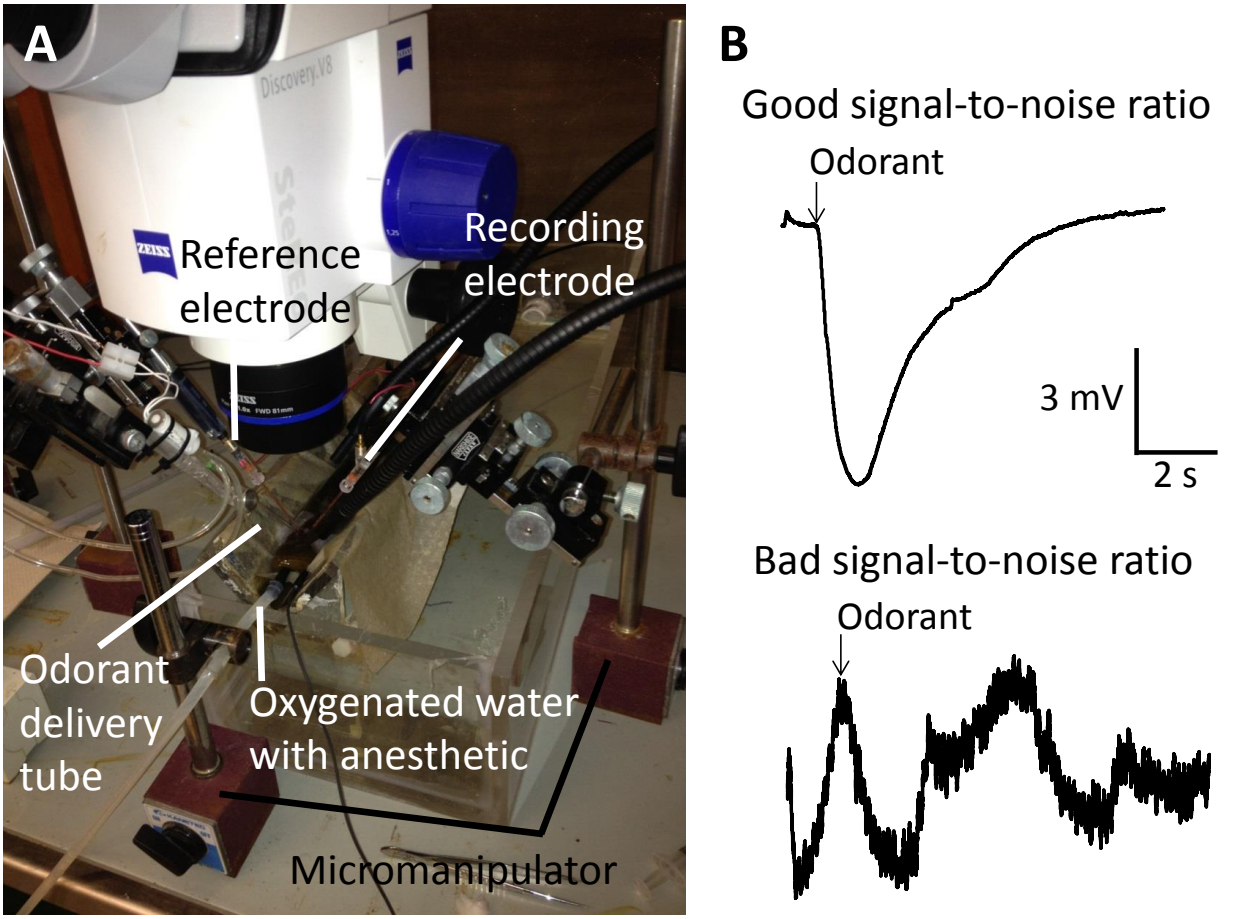

Figure 4-9. Electro-olfactogram (EOG) recording preparation and representative trace recordings. (A) An EOG preparation showing the exposed sea lamprey olfactory epithelium with the recording electrode, reference electrode, odorant delivery tube, oxygenated water with anesthetic, and micromanipulators. (B) Representative trace recordings demonstrating good (top) or bad (bottom) signal-to-noise ratios. Good signal-to-noise ratio is necessary for reliable odorant response recordings. The downward deflection of the EOG trace after odorant exposure is a negative potential.

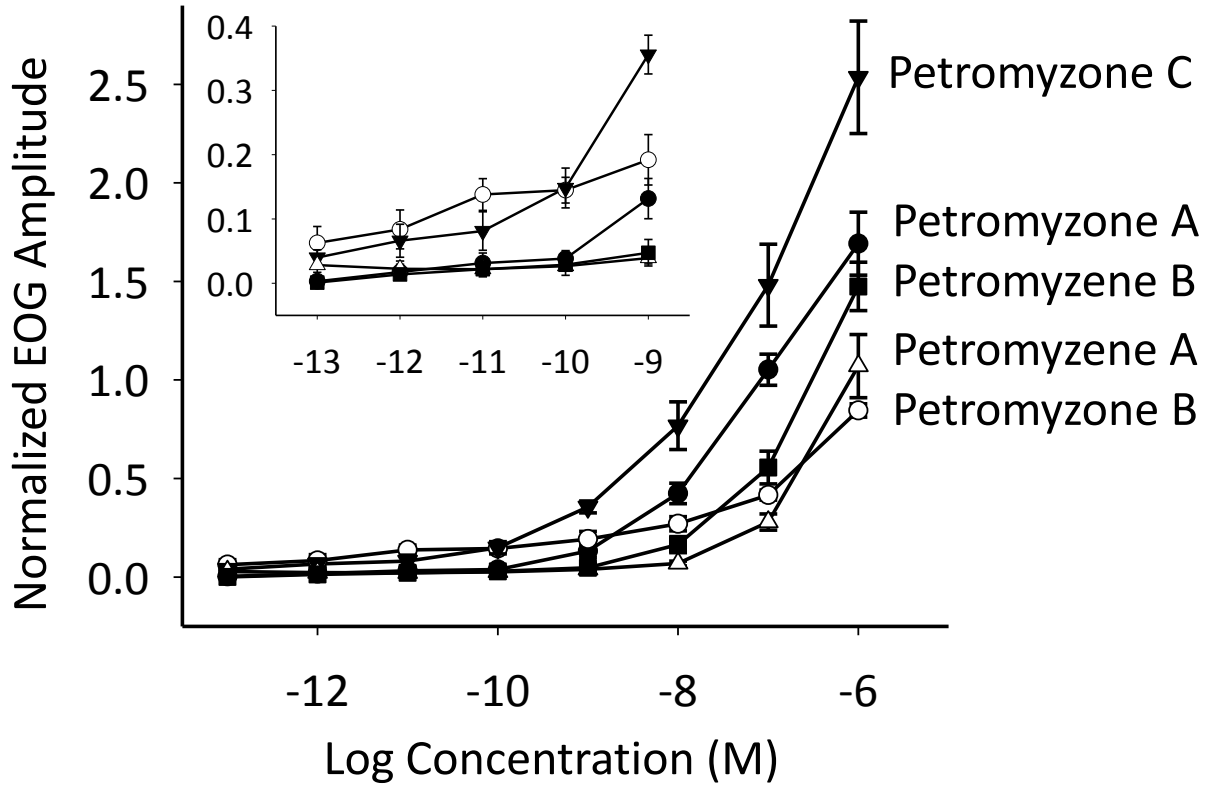

Figure 4-10. Semi-logarithmic plot of electro-olfactogram concentration response curves. Petromyzone A–C and petromyzene A and B were stimulatory to the adult sea lamprey olfactory epithelium and had low detection thresholds. Data are presented as the mean normalized EOG amplitude ± SEM. The sample sizes were as follows: petromyzone A, petromyzene A, and petromyzene B (n = 7); petromyzone B and petromyzone C (n = 5). Inset: Expanded view of EOG responses showing response threshold concentrations. This figure has been modified from Figure 3 in the reference (Li et al. 2017b) and Figure 4 in the reference (Li et al. 2017a).

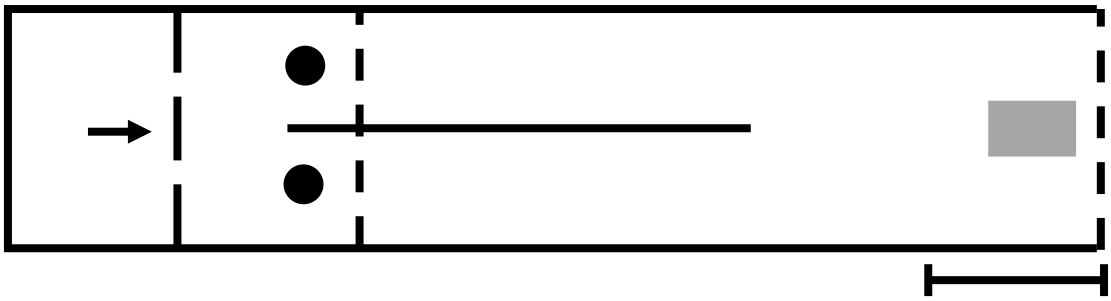

Figure 4-11. Schematic of two-choice maze used to evaluate behavioral responses of adult sea lampreys to odorants. Arrow represents the direction of water flow (0.07 m s^{-1} ± 0.01). The circles represent odorant administration points. The small dashed lines represent fine mesh used to restrict the movement of the sea lamprey to the confines of the maze. The large dashed lines represent flow boards used to reduce water turbulence. The gray rectangle represents the release cage. Scale bar = 1 m. This figure is from Figure S1 in the reference (Li et al. 2017a).

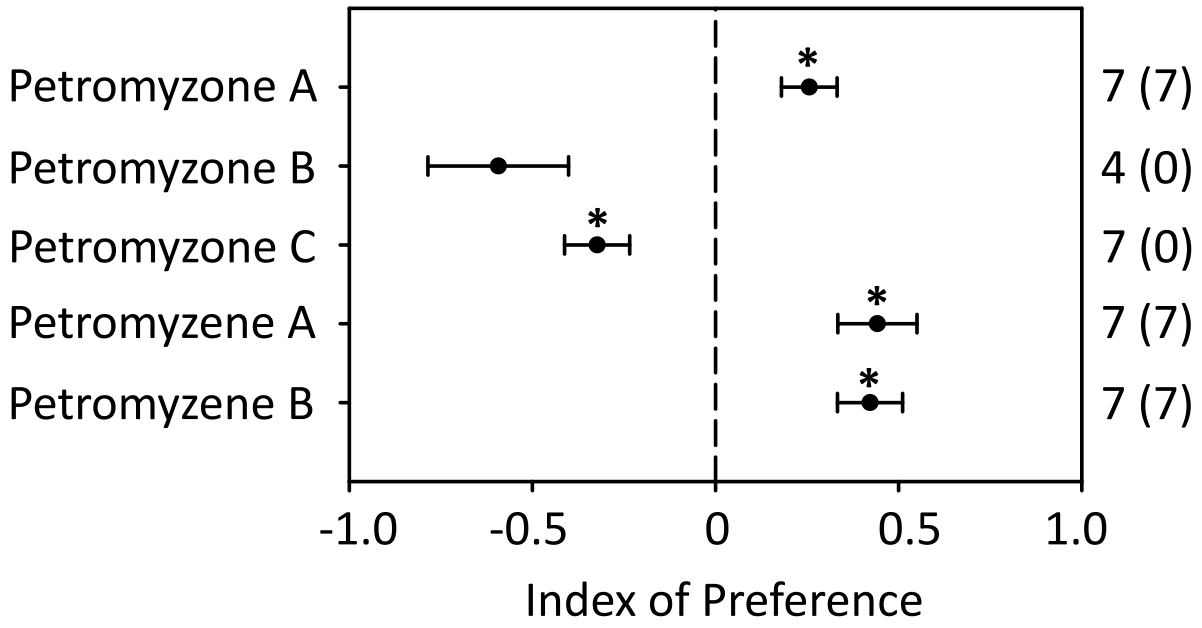

Figure 4-12. Ovulated female sea lamprey behavioral responses in a two-choice maze showing females were attracted to petromyzone A, petromyzene A, and petromyzene B. The time the lamprey spent in each channel of the maze before and after odorant exposure (10^{-12} M) was used to calculate an index of preference to assess its behavioral response to the odorant. A positive value of the index of preference indicates attraction. The sample size, n, is reported outside the parentheses and the number in the parentheses indicates the number of test subjects spending more time in the treatment side. Mean ± S.E.M. (*$p < 0.05$; Wilcoxon signed-rank test). This figure has been modified from Figure 4 in the reference (Li et al. 2017b) and Figure 5 in the reference (Li et al. 2017a).

DISCUSSION

Fish live in a chemical world full of compounds yet to be identified. Bioassay-guided fractionation has proven essential to identify and characterize the bioactive molecules that mediate many chemical interactions, such as those observed in masu salmon (Yambe et al. 2006), Asian elephants (Rasmussen et al. 1997), and sea lampreys (Fine and Sorensen 2008; Hoye et al. 2007; Sorensen et al. 2005). Bioassay-guided fractionation is an effective approach to accurately trace and pinpoint the bioactive compounds from the starting extract to the purified active compound. Using this approach, the identified bioactive compound can reveal a novel compound with a unique chemical skeleton that is unlikely to be predicted from the known biosynthetic pathways.

EOG recordings are conducted to determine which fractions or compounds elicit olfactory responses. Several technical considerations are imperative to accurately measure the olfactory responses to the putative pheromones with EOG recordings. First, in accordance with the Institutional Animal Care and Use Committee approved procedures, the fish must be deeply anesthetized with 3-aminobenzoic acid ethyl ester (MS222) and immobilized with an intra-muscular injection of gallamine triethiodide. The recording electrode will detect movement of the gills and heart beat due to insufficient immobilization, which can be a source of electrical noise. Second, the olfactory epithelium should be immediately exposed to charcoal-filtered water after the dissection to prevent desiccation. Adjusting the location of the recording and reference electrodes with the micromanipulators, the electrical ground, and odorant delivery tube can help maximize the response to the positive control odorant while minimizing response to the blank control (filtered water). Third, after identifying a sensitive recording location in the olfactory

epithelium, it is important to record from a similar position on the lamellae to minimize variation. To consistently record from a similar location, keep the tank and V-shaped plastic stand holding the fish, microscope, odorant delivery tube, and micromanipulators in the same position. The anesthetic tube must remain in the fish's buccal cavity for the duration of the experiment to ensure it remains anesthetized. However, the placement within the buccal cavity and the flow rate of the anesthetic can be modified if the recording electrode is detecting the movement of the water resulting in an erratic baseline of the electrical signal.

The design of the behavioral assay should be tailored to the behavioral ecology of the test subject and research question of interest. Two-choice maze behavioral assays are used to determine if any of the odorous fractions or compounds are also behaviorally active. Because purified compounds are often only available in minute amounts, the maze is a beneficial behavioral bioassay compared to the stream due to the lower discharge. We were interested in assessing the preference of females to putative sex pheromones released by mature males predicted to attract mature female mates in close proximity and to retain them on a nest for spawning. The behavioral assay was designed to replicate the natural conditions of an ovulated female choosing between the odorants of the mature males. Therefore, it was relevant for us to test ovulated females as the test subject in our behavior experiments. However, depending on the odorant tested, other test subjects (*i.e.*, different life stage or males) may be more appropriate. Variations on the dimensions of a two-choice maze may be necessary depending on the size of the test subject and the predicted active space of the pheromone (*i.e.*, near source versus long distance) (Buchinger et al. 2013). Likewise, behavioral responses are often concentration dependent. If a behavioral response is not observed when the putative pheromone is applied at the detection threshold concentration determined with EOG, the concentration of the pheromone

should be adjusted. However, it should be noted that even if a compound is a potent odorant, it may not necessarily induce a significant behavioral preference. Ultimately, the behavioral responses observed in the maze should be validated in a field setting with the synthetic compound to confirm the function of the putative pheromone.

One major limitation of bioassay-guided fractionation is the sequential testing of individual fractions or compounds in a bioassay (EOG or behavior). Previous work has shown insect sex pheromones are typically mixtures of multiple components at specific ratios (De Bruyne and Baker 2008) that function independently as components (Bradshaw et al. 1983) or synergistically as blends (Linn et al. 1987) to induce the appropriate responses in conspecifics. Therefore, compounds that are only active when present in specific mixtures may be overlooked with bioassay-guided fractionation because they require combinatorial tests to confirm the bioactivity. Other limitations of bioassay-guided fractionation include 1) A large quantity of starting material is necessary to have sufficient amount for structural analysis, EOG, and behavioral assays; 2) The process is time consuming due to the iterative purification process; and 3) Miniscule or unstable compounds are unlikely to be detected. In the future, overcoming some of the technical limitations of bioassay-guided fractionation may require a hybridized approach of bioassay-guided fractionation and metabolomics. Using a hybridized approach, additive or synergistic pheromone effects are more likely to be discerned (Kuhlisch and Pohnert 2015; Prince and Pohnert 2010) and unstable compounds are more likely to be detected.

The described bioassay-guided fractionation process is specifically designed for the identification of sea lamprey pheromones. However, chemical communication is ubiquitous in the animal kingdom (Wyatt 2014) and this process can be readily adapted to characterize the pheromones in a broad array of taxa. Pheromone identification and characterization is important

because pheromones can be applied to modulate behavioral responses resulting in the control of invasive species or restoration of imperiled native species.

ACKNOWLEDGMENTS

We thank the U.S. Geological Survey Hammond Bay Biological Station for use of research facilities and the staff of U.S. Fish and Wildlife Service and Fisheries and Oceans Canada for providing sea lampreys. This research was supported by grants from the Great Lakes Fishery Commission. My assistantship stipend was provided Great Lakes Fishery Commission with a grant to Dr. Leslie Kuhn and Dr. Weiming Li.

CONCLUDING REMARKS OF THE DISSERTATION

DISCOVERY OF A NEW CLASS OF PHEROMONE

In Chapter 1, a mechanism whereby females identify and seek potential mates through a sex pheromone emitted via seminal fluid of milt in sea lampreys was defined. Spermine is released by mature male sea lampreys through the emission of milt, induced olfactory responses at a sub-femtomolar concentration, and attracted ovulated females but not mature males. Ovulated females also showed a behavioral preference for milt that contains spermine. The olfactory detection of spermine and the subsequent behavioral responses were linked to the activation of two trace amine-associated receptors (TAARs) expressed in the olfactory epithelium of adult sea lampreys. Cyclen was identified as an antagonist to the spermine receptors. Cyclen reduced spermine olfactory responses and abolished female attraction to spermine.

Our results reveal an unexpected source and function of a pheromone present in milt. The identification of spermine in milt added a new chemical template to the repertoire of sea lamprey pheromones. The previously characterized sea lamprey pheromones include migratory cues excreted by larvae and sex pheromones released across the gills of spermiated males (Buchinger et al. 2015). Most of these compounds are bile acids with steroid scaffolds which differ from a polyamine like spermine, which is a linear organic compounds with amino groups. In addition, a novel secondary benefit of releasing gametes was identified; that is to recruit additional mates. It had been previously documented that semen is a source of chemical cues. In some insect species, males release anti-aphrodisiacs with their sperm to deter promiscuity and reduce sexual attractiveness of copulated females (Gilbert 1976; Kukuk 1985; Mann and Lutwak-Mann 1981). However, we found that a compound released with sperm rather than

functioning as an anti-aphrodisiac instead attracted additional mates and likely mediates interactions in spawning aggregations. We speculate that spermine is critical to male fertility and sperm mobility in sea lampreys, as has been shown in mammals (Stanger and Quinn 1982), and thus is a reliable indicator for sperm quality and quantity. This research added a new dimension to the study of milt compounds because we also de-orphanized two trace amine-associated receptors that are activated by spermine. These data have advanced the understanding of pheromones and further illustrate sea lamprey as a model to study pheromone communication. The discovery of spermine present in milt may instigate additional studies on the presence of pheromones in semen in sea lamprey and other animal taxa.

Unexpectedly, spermiated males were attracted to milt compared to the vehicle, but they showed no preference to 10^{-12} M spermine (Chapter 1). Other males may detect milt odors to cue in on spawning habitat and spawning activities, as male sea lamprey odorants are known to attract males en route to spawning grounds (Brant et al. 2015b); however, the milt chemical cues that attract males have not been identified. If the structure and biological responses of the compounds in milt that attract males are identified, this may provide insights on the function of chemical cues that mediate the inter- and intra-sexual interactions in the sea lamprey lek-like spawning aggregations.

CHARACTERIZING THE DIVERSITY OF SEA LAMPREY PHEROMONES

The sea lamprey is an ideal vertebrate model to study pheromone communication because this jawless fish relies heavily on the olfactory detection of chemical cues to mediate its anadromous life cycle. Bioassay-guided fractionation is an effective and interdisciplinary

approach to isolate and characterize the structure, olfactory potency, and behavioral response of putative pheromone compounds of sea lampreys (Chapter 4). This iterative approach is driven by the results of electrophysiological and behavioral bioassays that guide the isolation, purification, and identification of odorous and behaviorally active pheromone compounds. Eight novel bile salts or bile alcohols released from spermiated male sea lampreys were isolated and characterized using bioassay-guided fractionation (Appendices A−C). These eight compounds elicited concentration dependent olfactory responses with low detection thresholds typical of sea lamprey pheromones or cues. Most of the behavioral assay results from Appendices A−C are also consistent with previous pheromone studies (Johnson et al. 2009; Li et al. 2002). Low concentrations ($\sim 10^{-12}$ M) of most of the putative pheromones or cues elicited behavioral preferences in ovulated females. Bioassay-guided fractionation could be implemented to identify additional pheromones such as the unknown components of milt that attracted spermiated males.

While the maze assays are useful as an initial behavioral experiment to assess the preference for an odorant, the maze cannot replicate all of the physical, physiological, and social contexts associated with the natural stream environment (Johnson and Li 2010). To affirm the function of each of these putative pheromones, in stream assays under natural field conditions should be conducted. In addition, in stream experiments with side-by-side odorant sources should compare the full suite of sex pheromone components found in water conditioned with mature male sea lampreys versus a re-assembled pheromone blend containing the previously characterized male pheromone components (3kPZS, DkPES, and PAMS-24), the newly identified eight novel bile salts/alcohols, and spermine. Results from these experiments may provide an indication if there are additional male pheromone components in water conditioned with mature male sea lampreys yet to be identified. If the in stream behavioral results confirm

that the eight bile salts/alcohols characterized in Appendices A−C are pheromones or cues, they could serve as targets to identify additional antagonists.

Baiting sea lamprey traps with pheromones has been shown to increase trap efficacy. The addition of some of the eight novel bile salts or bile alcohols (Appendices A−C) or spermine (Chapter 1) released from spermiated male sea lampreys may further increase efficacy. 3kPZS has been shown to facilitate the trapping of adult sea lampreys, but its current applicability is limited. In a management scenario test, 3kPZS was applied at a final in stream concentration of 10^{-12} M to 18 streams with existing sea lamprey barrier integrated traps over three years. The baited traps captured on average 8% more adult sea lampreys than control traps (Johnson et al. 2013). The effectiveness varied substantially among streams, with the largest increase in capture rate found in streams with a low density of spawning adults. A follow-up study found sea lamprey traps placed in streams en route to the spawning grounds that were baited with water conditioned with mature male sea lampreys captured significantly more lampreys than the paired 3kPZS-baited traps (~10% increase) (Johnson et al. 2015b). These results suggest the addition of some of the eight novel bile salts or bile alcohols released from spermiated male sea lampreys characterized in Appendices A−C may increase catches when applied to traps placed en route to spawning grounds, especially in streams with a low density of spawning adults and limited spawning habitat.

IDENTIFICATION OF PHEROMONE ANTAGONISTS

Our results in Chapters 2 and 3 represent steps forward in utilizing pheromone antagonists as environmentally benign tools for reducing the reproductive success and

controlling invasive sea lampreys in the Great Lakes. Blocking the olfactory response to pheromones will likely disrupt spawning. In Chapter 2, PZS was identified as a naturally occurring inhibitor released by conspecifics that disrupted the olfactory and behavioral responses of female sea lampreys to 3kPZS, a sex pheromone component known to be indispensable for sea lampreys to successfully mate. PZS reduced the 3kPZS olfactory responses in a concentration dependent manner and reduced the behavioral preference for 3kPZS in the maze and in a natural spawning environment. These results represent the identification of the first vertebrate pheromone antagonist. Based on the results of virtual screening (Raschka et al. 2018), the biological assays described in Chapter 3 confirmed 3sPZS is a potent and effective synthetic 3kPZS antagonist that alters the electrophysiological and behavioral response of female sea lamprey to 3kPZS. 3sPZS selectively reduced the 3kPZS olfactory responses and induced avoidance when mixed with 3kPZS at a 1:1 ratio in the maze. 3sPZS reduced upstream movement, entry, and retention in artificial nest baited with 3kPZS in a field bioassay in a natural spawning environment. Four other polysulfated petromyzonol compounds when mixed with 3kPZS neutralized the attraction of ovulated females to 3kPZS in the maze. The neutralizing effect of these four compounds on 3kPZS-induced behavioral responses should be further investigated in a natural spawning environment.

Additional experiments should be conducted to further assess the utility of pheromone antagonists for sea lamprey control. Firstly, an effective formula of antagonists needs to be identified. The effective formula of antagonists should disrupt the upstream movement, nest approach, and spawning behaviors of ovulated females in response to pheromones. Based on our observations, PZS (Chapter 2) and 3sPZS (Chapter 3) when tested separately reduced upstream movement of ovulated females and averted most but not all females from entering the artificial

3kPZS-baited nest in a natural stream environment without spawning lampreys. In Chapter 1, cyclen was identified as an antagonist to the spermine receptors and abolished the female attraction to spermine in the maze. This experiment should be replicated in a spawning stream to determine if this compound should also be included in a formula of sea lamprey antagonists. After identifying an effective formula of antagonists that works in a stream lacking background pheromones (*i.e.*, above a sea lamprey barrier and only contains the females released in experiments), it would be necessary to determine the efficacy of the optimized antagonist formula in a natural spawning population below a sea lamprey barrier. Ideally, the optimized antagonist formula would prevent females from locating, interacting, and spawning with males and in effect halt reproduction of sea lamprey at the spawning grounds similar to results observed with naris plugged females (Johnson 2005). Understanding the mechanisms whereby the antagonists interact with pheromone receptors and subsequently alter olfactory responses to each pheromone component may facilitate the development of more effective antagonists.

Pheromone antagonists for management application should be versatile and easy to apply because they are not limited by the presence of infrastructure like other sea lamprey control efforts (*i.e.*, traps and barriers). Pheromone antagonists can likely be implemented to 1) disrupt sea lamprey mating at the spawning grounds; 2) re-direct sea lampreys away from rivers where lampricide application is prohibitively expensive; 3) re-direct sea lampreys into specific locations with poor habitat that would reduce fitness; and 4) enhance selective fish passages by selectively blocking sea lamprey upstream movement while allowing other valued species to pass. Together with other adult control initiatives, such as pheromone baited-trapping (Johnson et al. 2013), electrical guidance (Johnson et al. 2014a), or alarm substance application (Hume et al. 2015), we predict that it is achievable to reduce sea lamprey reproductive output.

CONCLUSION

In conclusion, my dissertation advances sea lamprey as a model for basic and applied pheromone research. The sea lamprey is a basal vertebrate with a well-developed olfactory system that detects a diversity of chemical cues and pheromones that have been further elucidated in this dissertation. Furthermore, the sea lamprey is a useful organism to study the behavioral ecology and evolution of multi-component chemical signals. The sea lamprey pheromone and pheromone antagonist research presented in the dissertation can help guide an effective sea lamprey control program in the Laurentian Great Lakes to sustain viable fisheries, rehabilitate the native biodiversity, and maintain a healthy ecosystem.

APPENDICES

APPENDIX A

BILE SALT-LIKE DIENONES WITH A NOVEL SKELETON OR A RARE SUBSTITUTED PATTERN FROM MALE SEA LAMPREY FUNCTION AS CHEMICAL CUES

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Bile Salt-like Dienones Having a Novel Skeleton or a Rare Substitution Pattern Function as Chemical Cues in Adult Sea Lamprey. Ke Li, Anne M. Scott, Cory O. Brant, Skye D. Fissette, Joseph J. Riedy, Thomas R. Hoye, and Weiming Li Organic Letters 2017 19 (17), 4444-4447

DOI: 10.1021/acs.orglett.7b01921.

The abstract included in this Appendix is from the original paper. The remainder of the Appendix only briefly highlights the chemistry procedures and results from extraction, isolation, purification, and structure elucidation of the original paper conducted by collaborators Dr. Ke Li (Michigan State University) and Dr. Tom Hoye (University of Minnesota). The Appendix focuses primarily on the biology portions of the original paper that I contributed.

ABSTRACT

Two novel sulfated bile salt-like dienones, featuring either a unique, rearranged side chain or a rare *cis*-11,12-diol on the steroidal B-ring, herein named petromyzene A (**1**) and B (**2**), respectively, were isolated from water conditioned with spawning male sea lamprey (*Petromyzon marinus*; a jawless vertebrate animal). The structures of these natural products were elucidated by mass spectrometry and NMR spectroscopy. Petromyzene A and B exhibited high olfactory potency for adult sea lamprey and strong behavioral attraction for spawning females.

INTRODUCTION

Bile salts are biosynthesized from cholesterol by a sequence of enzymatic modifications in vertebrate animals (Russell and Setchell 1992). The intermediates and the end products involved in the biosynthetic pathways show common structural features ─ a steroidal tetracyclic core (rings A-D) with a carbon upper side chain to form a skeleton containing 24 or 27 carbon atoms in total. Structural variants of bile salts, differing in the oxidation patterns throughout the carbon skeleton, have different functional roles in a variety of organisms (Martinez et al. 1998; Setchell et al. 1988).

Bile salt derivatives are known to be pheromones in sea lamprey (*Petromyzon marinus*) (Li et al. 2002), whose invasion represents a major ecological disaster in the Laurentian Great Lakes. This pheromone system offers the potential for a selective and environmentally benign approach for control of sea lamprey. We have previously reported five sulfated bile alcohol derivatives—namely, 3-keto petromyzonol sulfate (3kPZS, **3**) (Li et al. 2002), 3,12-diketopetromyzonene sulfate (DkPES, **4**) (Li et al. 2013), and petromyzone A-C (Li et al. 2017b)—in an extensive investigation of pheromones emitted by spawning male sea lamprey. All of these reported compounds conform to the basic skeleton of bile salts. Here we report (i) a bile salt-like dienone with a novel carbon skeleton petromyzene A (**1**) as well as (ii) a bile salt derivative with a rare hydroxylation pattern petromyzene B (**2**) (Figure A-1). We describe the olfactory and behavioral responses induced by **1** and **2** in adult sea lampreys.

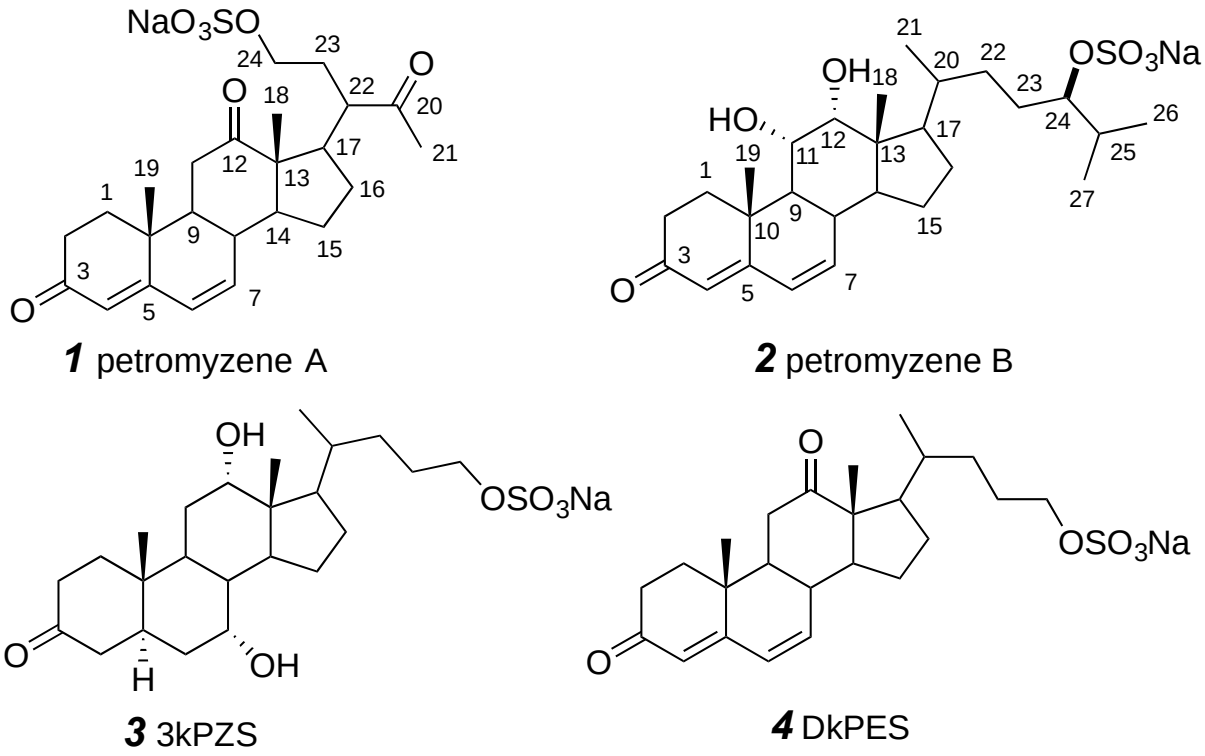

Figure A-1. Chemical structures of petromyzene A (1) and B (2), 3kPZS (3) and DkPES (4).

RESULTS

Petromyzene A (**1**) and B (**2**) were identified in water conditioned with spawning male sea lampreys (Figure A-1). The chemical extraction, isolation, purification, and structure elucidation of **1** and **2** were performed and the results were interpreted by collaborators Dr. Ke Li (Michigan State University) and Dr. Tom Hoye (University of Minnesota). The structures of these natural products were elucidated by mass spectrometry and NMR spectroscopy. The molecular formula of **1** was determined to be $C_{24}H_{32}O_7S$ (*m*/*z* 463.1813) and **2** was determined to be $C_{27}H_{42}O_7S$ (*m*/*z* 509.2592). Results of the chemical analyses are described in detail in the resulting publication (Li et al. 2017a).

Petromyzene A (**1**) and B (**2**) were both potent odorants that stimulated the adult sea lamprey olfactory epithelium in a concentration dependent manner, as measured in electro-olfactogram (EOG) assays (Figure A-2). The threshold of detection, the lowest concentration that elicited an olfactory response greater than the blank water control, for **1** was less than 10^{-13} M and for **2** was 10^{-11} M (paired *t* test, $p < 0.05$).

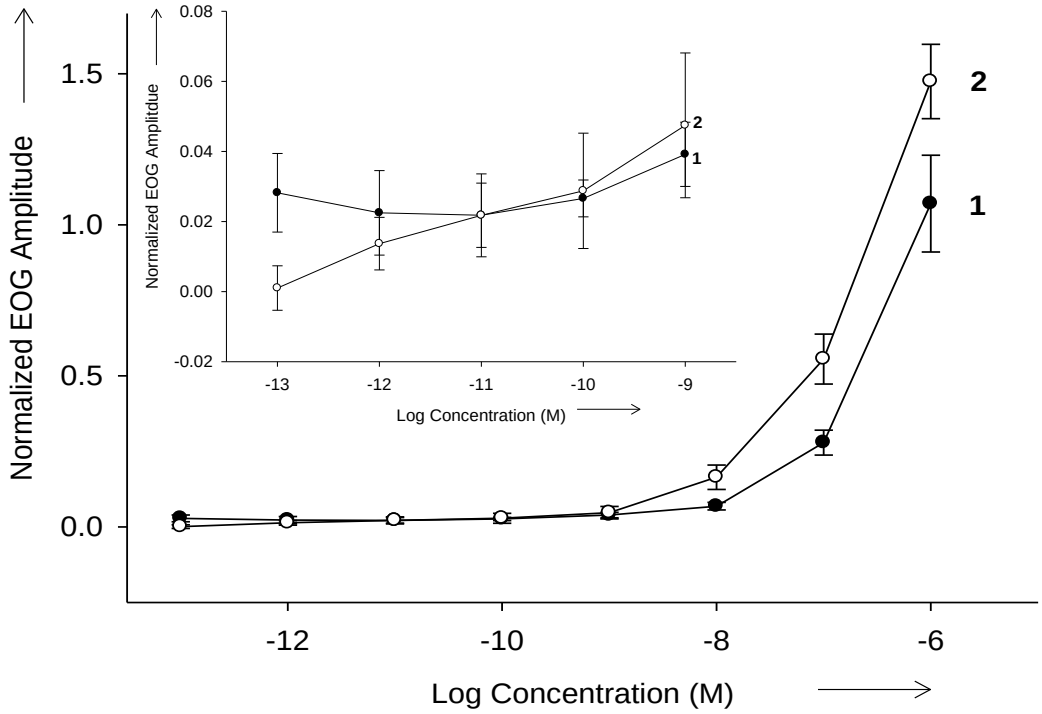

Figure A-2. Semi-logarithmic plot of electro-olfactogram (EOG) concentration response curves shows that 1 and 2 (petromyzene A and B, respectively) are stimulatory to the adult sea lamprey olfactory epithelium and have low detection thresholds. The numbers on the right of the figure correspond to each compound. Data are presented as the mean normalized EOG amplitude (n = 7). Vertical bars represent one standard error of the mean. Inset: Expanded view of responses showing response threshold concentrations.

Petromyzene A (**1**) and B (**2**) also attracted all spawning-phase females tested in two-choice behavioral trials. Of the 7 females assayed for each odorant, all 7 spent more time in the channel with compound **1** or **2** compared to the adjacent channel treated with the vehicle (50% methanol). Females were attracted to **1** at 10^{-12} M (mean index of preference ± S.E., 0.442 ± 0.108, n = 7) and to **2** at 10^{-12} M (mean index of preference ± S.E.; 0.422 ± 0.089, n = 7) (Wilcoxon signed-rank test, $p < 0.05$; Figure A-3). These levels of olfactory potency and behavioral responses suggest strongly that **1** and **2** are male pheromones.

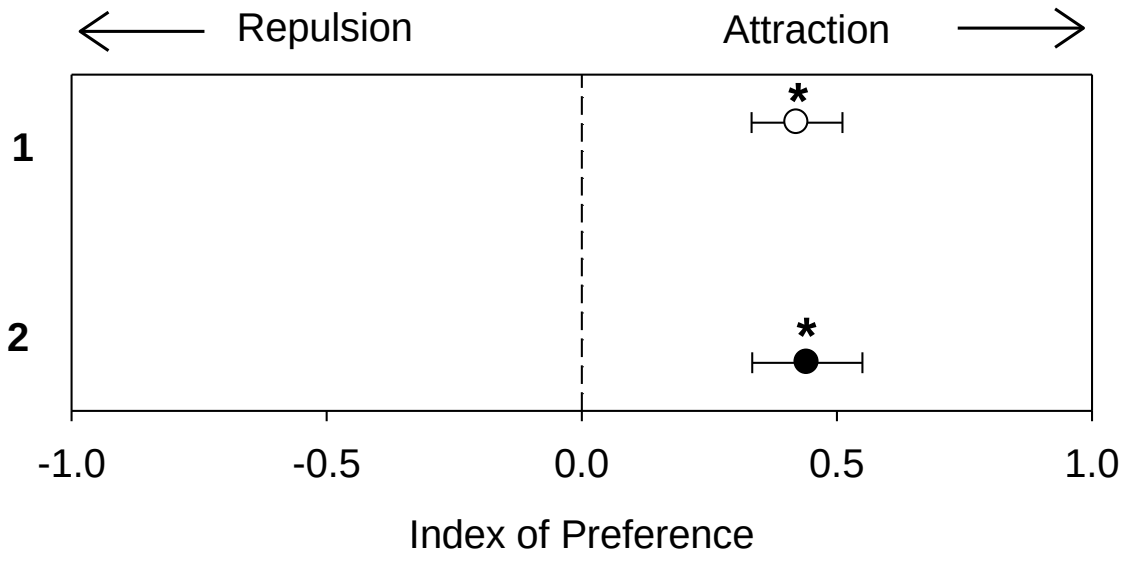

Figure A-3. Spawning phase female sea lampreys were attracted to petromyzene A and B (compound 1 and 2, respectively) in a two-choice maze. The time the lamprey spent in each channel of the maze before and after odorant exposure (10^{-12} M) was used to calculate an index of preference to assess its behavioral response to the odorant. A positive value of the index of preference indicates attraction. Data are presented as the mean ± S.E.M. (n = 7) and were evaluated using a Wilcoxon signed-rank test. ∗, $p < 0.05$.

In conclusion, two novel bile salts have been identified from the washing of sexually mature male sea lamprey. Among natural steroids, compound **1** possesses unprecedented connectivity within its side chain. Compound **2** contains a rarely occurring oxygenation pattern. Compounds **1** and **2** represent new additions to the structure diversity of the steroid family. Notably, the isolation of **1** and **2** from spawning-phase males and the behavior modification by **1**and **2** of spawning-phase females indicate that these two novel molecules may play an essential role in sea lamprey reproduction.

METHODS

Animals

All procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (IACUC) (Animal usage form number: 03/14-054-00). Adult sea lampreys were captured in tributaries of the Laurentian Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada according to approved scientific collection permits from those government agencies, transported to the U.S. Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, USA, and held in 500 or 1000 L aerated flow-through tanks maintained at 15–19 ^{o}C. Adult sea lamprey used for olfactory functional studies were transported to the University Research Containment Facility at Michigan State University, East Lansing, Michigan, USA. Sea lampreys were held in flow-through tanks (250 L) supplied with aerated, chilled well water maintained at 7–9 ^{o}C. Electro-olfactogram recordings were conducted in April and May 2016.

To produce sexually mature female test subjects for behavioral assays conducted in June and July 2016, immature female sea lampreys were transferred to acclimation cages constructed on polyurethane mesh and PVC pipe (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan to allow natural maturation. Sea lampreys were monitored daily for signs of sexual maturation including secondary sexual characteristics and expression of ovulated oocytes from the cloacal aperture following gentle pressure to the abdomen.

Extraction, isolation, purification, and structure elucidation

Details regarding the protocol for chemical extraction, isolation, purification, and structure elucidation of **1** and **2** are described in the resulting publication (Li et al. 2017a). Skye Fissette and Dr. Cory Brant collected the water conditioned with mature male sea lampreys. Dr. Ke Li (Michigan State University) and Dr. Tom Hoye (University of Minnesota) carried out the chemistry analyses.

Electro-olfactogram (EOG) recording

Electro-olfactogram recordings were conducted following a previously described procedure to determine the adult sea lamprey olfactory sensitivity to compounds **1** and **2** (Li et al. 2013). Sea lampreys for EOGs (243.9 ± 17.0 g, 502.4 ± 12.8 mm; mean ± S.E.M.) were anesthetized with 3-aminobenzoic acid ethyl ester (100 mg L^{-1}; MS222, Sigma-Aldrich, St. Louis, Missouri, USA), immobilized with an intra-muscular injection of gallamine triethiodide (3 mg/kg of body weight, in 0.9% saline; Sigma-Aldrich), and placed in a V-shaped plastic stand. Gills were continuously irrigated with aerated water containing 50 mg•L^{-1} tricaine methanesulfonate (MS222) throughout the experiment. The olfactory lamellae were surgically exposed by removing the skin on the surface of the olfactory capsule. A small capillary tube delivered the stimuli and charcoal-filtered water was continuously effused to the olfactory epithelium by gravity flow to prevent desiccation. The differential EOG response to each test stimulus was recorded using borosilicate electrodes filled with 0.04% agar in 0.9% saline connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, Hamden, Connecticut, USA) in 3M KCl. The recording electrode was placed between two olfactory lamellae and adjusted to maximize the response to $_{L}$-arginine standard while

minimizing the response to the blank control (the charcoal-filtered water). The reference electrode was placed on the external skin near the naris. Electrical signals were then amplified by a NeuroLog system (model NL102, Digitimer Ltd., Hertfordshire, England, United Kingdom), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized by Digidata 1440A (Molecular Devices LLC, Sunnyvale, California, USA), and recorded on a PC running AxoScope 10.4 software (Molecular Devices LLC).

For recording the concentration-response curves, the olfactory epithelium of the sea lamprey was exposed to 10^{-13} M to 10^{-6} M solutions of purified petromyzene A (**1**) and B (**2**). Stock solutions (10^{-3} M in 50% MeOH: deionized water) were prepared for each compound. These were stored at –20 ^{o}C and serially diluted with charcoal-filtered water to yield 10^{-13} M to 10^{-6} M working solutions. A 10^{-2} M stock solution of L-arginine (in deionized water) was prepared, stored at 4 ^{o}C, and then diluted with charcoal filtered water to yield a 10^{-5} M working solution. A 10^{-5} M $_{L}$-arginine standard was introduced to the olfactory epithelium for 4 s and the olfactory response was recorded as a reference. Then, the olfactory epithelium was flushed with charcoal-filtered water for 2 min. Subsequently, the blank control (charcoal-filtered water) was introduced to the olfactory epithelium and recorded to confirm the absence of a response in the charcoal-filtered water supply. Next, increasing concentrations of the test stimulus starting at 10^{-13} M to 10^{-6} M was applied to the olfactory epithelium, recorded, and flushed. Blank control and 10^{-5} M L-arginine standard was measured after every two concentrations. The EOG response magnitudes were measured in millivolts, normalized relative to 10^{-5} M L-arginine (2.910 ± 0.065; absolute raw value mean ± S.E), and blank-corrected (0.13 1± 0.004; absolute raw value mean ± S.E) as defined in Equation (1).

$$\text{Normalized EOG Amplitude} = [(Rt - Rb) / (Ra - Rb)] \qquad \text{(eq 1)}$$

where Rt is the response magnitude to the test stimulus, Rb is the response magnitude to the blank, and Ra is the response magnitude to L-arginine at 10^{-5} M. The L-arginine standard and blank control responses were comparable to previous studies (Fine et al. 2006; Li et al. 2013). The detection threshold was defined as the lowest concentration where the test stimulus elicited a larger response than the blank control (paired t-test, one tailed).

Behavioral assay

The preference of spawning female sea lamprey to 10^{-12} M purified petromyzene A (**1**) and B (**2**) was evaluated using a two-choice maze as previously described (Buchinger et al. 2013). A single lamprey was introduced to the furthest point downstream in the maze in a release cage. After 5 min of acclimation in the cage, the lamprey was released and the cumulative amount of time the lamprey spent in each channel was recorded. This time period before odorant application was used to assess side bias. After 10 min of recording, the test stimulus was introduced to a random channel and 50% methanol control (MeOH: deionized water) was introduced to the other channel using peristaltic pumps at constant rates of 200 ± 5 mL•min^{-1} (Masterflex 07557-00, Cole-Parmer, Vernon Hills, Illinois, USA). The odorants were pumped into the maze for 5 min without recording the lamprey's behavior. After the 5 min period, the behavior was recorded for an additional 10 min while odorants were continuously administered. The maze was flushed with water for 10 min before the start of the next experiment.

The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were recorded. An index of preference was calculated for each trial as defined in Equation (2).

$$\text{Index of preference} = [Ae/(Ae+Be) - Ac/(Ac+Bc)] \qquad \text{(eq 2)}$$

The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$). A positive value of the index of preference indicates attraction. The trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10 s during the 10 min period before the odorant was applied as this is an indication of strong side bias or inactivity.

ACKNOWLEDGMENTS

The staff of U.S. Geological Survey Hammond Bay Biological Station who provided research facilities are acknowledged. The U.S. Fish and Wildlife Service and Fisheries and Oceans Canada collected the sea lamprey. This work was supported by grants from the Great Lakes Fishery Commission to Dr. Weiming Li, Dr. Ke Li and Dr. Cory Brant. Dr. Tom Hoye was supported by National Institute of General Medical Sciences of the NIH (R01 GM108762). My assistantship stipend was provided Great Lakes Fishery Commission with a grant to Dr. Leslie Kuhn and Dr. Weiming Li.

APPENDIX B

THREE NOVEL BILE ALCOHOLS OF MATURE MALE SEA LAMPREY (PETROMYZON MARINUS) ACT AS CHEMICAL CUES FOR CONSPECIFICS

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Li, K., Scott, A.M., Riedy, J.J. et al. J Chem Ecol (2017) 43: 543.

https://doi.org/10.1007/s10886-017-0852-x, 2017.

The abstract included in this Appendix is from the original paper. The remainder of the Appendix only briefly highlights the chemistry procedures and results from the extraction, isolation, purification, and structure elucidation of the original paper conducted by a collaborator Dr. Ke Li (Michigan State University). The Appendix focuses primarily on the biology portions of the original paper that I contributed.

ABSTRACT

Sea lamprey, *Petromyzon marinus*, rely heavily on chemical cues that mediate their life history events, such as migration and reproduction. Here, we describe petromyzone A–C (**1**–**3**), three novel bile alcohols that are highly oxidized and sulfated, isolated from water conditioned with spermiated male sea lamprey. Structures of these compounds were unequivocally established by spectroscopic analyses and by comparison with spectra of known compounds. Electro-olfactogram recordings showed that **1** at 10^{-11} M was stimulatory to the adult sea lamprey olfactory epithelium, while **2** and **3** were stimulatory at 10^{-13} M. Behavioral assays indicated that **1** is attractive, **2** is not attractive or repulsive, and **3** is repulsive to ovulated female sea lampreys. The results suggest that these compounds may be putative pheromones or cues that mediate chemical communication in sea lamprey. The identification of these three components enhances our understanding of the structures and functions of sex pheromone components in this species and may provide useful behavioral manipulation tools for the integrated management of sea lamprey, a destructive invader in the Laurentian Great Lakes.

INTRODUCTION

The ecosystem of the Laurentian Great Lakes has been drastically altered by invasions of non-indigenous fishes during the last century (Herborg et al. 2007; Kolar and Lodge 2002; Mills et al. 1993; Pimentel et al. 2005; Rixon et al. 2005). Invasive species have caused great economic and ecological damage, thus motivating efforts to minimize or eliminate these threats (Pimentel et al. 2005). The arrival of sea lamprey, *Petromyzon marinus* L., a destructive jawless vertebrate, through shipping canals in the early 1900s contributed to the extirpation of several native fish species, including the top predator, lake trout (*Salvelinus namaycush*) from three of the five Great Lakes (Smith and Tibbles 1980). A plethora of strategies including lampricide application (Johnson 2014), physical removal by trapping (Holbrook et al. 2016), and construction of barriers to prevent access to spawning habitat (Lavis et al. 2003; Swink 1999) have been applied as part of an integrated management effort to control the invasive sea lamprey population and, therefore, to foster the recovery of the ecology of the Great Lakes. Application of chemical cues to manipulate adult sea lamprey behavior is among the options considered for new alternative sea lamprey control techniques in the Great Lakes. An impediment to the application of pheromones in the integrated sea lamprey management program stems from the lack of understanding of the structures and functions of sea lamprey sex pheromones (Twohey et al. 2003).

Sea lampreys rely heavily on chemical cues that mediate their anadromous life history to coordinate migration and reproduction. Stream resident larvae excrete larval pheromones that lure migratory adult sea lampreys from the Great Lakes to streams with suitable spawning habitat (Sorensen et al. 2005; Wagner et al. 2006; Wagner et al. 2009). After entering spawning streams,

sex pheromones are released by spermiated males and guide females upstream over long distances to nests (Johnson et al. 2009; Li et al. 2002; Siefkes et al. 2005). Previous research has demonstrated that a main component of the male sex pheromone, 7α,12α,24-trihydroxy-3-one-5α-cholan-24-sulfate (3kPZS) (Li et al. 2002), lures ovulated female sea lamprey upstream (Johnson et al. 2009). In addition to 3kPZS, spermiated males also release other bile salts in lower quantities including 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) (Li et al. 2013). A mixture that reconstructs the spermiated male-released component ratio of 3kPZS and DkPES of 30:1 (3kPZS: DkPES, molar: molar) was more effective than 3kPZS alone in attracting and retaining ovulated females in artificial nests (Brant et al. 2016). However, behavioral studies have indicated that the two component mixture of 3kPZS and DkPES does not induce all of the characterized behaviors elicited by water conditioned with spermiated male sea lamprey that contains the full suite of pheromone components (Johnson et al. 2009). Water conditioned with spermiated male sea lamprey resulted in greater attraction to the odor source, prolonged retention, and increased display of nesting behaviors such as tail fanning and rock movement in ovulated females in a quasi-natural in stream bioassay (Johnson et al. 2012).

These behavioral studies indicate spermiated male sea lamprey release other, unknown sex pheromone components that elicit these additional behaviors. It has been well documented that sex pheromones in insect are typically comprised of unique mixtures of multiple components at specific ratios (Cardé and Haynes 2004; De Bruyne and Baker 2008) that function independently (Bradshaw et al. 1983) or synergistically (Linn et al. 1987) to induce multiple behavioral responses in conspecific mates. Likewise, the male sea lamprey sex pheromone suite is hypothesized to comprise multiple components, some of which have yet to be characterized, and which have multiple functions.

In this study, we isolated and characterized the bioactivity of three novel compounds, petromyzone A−C, (**1**–**3**, respectively; Figure B-1) from water conditioned with spermiated male sea lamprey, each possessing a bile salt backbone. Here, we report the structure, olfactory potency, and behavioral activity of these three compounds.

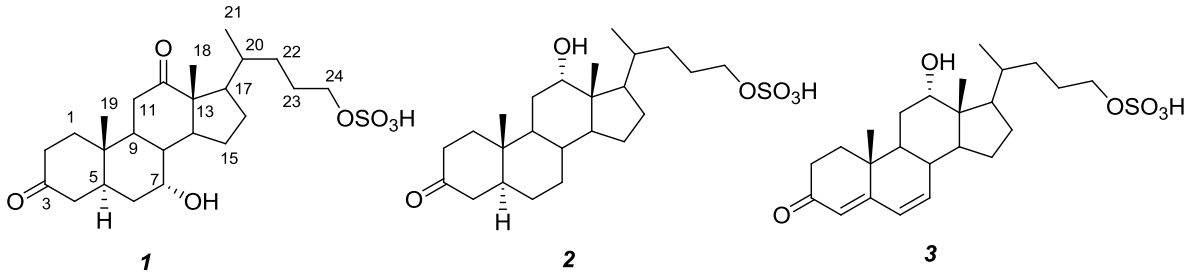

Figure B-1. Chemical structures of petromyzone A−C (1−3), respectively.

METHODS AND MATERIALS

Animals

All procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (Animal usage form number: 03/14-054-00). Adult sea lampreys were captured in tributaries of the Laurentian Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada according to approved scientific collection permits from those government agencies, transported to the U.S. Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station, Millersburg, Michigan, USA, and held in 1000 L capacity aerated flow through tanks supplied with Lake Huron water maintained at 15–19 ^{o}C. Adult sea lampreys used for electro-olfactogram recordings (weight: 238.5 g ± 15.1, length: 50.0 cm ± 1.1; mean ± S.E.) were transported to University Research Containment Facility at Michigan State University, East Lansing, Michigan, USA. Sea lampreys were held in flow through tanks (250 L) supplied with aerated, chilled well water maintained at 7–9 ^{o}C. Electro-olfactogram recordings were conducted in April and May 2016.

To produce ovulated female test subjects for behavioral assays conducted in June and July 2016, immature female sea lamprey were transferred to cages constructed on polyurethane mesh and polyvinyl chloride (PVC) pipes (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan to allow natural maturation. Sea lampreys were monitored daily for signs of sexual maturation including secondary sexual characteristics and expression of ovulated oocytes from the cloacal aperture following gentle manual pressure to the abdomen. Ovulated females were removed from the in-stream cages, returned to Hammond Bay Biological Station, and then transferred to the site of the behavioral assay for experimental use.

Extraction, isolation, purification, and structure elucidation

Details regarding the protocol for chemical extraction, isolation, purification, and structure elucidation of compounds **1**–**3** are described in the resulting publication (Li et al. 2017b). Skye Fissette collected the water conditioned with mature male sea lampreys and the chemistry analyses were carried out by Dr. Ke Li (Michigan State University) and Zoe Middleton under the direction of Dr. Ke Li.

Electro-olfactogram (EOG) recording

Electro-olfactogram recordings were conducted following the procedure as described previously (Li et al. 2013) to determine the olfactory concentration-response relationship and sensitivity to purified **1**, **2**, and **3**. Sea lamprey were anesthetized (100 mg/L 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich), immobilized (gallamine triethiodide 3 mg/kg of body weight, in 0.9% saline; Sigma-Aldrich), and placed in a V-shaped plastic stand. Gills were continuously irrigated with aerated water containing 50 mg/L MS222 throughout the experiment. The olfactory lamellae were surgically exposed. A small capillary tube delivered the stimuli and charcoal filtered water to the olfactory epithelium by gravity flow to prevent desiccation. The differential EOG response to each test stimulus was recorded using borosilicate electrodes connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, Hamden, Connecticut, USA). The recording electrode was placed between two olfactory lamellae and adjusted to maximize the response to $_{L}$-arginine standard while minimizing the response to the blank control (charcoal-filtered water), and the reference electrode was placed on the external skin near the naris. Electrical signals were then amplified by a NeuroLog system (model NL102, Digitimer Ltd., Hertfordshire, England, United Kingdom), filtered with a low-

pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized by Digidata 1440A (Molecular Devices LLC, Sunnyvale, California, USA), and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC).

For concentration-response relationships, the responses of the olfactory epithelia of sea lamprey exposed to 10^{-13} M to 10^{-6} M solutions of purified **1**, **2**, and **3** were recorded. 10^{-3} M stock solutions (in 50% MeOH: deionized water) were prepared for each compound, stored at −20 °C, and then serially diluted with charcoal filtered water to yield 10^{-13} M to 10^{-6} M working solutions. A 10^{-2} M stock solution of L-arginine (in deionized water) was prepared, stored at 4 °C, and then diluted with charcoal filtered water to yield a 10^{-5} M working solution. A 10^{-5} M L-arginine standard was introduced to the olfactory epithelium for 4 s and the olfactory response was recorded as a reference of the electrical activity, the olfactory epithelium was flushed with charcoal filtered water for 2 min, and the response to the blank control (charcoal filtered water) was recorded to confirm the absence of a response in the charcoal filtered water supply. Next, increasing concentrations of the test stimulus starting at 10^{-13} M to 10^{-6} M was applied to the olfactory epithelium, recorded, and flushed. Blank control and 10^{-5} M L-arginine standard were measured after every two concentrations. The EOG response magnitudes were measured in millivolts, normalized relative to that of 10^{-5} M L-arginine (2.769 ± 0.062; absolute raw value mean ± S.E), and blank-corrected (0.183 ± 0.009; absolute raw value mean ± S.E) as defined in Equation 1: Normalized EOG Amplitude = $(R_t - R_b)/(R_a - R_b)$, where Rt is the response magnitude to the test stimulus, Rb is the response magnitude to the blank, and Ra is the response magnitude to L-arginine at 10^{-5} M. The L-arginine standard and blank control responses were comparable to previous studies (Fine et al. 2006; Li et al. 2013). The detection threshold was calculated and defined as the lowest concentration where the test stimulus elicited a larger

response than the blank control (paired *t*-test, one tailed).

Two-choice maze behavioral assay

The preference of ovulated female sea lampreys to purified **1**, **2**, and **3** at 10^{-12} M was evaluated using a two-choice maze (Buchinger et al. 2013). A single lamprey was introduced to the furthest point downstream in the maze in a release cage. After 5 min of acclimation in the cage, the lamprey was released and the cumulative amount of time the lamprey spent in each channel was recorded for 10 min. After 10 min of recording, the test stimulus was introduced to a random channel and 50% methanol control (MeOH: deionized water) was introduced to the other channel using peristaltic pumps at constant rates of 200 ± 5 mL min^{-1} (Masterflex 07557-00, Cole-Parmer, Vernon Hills, Illinois, USA). The odorants were pumped into the maze for 5 min without recording the lamprey's behavior. After the 5 min period, the cumulative amount of time the lamprey spent in each channel was recorded for 10 min while the odorants were continuously administered. The maze was flushed with water for 10 min before the start of the next experiment. After the time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application was recorded, an index of preference was calculated for each trial as defined in Equation 2: Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$). The trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10s during the 10 min period before the odorant was applied as this is an indication of inactivity or strong side bias.

RESULTS

Structure of novel steroids

Using mass spectrometry and NMR, collaborator Dr. Ke Li (Michigan State University) elucidated the structures of **1**–**3** (Figure B-1) from water conditioned with mature male sea lampreys collected by Skye Fissette. The chemical structures were determined to be 3,12-diketo-7α-hydroxy-5α-cholan-24-sulfate (**1**) ($C_{24}H_{37}O_7S$*; m/z* 469.2234), 3-keto-12α-hydroxy-5α-cholan-24-sulfate (**2**) ($C_{24}H_{39}O_6S$; *m/z* 455.2458), 3-keto-12α-hydroxy-4,6-cholandiene-24-sulfate (**3**) ($C_{24}H_{35}O_6S$; *m/z* 451.2147), corresponding to the trivial names petromyzone A−C, respectively (Figure B-1). The results of the chemical analyses are described in detail in the resulting publication (Li et al. 2017b).

Olfactory potency

Petromyzone A–C (**1**–**3**, respectively) were potent odorants that stimulated the adult sea lamprey olfactory epithelium in the EOG assay. In the EOG assay, **3** was the most stimulatory compound and elicited larger responses than the other compounds at 10^{-10} M to 10^{-6} M, relative to normalized EOG amplitudes within each concentration, followed by **1** and then **2** (Figure B-2). The normalized EOG amplitudes of 10^{-6} M solutions of **1, 2**, and **3** were 1.691 ± 0.160 (mean ± S.E.; n = 7), 0.845 ± 0.034 (n = 5), and 2.536 ± 0.285 (n = 5), respectively. The thresholds of detection, the lowest concentration that elicited an olfactory response greater than the blank water control, for **1** was 10^{-11} M (paired t-test, t = −1.99, df = 6, P = 0.039), for **2** was $<10^{-13}$ M (t = −2.50, df = 4, P = 0.033), and for **3** was $<10^{-13}$ M (t = −4.42, df = 4, P = 0.006).

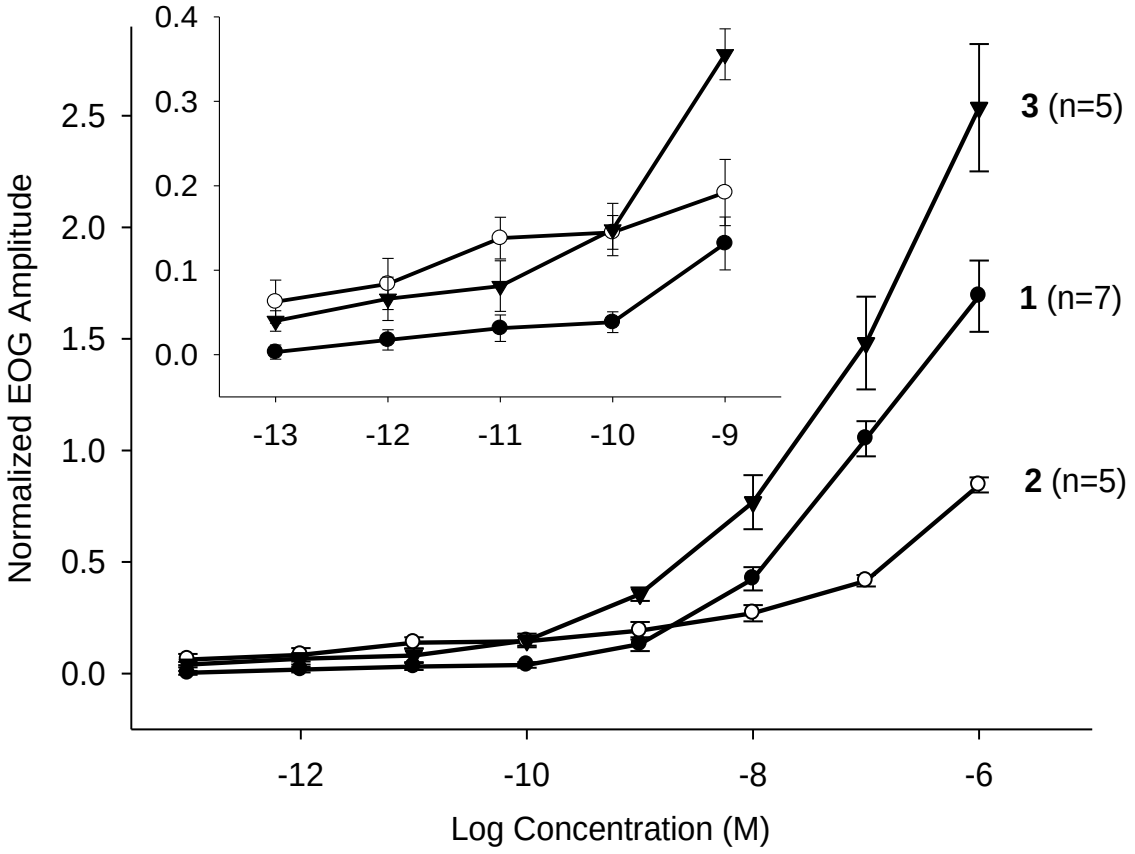

Figure B-2. Semi-logarithmic plot of electro-olfactogram (EOG) concentration response curves shows petromyzone A–C (1–3, respectively) are potent odorants for the adult sea lamprey olfactory epithelium and have low detection thresholds. The bolded numbers on the right of the figure correspond to each compound (filled circle, petromyzone A (**1**); open circle, petromyzone B (**2**); closed triangle, petromyzone C (**3**)) and the respective sample size (n) is shown in parentheses. Data are presented as the mean normalized EOG amplitude. Vertical bars represent the standard error of the mean. Insert: Expanded view of EOG responses showing response threshold concentrations.

Behavioral activity

In the two-choice maze behavioral assays, ovulated female sea lampreys were attracted to 10^{-12} M **1** (0.256 ± 0.076, mean index of preference ± S.E., n = 7) and repulsed from 10^{-12} M **3** (−0.323 ± 0.089, n = 7) (Wilcoxon signed-rank test, $P < 0.05$, Figure B-3). Ovulated female sea lamprey also appeared to be repulsed from 10^{-12} M **2**; however, the behavioral response to **2** was not significant (−0.593 ± 0.192, n = 4, P = 0.125, Figure B-3). A larger sample size was not possible due to the limited quantity of **2** available for the behavioral assays. Although these data suggest ovulated females may be behaviorally repulsed from **2**, additional trials should be conducted to test this.

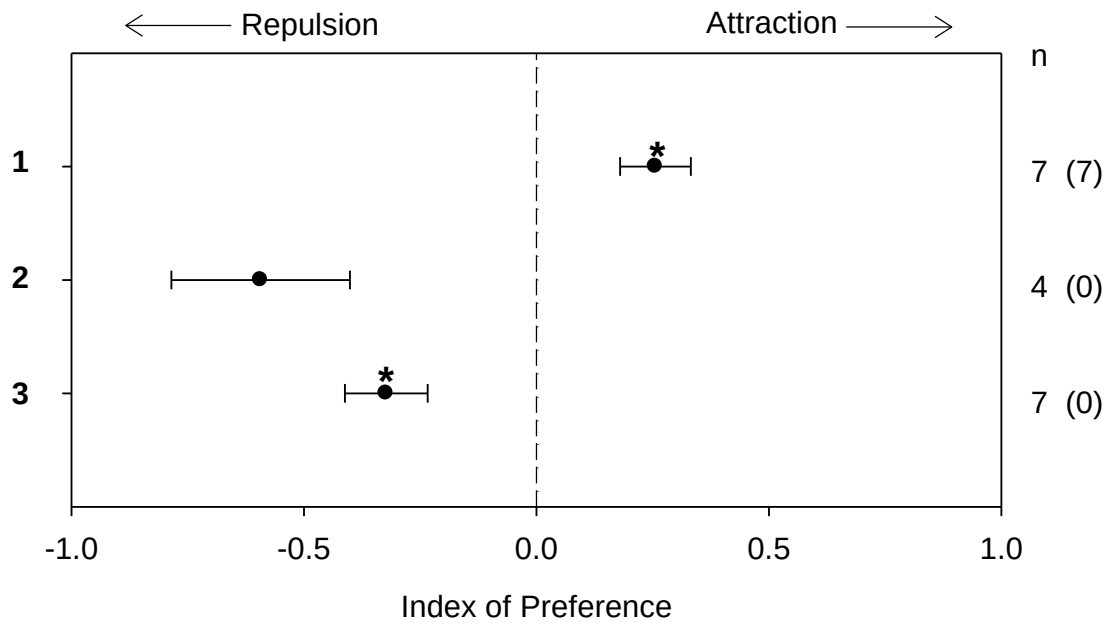

Figure B-3. Ovulated female sea lampreys were attracted to petromyzone A (1) and repulsed from petromyzone C (3) in the two-choice maze (*P* < 0.05). The time the lamprey spent in each channel of the maze before and after odorant exposure (10^{-12} M) was used to calculate an index of preference to assess its behavioral response to the odorant. A positive value of index of preference indicates attraction. A negative value of index of preference indicates repulsion. Data are presented as the mean ± S.E.M. and were evaluated using a Wilcoxon signed-rank test. n, sample size, with the number in the parentheses indicating the number of test subjects spending more time in the treatment side. ∗, $p < 0.05$.

DISCUSSION

We identified petromyzone A–C and characterized the olfactory potency and behavioral activity of each compound. These three novel bile salts were isolated from water conditioned with spermiated male sea lamprey. Compounds **1**–**3** are a series of variants of a class of C24 bile salts possessing different hydroxylation, oxidation, and double bond patterns, thus exemplifying the chemical diversity of bile salts. In terms of configuration and substitution pattern, these compounds show a high degree of similarity. Each compound has all-*trans* conjugated rings and 5α-H, except **3**, which lacks 5-H due to the ketene substitution pattern on C-3-C-6. The oxo and hydroxyl groups preferentially occupy C-3, C-7, and C-12 in known bile salts. The sulfated ester is formed on the terminal carbon of C-24 of bile salts and was confirmed for compounds **1**–**3**.

Results of the EOG assays show compounds **1**–**3** elicit concentration-dependent increases in olfactory responses with low detection thresholds, which are two characteristics consistent with electrophysiological results of previously identified sea lamprey pheromones. The concentration-response curve for each compound showed a steep increase in olfactory response as the concentration increased, which is a typical dynamic when a specific receptor is involved in the detection of an odorant (Li et al. 2013). Sea lamprey pheromone components that trigger physiological responses in conspecifics often have EOG detection thresholds between 10^{-8} M (Li et al. 2013) to 10^{-13} M (Brant et al. 2016). Therefore, the results of the EOG experiments for **1**–**3** fall within the anticipated range of the limit of detection for sea lamprey sex pheromones. Compound **1** was the least stimulatory compound tested in this study as it had the highest detection threshold of 10^{-11} M. The sea lamprey olfactory epithelium is

acutely sensitive to **2** and **3** with detection thresholds less than 10^{-13} M. The increased olfactory potency may be attributed to the hydroxyl group at C-12, a shared feature of **2** and **3** that differs from **1,** which has an oxo at C-12. This is consistent with previous experiments that demonstrated the olfactory response to 3kPZS, a compound with a hydroxyl at C-12, was much greater than that to DkPES, a structurally similar compound but with an oxo group at C-12 (Li et al. 2013).

Our behavioral tests provide evidence that compounds **1**–**3** may function as mating chemical cues. Experiments in the two-choice maze showed that compounds **1**–**3** induced behavioral responses in ovulated females. Our results are consistent with previous pheromone studies that demonstrated ovulated females are behaviorally responsive to low concentrations (10^{-12} M) (Johnson et al. 2009; Li et al. 2002). Ovulated females preferred the treatment side of the maze when **1** was used as the test odorant. In contrast, ovulated females avoided the treatment side of the maze when **2** and **3** were used as test odorants and instead spent more time in the control side of the maze conditioned with the vehicle. This experimental study describes odorant-mediated behaviors in a laboratory assay, however, warrants further examination in stream. Experiments in stream conditions under physical, physiological, and social contexts associated with natural environments are necessary to affirm the pheromonal functions of **1**–**3** (Johnson and Li 2010).

In summary, petromyzone A–C (**1**–**3**), three novel highly oxidized sulfated bile alcohols were identified from water conditioned with spermiated male sea lamprey and found to stimulate to the adult sea lamprey olfactory epithelium, as well as elicit behavioral responses in ovulated females. The identification of these three additional components released by spermiated male sea lamprey enhance our understanding of the structures and functions of sex

pheromone components in this species and may provide useful behavioral manipulation tools to be implemented with the integrated management of the destructive and invasive sea lamprey in the Laurentian Great Lakes.

ACKNOWLEDGMENTS

The staff of U.S. Geological Survey Hammond Bay Biological Station who provided research facilities are acknowledged. The U.S. Fish and Wildlife Service and Fisheries and Oceans Canada collected the sea lamprey. This work was supported by a grant from the Great Lakes Fishery Commission to Dr. Weiming Li and Dr. Ke Li. My assistantship stipend was provided Great Lakes Fishery Commission with a grant to Dr. Leslie Kuhn and Dr. Weiming Li.

APPENDIX C

BILE SALTS PETROMYLIDENE A−C ISOLATED FROM MALE SEA LAMPREY (PETROMYZON MARINUS) ACT AS PUTATIVE PHEROMONES

The abstract included in this Appendix is from the original draft paper. The remainder of the Appendix only briefly highlights the chemistry procedures and results from the extraction, isolation, purification, and structure elucidation of the draft paper conducted by a collaborator Dr. Ke Li (Michigan State University). The Appendix focuses primarily on the biology portions of the draft paper that I contributed.

ABSTRACT

Three novel bile acid derivatives, petromylidene A−C (**1**−**3**), were isolated from water conditioned with sexually mature male sea lampreys (*Petromyzon marinus*). Their structures were elucidated by mass spectrometry and NMR spectroscopy and by comparison to spectra data of related structures. The identification of compounds **1**−**3** further illustrates the structural diversity of the 5*α* bile salt family. Compounds **1**−**3** exhibited notable biological properties as well, including high olfactory potencies in adult sea lampreys and strong behavioral attraction of ovulated female sea lampreys. Electro-olfactogram recordings indicated the limit of detection for compound **1** was 10^{-9} M, **2** was 10^{-11} M, and **3** was less than 10^{-13} M. These results suggest compounds **1**−**3** are likely male pheromones that guide reproductive behaviors in the sea lampreys.

INTRODUCTION

The sea lamprey is an invasive, parasitic vertebrate in the Laurentian Great Lakes. One emerging option to control the invasive sea lamprey population is the application of the pheromones to modulate reproductive behaviors in this species (Buchinger et al. 2015; Siefkes 2017). Investigations of pheromone communication in sea lamprey have unveiled that sea lampreys, similar to many other fishes, have an olfactory system that is acutely sensitive to bile salts (Buchinger et al. 2014; Hara 1994). Furthermore, spermiated male lampreys (sexually mature males with expressible milt) produce unique sulfated bile alcohols with a C24 backbone and 5*α* configuration, which function to aggregate potential mates during spawning (Buchinger et al. 2015; Li et al. 2013; Li et al. 2002). In our ongoing investigation for structurally diverse and biologically active compounds released from spawning male sea lampreys, we identified three novel bile salts trivially named petromylidene A−C (**1**−**3**, Figure C-1). Here, we report the structures of petromylidene A−C (**1**−**3**) and describe the olfactory and behavioral responses induced by **1**−**3**.

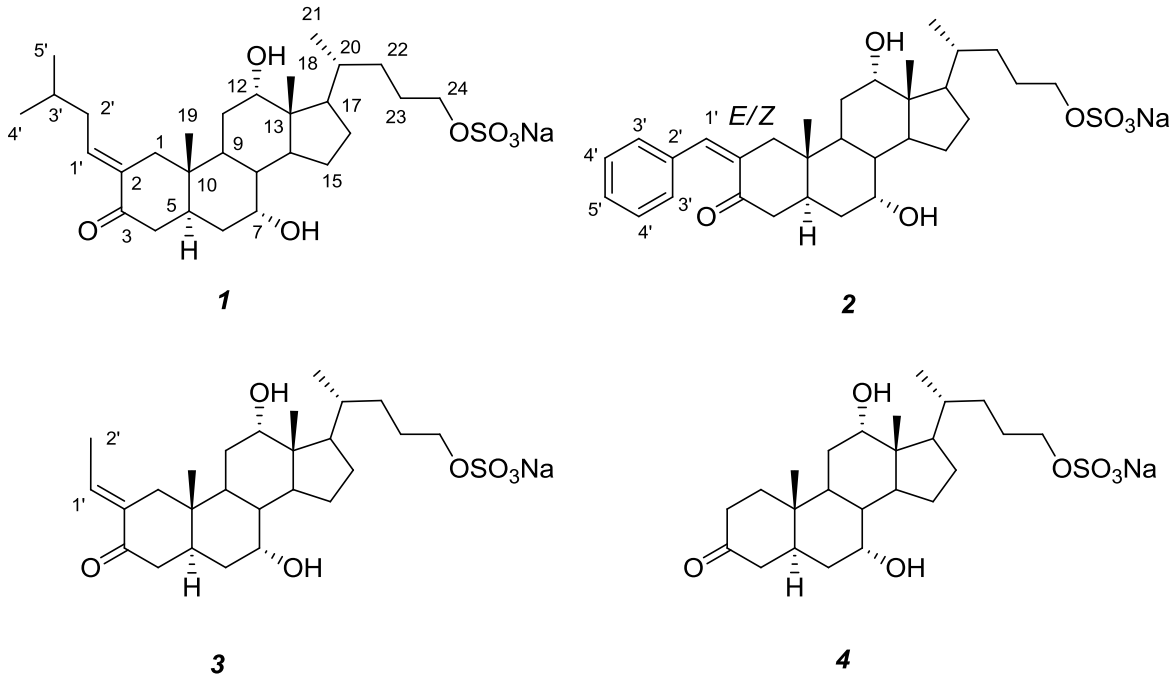

Figure C-1. Chemical structures of petromylidene A (1), B (2), C (3), and 3-keto petromyzonol sulfate (3kPZS, 4).

RESULTS

Petromylidene A−C (**1**−**3**) were isolated from water conditioned with sexually mature male sea lampreys (Figure C-1). Dr. Ke Li (Michigan State University) extracted the mature male sea lamprey conditioned water collected by Skye Fissette and Tyler Buchinger, separated it into **1**−**3,** and determined the structures of **1**−**3** by mass spectrometry and NMR spectroscopy. The structure of **1** was assigned as 2-(*E*)-(3-methylbutylidene)-7*α*,12*α*,24-trihydroxy-5*α*-cholan-3-one-24-sulfate and trivially named petromylidene A ($C_{29}H_{48}O_7S$*; m/z* 539.3058). Petromylidene B is a mixture of *Z*-(**2**) and *E*-(**2**) with approximate ratio of 3:1. The structures of **2** were assigned as 2-(*Z*)-benzylidene-7*α*,12*α*,24-trihydroxy-5*α*-cholan-3-one-24-sulfate and 2-(*E*)-benzylidene-7*α*,12*α*,24-trihydroxy-5*α*-cholan-3-one-24-sulfate, trivially named (*Z*)- and (*E*)-petromylidene B, respectively ($C_{31}H_{44}O_7S$; *m/z* 559.2729). The structure of **3** was assigned as 2-(*E*)-ethylidene-7*α*,12*α*,24-trihydroxy-5*α*-cholan-3-one-24-sulfate and trivially named petromylidene C ($C_{26}H_{42}O_7S$; *m/z* 497.2553).

Petromylidene A−C (**1**−**3**) were all potent odorants that stimulated the adult sea lamprey olfactory epithelium in the electro-olfactogram (EOG) assays. In the EOGs, **2** was the most potent and elicited larger responses than the other compounds across a range of concentrations (10^{-10} M to 10^{-6} M), followed by **1** and then **3** (Figure C-2). The threshold of detection, the lowest concentration that elicited an olfactory response greater than the blank water control, for **1** was 10^{-9} M (paired *t*-test, $t = -4.94$, $df = 6$, $p = 0.001$), for **2** was 10^{-11} M ($t = -3.53$, $df = 6$, $p = 0.006$), and for **3** was less than 10^{-13} M ($t = -2.52$, $df = 6$, $p = 0.023$) (Figure C-2).

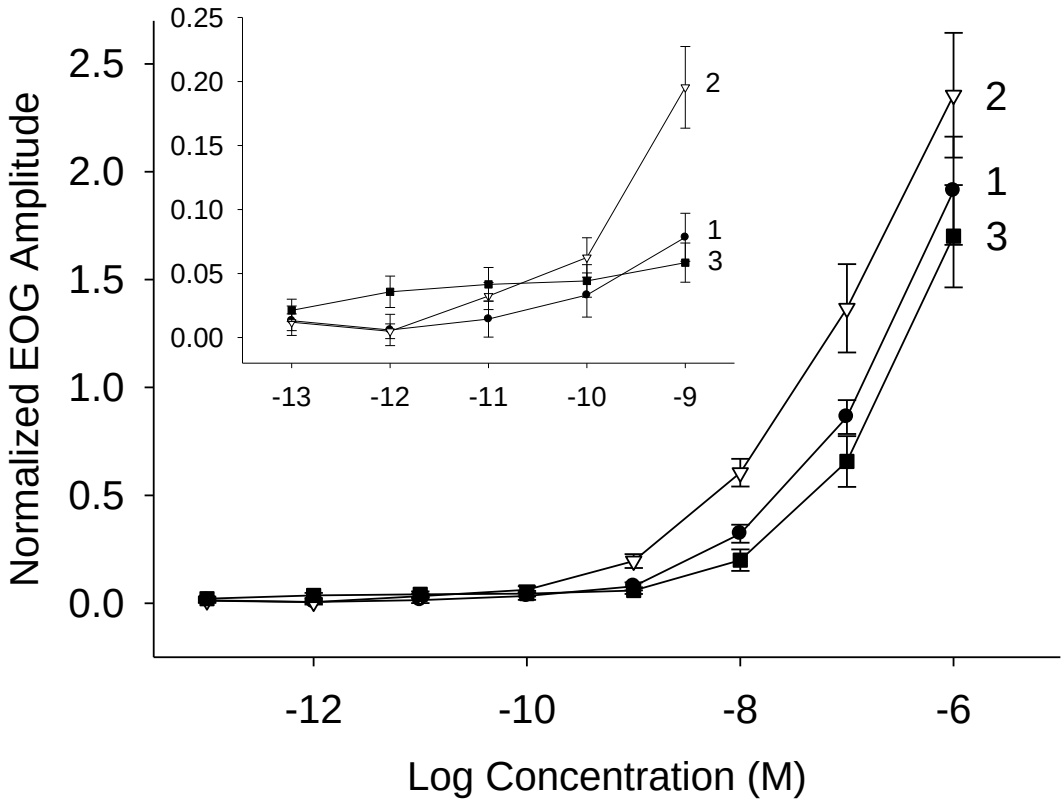

Figure C-2. Semi-logarithmic plot of electro-olfactogram (EOG) concentration response curves shows 1−3 (petromylidene A−C, respectively) are stimulatory to the adult sea lamprey olfactory epithelium and have low detection thresholds. The numbers on the right of the figure correspond to each compound (filled circle petromylidene A (1); open triangle petromylidene B (2); filled square petromylidene C (3)). Data are presented as the mean normalized EOG amplitude (n = 7). Vertical bars represent one standard error of the mean. Insert: Expanded view of responses showing response threshold concentrations.

In a two-choice maze behavioral assay, ovulated female sea lampreys were attracted to 10^{-12} M **1** (mean index of preference ± S.E.M; 0.232 ± 0.096, n = 11) and10^{-12} M **3** (0.488 ± 0.097, n = 7) (Wilcoxon signed-rank test, $p < 0.05$; Figure C-3). Ovulated females also appeared to be attracted to 10^{-12} M **2** (0.534 ± 0.054, n = 3, $p = 0.250$; Figure C-3); however, a larger sample size was not possible due to the limited quantity of **2** available for the behavioral assays.

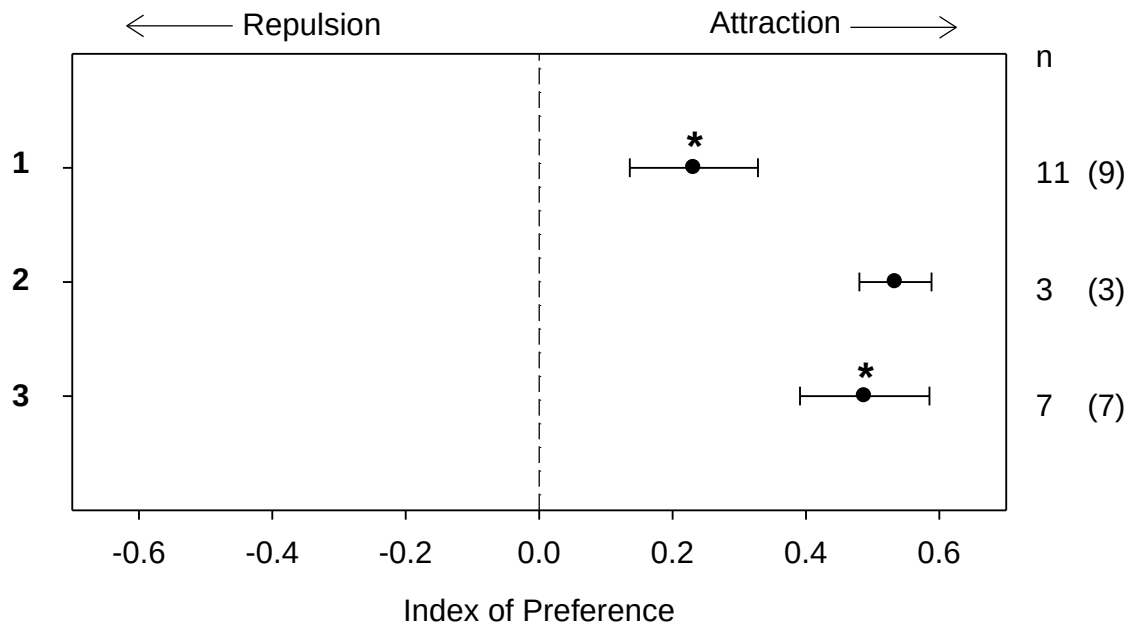

Figure C-3. Ovulated female sea lampreys were attracted to petromylidene A (1) and petromylidene C (3) in the two-choice maze ($p < 0.05$). The time the lamprey spent in the treatment or vehicle channel of the maze before and after odorant exposure (10^{-12} M) was used to calculate an index of preference. A positive index value indicates attraction and a negative index value indicates repulsion. Data are presented as the mean ± S.E.M. and were evaluated using a Wilcoxon signed-rank test. n, sample size, with the number in the parentheses indicating the number of test subjects spending more time in the treatment side. ∗, $p < 0.05$.

In conclusion, three novel bile salts were identified from water conditioned with sexually mature male sea lampreys. Compounds 1−3 represent three novel skeletons with unique additions on the A ring. Compounds 1−3 are highly similar in their configuration and substitution pattern, including a 5*α*-H typical of fully saturated rings and a sulfated ester on the terminal carbon. Together with 5*α*-myxinol-3*β*,27-disulfate found in hagfish (Hofmann et al. 2010) and 5*α*-cyprinol-27-sulfate found in Cypriniformes (Hagey et al. 2010), compounds 1−3 highlight the unique structural diversity of the 5*α* bile salt family. Notably, 1−3 are potent odorants that elicit attractive behavioral responses in the adult female sea lampreys, indicating they possibly function as pheromones. Continued research on 1−3 and all sea lamprey pheromone components may lead to novel tools with which to control the invasive populations and provide unique insights to the evolution of multi-component pheromones in vertebrates (Buchinger et al. 2017).

METHODS

Animals

All experimental procedures involving sea lampreys were approved by the Michigan State University Institutional Animal Use and Care Committee (IACUC) (Animal usage form number: 03/14-054-00). Adult sea lampreys were captured in tributaries of the Laurentian Great Lakes by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada according to approved scientific collection permits from those government agencies, transported to the U.S. Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, USA, and held in 500-1000 L aerated tanks supplied with Lake Huron water at 15–19 ^{o}C. Adult sea lampreys used for EOG recordings were transported to University Research Containment Facility at Michigan State University, East Lansing, Michigan, USA, and were held in flow-through tanks (250 L) supplied with aerated, chilled well water maintained at 7–9 ^{o}C.

To produce ovulated females as test subjects for the behavioral assays conducted in June and July 2016, immature female sea lampreys were transferred to acclimation cages constructed on polyurethane mesh and PVC pipe (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan to allow natural maturation. Sea lampreys were monitored daily for signs of sexual maturation including secondary sexual characteristics and expression of ovulated oocytes from the cloacal aperture following gentle pressure to the abdomen.

Extraction, isolation, purification, and structure elucidation

The protocol for chemical extraction, isolation, purification, and structure elucidation of compounds **1**-**3** were similar to those of Appendices A and B and the respective resulting

publications (Li et al. 2017a; Li et al. 2017b). Collaborators Skye Fissette and Dr. Tyler Buchinger collected the water conditioned with mature male sea lampreys. Dr. Ke Li (Michigan State University) carried out the chemistry analyses.

Electro-olfactogram (EOG) recording

Electro-olfactogram recordings were conducted following described procedures (Li et al. 2013; Li et al. 2017b) to determine the adult sea lamprey olfactory sensitivity to compounds **1**-**3**. Sea lampreys (243.9 g ± 17.0, 502.4 mm ± 12.8; mean ± S.E.M.) were anesthetized with 3-aminobenzoic acid ethyl ester (MS222; 100 mg L^{-1}; Sigma-Aldrich), immobilized with gallamine triethiodide (3 mg/kg of body weight, in 0.9% saline; Sigma-Aldrich), and placed in a V-shaped plastic stand. Gills were continuously irrigated with aerated water containing 50 mg L^{-1} MS222 throughout the experiment. The olfactory lamellae were surgically exposed. A small capillary tube delivered the stimuli and charcoal filtered water to the olfactory epithelium by gravity flow to prevent desiccation. The differential EOG response to each test stimulus was recorded. Electrical signals were then amplified (model NL102, Digitimer Ltd., Hertfordshire, England, United Kingdom), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized (Digidata 1440A, Molecular Devices LLC, Sunnyvale, California, USA), and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC). EOG recordings were conducted in April and May 2016.

For the concentration-response recordings, the olfactory epithelia of sea lampreys were exposed to 10^{-13} M to 10^{-6} M solutions of purified petromylidene A−C (**1**−**3**). 10^{-3} M stock solutions (in 50% MeOH: deionized water) were prepared for each compound, stored at −20 ^{o}C, and then serially diluted with charcoal filtered water to yield 10^{-13} M to 10^{-6} M solutions. A 10^{-2}

M stock solution of L-arginine (in deionized water) was prepared, stored at 4 °C, and then diluted with charcoal filtered water to yield a 10^{-5} M solution. A 10^{-5} M L-arginine standard was introduced to the olfactory epithelium for 4 s and the olfactory response was recorded as a reference, the olfactory epithelium was flushed with charcoal filtered water for 2 min, the response to the blank control (charcoal filtered water) was recorded to confirm the absence of a response in the charcoal filtered water supply. Next, increasing concentrations of the test stimulus starting at 10^{-13} M to 10^{-6} M was applied to the olfactory epithelium, recorded, and flushed. Blank control and 10^{-5} M L-arginine standard was measured after every two concentrations. The EOG response magnitudes were measured in millivolts, normalized relative to that of 10^{-5} M L-arginine (2.892 ± 0.061; absolute raw value mean ± S.E.M.), and blank-corrected (0.131 ± 0.005; absolute raw value mean ± S.E.M.) as defined in Equation (1): Normalized EOG Amplitude = $(R_t - R_b)/(R_a - R_b)$, where Rt is the response magnitude to the test stimulus, Rb is the response magnitude to the blank, and Ra is the response magnitude to L-arginine at 10^{-5} M. The L-arginine standard and blank control responses were comparable to previous studies (Li et al. 2013; Li et al. 2017a; Li et al. 2017b). The detection threshold was defined as the lowest concentration where the test stimulus elicited a larger response than the blank control (paired *t*-test, one tailed).

Behavioral assay

The behavioral responses of ovulated female sea lampreys to purified petromylidene A−C (**1**−**3**) at 10^{-12} M were evaluated using a two-choice maze previously described (Buchinger et al. 2013; Li et al. 2017b). A single lamprey was placed in a release cage at the furthest point downstream in the maze for 5 min, released, and the cumulative amount of time the lamprey

spent in each channel was recorded for 10 min. The test stimulus was introduced to a random channel and 50% methanol control (MeOH: deionized water) was introduced to the other channel using peristaltic pumps at constant rates of 200 ± 5 mL min^{-1} (Masterflex 07557-00, Cole-Parmer, Vernon Hills, Illinois, USA). The odorants were pumped into the maze for 5 min without recording the lamprey's behavior. Then, the cumulative amount of time the lamprey spent in each channel was recorded for 10 min while odorants were continuously administered. The maze was flushed with water for 10 min before the start of the next experiment. A 10-minute flushing period was deemed to be sufficient time in previous experiments (Li et al. 2002) and confirmed with a rhodamine dye test.

An index of preference was calculated for each trial as defined in Equation (2): Index of preference = [Ae/(Ae+Be) – Ac/(Ac+Bc)], where Bc is the time spent by the test animal in the control channel before odorant application, Be is the time spent in the experimental channel before odorant application, Ac is the time spent in the control channel after odorant application, and Ae is the time spent in the experimental channel after odorant application. The index results in a single number that can be either positive or negative. The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$) to determine if the index of preference was significantly different from zero. A significant positive value of the index of preference indicated attraction. A significant negative value of the index of preference indicated repulsion. A value not different from zero indicated neutral. The trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10 seconds during the 10 min period before the odorant was applied as this is an indication of inactivity or strong side bias.

ACKNOWLEDGMENTS

The U.S. Geological Survey Hammond Bay Biological Station provided facilities for holding sea lampreys. The staff of U.S. Fish and Wildlife Service and Fisheries and Oceans Canada collected sea lampreys. This work was supported by the Great Lakes Fishery Commission to Dr. Weiming Li and Dr. Ke Li. My assistantship stipend was provided Great Lakes Fishery Commission with a grant to Dr. Leslie Kuhn and Dr. Weiming Li.

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