CHARACTERIZATION OF SEA LAMPREY PHEROMONE COMPONENTS

By

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ABSTRACT

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Sea lamprey (*Petromyzon marinus* L.) rely upon chemical and environmental information to modulate key aspects of their single reproductive season, including; stream temperature, odor of juveniles, and a male-released mating pheromone containing a main component 3-keto petromyzonol sulphate (3kPZS). In this dissertation, I test the overall hypothesis that pheromone mediated behavior is dependent upon multiple factors (*i.e*. environmental, physiological and social cues, or pheromone components versus mixtures in specific ratios). In Chapter 1, field tests suggest 3kPZS functions as a migratory cue in pre-spawn sea lamprey specifically during cold (< 15 °C) stream temperatures. In Chapter 2, field tests confirm a functional shift of 3kPZS from a migratory cue to a proximal mating pheromone as spawning commences. Chapter 3 marks the next step in characterizing the behavioral function of a new mating pheromone component 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) that functions as a mating pheromone when mixed with 3kPZS. In Chapter 4, an additional male-released compound, identified as petromyzonamine-24-monosulfate (PAMS-24), is examined in field tests and hypothesized to function as a mating pheromone that advertises nest boundaries. In Chapter A-1, a novel dyhydroxylated tetrahydrofuran (THF) diol fatty acid, compiled of four stereoisomers, is currently under investigation for behavioral activity, and is therefore included as an appended chapter to this dissertation. Studies here advance our understanding of the multiple contexts at which pheromones modulate behavior, the origins of chemical communication, and the utility of pheromones for integrated invasive species control.

To my grandpa George W. Anderson who took me fishing.

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INTRODUCTION TO DISSERTATION

All organisms continuously emit non-communicative compounds into the environment as by-products of life and death. If released or excreted on a reliable basis under specific contexts, some of these compounds can become ritualized (Tinbergen, 1952) to a variety of evolutionary trajectories from ambient odor, to a chemical or mixture that serves as public information (cue), to a unique chemical signature that functions between members of the same species as a signal (Symonds and Elgar, 2008). I refer to a cue throughout this dissertation as a source of public information that only benefits the receiving individual. Cues can range from environmental/physical (*i.e.* changes in stream temperature which may deliver information to fishes that spring is approaching), to visual/audible (*i.e.* the sight of the turkey vulture (*Cathartes aura*) flying overhead which may indicate a potential food source to other scavengers), to chemical (*i.e.* the smell of compounds contained in the waste of juveniles which may indicate that successful reproduction has occurred in the past to adults). While cues have no direct benefit to the sender (*i.e.* vultures did not evolve to fly overhead to draw other animals to their food source), a signal is referred to in this dissertation as a form of communication between two members of the same species that evolved to benefit both parties (Symonds and Elgar, 2008). Pheromones are discussed throughout this dissertation as a chemical signal released from an individual that elicit a behavioral or physiological response in members of the same species (Karlson and Lüscher, 1959). Pheromone research over the past several decades has primarily focused on insects (over 80%) with Lepidoptera (butterflies and moths) as the predominant taxa (Symonds and Elgar, 2008). Studies of pheromone communication in vertebrates remain substantially less prominent (Symonds and Elgar, 2008). This creates large taxonomic gaps in our knowledge of the origins of chemical communication, the diversity of compounds that have evolved to function as pheromone mixtures and components, and the multiple factors

(environmental, physiological, and social) that influence behavioral responses to pheromones. The studies presented here focus on pheromone communication in sea lamprey (*Petromyzon marinus*, L.), a jawless fish that represents one of the most primitive, extant, vertebrates known to mankind (Oisi et al., 2013). I refer to the characterization of pheromone components throughout this dissertation as elucidation of chemical structure of new compounds, investigation of release and detection of these compounds in conspecifics, investigation of behavioral functions in the field, and investigation of the multiple factors (*i.e.* environmental, chemical, physiological, and social) that may influence behavioral activity of these compounds.

The invasive and destructive nature of sea lamprey in the Laurentian Great Lakes, coupled with their dependency upon chemical information to modulate their migration and mating season, has prompted many large scale investigations of sea lamprey pheromone communication (Li et al., 2007; Teeter, 1980). Yet only within the past decade have we truly begun to understand how important pheromones are in modulating the single migratory and mating season of sea lamprey. Parasitic adult sea lamprey detach from their host in early springtime, cease feeding and navigate to river plumes to stage their single reproductive migration (Figure I-1A). Sea lamprey do not home to their natal streams like Salmonids (Bergstedt and Seelye, 1995); yet, anosmic sea lamprey are unsuccessful in locating the river mouth, emphasizing their dependency upon olfactory senses at this time (Vrieze et al., 2010). Adults are hypothesized to implement a series of search tactics to navigate the coast and locate a river plume, navigate the river mouth relying partially on olfactory information provided by conspecific juveniles, and enter a tributary (Meckley et al., 2014; Vrieze et al., 2011). The odor of juvenile larvae is hypothesized to indicate the presence of high-quality reproductive habitat upstream (Bjerselius et al., 2000; Sorensen et al., 2005; Wagner et al., 2009). Sea lamprey

migrate upstream in the springtime where they sexually mature and establish spawning nests (Figure I-1B). Migration primarily occurs during the night (Applegate, 1950; Manion and Hanson, 1980), and migrants navigate the stream using larval odor (Bjerselius et al., 2000; Teeter, 1980; Wagner et al., 2009), stream temperatures (Binder and McDonald, 2008), and likely other environmental cues (Binder et al., 2010). Males establish spawning nests in rocky/riffle areas of stream (Applegate, 1950; Manion and Hanson, 1980), and begin to release a mating pheromone (Li et al., 2002; Siefkes et al., 2005; Teeter, 1980). The main component of the male mating pheromone has been identified as a bile alcohol 3-keto petromyzonol sulphate, or 3kPZS (Li et al., 2002; Yun et al., 2003). The spawning season lasts for roughly two weeks in early summer (June) followed by the death of spent adults (Applegate, 1950; Manion and Hanson, 1980). Upon hatching, juvenile larvae burrow into stream sediments (Figure I-1C) where they can remain for 4 – 17+ years (average 4 – 5 years), filter-feeding on algae and other plant particles carried by stream flow during this time while subsequently releasing a suite of bile acids and steroids as metabolic by-products of their feeding which constitute the larval odor (Li et al., 1995; Sorensen et al., 2005). In the winter months, larvae transform into parasitic adults and complete a migration downstream into the lake or ocean (Figure I-1D). Parasitic adults attach and feed upon other fishes for roughly 1.5 years while steadily increasing their body mass (Applegate, 1950), thus completing the life cycle.

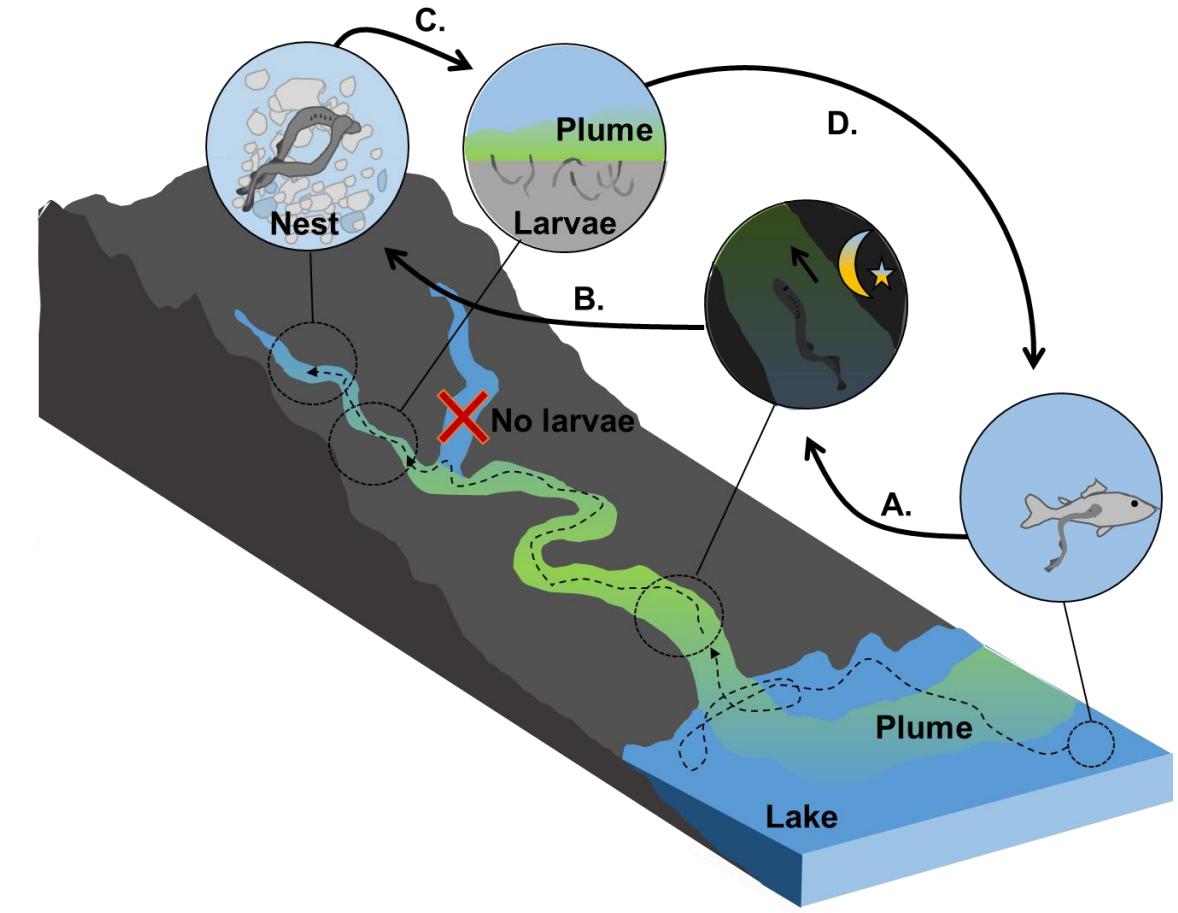

Figure I-1. Hypothesized role of pheromones in modulating the life history of the sea lamprey. (A) Parasitic adults detach from host fish, cease feeding, navigate the coast, and migrate at night into freshwater tributaries that are activated with the smell of juvenile conspecifics (Plume) in the early spring (Feb.-April). **(B)** Nocturnal migrating adults continue to move upstream in the spring (May-June), guided in part by the smell of conspecific larvae. Males move upstream, mature sexually, establish nests in rocky/riffle areas of the stream, and begin to release a mating pheromone (June-July). The main component of the male mating pheromone consists of a bile alcohol identified as 3-keto petromyzonol sulphate (3kPZS), which functions to draw ovulated females to the male for courtship. Spent adults die after a single spawning season. **(C)** Eggs hatch and juvenile larvae remain burrowed in upstream sediments for 4 – 17+ years where they filter-feed on algae and plant particles carried with the current, releasing the migratory odor as a metabolic by-product with their waste (comprised of bile acids and steroid derived compounds). **(D)** During winter months, larval sea lamprey transform into parasitic adults, and move downstream into the lake or ocean where they feed on fish for roughly 1.5 years, thus completing the cycle.

Much of the research within the last decade regarding chemical communication in sea lamprey, including the studies presented in this dissertation, has been supported by the Great Lakes Fishery Commission, a binational treaty formed in between the U.S. and Canada (1954) with a mission to control the destructive sea lamprey in the Laurentian Great Lakes and protect the fishery (Commission, 2001). The fishery in the Great Lakes is worth an estimated $7 billion annually, and the sea lamprey invasion undoubtedly added to the collapse of the fishery throughout the mid-20th century (Commission, 2001; Smith and Tibbles, 1980). Controlling sea lamprey populations in the Great Lakes is an ongoing battle. Fuelled by a mission to identify and integrate new pheromones into the current arsenal of control techniques used for sea lamprey in the Great Lakes (see Control Implications for a description of the current control techniques), our laboratory developed a bioassay guided fractionation system at Michigan State University that would allow us to further characterize the structure and function of known pheromone components and identify new components that elicit behavioral or olfactory responses in sea lamprey (Figure I-2). We assembled an integrated team of researchers at Michigan State University to develop the bioassay guided fractionation system. Led by primary investigator Dr. Weiming Li, our team is comprised of Dr. Ke Li (analytical and theoretical chemist), Dr. Mar Huertas (electrophysiologist), and myself (behavior). Several new compounds have been identified and published by our team using this system, and more are continuing to show stereochemistry across a broad range of chemical diversity (Appendix, Publication List).

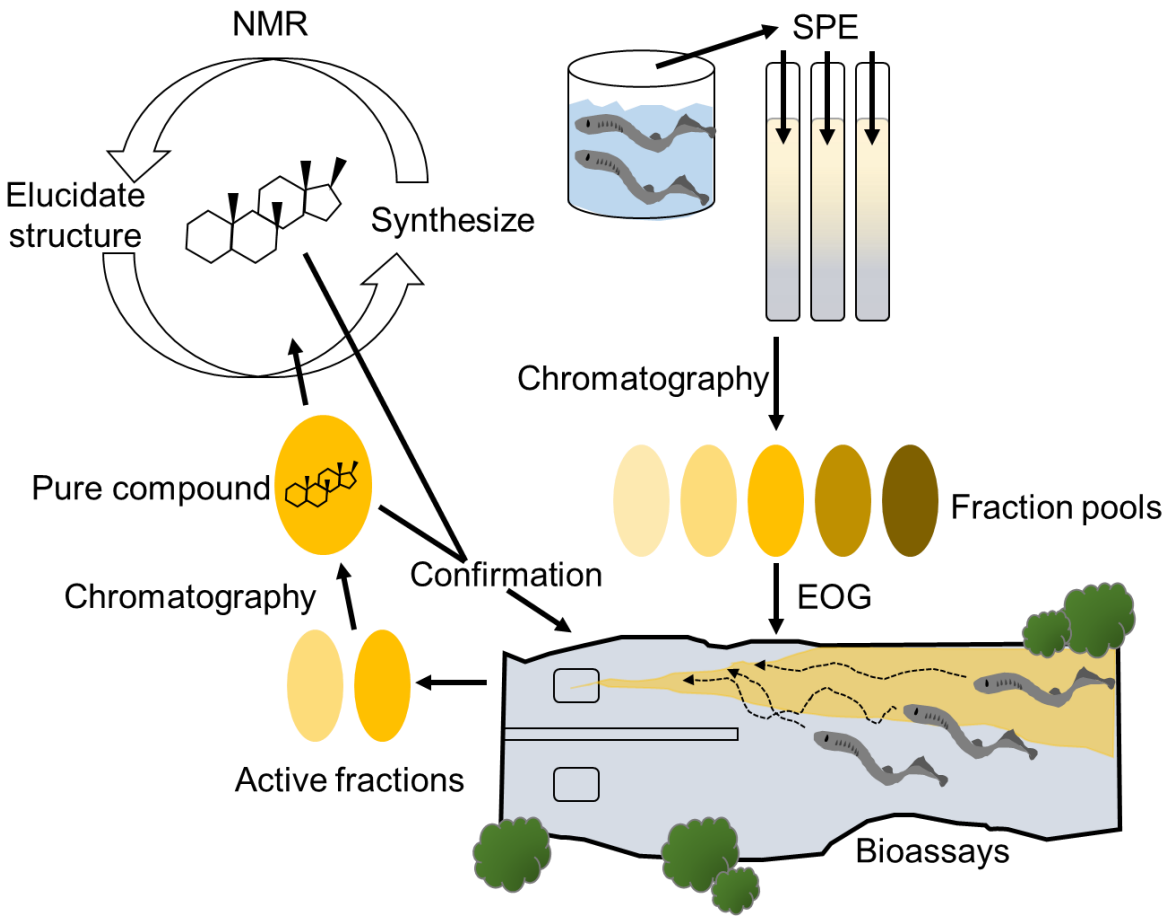

Figure I-2. Details of the bioassay guided fractionation system developed by our laboratory at Michigan State University for identification and characterization of sea lamprey pheromone components. Subjects are kept in holding tanks for collection of sea lamprey conditioned wash-water. Wash-water is extracted with solid-phase extraction (SPE) across a bed of aliphatic adsorbent resin (Fine et al., 2006). Extracts from resin are subjected to chromatography to separate fractions or pools of fractionated compounds (Li et al., 2012). Fractions are tested for olfactory potency using electro-olfactogram (EOG) assays (Li et al., 2014). Active pools, fractions, or components at the olfactory level are tested in the field to evaluate behavioral activity (Li et al., 2013b). Further separation into pure compounds is conducted with chromatography, and nuclear magnetic resonance (NMR) is used to identify chemical structure (Li et al., 2013b). Upon purification or chemical synthesis, behavioral activity is confirmed in the field (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005).

My overall hypothesis tested in this dissertation is that behavioral responses to pheromones are modulated by multiple environmental, physiological, chemical, and social factors. Specifically, I predict animals are faced with a hierarchy of information based on certain contexts – from environmental factors such as temperature, physiological factors such as sexual maturation, chemical factors such as pheromone components or mixtures of pheromones, and social factors such as inter- and intraspecific interactions with others, which ultimately must be sorted for optimal fitness payoffs on the signaller and receiver ends. Over the past decade, the hypothesized main mating pheromone component, 3kPZS, was extensively studied in the field for its mating function in sea lamprey, as well as for its applicability for sea lamprey control. Male sea lamprey were found to undergo a massive upregulation of bile salt synthesis in their livers upon sexually maturing (Brant et al., 2013), at which point 3kPZS was found to be released across specialized gill epithelia cells at a rate of 0.5 mg/hr (Siefkes et al., 2003). In the field, 3kPZS was found to draw significant numbers of ovulated females to the odorant source point consisting of 1 m^2 area of the stream (Johnson et al., 2006; Johnson et al., 2012; Johnson et al., 2009; Siefkes et al., 2005). Therefore 3kPZS was targeted as a potential lure to increase trap efficacy (Johnson et al., 2013). Extensive field tests of 3kPZS began to suggest that the component may not solely function in mating, but may have a behavioral function in pre-spawn adults as well which interacts with water temperature (Brant, 2011 MSc Thesis, Michigan State University).

Environmental factors modulate movement in organisms across the animal kingdom. In aquatic organisms, environmental cues such as water temperature, river discharge, turbidity, and lunar cycle (Forsythe et al., 2012; Quinn and Adams, 1996) have been shown to modulate behavioral aspects of fish migration. Temperature fluctuations have been hypothesized as a

primary migratory cue in teleost fishes (Moore et al., 2012; Skov et al., 2010), as well as in lampreys including Pacific lamprey (*Entosphenus tridentatus*) (Clemens et al., 2012), river lamprey (*Lampretra fluviatilis*) (Kemp et al., 2011), and sea lamprey (Binder and McDonald, 2008; Binder et al., 2010). However, interactions between environmental information and pheromone components in modulating behavior of animals has not been described, and little is known regarding other contexts in which pheromone components function.

In Chapter 1 of my dissertation, titled: "A pheromone outweighs temperature in influencing migration of sea lamprey," I hypothesized that pheromone component 3kPZS – a compound previously only considered as a mating pheromone, and stream temperature – previously shown to influence navigation of migrating sea lamprey (Binder and McDonald, 2008; Binder et al., 2010), interact to modulate pre-spawn migratory behavior of sea lamprey. During cold stream temperatures (< 15 °C) when migrating sea lamprey did not often move upstream, presence of 3kPZS overrode temperature-induced inactivity and increased upstream movement by over 40%. Chapter 1 presents an example of the hierarchy of contradictory information and context that animals face when making decisions. Chapter 1 is published in Royal Society Open Science.

Upon discovering the overriding effect of 3kPZS on stream temperature in migrating female sea lamprey, I hypothesized that 3kPZS must have undergone a functional shift from a cue during migration to a more directionally proximal and reliable mating signal during the spawning season, given that the mating pheromone function of 3kPZS has been well documented and the mating season for sea lamprey is highly synchronized. In Chapter 2, titled: "Functional transition of a chemical cue into a chemical signal in sea lamprey," I predicted that the functional shift of 3kPZS (*i.e.* from a cue for migration to a directed and coevolved signal for mating) can

still be seen by examining the behavior of female sea lamprey to sources of 3kPZS during three key stages of their life history: early migration (May), late migration (June), and spawning (June/July). Using a field test, I provided behavioral evidence that pre-spawn female sea lamprey shift their preference towards a sub-channel activated with 3kPZS (up to 70%) specifically during late migration. Histological evidence suggested subjects' oocytes were less than 3 days from ovulation once a preference for the 3kPZS activated sub-channel began to appear. Chapter 2 provides insights into the origins of stable communication signalling systems, and is currently submitted to Behavioral Ecology. Chapter 2 is formatted in accordance with the respective Behavioral Ecology guide for authors.

Given the life history of the sea lamprey in relation to pheromones (Figure I-1), and results from Chapters 1 and 2, it becomes apparent that females continue to become more proximally bias to the odor of mature males, likely to insure synchrony of reproduction. Since vison becomes increasingly degenerated during the spawning phase of adult sea lamprey (Binder and McDonald, 2007), and a background concentration of 3kPZS remains high within spawning grounds during the mating season (Xi et al., 2011), I hypothesize that additional components released from males provide further information required for approaching females to locate a mate with accuracy. I predicted that mixtures of minor components with the main mating pheromone 3kPZS provide additional information to females that allow them to locate the releaser male with high accuracy on spawning grounds. Insects such as moths have been shown to broadcast mixtures of pheromone components that are emitted at specific ratios (Linn and Roelofs, 1989; Reyes-Garcia et al., 2014; Wyatt, 2010). Individual components and mixtures that are inconsistent with the natural ratio still often yield behavioral response in moths, yet test subjects often show a peak preference to the ratio that best reconstructs that of the ratio emitted

by conspecific senders (Cardé et al., 1977; Coracini et al., 2001; Reyes-Garcia et al., 2014). The specificity of pheromone components and their mixtures in specific ratios have not been evaluated in vertebrates.

In recent years our bioassay guided fractionation system (Figure I-2) has yielded several components from sea lamprey that are highly stimulatory in the olfactory epithelia of conspecifics and are released at lower rates than 3kPZS. Recent discoveries include a new polyhydroxysteroid named petromyzestrosterol (Li et al., 2012), several fatty acid-derived hydroxylated tetrahydrofurans (Li et al., 2014; 2015), a new hexahydrophenanthrene sulfate named petromyzonin (Li et al., 2013a), and a new sulphated bile alcohol identified as 3,12-diketo-4,6-petromyzonene-24-sulfate, or DkPES (Li et al., 2013b). Field tests suggest that DkPES increases ovulated female preference for the odorant source when combined with 3kPZS (Li et al., 2013b), but details of ratios and their influence on behavioral responses remained unknown.

In Chapter 3, titled: "Mixtures of two bile alcohol sulfates function as a proximity pheromone in sea lamprey," I hypothesized that DkPES functions a proximal pheromone in ovulated females when mixed with 3kPZS, allowing them to efficiently locate the odorant source. In the field, I provided evidence that ovulated females showed the greatest preference to a source of 3kPZS and DkPES when combined at the ratio that best matches that which is naturally occurring in wash-water collected from spermiated males (SMW, 30:1, 3kPZS:DkPES). Further, I characterize search behavior (sinuosity of swim paths) of females approaching a range of 3kPZS:DkPES ratios. An increase in search activity occurs when subjects approach the SMW and the 30:1 ratio source. Unique pheromone ratios may provide utility in pheromone-integrated control of invasive sea lamprey in the Great Lakes. Chapter 3 is the first

example of pheromone ratios as mating pheromones in animals outside of insects, and is currently submitted to PLOS ONE. Chapter 3 is formatted in accordance with the respective PLOS ONE guide for authors.

Recently, a sulfated steroid identified as petromyzonamine-24-monosulfate (PAMS-24) was also discovered in SMW. In Chapter 4, titled: "A territorial pheromone that defines nest boundary in the sea lamprey," I hypothesized that PAMS-24 also functions as a proximal pheromone in ovulated females when mixed with 3kPZS, similarly to DkPES. Further, I predicted that PAMS-24 may dual-function as a component of a territorial pheromone that delineates nest boundaries among males. Behavior of male sea lamprey to mating pheromone components was virtually unknown in the field up to this point. Beginning in 2013, the U.S. Fish and Wildlife Service ceased their sterile male release program (Bergstedt and Twohey, 2007) allowing male sea lamprey to become available for field research. Preliminary field tests using sexually mature male subjects suggested that males were drawn to a source of 3kPZS, yet avoid a source of SMW. Combining these results from preliminary field tests with observations that nests can be constructed in high densities, often contain only one male, and nesting males are known to be aggressive to intruder males, evidence was mounting for the existence of a territorial pheromone that is released from sexually mature males (Teeter, 1980). I provided behavioral evidence that SMW, 3kPZS and PAMS-24 treatments applied to artificial nests in the field were preferred by females, yet PAMS-24 and SMW averted males. Behavioral assays conducted thus far suggest that PAMS-24 may function as the first identified territorial pheromone component in a vertebrate. Chapter 4 is in preparation for Science and is formatted in accordance with the respective guide for authors.

Chapter A-1, titled "Fatty acids with stereochemistry function as a pheromone in sea lamprey," was placed in the appendix of this dissertation because the characterization of these compounds is currently underway. These fatty acids were originally extracted from wash-water conditioned with larval sea lamprey, and thought to be a single compound. Upon further chemical investigation, it was learned that four compounds existed; two stereoisomers; 9,(12)-oxy-10,13-dihydroxystearic acid (1a) and 10,(13)-oxy-9,12-dihydroxystearic acid (1b) consisting as a mixture of (+)-1a (973), (−)-1a (971), (+)-1b (974), and (−)-1b (972). Compounds 972 and 974 were not detected in olfactory epithelia of adult sea lamprey, and so were not tested in the field. Compounds 971 and 973 were olfactory stimulants and were therefore tested in the field during the migratory season at the field site shown in Chapter 1 and 2. Migrating females began to show a preference for 973 alone and 971:973 mixtures (1:1, molar:molar) during late migration (June), but no response was seen when 971 alone was applied. To my surprise during these trials, old subjects that were released during previous trials that spring (*i.e.* old subjects that were still "at large" in the system and retained a surgically-implanted passive integrated transponder [PIT] tag) began swimming back within the field site each night, locating the odor source (1 m^2 PIT antenna) specifically when a mixture of 971:973 (1:1, molar:molar) was administered into the center of the 1 m^2 antenna, and remaining on the source (averaging 530 ± 105 sec. inside the 1 m^2 nest emitting the 971:973 mixture). Over 70 subjects returned to the source of 971:973. Four subjects were hand-grabbed while responding to the 971:973 mixture. Histological examination of oocytes confirmed these four subjects were ovulated. The four females spent an average of 15 ± 1.6 days "at large" in the river system before returning to the site. Compounds 971 and 973 were later found to be released by mature male sea lamprey, but field tests presented in the appended Chapter A-1 were not designed to evaluate a mating

function of these fatty acids. Field tests similar to those in Chapters 3 and 4 are underway to further evaluate behavioral activity of 971:973 in spawning phase sea lamprey, and determine whether stereochemistry of compounds represents yet another context that influences pheromone function in sea lamprey (see Appendix, Chapter A-1).

Studies presented in this dissertation describe several lines of evidence that directly support the overarching hypothesis of this dissertation. Environmental, physiological and social information interact with pheromone mixtures and components in modulating behaviors, and these interactions may not be observable in laboratory conditions. Additional minor pheromone components in combination with 3kPZS may serve as additional information used by females to locate a nesting male. Understanding the context in which pheromones function is applicable for research in behavioral ecology, chemical communication, and olfaction, and will provide insights into the origins of chemical signals. Finally, it is imperative that we understand the multiple contexts in which pheromones function if we are to integrate pheromones into control techniques of invasive sea lamprey in the Laurentian Great Lakes (Johnson et al., 2013; Li et al., 2007).

CHAPTER 1

A PHEROMONE OUTWEIGHS TEMPERATURE IN INFLUENCING MIGRATION OF SEA LAMPREY

Brant C.O., Li K., Johnson N.S., Li W. 2015. A pheromone outweighs temperature in influencing migration of sea lamprey. *R. Soc. Open Sci.* 2: 150009. http://dx.doi.org/10.1098/rsos.150009

ABSTRACT

Organisms continuously acquire and process information from surrounding cues. While some cues complement one another in delivering more reliable information, others may provide conflicting information. How organisms extract and use reliable information from a multitude of cues is largely unknown. We examined movement decisions of sea lampreys (*Petromyzon marinus* L.) exposed to a conspecific and an environmental cue during pre-spawning migration. Specifically, we predicted that the mature male-released sex pheromone 3-keto petromyzonol sulfate (3kPZS) will outweigh the locomotor inhibiting effects of cold stream temperature (< 15°C). Using large-scale stream bioassays, we found that 3kPZS elicits an increase (> 40%) in upstream movement of pre-spawning lampreys when the water temperatures were below 15°C. Both warming temperatures and conspecific cues increase upstream movement when the water temperature rose above 15°C. These patterns define an interaction between abiotic and conspecific cues in modulating animal decision making, providing an example of the hierarchy of contradictory information.

INTRODUCTION

Environmental cues (*e.g.* abiotic information) and signals (*e.g.* an entity evolved by a sender that elicits an evolved behavioral response in a receiver) provide a constant input of information used by organisms in decision making processes (Davies et al., 2012; Laidre and Johnstone, 2013). Exactly when certain information becomes more reliable in the decision making process is likely shaped by natural selection (*e.g.* decisions that ultimately impact the recipient's fitness) (Laidre and Johnstone, 2013). How organisms distinguish reliable information from a multitude of environmental, conspecific, and heterospecific cues remains largely unknown.

Among abiotic cues, water temperature is critical in influencing the timing of fish migration (Lucas et al., 2001). Pheromones (Karlson and Lüscher, 1959; Wyatt, 2010) are signals that may inform fish of mate readiness and/or habitat suitability (Wyatt, 2010). The sea lamprey relies on environmental and conspecific cues during reproduction. Specifically, when water temperatures drop below 15 °C, sea lamprey become increasingly less active. Warmer water temperatures correlate with increases in locomotor activity of pre-spawn migrating sea lamprey, likely as an adaptation to reach spawning grounds before full maturation of gametes occurs. (Binder and McDonald, 2008). In addition, the odor of stream-resident larvae guides pre-spawning sea lamprey in selecting suitable spawning habitat (Bjerselius et al., 2000; Wagner et al., 2009). Later, sexually mature female sea lamprey rely on a sex pheromone released by mature males (3-keto petromyzonol sulphate or 3kPZS) (Li et al., 2002) to locate nesting mates (Johnson et al., 2009).

We hypothesized that the conspecific male sex-pheromone (3kPZS) contains more reliable information compared to water temperature regarding spawning conditions upstream.

Here we report evidence that both male and female pre-spawn adults move upstream in the presence of 3kPZS within the otherwise locomotor inhibitory temperature range (< 15°C), which is consistent with the hypothesis. While test subjects move upstream, they do not bias towards a channel-side containing the source of 3kPZS during our studies, a previously described behavior in mature female conspecifics (Johnson et al., 2009; Li et al., 2002).

METHODS

Animals and tagging

Procedures involving sea lamprey were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF# 05/09-088-00). Immature adult sea lamprey were captured by the United States Fish and Wildlife Service and Fisheries and Oceans Canada from tributaries to Lake Michigan and Lake Huron, in May-June 2009, 2010 and 2012, and transported to the United States Geological Survey Hammond Bay Biological Station (HBBS) for further procedures. Sex and maturity determination, and animal housing, followed procedures described (Johnson et al., 2009). Pre-spawn sea lamprey refers to river-migrating adults that are non-feeding, yet not fully sexually matured. Pre-spawn sea lamprey where implanted with a 23 mm-long half duplex passive integrated transponder (PIT) tag (Oregon RFID, Portland, Oregon, U.S.A.) through a 3 mm lateral incision in the mid-abdominal region.

Pheromones

Use of 3kPZS (synthesized by Bridge Organics, Vicksburg, Michigan, U.S.A.; purity >97%) in the stream was permitted by the United States Environmental Protection Agency (experimental user permit 75437-EUP-2). A 10 mg ml^{-1} stock solution of synthesized 3kPZS (in 50% methanol) was prepared. 3kPZS stock solution was stored at -80°C until use in the field. Larval extracts, extracts of water conditioned with larval sea lamprey (Fine et al., 2006; Li et al., 2013a), were used as a positive control to validate the experimental system (Bjerselius et al., 2000; Wagner et al., 2009). To collect extracts of water conditioned with larval sea lamprey, over 20,000 larval sea lamprey were held in flowing 500 litre-capacity tanks at HBBS from April-August 2008. Larvae were given a sand substrate for refuge and fed yeast weekly. Tank

flows were shut off between ~2000 – 0800 hours (h) allowing larval odor to concentrate. Larval-conditioned water was passed through vertical columns containing 500 g of methanol-activated Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) using peristaltic pumps (Masterflex 7553-70, Cole-Parmer, Vernon Hills, Illinois, U.S.A.). Loading speed was ~300 ml min^{-1}. Three columns were loaded for up to 24 hours at a time. Each column was then eluted with 4-l of methanol. Eluents were concentrated using a model R-210 roto-evaporator (Buchi Rotovapor, Flawil, Switzerland) and stored at -80°C. All larval extracts were fully thawed, pooled, and thoroughly mixed before further analyses were conducted. Petromyzonamine disulphate (PADS), a component of larval extract (Sorensen et al., 2005), was used as a benchmark when calculating the volume of larval extract needed to activate the stream with PADS at a concentration of $5x10^{-14}$ molar (M). The concentration of PADS in the larval extract was determined using high performance liquid chromatography–tandem mass spectrometry (Li et al., 2013a).

Field behavioral tests

Subjects were tested at night, when migrating sea lamprey move upstream (Stier and Kynard, 1986), in experimental sites shown in Figure 1-1. Barriers near each river-mouth prevent wild migrating sea lamprey from entering the upper reaches. Females were tested in a separate field site (Figure 1-1a) than males (Figure 1-1b) to prevent unwanted reproduction of this invasive species above each barrier. The most upstream section of each site was bifurcated by a natural island, which separated two sub-channels of similar hydrologic and physical qualities. Test odorants were diluted with 30 L of river water in large mixing bins. Bins were kept consistent for each test odorant to reduce the potential for contamination during dilution. Each solution was pumped into respective sub-channels through separate latex tubes at a rate of 167

ml min^{-1} (± 5 ml min^{-1}) over the span of three hours using peristaltic pumps (Cole-Parmer). A test odorant was administered to one sub-channel (activated channel, herein) while an equal volume of methanol was administered into the adjacent sub-channel (control channel, herein), and the activated and control channels were alternated each trial.

Trials were conducted between 13-May and 11-June 2009, between 3-May and 8-June 2010 (females), and between 19 and 31-May 2012 (males) at night. Stream discharge was estimated every three days, or after every precipitation event, at a fixed location in the stream using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, Maryland, U.S.A.) to determine the amount of odorant stock solution to apply to the stream and maintain consistent concentrations across trials. Stream flow was relatively uniform within each site, and flows were lower on average in the male-only site (950-1200 m $sec.^{-1}$ in Figure 1-1a, and 200-320 m $sec.^{-1}$ in Figure 1-1b).

Up to two trials were conducted each night, depending upon animal availability. The early trial was conducted from sundown ~ 2030 – 2320 h, and a late trial was then run from ~ 0010 – 0310 h (trial times were dependent upon when sundown occurred). Twenty or 30 PIT-tagged sea lamprey (depending on animal availability) were removed from holding tanks at HBBS and transported to their respective stream acclimation cage at the release point (Figure 1-1) between 0300 – 0500 h the night prior to experimentation. Acclimation/release cages were mesh aluminium (~ 0.25 m^3) consisting of a sliding door that was removed manually upon release. Animals were then allowed an acclimation period in the stream for 15+ hours. Mortality during acclimation was < 0.5%.

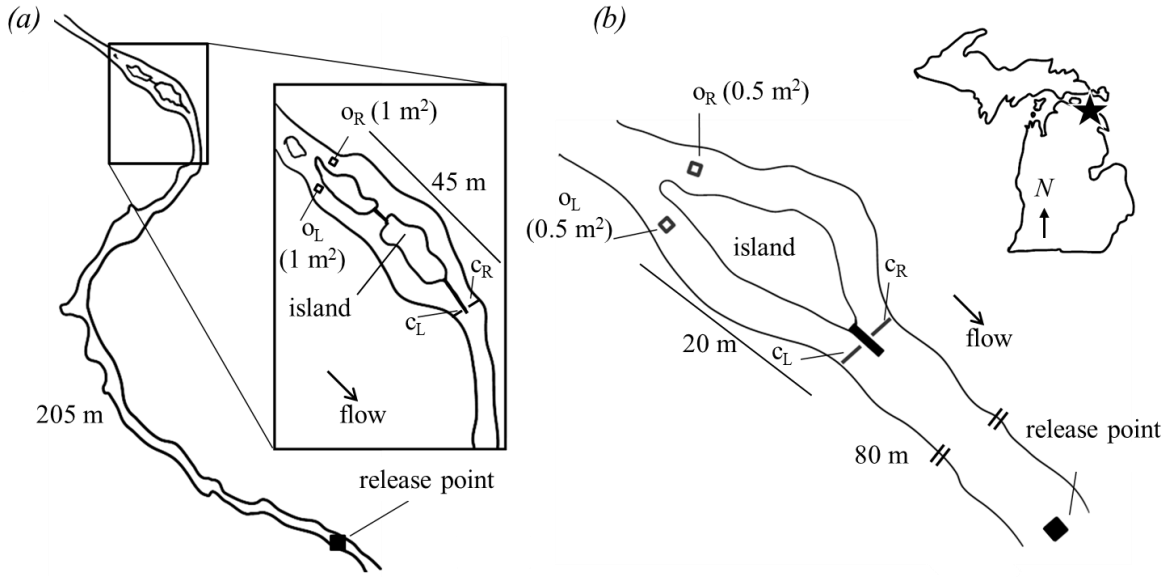

Figure 1-1. (*a*) Schematic of the Upper Ocqueoc River, Millersburg, MI, U.S.A. field for female testing only. (*b*) Schematic of Upper Trout River, Rogers City, MI, U.S.A. field site for male testing only. Transecting right (C_R) and left (C_L) PIT antennas recorded the number of test subjects that move into the treatment sub-channel (alternated each trial). Square-frame O_R and O_L PIT antennas recorded animals entering near the treatment source.

Each trial was three hours long. Stream temperature was recorded at the start of each trial. In the first hour of each trial, the test odorant was administered to the stream. At the start of the second hour, test animals were released. During the remaining two hours, animal behaviors were monitored while odorants were administered. The second trial began 30 minutes after the first. Test odorants were kept consistent for each night of trials. As an example, if 3kPZS was tested during the early trial, 3kPZS was also tested in the opposite sub-channel during the late trial to prevent the possibility of any unwanted contamination from other test odorants. No animals were recovered from the stream after a trial. Each test subject's unique PIT tag identification number prevented any pseudo-replication from test subjects released during previous nights. Movement data were consolidated and stored using a multiplexor (Oregon RFID, Portland, Oregon, U.S.A.). Data were uploaded each trial night using a hand-held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

Treatments were administered from a fixed point in the centre of square-frame PIT antennas at the upstream end of each sub-channel (Figure 1-1). PIT antennas were tuned to a detection sensitivity of roughly 0.3 m from the edges. Scan frequencies were programmed to 3 scans sec.$^{-1}$. Treatments included: (1) 3kPZS ($5x10^{-13}$ M) administered into one sub-channel (methanol into the adjacent sub-channel), (2) larval extract ($5x10^{-14}$ M PADS) administered into one sub-channel (methanol into the adjacent sub-channel), and (3) 50% methanol (in de-ionized water) into both sub-channels for vehicle controls. Molar concentrations were the estimated final concentrations in the activated sub-channel of the stream.

Data analysis

The dependent variable, proportions that moved upstream for 205 m to the channel confluence, were arcsine transformed and examined for violations of assumptions of normality and variance homogeneity using the Univariate Procedure in SAS (SAS Incorporated, Cary, North Carolina, U.S.A.) before conducting statistical analyses. Upon observing no violations of the assumptions, analysis of covariance (ANCOVA, $\alpha = 0.05$) was used to test which explanatory variables influenced the upstream movement of pre-spawn female sea lamprey. The explanatory variables tested were: (1) treatment (fixed), (2) date (including year: random), (3) stream temperature (fixed), (4) rate stream temperature decreased per trial (fixed), (5) time when trial was conducted (fixed), and (6) the number of animals released per trial (fixed). The final model was selected based on residual log likelihood (LogLik) and weighted AIC (w_i) values (Binder et al., 2010). The difference between the model with the lowest AIC and each additional model presented was calculated using equation (*1*):

(*1*) $$\Delta_i = AIC_i - AIC_{min}$$

where Δ_i is the difference between the best fitting model (AIC_{min}) and each model (AIC_i). The normalized relative likelihood values were calculated using equation (*2*):

(*2*) $$w_i = \frac{exp(-0.5 * \Delta_i)}{\sum_{r=1}^{R} exp(-0.5 * \Delta_r)}$$

where w_i is the weighted *AIC* value (Akaike weight) determined by dividing the relative likelihood of each model ($exp(-0.5*\Delta_i)$) by the sum of relative likelihoods of all models.

Differences of least squares means (two-tailed *t* test, $\alpha = 0.05$) of the proportions of animals moving upstream per trial were examined at the lower (q_1), median (q_2), and upper (q_3) quartiles of temperature across treatments (SAS). Quartiles in Figure 1-1 were determined by

first dividing the temperature data into two halves at the median (which was itself calculated as a mean of the two middle data). The median of the lower half of the data (lower quartile) and the median of the upper half (upper quartile) were then calculated. To determine whether pre-spawn conspecifics showed a more proximal preference towards treatments, we used logistic regression with a binomial distribution (R version 2.11.1, Vienna, Austria) to examine: (1) total number that moved upstream to the confluence of the sub-channels, (2) total number that entered the sub-channel activated with each treatment, and (3) total number in the treatment channel that entered the square administration point. Details of similar statistical analyses have been described (Johnson et al., 2009; Li et al., 2013b). No signs of nonlinearities or overdispersion were observed. All responses were compared to those of methanol control trials.

RESULTS

Movement upstream

Female responses to 3kPZS during 2009 and 2010 were consistent ($F_{1, 7}$ = 3.59, P = 0.100) and combined in the analysis. Data from males were analysed separately because they were tested in a separate stream with different dimensions. The following variables did not influence the proportion of migrating females moving upstream across trials in our experimental system: the rate of stream temperature decrease per trial (°C hr^{-1}), time of trial (early or late), or the number of immature adult females released per trial (20 or 30) (ANCOVA: $F_{1,10}$ = 0.97, P = 0.348; $F_{1,12}$ = 0.42, P = 0.530; $F_{1,11}$ = 0.01, P = 0.919; $F_{1, 12}$ = 0.40, P = 0.540, respectively), and were removed from the final model. The final model considered effects of treatment, stream temperature, and their interaction as best explaining variability in the proportion of pre-spawn adult sea lamprey moving upstream (females: $F_{2, 19}$ = 9.36, P = 0.002, males: $F_{2, 3}$ = 11.26, P = 0.040, Figure 1-2). Statistical fitness values were lowest in the final model (Females: *logLik* = -25.3, *AIC* = -21.3, w_i = 0.22; Table 1-1). This model was then used for analyses of male responses. *Post hoc* tests compared upstream responses at each temperature quartile. Overall, higher numbers of pre-spawn sea lamprey moved upstream in the presence of 3kPZS compared to methanol controls, specifically at the lower quartiles of temperature (~15 °C) as seen in Figure 1-2 (females: t_{19} = 5.16, P < 0.001, males: t_3 = 1.22, P = 0.310, Table 1-2).

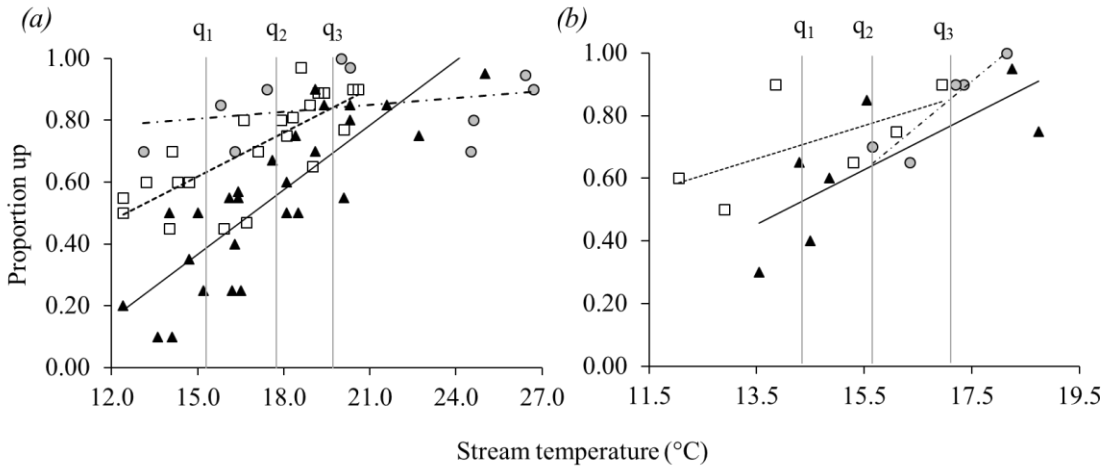

Figure 1-2. Upstream movement of pre-spawn female (*a*) and male (*b*) sea lamprey. □ = trials where 3kPZS was administered to the stream (dashed regression line, females $R^2 = 0.59$, males $R^2 = 0.38$), ▲ = trials where 50% methanol (control) was administered to the stream (solid regression line, females $R^2 = 0.68$, males $R^2 = 0.57$), and ◯ = trials where larval extract was administered to the stream (dot-dashed regression line, females $R^2 = 0.10$, males $R^2 = 0.85$). q_1, q_2, and q_3 = the lower, median, and upper quartiles of temperatures. See Table 1-2 for statistical comparisons at each quartile of temperature.

Table 1-1. Multiple effects, their interactions, and fit statistics for each model examined for best explaining the variation in the proportion of sea lamprey that moved upstream across treatments.

Effect(s)	Interaction(s)	*logLik*	*AIC*	Δ_i *AIC*	exp(-0.5*Δ_i)	w_i
odorant; temp	odorant*temp	-25.3	-21.3	0.00	1.00	0.22*
temp		-12.6	-6.6	14.70	0.48	0.10
	odorant*period	-4.8	1.2	22.50	0.32	0.07
	temp*period	-6.8	-0.8	20.50	0.36	0.08
odorant; temp; period	odorant*temp; odorant*period; odorant*temp*period	-5.8	-1.8	19.50	0.38	0.08
temp; period	temp*period	-5.8	0.2	21.50	0.34	0.07
	odorant*temp	-7.9	-3.9	17.40	0.42	0.09
odorant; period	odorant*period	-4.8	1.2	22.50	0.32	0.07
period		-1.6	4.4	25.70	0.28	0.06
odorant		-12.6	-6.6	14.70	0.48	0.10
date		3.7	7.7	29.00	0.23	0.05

*Final model selected with corresponding *AIC* and *logLik* values. Δ_i *AIC* and Akaike weights (w_i) were calculated using equations (*1*) and (*2*) above. Relative likelihoods were calculated with the following equation: exp(-0.5*Δ_i). Odorant refers to 3kPZS, methanol (control), or larval extract treatments. Period refers to early (2000-2300h) or late (0000-0300h) trials. Temp refers to stream temperature (°C).

Table 1-2. Treatment comparisons at the lower, median, and upper quartiles of stream temperature in Figure 1-2.

Females	methanol vs. 3kPZS		3kPZS vs. LE		methanol vs. LE	
temperature	t_{19}	*P*	t_{19}	*P*	t_{19}	*P*
q_1 15.5°C	5.16	*<0.001*	2.61	*0.017*	5.96	*<0.001*
q_2 17.9°C	4.59	*<0.001*	1.58	0.130	4.98	*<0.001*
q_3 19.7°C	2.76	*0.012*	0.36	0.726	3.13	*0.006*

Males	methanol vs. 3kPZS		3kPZS vs. LE		methanol vs. LE	
temperature	t_3	*P*	t_3	*P*	t_3	*P*
q_1 14.4°C	1.22	0.310	1.86	0.161	1.16	0.330
q_2 15.6°C	0.66	0.558	0.89	0.439	0.49	0.657
q_3 17.1°C	0.46	0.678	1.57	0.214	1.51	0.229

Odorant treatments include methanol (control), synthesized 3kPZS ($5x10^{-13}$ M), and larval extract (LE; $5x10^{-14}$ M PADS). Italicized *P*-values indicate a significant difference (two-tailed *t* test, $\alpha = 0.05$) in upstream movement between treatments at each quartile of the temperature range (Figure 1-2).

Movement towards treatment channels

Pre-spawn sea lamprey did not prefer the 3kPZS treatment channel over the adjacent vehicle (methanol) channel across a full migratory season, yet both preferred the channel with larval extract over the adjacent vehicle channel. The same was true for within 0.5-0.25 m of the treatment source (Table 1-3).

Table 1-3. Behavior responses (percentage) of pre-spawn sea lamprey to treatments.

Sex	Treatment	Trials	Released (*N*)	Upstream (*n*)	Treatment channel (*n*)	Treatment source (*n*)
Female	Control	27	617	55% (342) a	50% (171) a	15% (25) a
Female	Larval extract	6	140	85% (119) b	86% (102) b	61% (62) b
Female	3kPZS	22	489	71% (346) c	42% (146) a	18% (27) a
			X^2	59.56	67.03	53.21
			df	2	2	2
			P-value	< 0.001	< 0.001	< 0.001
Male	Control	7	140	64% (90) a	46% (41) a	76% (31) a
Male	Larval extract	5	100	83% (83) b	71% (59) b	90% (53) a
Male	3kPZS	6	120	72% (86) a	55% (47) a	77% (36) a
			X^2	10.58	11.94	4.72
			df	2	2	2
			P-value	0.005	0.003	0.095

Treatments: 3-keto petromyzonol sulfate (3kPZS, dissolved in 50% methanol), extracted water conditioned with larvae (larval extract, dissolved in 50% methanol), and control (vehicle, 50% methanol). Response variables: percentage swimming upstream (Upstream), percentage entering the sub-channel containing each treatment (Treatment channel), and the percentage entering within proximity of the treatment source (Treatment source). Different lower-case letters within each response variable within each sex indicate statistical differences (logistic regression; $\alpha = 0.05$).

DISCUSSION

The male pheromone, 3kPZS outweighed the effects of cold temperatures in modulating upstream movement of pre-spawning female sea lamprey. These results suggest that, in addition to functioning as a male-released sex pheromone (Johnson et al., 2009), 3kPZS also functions as an indicator of the onset of spawning. Pre-spawn migrating sea lamprey showed a positive directional response towards proximity of the larval extract source, which remained consistent with previous studies (Bjerselius et al., 2000; Wagner et al., 2009). Upstream movement at the lower quartile of temperature in males was not significant, yet the trend remained. Males, as releasers of 3kPZS, likely rely on a slightly different multitude of cues to establish nesting sites and begin to signal females.

Selective pressures favour the use of multiple sources of abiotic and biotic information to promote synchrony and reproductive success in animals that migrate long distances. Environmental cues such as temperature and stream flow (Binder et al., 2010), and conspecific cues such as larval odors and information regarding spawning ground size and quality (*i.e.* 3kPZS) may form a hierarchy of contradictory information that, when received in specific context, allow individuals to optimize timing of aggregation for reproduction. This study compares reliability of a particular abiotic cue and conspecific signal 3kPZS, and provides insights as to why pheromone signals appear to be informative for migratory animals such as the sea lamprey.

Data accessibility. Data available in the Electronic Supplementary Material: Raw data.

Competing interests. The authors declare no competing interests.

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CHAPTER 2

FUNCTIONAL TRANSITION OF A CHEMICAL CUE INTO A CHEMICAL SIGNAL IN SEA LAMPREY

ABSTRACT

The sensory trap model of signal evolution hypothesizes that signallers adapt to exploit a cue used by the receiver in another context. While exploitation of receiver biases can result in conflict between the sexes, deceptive signalling systems that are mutually beneficial drive the evolution of reliable communication systems. However, female responses in the non-sexual and sexual context must be uncoupled for communication systems originating through a sensory trap to be reliable. Male sea lamprey (*Petromyzon marinus*) signal with a mating pheromone, 3-keto petromyzonol sulfate (3kPZS), which previous phylogenetic comparisons indicate to be a match to female preference for juvenile odor during migration. Upstream movement of migratory lampreys is partially guided by 3kPZS, but females only move towards 3kPZS with proximal accuracy during spawning. Here, we use instream behavioral assays paired with gonad histology to document the transition of female preference for juvenile- and male-released 3kPZS that coincides with the functional shift of 3kPZS as a migratory cue to a mating pheromone. Females became increasingly bias towards the source of synthesized 3kPZS as their maturation progressed into the reproductive phase, at which point, a preference for juvenile odor (also containing 3kPZS naturally) ceased to exist. Uncoupling of female responses during migration and spawning makes the 3kPZS communication system a reliable means of synchronizing mate search. The present study offers a rare example of a functional shift from a non-sexual migratory cue into a mating pheromone, and provides insights into the origins of stable communication signalling systems.

Key words: pheromone, 3kPZS, conspecific cue, receiver bias, semiochemical.

INTRODUCTION

The sensory trap model of signal evolution hypothesizes that a male trait evolved as a match to a cue used by females in another context (Ryan and Cummings, 2013). While exploitation of receiver biases can result in conflict between the sexes, deceptive signals that are mutually beneficial drive the evolution of stable communication systems (Garcia and Ramirez, 2005). However, females must overcome the deception and adjust their responses to fit the sexual context, functionally uncoupling the responses to the trait in the non-sexual and sexual contexts (Stuart-Fox, 2005). Although many empirical studies implicate a role of sensory traps underlying female preference for male signals (reviewed by (Ryan and Cummings, 2013), the transition associated with uncoupling responses in the non-sexual and sexual contexts remains poorly documented.

Chemical communication is a widely employed sensory modality that offers opportunity to examine the evolution of signaling systems. Previous research on the evolution of animal communication systems has focused primarily on visual and vocal stimuli (Christy, 1995; Gasparini et al., 2013; Hurd and Enquist, 2005; Kelley and Kelley, 2014; Stoddard and Prum, 2011; Tibbetts, 2002), perhaps because they are often within the range of human perception. The evolution of chemical signals remains poorly understood (Symonds and Elgar, 2008; Wisenden, 2014), but is hypothesized to involve a transition of a chemical cue into a signal (Wisenden, 2014). For example, stimuli can originate as byproducts of physiological processes such as "leaking" of hormones or metabolites, or secretion of cuticular defensive or protective compounds (Davidson et al., 2011; Døving et al., 1980; Weiss et al., 2013). Recent phylogenetic comparisons using behavioral bioassays suggest the evolution of the pheromone communication

system in sea lamprey (*Petromyzon marinus*) involved the functional transition of chemical cue into a signal (Buchinger et al., 2013).

Sea lamprey use pheromones to modulate key aspects of their life history. Pheromones are discussed here as chemical signals that are produced by an organism and elicit a physiological or behavioral response in conspecifics (Karlson and Lüscher, 1959). Adult sea lampreys cue onto odors of stream-residing juvenile larvae to navigate towards suitable spawning habitat during their springtime migration from freshwater lakes, or the Atlantic Ocean, into freshwater streams (Bjerselius et al., 2000; Teeter, 1980; Wagner et al., 2009). Males typically move upstream before females (Applegate, 1950), establish nests (Manion and Hanson, 1980), and begin to release a bile salt, 3-keto petromyzonol sulfate (3kPZS), that functions as a sex pheromone (Johnson et al., 2006; Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005).

Female preference for 3kPZS is hypothesized to have originated in a non-sexual context (Buchinger et al., 2013). Female silver lamprey (*Ichthyomyzon unicuspis*), a close relative of sea lamprey, show an upstream migratory response to 3kPZS, but only stream-resident juveniles, not males, release 3kPZS into the water (Buchinger et al., 2013). Furthermore, general upstream movement of female sea lamprey is initiated by 3kPZS during migration (Brant et al., 2015). Female preference for 3kPZS was matched by male sea lamprey. Males dramatically upregulate the 3kPZS biosynthetic pathway (up to 8000-fold increase) in their livers (Brant et al., 2013; Yeh et al., 2012), and release 3kPZS at high rates (~ 0.5mg/h) across specialized cells in the gill epithelia (Siefkes et al., 2003) upon reaching sexual maturation. Since during migration 3kPZS only elicits general upstream movement (Brant et al., 2015), while during spawning 3kPZS elicits a highly targeted and proximate preference in females (Johnson et al., 2009; Siefkes et al.,

2005), female response to 3kPZS in each context had to be uncoupled. Hence, a transition in the function of 3kPZS must occur between the migration and the spawning periods.

Here, we document the functional transition of 3kPZS from a migratory cue to a sexual signal. Using a field study paired with gonadal histology, we track the uncoupling of migratory female responses to juvenile-released 3kPZS and spawning female responses to male-released 3kPZS. Female sea lamprey gradually adjusted their proximal attraction to 3kPZS, which coincides with progression of oocyte maturation. Further, to support a new conceptual model, we confirm that 3kPZS is released by stream-residing larval sea lamprey, and that similar larval-released compounds with similar detection thresholds in the olfactory epithelium (Fine and Sorensen, 2008; Li et al., 1995; Li et al., 2002) do not yield the same behaviors as 3kPZS elicits. Describing the adaptations in male and female sea lamprey associated with 3kPZS signaling provides insights into the evolution of communication in vertebrates.

METHODS

Field behavioral assay

All procedures involving sea lamprey were approved by the Michigan State University Institutional Animal Care and Use Committee prior to the start of the study (Nos. 05/06-066-00, 03/11-053-00 and 03/14-054-00). Pre-spawn migratory adult (immature, herein) sea lamprey were captured by the United States Fish and Wildlife Service and Department of Fisheries and Oceans Canada throughout tributaries to Lake Michigan, Lake Superior and Lake Huron, transported to the United States Geological Survey-Hammond Bay Biological Station, Millersburg, MI, USA (HBBS), separated by sex, and held separately in 500-1000 L flow through tanks. Sex of each individual was later confirmed during surgical tagging procedures by visual observation of eggs. Males were removed from all treatment animals, as only females could be released for testing during this study (*i.e.* to avoid repopulation of the invasive species in barrier-controlled tributaries, see Field site for experimental tests). Ovulated female lamprey (mature, herein) were matured in natural conditions using stream acclimation cages. To accomplish this, 10-15 immature adults were placed in stream acclimation cages (~ 0.25 m^3) that were submerged in the Lower Ocqueoc River near the highway 23 bridge, Millersburg, MI. Acclimation took between 5-10 days for subjects to reach sexual maturity. Sexual maturity was determined by applying gentle pressure to the abdomen and observing for expression of ovulated gametes from the cloacal aperture (Manion and Hanson, 1980; Siefkes et al., 2005). Juvenile sea lamprey (larval, herein) for washings experiments were collected in tributaries to Lake Michigan and Lake Huron year-round using electrofishing gear following published methods (Steeves et al., 2003).

Passive integrated transponder (PIT, Oregon RFID, Portland, Oregon, USA) tagging procedures for migratory female subjects followed those procedures described in Johnson et al. (2009), and those for ovulated females followed Li et al. (2013b). The procedure typically took less than 30 seconds per subject. Implanted animals were immediately transferred into aerated holding tanks with a constant flow of Lake Huron water for up to 24 hours, until they were stocked into stream release cages. Subjects were monitored throughout the day for signs of distress or mortality.

The experimental site for migratory trials consisted of a 250 m-long section of the Upper Ocqueoc River located in Millersburg, Michigan, USA (T35N, R3E, Sec. 27). The upper reaches of the Ocqueoc River consist of suitable spawning substrate and larval habitat, and were historically infested with nesting sea lamprey (Applegate, 1950). Wild populations are now physically barred from entering the Upper Ocqueoc River due to a trap-integrated weir located ~20 km downstream, providing a controlled field site with no naturally occurring background pheromones. The most-upstream 45 m of the site is divided by a naturally occurring island, which separates two similar sub-channels. The center island between the two sub-channels was stabilized using sand bags to insure no treatment odorants seeped between sub-channels during trials. Treatments were administered into the center of a 1 m^2 PIT antenna placed in the center of the stream at the upstream end of each sub-channel. At the confluence of each sub-channel, a transecting PIT antenna, 0.5 m high x 6 m long, independently monitors each mouth to count the numbers of test subjects that enter each sub-channel. The detection range outside of each PIT antenna was 0.3 m, and scan frequencies were 3 scans/sec. Downstream 205 m from the confluence of sub-channels, release cages (~ 0.25 m^3) were anchored in the center of the main

channel (Figure 2-1a). Details of the field site for sexually mature female trials are outlined in Li et al. (2013b) with slight modification seen in Figure 2-1b.

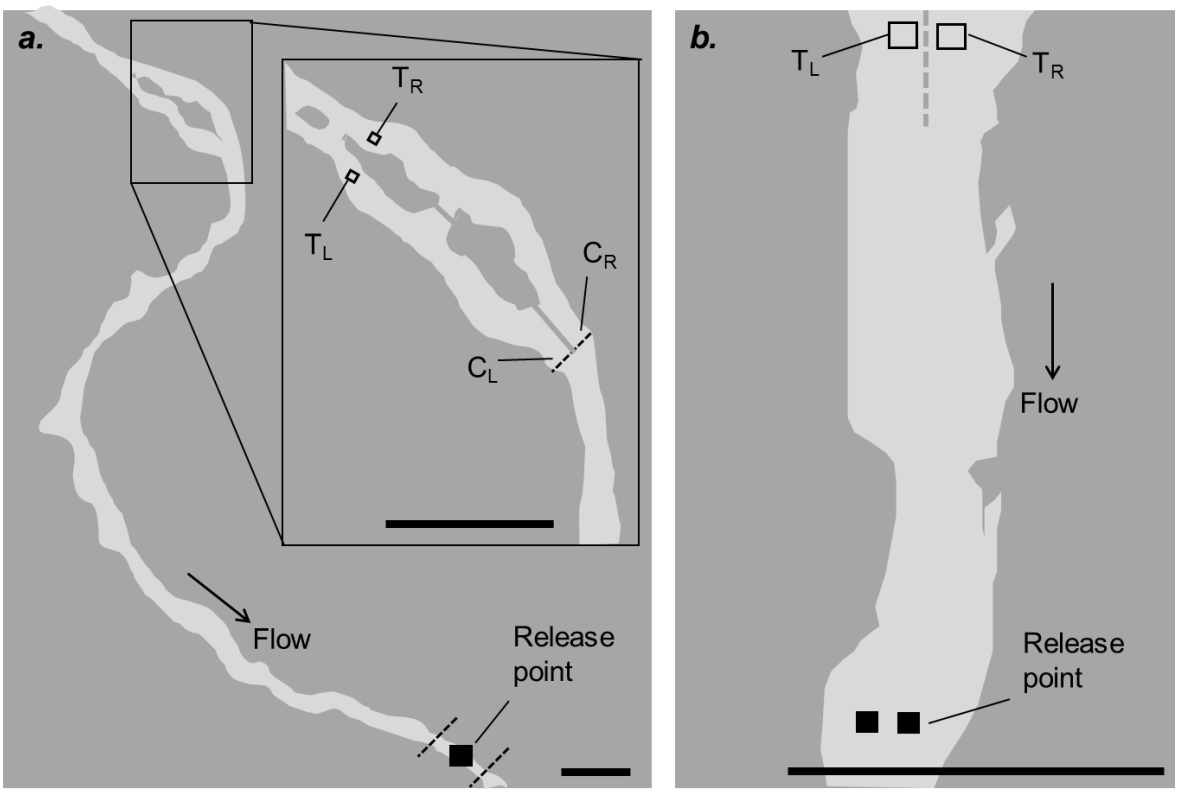

Figure 2-1. The section of the Upper Ocqueoc River, Millersburg, MI, U.S.A (T35N, R3E, Sec. 27) used to examine behaviors of sea lamprey in relation to pheromone treatments. (*a*) Full 250 m-long section and details of the naturally bifurcated sub-channels used for examining behaviors of migratory female subjects. Black-dashed lines indicate channel transecting PIT antennas placed at the confluence of the left (C_L) and right (C_R) sub-channels, and upstream and downstream of the release point. **(*b*)** The 45 m-long section used for examining behaviors of sexually mature female subjects. Hollow boxes represent left (T_L) and right (T_R) 1 m^2 PIT antennas where, in the center of each, treatments were administered. Scale bars = 25 m.

Permission to administer 3kPZS and similar compounds/raw odor of lamprey into the stream was obtained from the United States Environmental Protection Agency through experimental user permits 75437-EUP-1 and 75437-EUP-2 prior to any field testing. 3kPZS was custom synthesized by Bridge Organics (Vicksburg, Michigan, USA; purity >97%) and stored in powder form at -80^{o}C until stock solutions were made. Purity was determined using a Waters ACQUITY LC System coupled with the Waters Quattro Premier XE tandem quadrupole mass spectrometer (LC–MS/MS, Milford, MA, USA). Nuclear magnetic resonance (^{1}H NMR) was used to confirm the chemical structure of 3kPZS. A 10 mg/mL stock solution of synthesized 3kPZS (in 100% methanol) was prepared and transferred into five vials of 10 mL aliquots, each. 3kPZS stock solution was stored at -80^{o}C until use in the field.

Extracted wash-water from larvae (larval extract) was used as a positive control to validate the experimental system. Larval extracts have been shown to induce strong targeted preferences towards the odorant source from migratory female sea lamprey during past studies (Bjerselius et al., 2000; Wagner et al., 2009). Methods of extraction followed those outlined by Fine et al. (2006) . PADS, a component of larval extract with known chemical structure (Sorensen et al., 2005), was used as a benchmark compound when calculating the volume of extract to apply to the stream and maintain consistent full stream concentrations. The concentration of PADS was determined using HPLC–tandem mass spectrometry (HPLC-MS/MS) following Li et al. (2013a). Treatments included; (1) synthesized 3kPZS ($5x10^{-13}$ molar, M) administered into one sub-channel (methanol vehicle into the adjacent sub-channel), (2) larval extract ($5x10^{-14}$ M benchmark PADS) administered into one sub-channel (river water vehicle into the adjacent sub-channel), and (3) vehicle control (methanol into both sub-channels).

A typical riverine migratory season for sea lamprey lasts from May through late June. To specifically examine whether responses to 3kPZS change in female sea lamprey as they approach ovulation, and subsequently the spawning season, we divided field trials into an *Early* migration, *Late* migration and *Mature* season. *Mature* trials were conducted with sexually mature subjects similarly to previous studies (Johnson et al., 2006; Siefkes et al., 2005). *Early* migratory season trials were conducted 08 – 27 May, and *Late* trials were conducted 13 – 27 June, 2013, at night when migratory sea lamprey are most active (Applegate, 1950). *Mature* trials were conducted 3 – 19 July, 2013 during the day when mature sea lamprey are active and nesting (Applegate, 1950).

Pheromone treatments were diluted with 30 L of river water in large mixing bins. Each solution was pumped into the center of each PIT antenna in respective sub-channels through latex tubes at a rate of 167 ± 5 mL/min over the span of 2.5 hours using peristaltic pumps (Masterflex 7553-70, Cole-Parmer, Vernon Hills, Illinois, USA). A treatment was administered to one sub-channel (treatment channel) while a methanol vehicle was administered into the adjacent sub-channel. The treatment and control sub-channels were alternated each trial to avoid artifacts of channel bias. Stream discharge was estimated following published methods (Murphy and Willis, 1996) throughout the migratory and mating season (taken every three days, or after every precipitation event) to determine the volume of treatment stock solution to apply to the stream each trial and maintain consistent full-stream concentrations. Stream discharge ranged from 3.09 – 1.50 meters^3/sec during migratory trials and from 0.85 – 0.40 meters^3/sec. during mating trials.

Two trials were conducted each night or day. For migratory trials, no more than 20 PIT-tagged sea lamprey were released per trial. For mating trials, no more than 10 ovulated females were released per trial because mature female sea lamprey are more difficult to attain. Subjects

were allowed an acclimation period in the stream of 10 – 15 hours prior to a trial. Subjects for each trial were acclimated in separate release cages (2 cages total). Minimal mortality occurred during acclimation of migratory subjects (< 1%). Mortality is more common in sexually mature subjects during acclimation (7%), as they are in their final life stage at this time (*i.e.* a semelparous species). Therefore, two extra mature subjects were often stocked (12 total) for each trial. If no mortality occurred, extra subjects were removed from the release cage prior to release. Release cages were solid aluminum and stainless steel (~ 0.25 m^3), consisting of a sliding door that was removed manually upon release. Migratory trials were conducted from ~ 2020 h (starting at sundown) through ~ 0310 h, and mating trials from ~ 0700 h – 1200 h.

Each trial was 2.5 hours long. In the first ½ hour, the treatment was administered to the stream allowing the current to carry the compound to the downstream acclimation cage containing test subjects. Subjects were released, and during the remaining two hours, animals were free to swim throughout the experimental system while treatments were administered. The second trial started 15 – 30 minutes after the first. Treatments were kept consistent for each day or night of trials (*i.e.* if 3kPZS was tested during the early trial, 3kPZS was also tested during the late trial to prevent the possibility of any unwanted contamination from other treatments that day/night). All equipment was thoroughly rinsed with stream water prior to a new trial. No animals were recovered from the stream after a trial. Unique PIT identification for each subject prevented pseudoreplication during trials. Movement data were consolidated and stored using a multiplexor (Oregon RFID, Portland, Oregon, U.S.A.). Data were uploaded each trial night using a hand-held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

Subjects were randomly selected and gonads were dissected for histology during field trials (Supplemental Table S2-1). Samples of oocytes were collected from the posterior, medial, and anterior locations of the ovary. Oocytes were fixed in a 4% paraformaldehyde solution and placed in 4^{o}C until sectioning, hemotoxylin, and eosin staining procedures could be conducted. Histological examination of oocytes and determination of maturity (Days from ovulation, Supplemental Table S2-1) followed published procedures (Yorke and McMillan, 1980). All oocytes on each slide were examined to confirm homogeneity of maturational state throughout each ovary. Days from ovulation were estimated by comparing oocyte morphology and time of year to those estimates of days until ovulation in the literature (Larsen, 1980; Lewis and McMillan, 1965; Yorke and McMillan, 1980).

Three known larval-released compounds, PZS, PADS, and PSDS, with similar olfactory sensitivity to 3kPZS (Fine and Sorensen, 2008; Li et al., 1995; Li et al., 2002), were compared to 3kPZS in behavioral field trials to confirm behavioral responses to 3kPZS aren't simply based on familiarity to compounds released from conspecifics. Trials were conducted only during *Late* migration, from 31 May – 12 June 2008. All experimental procedures were conducted in the same experimental system (Figure 2-1a), with slight modification. Three trials were conducted per night instead of two. Females were pre-exposed to the test article 1 h prior to release, and their movement and distribution in the stream were monitored for 2 h after release. Additional treatments included: (1) a mixture of PADS ($1x10^{-12}$ M), PSDS ($5x10^{-13}$ M), PZS ($5x10^{-13}$ M), and 3kPZS ($5x10^{-13}$ M), (2) a mixture of PADS ($1x10^{-12}$ M), PSDS ($5x10^{-13}$ M), and PZS ($5x10^{-13}$ M), and (3) synthesized 3kPZS ($5x10^{-13}$ M). Vehicle and larval extract control trials were conducted consistently across all behavioral trials. Ratios of PADS, PSDS, and PZS mixtures were kept consistent with those in published literature (Fine and Sorensen, 2008; Sorensen et al.,

2005). Oocytes were sampled on 03 June (n = 12) and on 12 June 2008 (n = 12, Supplemental Table S2-1). Histological analyses of oocytes were consistent for all trials (data not shown). PADS, PSDS, PZS and 3kPZS were synthesized by Bridge Organics in 2008 (Vicksburg, Michigan, USA) with purity greater than 95%.

Previously, 3kPZS had been considered to be released exclusively from mature male sea lamprey during the mating season to draw mature females towards the nest for courtship (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005). To examine whether larval sea lamprey release 3kPZS specifically during the migratory season when immature migrating sea lamprey would be exposed, we sampled populations of wild larval sea lamprey to analyze larval-conditioned wash-water over that time period. Five collections were completed from 08 December 2012 – 28 April 2013 (Table 2-3). Ice and flooding made collections difficult for every month, yet our collections were considered representative of the onset of migration through pre-spawn migration in the Great Lakes region (Manion and Hanson, 1980). Larvae were collected with assistance from the U.S. Fish and Wildlife Service in streams using electrofishing gear following published methods (Steeves et al., 2003). Details of larval collections can be seen in Table 2-3. Details of wash-water collection from larvae can be seen in Supplemental Methods, Supplemental Figure S2-1.

Data analyses

Statistical analyses for all in-stream migratory and sexually mature female behaviors during field studies follow those previously described by our laboratory (Johnson et al., 2009; Li et al., 2013b) with slight modification. During 2013 migratory trials, four response variables were examined: (1) the number of subjects that moved downstream of the release cage, and did not move back up during the trial (*Down*), (2) the number of subjects that moved upstream from

release cages to the confluence of the two sub-channels (205 m, *Up*), (3) of the total number of subjects that moved upstream to the confluence of the two sub-channels, the number of subjects that then entered a sub-channel activated with a pheromone treatment (*Treatment channel*), and (4) of the subjects that entered the treatment channel, the number that entered within 0.5 m of the treatment source (*Treatment source*). In 2008 migratory trials, all response variables were consistent except "*Down*," which could not be examined due to a release-point antenna failure. In sexually mature subjects, our field site was slightly modified from published experimental systems (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005). A single response variable was examined in mature subjects: the number of subjects that entered within 0.5 m of the treatment source (Treatment source), which was adjacent to another source (1 m^2 PIT antennas, 1.5 m apart). These side-by-side nests represented the left and right sub-channels in statistical analyses when comparing to migratory trials. Sexually mature females had to be tested in a smaller system than the system used for testing migratory females because subjects were ovulated and therefore physically incapable of moving upstream on such a large scale within the allotted swim time (2 – 3 hours). For all behavior data, upon observing no signs of over dispersion or nonlinearities, logistic regression (generalized linear model) with a binomial distribution was examined for each response variable in relation to each treatment (R version 2.11.1).

Pheromone concentration data from monthly larval wash-water collections were standardized by weight (ng/g-larvae/hr). All values from LC–MS/MS analyses associated with a signal-to-noise ratio less than or equal to 10 were considered below the limit of quantitation and removed from the data set. The Levene's test for homogeneity of variance was used to examine any violations of assumptions normality across variance before further statistical analyses were

conducted. Data that were not normally distributed or showed heterogeneity across variance were log-transformed. Once homogeneity of variance was observed, an ANOVA and post-hoc Tukey's HSD ($\alpha = 0.05$) was conducted for all statistical comparisons (R version 2.11.1, Vienna, Austria).

RESULTS

Preference for 3kPZS becomes increasingly targeted as females approach ovulation

Immature female subjects incrementally increased their directional preference towards the sub-channel activated with 3kPZS as their oocytes approached ovulation. During the early migratory season (08 – 15 May, 2013) large numbers of females moved upstream (74-86%). Females did not show a bias towards the sub-channel activated with 3kPZS ($Z_5 = 0.34$, $P = 0.737$), while a positive bias was seen towards the sub-channel activated with positive control larval extract ($Z_5 = 2.53$, $P = 0.012$), see Table 2-1 for statistical comparisons. Histology of oocytes from these subjects yielded eggs to be pre-ovulated, with intact follicular cell layer encompassing an adhesive cell layer, pronounced nucleus, and a buildup of fluid of ovulation (Figure 2-2a - Early). These subjects are estimated to be more than 10 days from ovulation based on previous studies examining structural and developmental aspects of the ovary in sea lamprey (Supplemental Table 2-1).

During the late half of the migratory season (13 – 26 June, 2013), the bias towards 3kPZS matched that of larval extract when significant numbers (up to 75%) of subjects began moving into the sub-channel (3kPZS: $Z_5 = 2.23$, $P = 0.026$, Larval Extract: $Z_5 = 3.07$, $P = 0.002$). High numbers of subjects moved upstream (77-90%) and approached the sources of treatments during this time (Table 2-1). Histology on subjects during late migration showed oocytes to be closer to ovulation, yet still pre-ovulated, with intact follicular cell layer, a present yet grainy nucleus, and compression of the adhesive cells due to tightening of the follicular cell layer from increasing buildup of fluid of ovulation (Figure 2-2a - Late). These subjects are estimated to be within 4 days from ovulation (Supplemental Table S2-1).

Mature (ovulated) subjects (tested 07 – 19 July, 2013) showed a strong preference towards a 3kPZS source as expected (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005), where 100% entered the 1 m^2 3kPZS source ($X^2_2 = 57.25$, $P < 0.001$). Histology confirmed that oocytes were ovulated in a sub-sample of these test subjects, showing a broken follicular cell layer and exposed adhesive cells on the distal half of each oocyte and no nucleus present. Egg development did not vary between the posterior, middle, or anterior regions of the ovary in any of our samples (Figure 2-2b). Also expected yet previously undocumented, a preference for larval extract treatments did not occur by mature female subjects (Figure 2-2b).

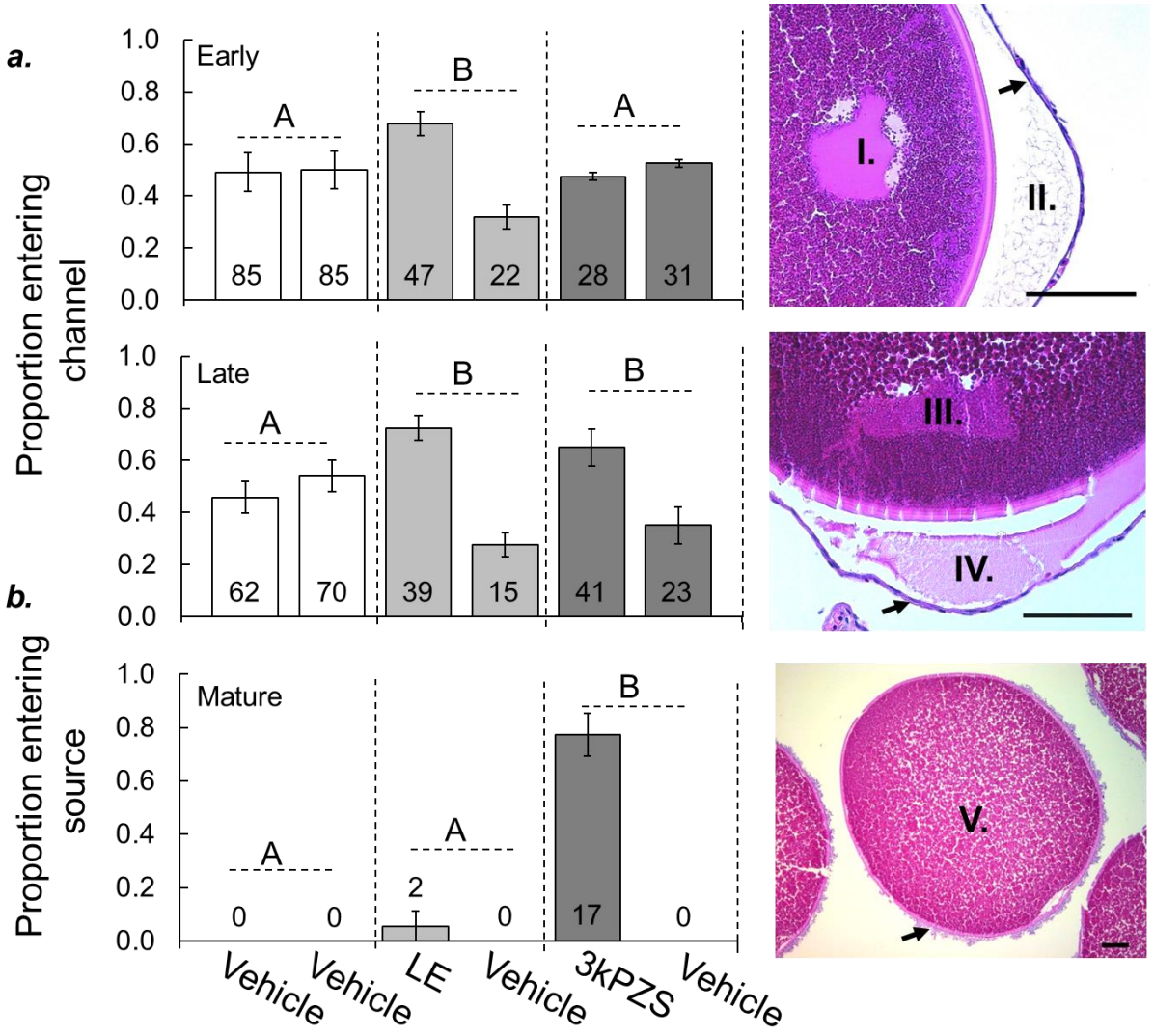

Figure 2-2. Preference response of sea lamprey to conspecific-released compounds. Treatments included methanol vehicle controls (vehicle vs. vehicle), larval extract (LE) at $5x10^{-14}$ molar benchmark PADS vs. vehicle, and synthesized 3kPZS (3kPZS) at $5x10^{-13}$ molar vs. vehicle. (***a.***) The proportion of immature female sea lamprey entering the sub-channel containing each treatment during the *Early* (May, 2013) and *Late* (June, 2013) migratory season. Different upper-case letters indicate a significant proportion moved into the activated treatment sub-channel (logistic regression: $X^2_5 = 20.35$, $P = 0.001$). Key histological features examined in immature oocytes included the follicular cell layer (**arrow**) that encompasses an adhesive cell layer, nucleus (**I.** in *Early*, **III.** in *Late*), and build-up of fluid of ovulation (**II.** in *Early*, **IV.** in *Late*). (***b.***) The proportion of *Mature* ovulated female sea lamprey entering the treatment vs. vehicle source (1 m^2) during the mating season in early July. Different upper-case letters indicate a significant proportion moved into the activated treatment source ($X^2_2 = 57.25$, $P < 0.001$). Key features examined in *Mature* oocytes included a broken (absent) follicular cell layer with exposed, lysed, adhesive cells (**arrow**), and lack of nucleus (**V.**). Scale bars = 100µm.

Table 2-1: Additional preference responses (in addition to Figure 2-2a) of migratory female sea lamprey to conspecific-released compounds.

Treatment	Trials	Released	Down (*n*)	Up (*n*)	Treatment source (*n*)
Vehicle **Early**	11	218	17% (*36*) A	78% (*170*) A	14% (*12*) A
Larval Extract **Early**	4	80	4% (*3*) B	86% (*69*) AB	43% (*20*) BC
3kPZS (5E-13 M) **Early**	4	80	16% (*13*) A	74% (*59*) A	11% (*3*) A
Vehicle **Late**	9	171	6% (*11*) B	77% (*132*) A	29% (*18*) B
Larval Extract **Late**	3	60	5% (*3*) B	90% (*54*) B	54% (*21*) C
3kPZS (5E-13 M) **Late**	4	80	5% (*4*) B	80% (*64*) A	32% (*13*) BC
		X^2	26.05	15.03	27.87
		df	5	5	5
		P-value	< 0.001	0.010	< 0.001

See Figure 2-2 for explanation of treatments. Trials were conducted during *Early* (May) and *Late* (June) 2013 migratory season. Additional preference response *Down* shows the percentage (*n*) of subjects that moved down from release cages, and did not come back upstream during trials. *Up* refers to subjects that moved upstream from the release cage and continued to swim 205 m to the confluence of the two sub-channels. *Treatment source* refers to subjects that entered within 0.5 m of the treatment source after entering the sub-channel activated with each treatment. Responses that share a letter across treatments are not significantly different (logistic regression; $\alpha = 0.05$).

Similar compounds did not yield a behavioral response in migratory subjects

Behavioral trials testing mixtures of similar compounds PZS, PADS, and PSDS with an addition or subtraction of 3kPZS yielded 3kPZS to be responsible for behavioral responses seen during late migration in our trials (Table 2-2). Late migratory female responses to treatments did not vary between years (vehicle controls: $X^2_1 = 1.12$, $P = 0.289$; 3kPZS: $X^2_1 = 0.39$, $P = 0.531$; and larval extract controls: $X^2_1 = 0.12$, $P = 0.729$), and so were combined. Greater numbers of subjects again moved upstream towards the sources of 3kPZS and larval extract (68-75%), and moved upstream less during vehicle controls ($X^2_4 = 13.24$, $P < 0.001$). Both larval extract and all treatments specifically containing 3kPZS increased the numbers of migratory subjects that entered the treatment sub-channel by up to 33% from that of vehicle controls ($X^2_4 = 16.24$, $P < 0.001$). Both larval extract and all treatments specifically containing 3kPZS increased the numbers of migratory subjects also increased the numbers that entered within 0.5 m of the treatment source by up to 19% compared to vehicle controls ($X^2_4 = 43.32$, $P < 0.001$, Table 2-2).

Table 2-2: Preference responses of migratory female sea lamprey to similar conspecific-released compounds.

Treatment	Trials	Released	Up (*n*)	Treatment channel (*n*)	Treatment source (*n*)
Vehicle	8	160	41% (*65*) A	42% (*27*) A	9% (*2*) A
Larval Extract	8	160	71% (*114*) BC	75% (*86*) B	60% (*52*) C
3kPZS, PZS, PADS, PSDS	6	120	75% (*90*) C	69% (*62*) B	28% (*17*) B
PZS, PADS, PSDS	8	160	42% (*67*) A	43% (*29*) A	16% (*5*) A
3kPZS	6	120	59% (*71*) B	68% (*48*) B	32% (*15*) B
		X^2	13.24	16.24	43.32
		df	4	4	4
		P-value	< 0.001	< 0.001	< 0.001

Treatments included methanol vehicle controls (vehicle vs. vehicle), larval extract at $5x10^{-14}$ molar (M) benchmark PADS vs. vehicle, a mixture of PADS ($1x10^{-12}$ M), PSDS ($5x10^{-13}$ M), PZS ($5x10^{-13}$ M) and 3kPZS ($5x10^{-13}$ M) vs. vehicle, the same mixture minus 3kPZS vs. vehicle, and synthesized 3kPZS (3kPZS) at $5x10^{-13}$ molar vs. vehicle. Response variables are consistent with those described in Figure 2-2, Table 2-1. Responses that share a letter across treatments are not significantly different (logistic regression; $\alpha = 0.05$).

Larval sea lamprey release 3kPZS

Analysis of the monthly wash-water collection revealed that 3kPZS is released from larval sea lamprey in the months leading up to the spawning season. While little variation was seen in PZS release rates through the sampling months, 3kPZS showed an increase in release rates during the migratory season. Specifically, 3kPZS release rates were highest from larvae towards the end of March (ANOVA: $F_4 = 27.35$, $P = 0.001$). Mean (± 1 SEM) 3kPZS release rate from larvae for all months was 0.107 ± 0.05 ng/g-larvae/hr. Compound PZS was also released from larvae during this time, and did not vary between months ($F_4 = 0.54$, $P = 0.750$). Mean PZS release rate from larvae for all months was 0.422 ± 0.07 ng/g-larvae/hr, which was significantly higher than that of 3kPZS (Two-way *t*-test: $t_8 = 3.41$, $P = 0.009$). Compounds PADS and PSDS were not detected from larvae in our samples during these months (Table 2-3).

Table 2-3: Release rates of larval-released compounds.

Date	River	*n*	Temp (°C)	Batch weight (g)	3kPZS release (± 1 SEM)	PZS release (± 1 SEM)	PADS release	PSDS release
8-Dec-12	Betsie	168	6.3	210	0.01 (0.01) A	0.25 (0.05) A	*ND*	*ND*
12-Jan-13	Betsie	241	3.7	369	0.09 (0.03) A	0.46 (0.26) A	*ND*	*ND*
4-Mar-13	White	169	1.7	254	0.03 (0.02) A	0.38 (0.25) A	*ND*	*ND*
31-Mar-13	Silver	82	3.8	111	0.31 (0.16) B	0.69 (0.60) A	*ND*	*ND*
28-Apr-13	Carp	127	6.0	177	0.10 (0.05) A	0.33 (0.12) A	*ND*	*ND*
				F	27.35	0.54	*NA*	*NA*
				df	4	4	*NA*	*NA*
				P-value	0.001	0.750	*NA*	*NA*

Larval sea lamprey were sampled from for tributaries of Lakes Michigan and Huron, located in the northern lower peninsula of Michigan. Compounds examined included: 3-keto petromyzonol sulfate (3kPZS), petromyzonamine disulfate (PADS), petromyzosterol disulfate (PSDS), and petromyzonol sulfate (PZS). Mean release rates (ng/g-larvae/hr) from triplicate samples of each batch of larvae (*n*) are shown for each date of collection. Release rates within each compound that share a letter are not significantly different (ANOVA; $\alpha = 0.05$). Compounds PADS and PSDS were not detected (*ND*) in our samples during these dates.

DISCUSSION

Data presented in this study, when taken together with evidence from previous studies, document the transition of 3kPZS from a navigational cue to a mating pheromone in sea lamprey. Migratory sea lamprey use 3kPZS as a navigational cue that initiates non-targeted upstream movement (Brant et al., 2015). During spawning, however, male-released 3kPZS functions as a sexual signal that guides females upstream to the proximity of a nest (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2003; Siefkes et al., 2005). Phylogenetic studies indicate that the migratory response to 3kPZS predates the sexual response to 3kPZS (Buchinger et al., 2013). The pre-existing bias of females to move upstream to 3kPZS likely created selective pressure on males to massively upregulate 3kPZS synthesis (Brant et al., 2013) and amplify 3kPZS as a signal. Although deceptive signals can lead to conflicts of interest between the sexes, the mutual drive of males and females to efficiently find mates likely precludes any conflict associated with chemical communication during spawning. However, females must adjust their responses to 3kPZS during spawning for the communication system to be stable. Here, we document the functional transition of 3kPZS as a migratory cue to a sexual signal by tracking female gonad maturation alongside their behavioral responses to 3kPZS.

If a sensory trap leads to a mutually beneficial communication system, female response must be uncoupled between the non-sexual and sexual contexts, enabling females to respond to the male trait in a way appropriate to the sexual context (Garcia and Ramirez, 2005). For example, male signaling with a terminal yellow band on the caudal fin in Goodeinae fishes appears to match an existing foraging preference in females, but females of some species have further evolved to use the trait to evaluate male quality. However, females that use the terminal

yellow band to select mates have lessened their responses to the yellow band as a foraging cue, effectively uncoupling the responses in the foraging and sexual contexts (Garcia and Ramirez, 2005). Likewise, female sea lamprey were likely selected to adjust their response to 3kPZS between the migratory and spawning contexts. 3kPZS initiates non-targeted upstream movement of migratory sea lamprey, a behavior fitting the large-scale navigations necessary to locate rivers and tributaries in which to spawn (Applegate, 1950; Manion and Hanson, 1980). During spawning, 3kPZS elicits a highly targeted bias towards the 3kPZS source, and resumed search behavior when females exit the odor plume, to locate mates (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005). Incorrectly responding to 3kPZS in either context is maladaptive for females. Hence, the integration of 3kPZS into the sexual communication system occurred through male adaptations for signaling and female adaptions to appropriately respond to 3kPZS. Interestingly, non-sexual and sexual female preferences in sea lamprey became uncoupled by an increase in female preference in the sexual context, whereas non-sexual and sexual female preferences in Goodeinae fishes became uncoupled by a decrease in female preference in the non-sexual context (Garcia and Ramirez, 2005).

The transition of 3kPZS from a navigational cue to a mating pheromone in sea lamprey is thus far specific to 3kPZS. Other larval-released compounds, PZS, PADS, and PSDS have been shown to have similar response thresholds in the olfactory epithelium compared to 3kPZS during electro-physiological analyses (Li et al., 1995; Sorensen et al., 2005), and all appear to share similar biosynthetic pathways (Brant et al., 2013; Venkatachalam, 2005), yet only 3kPZS induced behavioral responses consistent with a shift from chemical cue to chemical signal as females mature. Compounds PADS and PSDS were not detected from larvae in our samples, nor were they behaviorally active in our field studies. Behavioral results testing these compounds in

the field are consistent with previous field evaluations (Meckley et al., 2012), and inconsistent with previous laboratory evaluations (Sorensen et al., 2005), suggesting discontinuity between laboratory and field studies for evaluation of behaviors to pheromones in fishes (Johnson and Li, 2010). Interestingly, PZS was released at significantly higher rates than 3kPZS from larvae, yet females became ritualized to 3kPZS over PZS. The reason 3kPZS was selected over PZS as a pheromone may be attributable to a mechanism of release across gill epithelia in male senders (Brant et al., 2013; Li et al., 2002; Siefkes et al., 2003), yet the exact reason remains unknown.

In summary, we document a missing behavioral link of the functional transition of 3kPZS from a navigational cue to a pheromone. Results here support the theory that female responses must be uncoupled between the non-sexual and sexual contexts, enabling females to respond to the male trait in a way appropriate to the sexual context (Garcia and Ramirez, 2005). While our observation of increasingly targeted preferences as female maturation progressed into the spawning phase tracks the uncoupling of the responses in each context, the mechanisms underlying the transition remain unknown. We document 3kPZS release by larval sea lamprey, and conclude that 3kPZS was selected to become a conspicuous signal over additional larval-released compounds with similar detection thresholds in the olfactory epithelium (Fine and Sorensen, 2008; Li et al., 1995; Li et al., 2002). Sea lamprey represent a useful model for understanding the origins of chemical signals in vertebrates (Figure 2-3).

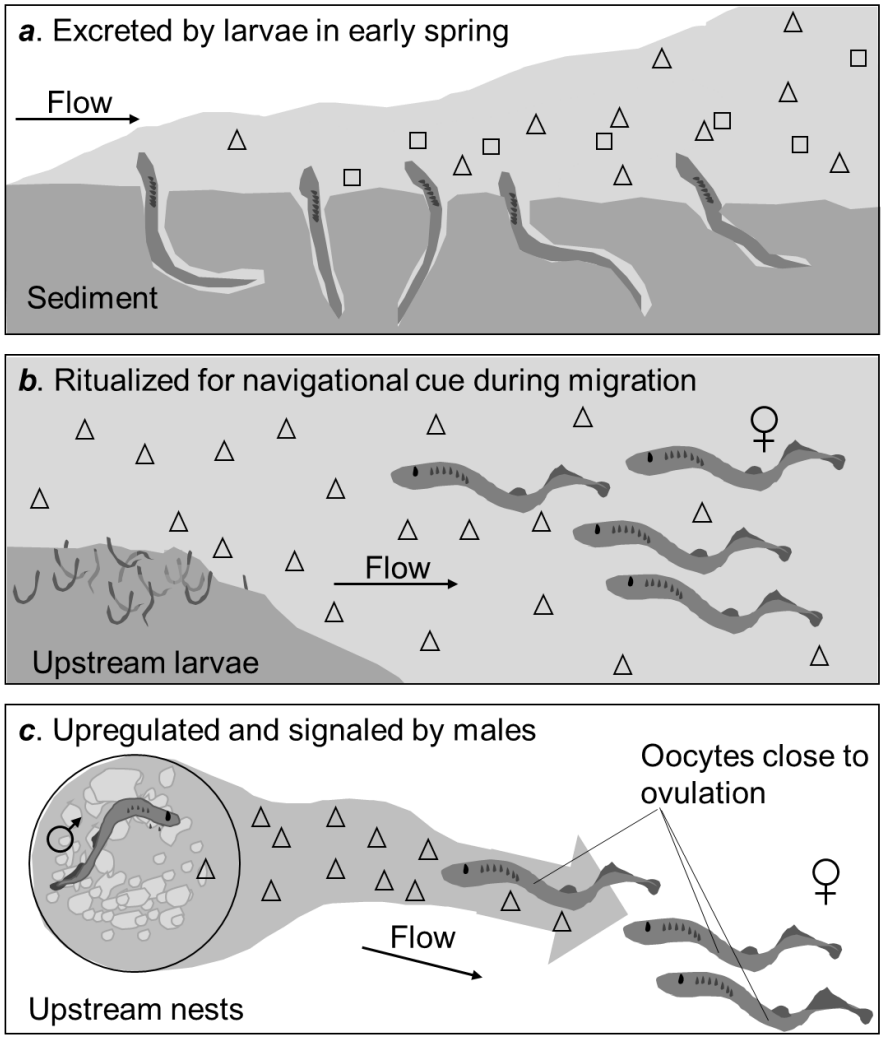

Figure 2-3. Conceptual model of the evolution of pheromone 3-keto petromyzonol sulfate (3kPZS) in *P. marinus*. (***a.***) 3kPZS (Δ) is excreted as a metabolic by-product by larvae residing and feeding in streams, along with a suite of other compounds (□). (***b.***) 3kPZS (Δ) became ritualized as a navigational cue to migrating adults. (***c.***) Males adapted to massively upregulate and release of 3kPZS (Δ) as a signal to benefit from a pre-adapted bias to 3kPZS in female receivers. Females adapted to fine tune their movement towards a 3kPZS source (nest) to synchronize reproduction upon maturation of their gonads. Both parties continued to mutually benefit as 3kPZS evolved from a cue to a signal.

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CHAPTER 3

MIXTURES OF TWO BILE ALCOHOL SULFATES FUNCTION AS A PROXIMITY PHEROMONE IN SEA LAMPREY

ABSTRACT

Unique mixtures of pheromone components are commonly identified in insects, and have been shown to increase attractiveness when reconstructed at the natural ratio released by the signaler (Cardé et al., 1977). In previous field studies of pheromones that attract female sea lamprey (*Petromyzon marinus*, L.), putative components of the male-released mating pheromone included the newly described bile alcohol 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) and the well characterized 3-keto petromyzonol sulfate (3kPZS). Here, we show chemical evidence that unequivocally confirms the elucidated structure of DkPES, electrophysiological evidence that each component is independently detected by the olfactory epithelium, and behavioral evidence that mature female sea lamprey prefer artificial nests activated with a mixture that reconstructs the male-released component ratio of 30:1 (3kPZS:DkPES, molar:molar), and characterize search behavior (sinuosity of swim paths) of females approaching ratio treatment sources. Unique pheromone ratios may underlie reproductive isolating mechanisms in vertebrates, as well as provide utility in pheromone-integrated control of invasive sea lamprey in the Great Lakes.

Key words: pheromone ratio, sea lamprey, bile alcohol, chemical signal

INTRODUCTION

A common source of conspecific information used in orientation strategies and mate location across the animal kingdom is provided by pheromones, or unique chemical signatures that are released by animals and influence behavior or development of members of the same species (Karlson and Butenandt, 1959; Wyatt, 2010). Together, the sending and receiving of pheromones result in movement patterns that reduce the distance between conspecifics across their odor landscape (attraction) and/or maintain individuals in place (arrestant) to gain advantage in mating or feeding (Steiger et al., 2011; Wyatt, 2010). In insects, pheromones that function in sex and aggregation are often comprised of multiple components at specific ratios (Cardé et al., 1977; Coracini et al., 2001; Reyes-Garcia et al., 2014). For pheromones of unique ratios to function as species-specific attractants, a level of discrimination against individual components must occur at the sensory and behavioral level. In fishes, olfactory systems have been shown, via electrophysiological cross-adaptation experiments, to detect and discriminate between compounds with separate receptors (Hara, 1992; Keller-Costa et al., 2014; Lipschitz and Michel, 1999). Detection and discrimination of individual compounds in phylogenetically similar groups of fishes has been proposed as an adaptation involved in speciation (Keller-Costa et al., 2014). However, behavioral evidence is rarely presented to evaluate the specificity of pheromone ratios in vertebrates (Wyatt, 2010).

The olfactory epithelium of the sea lamprey (*Petromyzon marinus*, L.) has been shown to detect and discriminate multiple compounds that are structurally similar (Li et al., 2014; Li et al., 1995; Sorensen et al., 2005). Using pheromones for key aspects of their life history (Bjerselius et al., 2000; Johnson et al., 2009; Teeter, 1980; Wagner et al., 2009), sea lamprey present a useful

vertebrate model for studies regarding pheromone ratio specificity. Sea lamprey begin their single reproductive season by migrating into freshwater tributaries to the Atlantic Ocean (native range), or the Laurentian Great Lakes (invasive range), that are activated with compounds released by stream-residing conspecific larvae (Bjerselius et al., 2000; Wagner et al., 2009). Males often move upstream in greater numbers earlier in the migratory season through April – May, and establish nests in suitable spawning habitat in early June (Johnson et al., 2015b; Manion and Hanson, 1980). Upon reaching sexual maturation in streams, males release a pheromone that includes a main component, 3-keto petromyzonol sulfate (3kPZS), across gill epithelia (Li et al., 2002; Siefkes et al., 2003). Synthesized 3kPZS alone draws significant numbers of mature females upstream towards the source (Johnson et al., 2009; Siefkes et al., 2005). However, 3kPZS as a single component is often less attractive than the whole male odor (Johnson et al., 2009). Upon analyzing whole male odor (termed spermiated male washings, or SMW, herein), the chemical structure of a new sulfate-conjugated compound, 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) was elucidated (Li et al., 2013b). Mature females were shown to increase their preference for mixtures of 3kPZS and DkPES compared to 3kPZS alone. However, many critical issues have not been addressed regarding the identity and function of DkPES (Li et al., 2013b), including chemical synthesis of DkPES and confirmation of the elucidated structure, discrimination of DkPES from 3kPZS by the olfactory epithelia, the orientation mechanisms used by sea lamprey to locate a mixture of these two compounds, and the effective range of ratios between the two pheromone components required for attraction.

This study reports the next step in identifying the function of DkPES as a pheromone component in sea lamprey. Here, we confirm the structure elucidated for DkPES with its synthesized copy, show the olfactory epithelium of sea lamprey discriminates DkPES from

3kPZS, demonstrate that females are attracted to a reconstructed ratio similar to that seen in extracts from mature male sea lamprey (Li et al., 2013b), and further define the orientation strategies of mature females to the mixture of DkPES and 3kPZS. Based on the results, we provide a conceptual model outlining the importance of specific pheromone ratios in guiding chemo-orientation strategies in sea lamprey.

RESULTS

Spectra of synthesized DkPES match those of purified DkPES

The compound DkPES ammonium salt applied in this study was synthesized by Apeloa Kangyu Pharmaceutical Co. (Dongyang, Zhejiang, China) according to the structure deduced in the preceding study (Li et al., 2013b). Differential scanning colorimetry (DSC) analysis (Giron and Goldbronn, 1995; Van Dooren and Müller, 1984) of the synthetic product indicated that its purity was 97.0% (Supplementary Information Figure S3-1). The chemical structure of DkPES was further confirmed by comparison of high resolution mass and NMR spectra of the purified compound (Li et al., 2013b) and the synthesized copy. The pseudo-molecular formula determined by high resolution mass spectrometry (*m/z* 449.2012 [M – NH_4]–) of the synthetic product was $C_{24}H_{33}O_5S$, which was in good agreement with the calculated mass (*m/z* 449.1998, ΔmDa 1.4, ppm 3.1, Supplementary Information Table S3-1). The purified and the synthesized compounds showed overlapped NMR spectra (Figure 3-1), indicating that they are identical in their chemical structure.

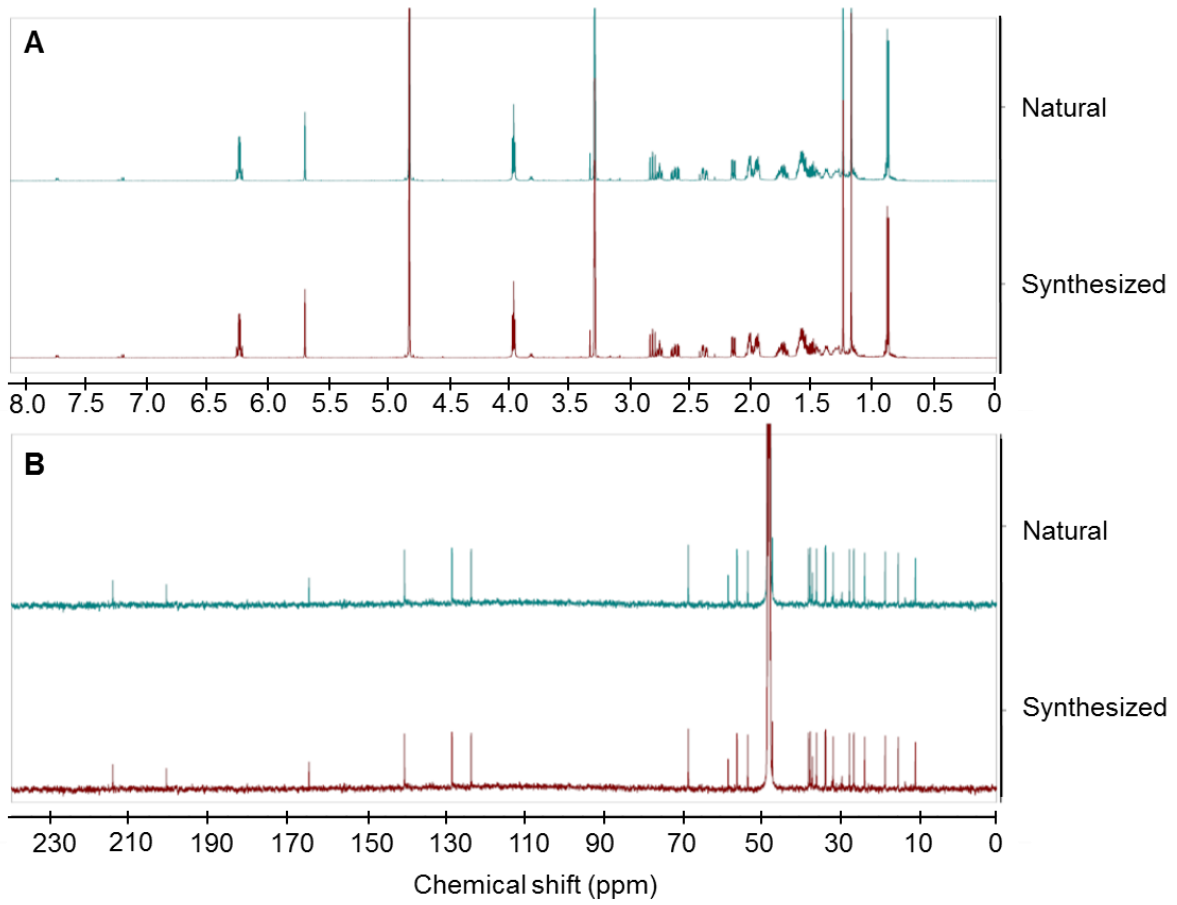

Figure 3-1. Spectral comparisons of synthesized and natural (purified) DkPES. (A) Comparison of ^{1}H NMR spectra of natural and synthesized DkPES obtained from 600 MHz NMR spectrometry (Varian Inova) in methanol-d_4. **(B)** Comparison of ^{13}C NMR spectra of natural and synthesized DkPES obtained from 600 MHz NMR spectrometry (Varian Inova) in methanol-d_4.

DkPES is discriminated from 3kPZS in sea lamprey olfactory epithelia

Electro-olfactogram (EOG) recording showed that both synthesized 3kPZS and DkPES are highly stimulatory for the olfactory epithelia of adult sea lamprey (Figure 3-2A). At each concentration tested, the amplitude of the olfactory response elicited by 3kPZS was larger than that elicited by DkPES. The threshold of detection for 3kPZS was 10^{-13} Molar (M) and for DkPES was 10^{-10} M. The difference in the magnitude and slope in the concentration-response relationships for 3kPZS and DkPES suggested that the olfactory receptor mechanisms for these two compounds may differ (Kang and Caprio, 1997). This hypothesis was further supported by a set of cross-adaptation experiments (Kang and Caprio, 1997), in which preadaptation of the olfactory epithelium to one compound did not suppress the olfactory responses to the other compound (ANOVA: $F_{320} = 7.33$, $P = 0.02$; Figure 3-2B). In particular, when the sensory epithelium was subjected to prolonged perfusion (pre-adaptation) with 3kPZS, the normalized EOG response to DkPES was larger than that to 3kPZS (Figure 3-2B, Student's *t*-test, $t = 3.78$, $P = 0.004$). Vice versa, when the epithelium was pre-adapted to DkPES, the EOG response to 3kPZS was larger than the response to DkPES (Figure 3-2B, $t = 2.81$, $P = 0.019$). These data indicated that the olfactory epithelium distinguished DkPES and 3kPZS by at least two different receptors.

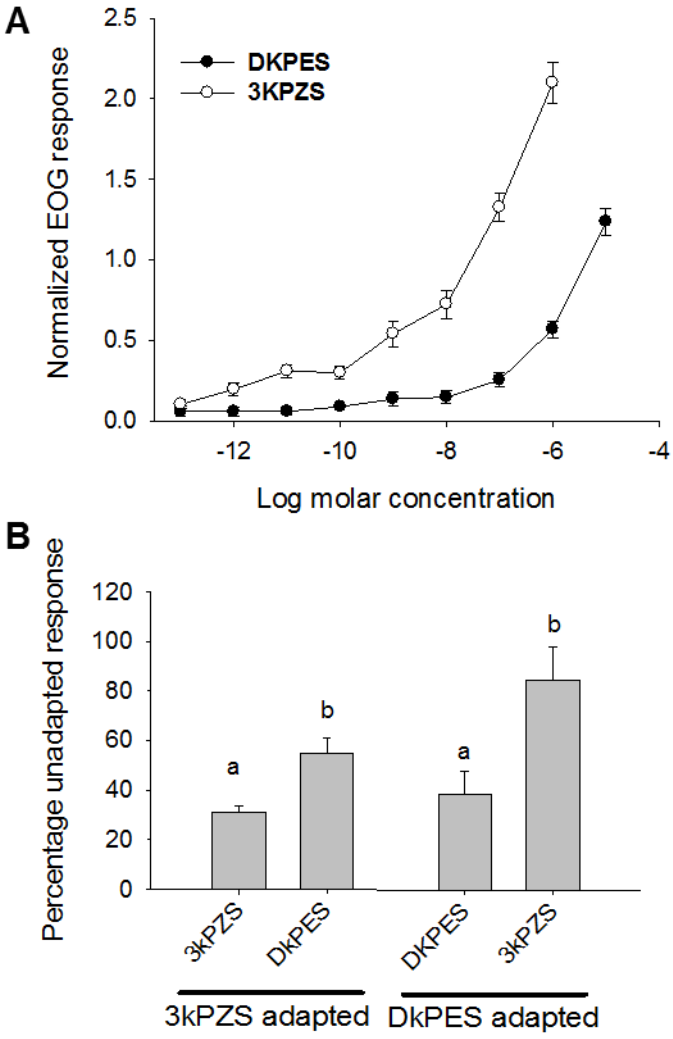

Figure 3-2. Olfactory detection and discrimination of DkPES and 3kPZS by adult sea lamprey. (A) Semi-logarithmic plots of electro-olfactogram (EOG) responses to different concentrations of DkPES and 3kPZS. The response amplitude was corrected for the blank response amplitude and normalized against the response amplitude of a standard odorant, L-arginine at 10^{-5} Molar. **(B)** Qualitative differences in DkPES and 3kPZS assessed by cross-adaptation. Percentage unadapted response is the response amplitude to an odorant (treatment) when the olfactory epithelium was pre-adapted to an odorant, expressed as a percentage of the response amplitude to the same testing odorant when the olfactory epithelium was not pre-adapted. 3kPZS adapted and DkPES adapted indicate when the olfactory epithelium was pre-adapted to 3kPZS and DkPES, respectively. Different letters indicate significant differences in responses amplitude with respect to the corresponding adaptation (DkPES: $t = 3.78$, $P = 0.004$; 3kPZS: $t = 2.81$, $P = 0.019$). Vertical bars represent one standard error, $n = 6$.

Females are attracted to the 3kPZS and DkPES mixture at ratios similar to those identified in SMW extracts

A ratio of 3kPZS:DkPES observed in spermiated male wash-water (SMW) extracts from a group of mature males was found to be 30:1 (3kPZS:DkPES) in our previous work (Li et al., 2013b), and thus the 30:1 ratio was examined along with other ratios during these trials. The point in the stream at which the molar concentration of our treatments reached that of our target whole-stream concentration of $5x10^{-13}$ M for 3kPZS (as indicated by *S* in Figure 3-3A) was calculated based on rhodamine dye concentrations as previously described (Johnson et al., 2009) and determined to be roughly 15 m downstream from the experimental nests (Figure 3-3A). Swim tracks overlain onto rhodamine plume maps indicated that subjects showed a clear preference for the 30:1 3kPZS:DkPES mixture compared to the 1:1, 10:1, and 20:1 mixtures (Figure 3-3B and Supplemental Figure S3-2). Sinuosity, calculated as the total track length (starting at point *S*) divided by the shortest distance between the start and end of the track, was highest when subjects were exposed to SMW. Mean sinuosity of swim tracks was lowest during 1:1 mixture treatments, and highest during SMW treatments (ANOVA and *post-hoc* Tukey's HSD: $F_{5, 74} = 3.19$, $P = 0.012$). Finally, mean sinuosity did not differ between SMW and 30:1 treatments (HSD: $P = 0.766$), yet was lower for 3kPZS treatments compared to SMW treatments (HSD: $P = 0.023$, Figure 3-3C). Full swim tracks for all treatments can be seen in Supplemental Figure S3-1.

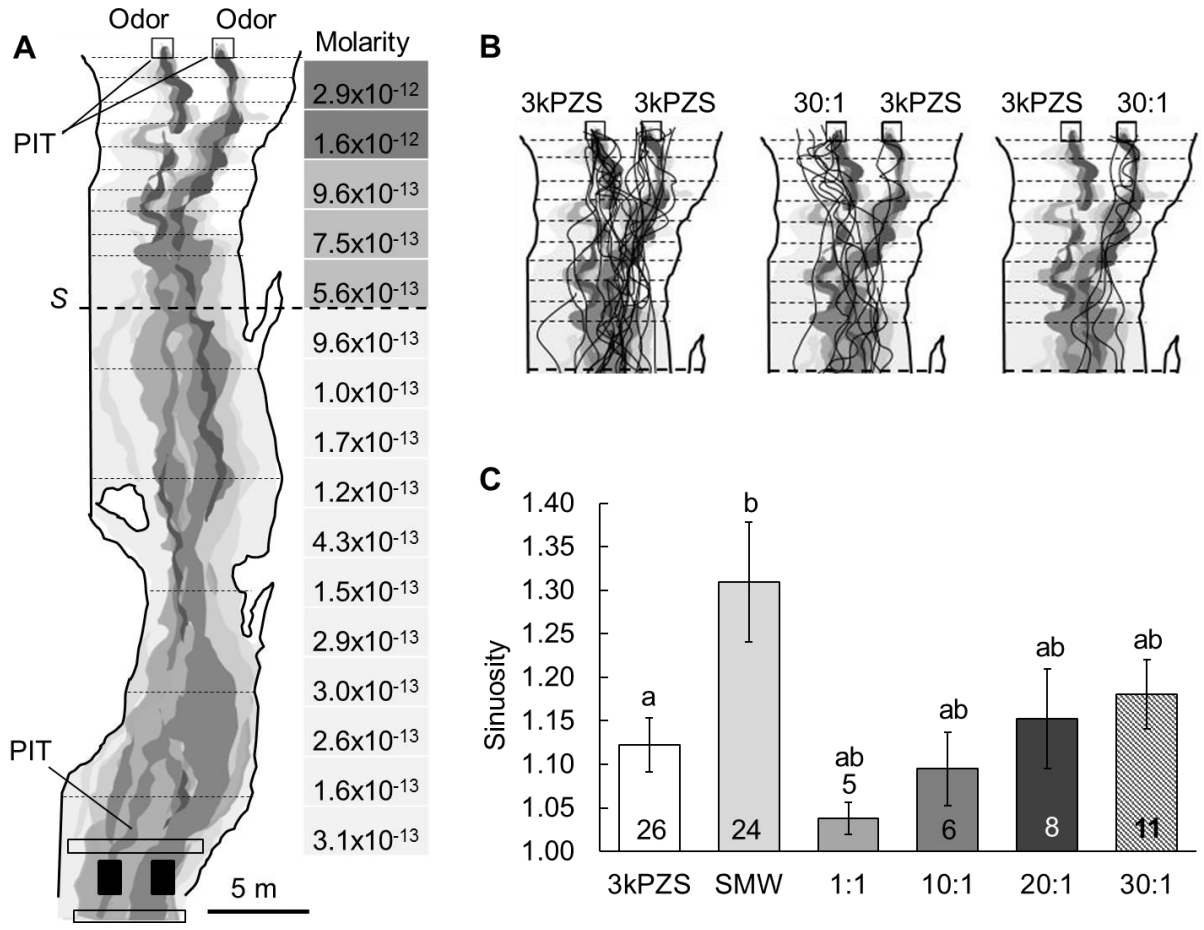

Figure 3-3. Behavioral responses of mature female sea lamprey to DkPES in a natural spawning stream. (A) Schematic of the 45 m-long section of the Upper Ocqueoc River used for field bioassays. The point at which treatment concentrations reached that of our target whole-stream molarity was calculated based on rhodamine concentration, and is indicated at *S*. The plume map and estimated treatment concentrations (Molarity) are shown, and were mapped following described methods (Johnson et al., 2009). Locations of passive integrated transponder (PIT) antennas are shown. Downstream release cages are indicated with solid black boxes. **(B)** Swim tracks of individual test subjects during 3kPZS ($5x10^{-13}$ M) vs. 3kPZS ($5x10^{-13}$ M) control treatments and 3kPZS ($5x10^{-13}$ M) vs. ratio 30:1 ($5x10^{-13}$ M 3kPZS:$1.7x10^{-14}$ M DkPES) treatments are shown starting at point *S*. **(C)** Mean sinuosity (track length/shortest connecting line) of tracks for each treatment (± 1 SEM) was calculated from point *S* up to adjacent nests (~ 15 m) during treatments: 3kPZS ($5x10^{-13}$ M), spermiated male washings (SMW, applied at $5x10^{-13}$ M 3kPZS benchmark), ratio 1:1 ($5x10^{-13}$ M 3kPZS:$5x10^{-13}$ M DkPES), ratio 10:1 ($5x10^{-13}$ M 3kPZS:$5x10^{-14}$ M DkPES), ratio 20:1 ($5x10^{-13}$ M 3kPZS: $2.5x10^{-14}$ M DkPES), and ratio 30:1. Treatments that share a letter are not significantly different (ANOVA and *post-hoc* Tukey's HSD: $F_{5,74} = 3.19$, $P = 0.012$). The number of responding subjects (*n*) are indicated within each column.

From passive integrated transponder (PIT) telemetry data, the proportion of mature female subjects that moved upstream was not different across treatment levels (Logistic regression: $X^2_5 = 5.85$, $P = 0.322$, Supplemental Table S3-2). This is likely due to the fact that all treatments contained 3kPZS, a pheromone component known to induce upstream movement of mature female sea lamprey (Johnson et al., 2009). The proportion of subjects entering the treatment nest (within 0.5 m of the treatment source) during SMW or 30:1 treatments did not differ from one-another (Two-tailed *t*-test: $t = 1.52$, $P = 0.132$), and both showed highest entry rates ($X^2_5 = 25.51$, $P < 0.001$, Figure 3-4A) compared to all other treatments. However, the mean time spent within the nest by mature females differed between SMW and the 30:1 treatments. Specifically, subjects showed higher retention (minutes) inside nests when SMW was administered ($F_{11, 123} = 3.55$, $P < 0.001$) compared to all other treatments. No differences were observed in retention time across all other treatments (see Figure 3-4B for all statistical comparisons).

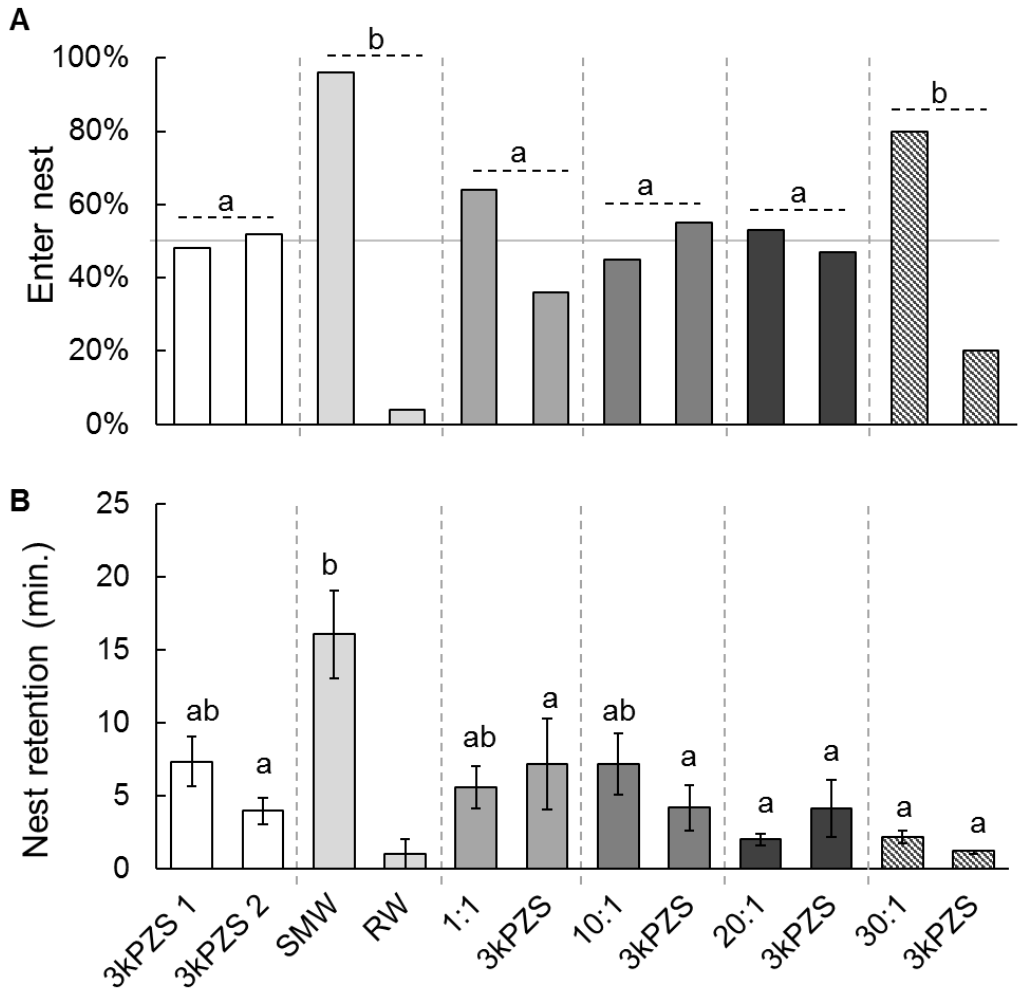

Figure 3-4. Pair-wise comparison of male pheromone components at various ratios for their induction of preference response in mature female sea lamprey. Treatments included: 3kPZS ($5x10^{-13}$ M, n = 16) vs. 3kPZS ($5x10^{-13}$ M, n = 17), spermiated male washings (SMW, applied at $5x10^{-13}$ M 3kPZS benchmark, n = 26) vs. river water (RW, n = 1), ratio 1:1 ($5x10^{-13}$ M 3kPZS: $5x10^{-13}$ M DkPES, n = 16) vs. 3kPZS ($5x10^{-13}$ M, n = 9), ratio 10:1 ($5x10^{-13}$ M 3kPZS:$5x10^{-14}$ M DkPES, n = 9) vs. 3kPZS ($5x10^{-13}$ M, n = 11), ratio 20:1 ($5x10^{-13}$ M 3kPZS: $2.5x10^{-14}$ M DkPES, n = 8) vs. 3kPZS ($5x10^{-13}$ M, n = 7), and ratio 30:1 ($5x10^{-13}$ M 3kPZS:$1.7x10^{-14}$ M DkPES, n = 12) vs. 3kPZS ($5x10^{-13}$ M, n = 3). Dashed vertical grey lines separate pair-wise comparisons. **(A)** Percentage of subjects that entered each treatment nest. Horizontal grey line indicates 50%. Treatments that share a letter are not significantly different (Logistic regression: $X^2_5 = 25.51$, $P < 0.001$). **(B)** Mean (± 1 SEM) retention (min.) of subjects inside respective treatment nests. Treatments that share a letter are not significantly different (ANOVA and *post-hoc* Tukey's HSD: $F_{11,123} = 3.55$, $P < 0.001$).

DISCUSSION

This study confirms the structure of DkPES and defines the function of DkPES and 3kPES as a pheromone mixture in sea lamprey. Our previous work suggested that certain ratios of DkPES and 3kPZS may be important for orientation of receiver females to the male odorant source, and mature females showed a behavioral preference to the mixture at multiple ratios (Li et al., 2013b). In the present study, we unequivocally confirmed the structure of DkPES through chemical synthesis, showed that DkPES was discriminated from 3kPZS in the olfactory epithelia, and elucidated orientation strategies of receiver females exposed to mixtures of DkPES and 3kPZS in a range of ratios.

From the data on locomotion patterns of mature females released in a sea lamprey spawning stream, both the behavioral preference and the sinuosity analyses indicate that a range of mixtures is likely to be effective at drawing receiver females towards the source, provided that DkPES remains the more minor component in the mixture (Li et al., 2013b). Thus, we postulate that minor components such as DkPES allow receivers to gauge distance to the source, as DkPES is likely not detectable until the receiver is within proximity of a signaler (Figure 3-5). Further, we postulate that the mixture with peak behavioral activity (30:1, 3kPZS:DkPES) remains within a range similar to that released by mature males. Our previous study observed an average 30:1 ratio of 3kPZS:DkPES (10^{-10} M 3kPZS:$3.3x10^{-12}$ M DkPES) in wash-water extracts collected from a group of mature males (Li et al., 2013b). In the future, it will be useful to test individual male variation in ratios, and examine variables that may influence these ratios.

The increasing behavioral preference for a mixture of pheromone components that approaches that of the natural ratio released from a signaler sea lamprey is consistent with

research on insects such as moths to mixtures of pheromone components (Linn and Roelofs, 1989; Reyes-Garcia et al., 2014; Wyatt, 2010). While several components and variations of mixtures can yield attractive behaviors in moths, individual moth species often show a peak preference to the ratio that best reconstructs that of the natural ratio emitted by conspecific senders (Cardé et al., 1977; Coracini et al., 2001; Reyes-Garcia et al., 2014). Behavioral data here suggests a similar relationship in sea lamprey. Unique ratios that vary between members of similar Lepidopteran species have been theorized to act as reproductive isolating mechanisms (Cardé et al., 1977). While there appears to be some overlap in sex pheromone components used among phylogenetically similar lamprey species (Buchinger et al., 2013), specific ratios of sex pheromones have not yet been identified in other lampreys.

The detection thresholds for the two pheromone components varied between our previous and current studies. The threshold of detection of isolated DkPES in our previous study was within a range of 10^{-7} – 10^{-8} M in our subjects (Li et al., 2013b), while the current study showed a EOG detection threshold of 10^{-10} M. Similarly, compound 3kPZS showed an EOG limit of detection of 10^{-10} M in the previous study (Li et al., 2013b), and 10^{-13} M in the current study. In a separate study (Siefkes 2002), the detection threshold for 3kPZS was determined as 10^{-12} M. This variation may be attributable to sea lamprey conditions during the time of research. In our experience, longevity and sensitivity can vary based upon stream temperatures, date of capture, time held in traps by government agencies, holding conditions before transport to researchers, and length of transport. Additionally, physiological processes degrade as the spawning season progresses and sea lamprey approach natural senescence (Manion and Hanson, 1980), which likely impacts their detection threshold of compounds and ability to respond. Finally, variability

in detection thresholds may be due to an updated and more sensitive electro-olfactogram (EOG) recording system in our laboratory since our previous study.

In summary, our data suggest that mature females detect DkPES and 3kPZS with independent receptors while making orientation decisions based on the odorant plume (Johnson et al., 2012). The sinuous pattern of movement and casting into and out of the odorant plume seen here are consistent with behaviors observed in birds and fishes when tracking odors (DeBose and Nevitt, 2008). Our data suggest that female sea lamprey encounter a plume structure, discriminate between 3kPZS and minor components such as DkPES at the olfactory level, and utilize stream flow using odor-conditioned rheotaxis (Johnson et al., 2012), to gauge location of a conspecific signaler (Figure 3-5). We hypothesize that additional components or their mixtures with 3kPZS and DkPES function to arrest females inside the nest boundaries for courtship with signaler males. Taken together, examination of chemical communication in sea lamprey may provide insights into the independent detection mechanisms of pheromone mixtures at various ratios (Kang and Caprio, 1997; Keller-Costa et al., 2014), the underlying role of these ratios as reproductive isolating mechanisms in vertebrates (Keller-Costa et al., 2014), and provide a useful tool for the integrated pest management of invasive sea lamprey (Johnson et al., 2013; Teeter, 1980).

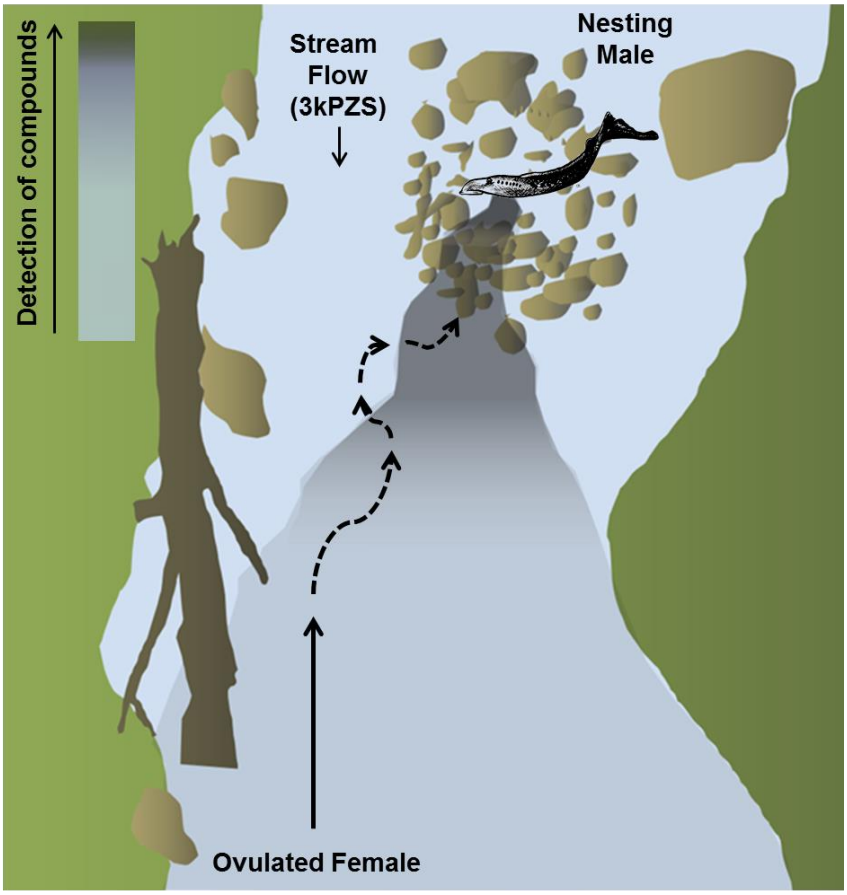

Figure 3-5. A conceptual model for a female sea lamprey encountering the conspecific male pheromone. Nesting males release compound 3kPZS that induces upstream movement in mature (ovulated) females (Johnson et al., 2009; Li et al., 2002). Background 3kPZS concentrations remain very high throughout spawning grounds (Xi et al., 2011), indicated by (3kPZS) under stream flow. Females are able to detect and discriminate between 3kPZS and minute compounds such as DkPES at the olfactory level, while increasing the sinuosity of their swim path around the odor plume (Johnson et al., 2012), as they approach the source.

METHODS

Test Subjects

All procedures using sea lamprey presented in this manuscript were approved by the Michigan State University Institutional Animal Care and Use Committee prior to any experimentation (AUF# 05/09-088-00 and 03/11-053-00). Adult sea lamprey used for EOG recordings were collected in spring 2012 by commercial fishing companies in Lake Huron and transported to United States Geological Survey and Great Lakes Science Center – Hammond Bay Biological Station (HBBS), and later shipped to Michigan State University, East Lansing, Michigan. Migrating adult sea lamprey were captured by the United States Fisheries and Wildlife Service and Department of Fisheries and Oceans Canada from tributaries to Lake Michigan and Lake Huron in May – June 2012, following animal use and care protocols established by those agencies. Live lamprey were transported to HBBS and held in 500-1000 L-capacity flow through tanks until use. Males were kept separate from females in tanks and never used for this study. Sea lamprey are a non-native invader of the Laurentian Great Lakes and their tributaries. Hence, males and females could not be released together upstream of sea lamprey barriers to reduce the risk of repopulation of the river system.

To produce sexually mature ovulated female (MF) test subjects, lamprey were transferred to acclimation cages constructed of polyurethane mesh and PVC pipe (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan, to allow natural maturation *in situ*. Sea lamprey were monitored daily for signs of sexual maturation. Briefly, MFs were identified by first checking for secondary sexual characteristics (Applegate, 1950), then applying gentle pressure to the abdomen and checking for expression of ovulated oocytes from the cloacal aperture.

Purity analysis of DkPES

Quality of synthetic DkPES in chemical structure and purity were examined using a series of chemical analyses by high resolution mass spectrum (HR-ESI-MS) , ^{1}H, ^{13}C NMR spectra and DSC analyses. ^{1}H-NMR and ^{13}C NMR experiments were performed on the synthetic DkPES ammonium salt using a Varian Inova 600 MHz NMR spectrometer at MSU Max T. Rogers NMR facility. Samples (ca. 5.0 mg) was prepared in CD_3OD and subjected to NMR analysis. The results were compared with original DkPES and displayed in Figure 3-1.

HR-ESI-MS of synthetic DkPES ammonium salt was performed on a TQ-S TOF LC mass spectrometer (Waters Corporation, Milford, MA, USA). The solution (10 μL) of synthetic DkPES (1.0 μg/ml, MeOH/H_2O = 1:1, v/v) was injected by auto-sampler. The mobile phase consisted of water as (A), and methanol (B).The isocratic gradient (30%A and 70%B) was used a eluant. The UHPLC effluent was introduced into the mass spectrometer with electrospray ionization in the negative mode. The ESI-MS parameter was set as capillary voltage, 2.60 kV; extractor voltage, 5 V; source temperature, 150 ^{o}C; desolvation temperature, 500 ^{o}C; desolvation gas flow, 800 L/h (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. Data were collected in centroid mode with a scan range of 50–1000 *m/z*. Data process performed on MassLynx 4.1.

DSC were used to evaluate the absolution purity of synthetic DkPES without standard. The DSC experiment was carried out on DSC Q2000 (TA Instrument, New Castel, DE, USA). Sample (ca. 3.2 mg) was set in a Tzero^{TM} pan with lid (TA Instrument, New Castel, DE, USA) and stored on oven by auto-sampler. The temperature ramp was set as start from 20 ^{o}C to 250 ^{o}C, heating rate is 1.0 ^{o}C/min. The absolute purity of DkPES was analyzed by software TA Instruments universal analysis 2000 provided by the manufacturer.

Synthesized Pheromone Components

Compound 3kPZS was custom synthesized by Bridge Organics Co. (purity = 97%, Vicksburg, MI) as a white powder salt. DkPES was chemically synthesized by Apeloa Kangyu Pharmaceutical Co. (purity = 97%, Dongyang, Zhejiang, China) as a white powder salt. The synthetic compound exhibits the same spectral characteristics and biological activity as the published natural compound (Li et al., 2012). A 1 mg/mL stock solution of each compound (in 50% methanol:deionized water) was prepared. Stock solutions were stored at -20°C until use.

Electro-olfactogram (EOG) Recording

Electro-olfactogram recordings were obtained from lamprey in spring 2012. Our procedures for EOG are detailed in Li et al. (2013b). Briefly, sea lamprey were anesthetized with 3-aminobenzoic acid ethyl ester (100 mg/L; MS222, Sigma-Aldrich Chemical Co.), immobilized with an intra-muscular injection of gallamine triethiodide (3 mg .kg^{-1} in 0.9% saline), and placed in a partially inundated V-shaped Plexiglas cradle. Gills were continuously irrigated with aerated water containing 50 mg/L MS222. The olfactory rosette was surgically exposed and olfactory responses to stimuli were recorded by borosilicate electrodes filled with agar 0.04% in saline 0.9 % connected to solid-state electronics with Ag/AgCl pellets in 3mol/L KCl. Electrodes where placed between olfactory lamella (recording electrode) and external skin (reference electrode). Olfactory responses were filtered and amplified by a NeuroLog system model NL102, filtered with a low-pass 60Hz, model NL125 (Digitimer Ltd., Hertfordshire, England), digitized by a Digidata 1550 (Molecular Devices LLC., Sunnyvale, California), and stored on a PC running AxoScope 10.4 software (Molecular Devices LLC.).

For concentration-response curves, stimuli were serially diluted in charcoal filtered water from a 10^{-3} M stock solution of synthesized 3kPZS and DkPES, respectively. Responses were

measured in mV, blank subtracted and normalized to those of L-arginine at 10^{-5} M. Response thresholds were determined from the concentration with no significant difference with the blank normalized response (Student's *t*-test, $\alpha = 0.05$).

Cross-adaptation experiments followed the protocol of Huertas et al. (Huertas et al., 2007). Dilutions of 3kPZS and DkPES that evoked the same EOG amplitude (10^{-9} M 3kPZS and 10^{-7} M DkPES) were recorded as normal, the 'unadapted' response. Then the olfactory rosette was continually exposed to unadapted 3kPZS solution for at least 1 min and the response to a sample $2x10^{-9}$ M of 3kPZS was recorded, the 'self-adapted control'. The responses to a mixture of 3kPZS and DkPES at 10^{-9} M and 10^{-7} M respectively was then recorded, the 'adapted' response. The amplitudes of self-adapted control and adapted responses were then showed as a percentage of the appropriate unadapted response. The olfactory epithelium was then exposed to charcoal filtered water for 10 min, and the process repeated using DkPES as the adapting solution and 3kPZS and the mixture as stimuli. The sequence of adaptation of 3kPZS and DkPES were randomized. Overall treatment effects were tested with an analysis of variance (ANOVA), and significant differences between responses of test odorants when the epithelium was adapted to particular odorants were tested by Student's *t*-test ($\alpha = 0.05$).

Passive Integrated Transponder (PIT) Tagging Procedures

Passive integrated transponder (PIT) tagging procedures for MFs followed Johnson et al. (Johnson et al., 2009). Each PIT tag was fitted into a latex sleeve and attached to the mid-dorsal region of each MF using a suture on both sides (Size 3-0, Ethicon Inc., Cornelia, GA). Subjects were also fitted with unique color combinations of ribbon tags (Hallprint, Hindmarsh Valley, South AU) through each dorsal fin to identify individuals for visual tracking during trials.

Tagged animals were immediately transferred into aerated holding tanks with a constant flow of Lake Huron water for up to 24 hours, until they were stocked into stream acclimation cages.

Field Bioassays

Trials were ran from 12 June – 27 July 2012, a time-frame representative of a typical spawning season for sea lamprey (Applegate, 1950; Manion and Hanson, 1980), in a 45 m-long stretch of the Upper Ocqueoc River, Millersburg, MI. The field site is consistent with our previous study (Li et al., 2013b), with slight modification (Figure 3-3A). Two 1 m^2 nest antennas were placed side-by-side to one another on the upstream end of site, laid flat on the stream bed, 1.5 m apart. These antennas monitored the proportion of subjects that entered a particular "nest" containing treatments. Downstream 45 m, two aluminum-mesh release cages (0.25 m^3) equipped with sliding release doors were positioned in the center of the stream channel. A PIT antenna roughly 0.5 m-high x 6 m-long was positioned 5 m upstream of the release cages to monitor individuals that exit the cage and move upstream (Figure 3-3A).

Stream temperatures were recorded at the start of and end of each trial. Stream discharge was estimated every three days, or after every precipitation event, at a fixed location in the stream using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, MD) to determine the amount of treatment stock solution to apply to the stream and maintain consistent concentrations across trials. Treatments were diluted with 20 L of river water in large mixing bins on shore. Bins were kept consistent for each test treatment, and rinsed in the stream several times before each new trial, to reduce the potential for contamination during mixing. Each treatment solution was then pumped from bins into the stream at the center of each "nest" antenna at a rate of 167 mL/min (± 3 mL/min) using peristaltic pumps (Cole-Parmer). Trials were a total of two hours long. In the first half-hour of each trial the treatments were

administered to the stream while subjects remained in the release cage. At the start of the following 1.5 hours, subjects were released and their movements were monitored with PIT antennas until the trial ended. No animals were recovered from the stream after a trial.

Copper wire was wrapped around each antenna frame twice during the construction of PIT antennas for a more focused read range. Antennas were wired to a multiplexor in the field for consolidation and storage of data (Oregon RFID, Portland, OR). Antennas were tuned to a detection sensitivity of roughly 0.3 m from the frame edges. Scan frequencies of each antenna were programmed to three scans/sec. Data for each trial were uploaded each day using a hand-held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

Details of Treatments

Treatments included: (1) 3kPZS ($5x10^{-13}$ M) vs. 3kPZS ($5x10^{-13}$ M), (2) spermiated male washings (SMW, applied at $5x10^{-13}$ M 3kPZS benchmark) vs. river water (RW), (3) ratio 1:1 ($5x10^{-13}$ M 3kPZS:$5x10^{-13}$ M DkPES) vs. 3kPZS ($5x10^{-13}$ M), (4) ratio 10:1 ($5x10^{-13}$ M 3kPZS:$5x10^{-14}$ M DkPES) vs. 3kPZS ($5x10^{-13}$ M), (5) ratio 20:1 ($5x10^{-13}$ M 3kPZS: $2.5x10^{-14}$ M DkPES) vs. 3kPZS ($5x10^{-13}$ M), and (6) ratio 30:1 ($5x10^{-13}$ M 3kPZS:$1.7x10^{-14}$ M DkPES) vs. 3kPZS ($5x10^{-13}$ M). Treatments applied to each "nest" were alternated each trial. Up to two trials were conducted each day depending upon the availability of mature animals. The early trial was conducted from ~0700h – 0900h, and a late trial was then run from ~0930h – 1130h. Ten PIT-tagged MFs were transferred to respective acclimation/release cages for each trial between 2000 – 2200h the night prior to experimentation. Subjects were then allowed an acclimation period in the stream for a minimum of 9 hours.

Swim Track Mapping

Swim tracks were mapped during trials following Johnson et al. (Johnson et al., 2009), with slight modification stated here. The stream section seen in Figure 3-3A was fixed with transecting strings every 1 meter downstream of "nests" for the first 10 m, and every 5 m down after that until reaching release cages. Each transecting string was divided into tenths (of the total stream width). Given that each test subject was marked with a unique color combination of ribbon tags, we were able to visually observe and record individuals onto scale maps by hand as they swam upstream. Observers followed each subject until reaching the nests, using transecting strings as reference markers. Only subjects that were observed exiting the release cage were followed. Preference responses for the rest of the subjects that exited unseen by observers were recorded via PIT antennas.

To map the odor plume, rhodamine dye was administered to the stream at our treatment pumping rate (167 mL/min) when streamflow was 537 L/sec (which fell into the range of our average stream flow across all trials of 598 ± 76 L/sec). Stream samples were taken following Johnson et al. (Johnson et al., 2009) with one modification stated here. Rhodamine concentrations were detected and recorded at each sample point (*i.e.* every tenth of the stream widths, marked along transecting strings) with a hand-held DataBank datalogger and Cyclops-7 Optical Rhodamine Dye Tracer (Turner Designs, Sunnyvale, CA), instead of hand-grabbing and analyzing water samples with a laboratory spectrophotometer (Johnson et al., 2009). All swim tracks were traced onto a digital map using a tablet computer (Lenovo X201 Tablet). Swim tracks and the odor plumes were both mapped to scale, independently, and tracks were later overlain onto plumes in a double-blind design for all track figures (Supplemental Figure S3-1).

Statistical Analyses of Behavioral Data

Rhodamine concentrations were used to estimate the downstream point (transecting line) at which average treatment molarity reached that of our instream target concentration and became detectable from bank-to-bank. From this transect, indicated as *S* in Figure 3-3A, sinuosity of each swim track was calculated by dividing the track length by the length of a straight line connecting the start and end of each track. Transect *S* was chosen for a sinuosity calculation start point because it was the point at which lamprey would begin exposure to a gradual increase in odorant concentration and plume edges that would allow subjects to cast into and out of the plume structure. Since sinuosity values are proportions (*i.e.* a value of 1 is a straight line), values were square root-transformed. The Levene's test for homogeneity of variance was used to examine variance of newly transformed data. Once homogeneity of variance was observed, an ANOVA and *post-hoc* Tukey's HSD ($\alpha = 0.05$) was conducted for statistical comparisons of retention data across treatments using R-software (R version 2.11.1 Vienna, Austria)

For all PIT telemetry data, logistic regression with a binomial distribution was examined for each response variable across treatments (R version 2.11.1). No signs of nonlinearities or over dispersion were observed in the models. All behavioral statistics reported are two-tailed analyses ($\alpha = 0.05$). Two main binary response variables were examined from PIT data: (1) the distribution of subjects that swam upstream from release cages and did not move back down (*Up*: 1 = hit on upstream release antenna and continued towards nests, 0 = did not hit on upstream release antenna) and (2) of those animals that hit on the upstream antenna, the distribution that entered the "nest" containing the test treatment (*Enter nest*: 1 = hit treatment nest, 0 = hit control nest). Since 3kPZS was administered to both nests during control trials, one

nest was randomly assigned for statistical purposes to be the "treatment" nest. The "treatment" nest was randomly chosen to be the right nest, and alternated every trial to follow the same pattern of the other treatments. Each test subject's unique PIT tag identification number prevented any pseudo-replication from test subjects released during trials.

When a subject entered a nest, observers recorded the amount of time spent inside the 1 m^2 area (retention, min.) until respective subjects moved on. All retention data was examined for violation of assumptions of normality and homogeneity across variance before further statistical analyses were conducted. Retention data that were not normally distributed or showed heterogeneity across variance were log-transformed. The Levene's test for homogeneity of variance was used to examine variance of newly transformed data. Once homogeneity of variance was confirmed, an ANOVA and *post-hoc* Tukey's HSD ($\alpha = 0.05$) was conducted for statistical comparisons of retention across treatments (R version 2.11.1).

ACKNOWLEDGEMENTS

This work was supported by the Great Lakes Fishery Commission, Ann Arbor, MI. We are grateful to personnel of the U.S. Geological Survey Hammond Bay Biological Station for use of facilities, the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada for providing sea lamprey, and to Dolly Trump and Lydia Lorenz for the use of their private land for stream access. Special thanks to field technicians; Ethan Buchinger, Elizabeth Racey, Brian Grieve, Zak Smillie, and Kyle Hill, for their help with tracking and mapping sea lamprey movement.

SUPPLEMENTAL INFORMATION

Table S3-1. High resolution mass spectrum report for synthesized DkPES ammonium salt (HR-ESI-MS)

Elemental Composition Report
Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%
Monoisotopic Mass, Odd and Even Electron Ions
13 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Minimum:			-1.5			
Maximum:		200.0	50.0		100.0	
Mass	Calc. Mass	mDa	PPM	DBE		
Score	Formula					
449.2012	449.1998	1.4	3.1	8.5		1
	C24 H33 O6 S					

Table S3-2. Percentage of sexually mature female sea lamprey that moved upstream 45 m (Up) to side-by-side nest antennas activated with pheromone treatments.

Treatment nest	Adjacent nest	Trials	Released	Up	
3kPZS (5E-13M)	*3kPZS (5E-13M)*	12	112	*29%*	(33) A
SMW	*River water*	8	81	*33%*	(27) A
1:1	*3kPZS (5E-13M)*	9	90	*28%*	(25) A
10:1	*3kPZS (5E-13M)*	7	69	*29%*	(20) A
20:1	*3kPZS (5E-13M)*	6	66	*23%*	(15) A
30:1	*3kPZS (5E-13M)*	7	81	*19%*	(15) A
				X^2	5.85
				df	5
				P-value	0.322

Treatments included: 3kPZS (5E-13 M) vs. 3kPZS (5E-13 M), spermiated male washings (SMW, applied at 5E-13 M 3kPZS benchmark) vs. river water, ratio 1:1 (5E-13 M 3kPZS:5E-13 M DkPES) vs. 3kPZS (5E-13 M), ratio 10:1 (5E -13 M 3kPZS:5E-14 M DkPES) vs. 3kPZS (5E-13 M), ratio 20:1 (5E-13 M 3kPZS: 2.5E-14 M DkPES) vs. 3kPZS (5E-13 M), and ratio 30:1 (5E-13 M 3kPZS:1.67E-14 M DkPES) vs. 3kPZS (5E-13 M).

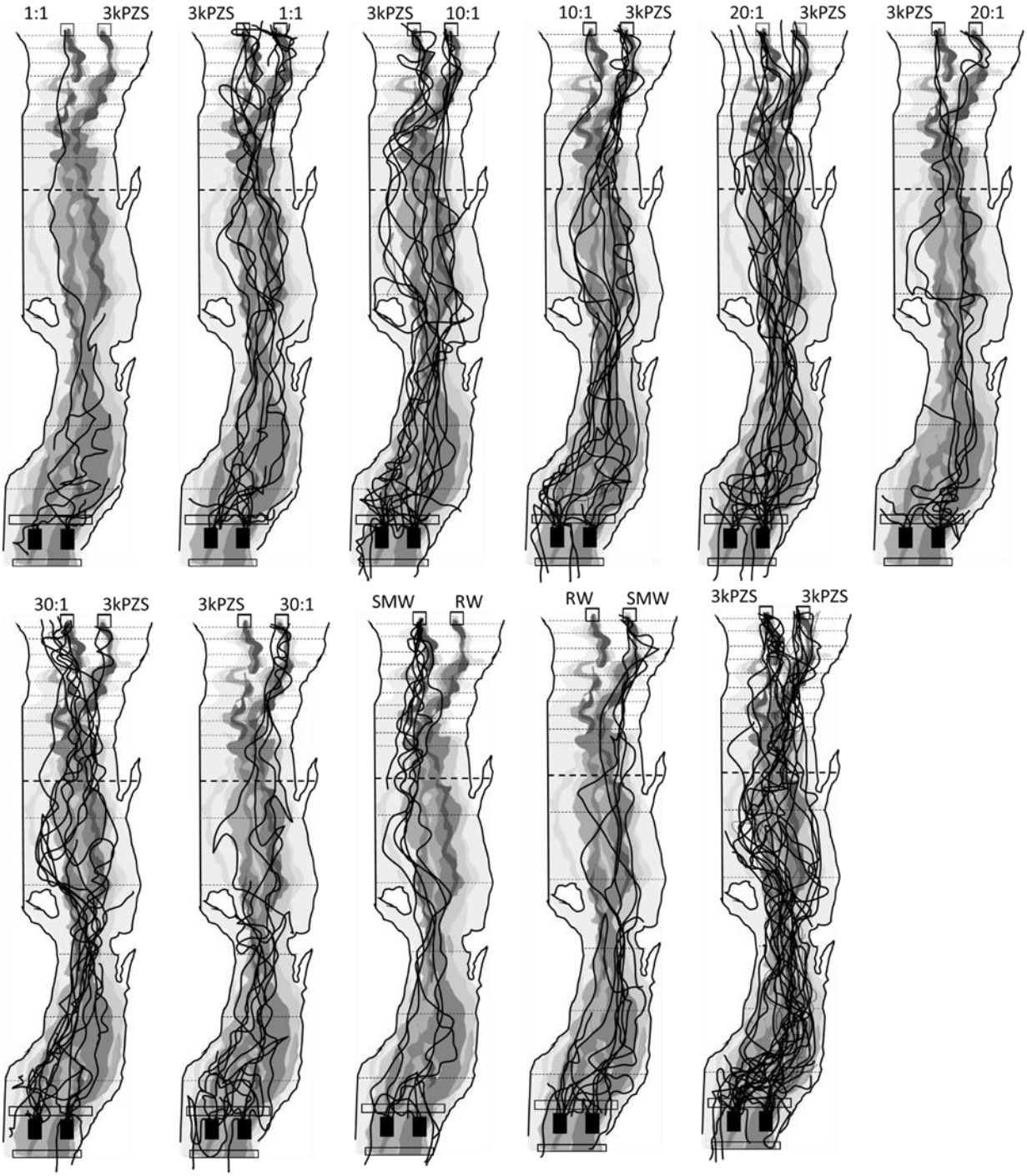

Figure S3-1. All tracks and plumes for all treatments during field trials. Treatments and ratios are described in Figure 3-3.

CHAPTER 4

A TERRITORIAL PHEROMONE THAT DEFINES NEST BOUNDARY IN THE SEA LAMPREY

ABSTRACT

Territorial pheromones, substances that advertise territory ownership and avert intruders of the same species, have been hypothesized but in no species have they been identified. We found that individual spawning male sea lamprey can host many mature females, but rarely males, in their nests. Intruding males were immediately attacked and cast out of the nest. From spawning male washings a novel steroid, PAMS-24, was identified and shown to be a distinct odorant in adults. PAMS-24 release was exclusive to spawning males, and was released with a mixture of 3kPZS (a known mating pheromone in sea lamprey) in a ratio of 100:1 (3kPZS:PAMS-24, molar:molar). Spawning male wash-water and mixtures of synthetic 3kPZS:PAMS-24 (100:1) applied to artificial nests in a spawning habitat lured females and often averted males. Some large males, after increasing search activity in the vicinity, entered 100:1 treated nests. We conclude that PAMS-24 advertises nest ownership and reduces intrusion as a partial territorial pheromone.

INTRODUCTION

A territory is an area occupied by an animal, often exclusively, through overt defense or advertisement (Wilson, 1970). Many animals defend territories to secure resources such as mates, but do so at the risk of aggressive intrusions. Selection should favour the evolution of signals that advertise territories and minimize such risk. Indeed chemical signals are used throughout the animal kingdom to convey territory ownership. However, a "true territorial pheromone", or "a substance deposited on a portion of the home range that induces aversive or agonistic behavior in intruders belonging to the same species" (Hölldobler and Wilson, 1977), has not been identified. We sought to identify the structure and function of a territorial pheromone in mature male sea lamprey (*Petromyzon marinus*) that establish and defend territories for nesting (Manion and Hanson, 1980; Teeter, 1980). When the spawning season commences in early summer, mature males (at the onset of spermiation) congregate in gravel patches of spawning streams, build nests (~ 0.5 m^2), and reproduce within the final weeks of their life (Manion and Hanson, 1980). Nests can be evenly spaced, yet as close as a few meters to one-another. Once nesting, the male releases a sex pheromone 3-keto petromyzonol sulfate (3kPZS)(Li et al., 2002; Yun et al., 2003) that draws females to the nest (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005; Yun et al., 2003). Mature female sea lamprey are polyandrous (Applegate, 1950; Manion and Hanson, 1980), often moving among multiple active nests and mates. In stark contrast, mature males defend their nests from male intruders, often times casting them from their nest (Applegate, 1950; Manion and Hanson, 1980; Teeter, 1980).

RESULTS

We hypothesize that sea lamprey have evolved an effective mechanism that communicates occupancy (territory) inside a nest within the nest cluster to avoid constant intrusion by rival males and broadcast mate-readiness to gravid females. To determine if the male territorial signal is chemical, we tracked movement patterns of mature adults in spawning grounds in the presence of male odors (Supplementary Figure S4-1). Mature females moved to the exact source of spermiated male washings (SMW), as expected (Teeter, 1980), while mature males often avoided SMW (Supplementary Figure S4-2). We then examined whether male sea lamprey protect a nest-sized territory in their natural habitat (Ocqueoc and Cheboygan Rivers, MI, USA). The close proximity of the responses shown in Supplementary Figure S4-2 (0.5 - 1 m^2) were similar to the average size of natural nests (0.65 ± 0.19 m^2, n = 18). Of the 51 occupied nests and 116 nesting sea lamprey observed in those nests in 2009, only 10 male-male interactions were observed: three intruders avoided the resident male while the other seven intruders were attacked and cast from the nest (Supplementary Movie S4-1). In all observed male-male conflicts, the resident male cast the intruder male from the nest. The number of females accompanying a male on a nest ranged from zero (Supplementary Figure S4-3a) up to seven (Supplementary Figure S4-3b). Larger males courted with a greater number of females at one time compared to smaller males (Supplementary Figure S4-4a). Nests were maintained almost always by a single male (Supplementary Figure S4-4b).

Having shown that SMW may indeed contain a territorial odor, we fractionated SMW using a bioactivity-guided strategy. Solid phase extract of SMW was subjected to liquid chromatography over silica gel and detected by Thin Layer Chromatograph (TLC), subsequently yielded nine fractions. Fraction 8 (30 mg) was found to contain two main molecular masses (*m/z*

at 471 and 623) by ESI mass spectra (full scan). The compound with *m/z* 471 was confirmed as the known sex pheromone 3kPZS high resolution mass spectrometry. The other compound was further purified by successive chromatography, yielding a compound ***1*** (Figure 4-1a, 1.4 mg, purity ca. 99.5% by HPLC analysis). The high-resolution negative ESI-MS at *m/z* 623.4108 $[M - H]^-$ (calculated for $C_{34}H_{59}N_2O_6S$, 623.4049) implied a molecular formula of $C_{34}H_{60}N_2O_6S$. The ^1H NMR spectrum (Supplementary Table S4-1) suggested that ***1*** was a steroid with characteristic appearance of a side chain similar to cholesterol. Comparison of the carbon resonance of ***1*** with those of PADS and squalamine (Supplementary Figure S4-5) showed ***1*** as (3*β*,5α,7α,24*R*)-1-[3-[[24-sulfooxy-cholestan-3-yl]amino]-propyl]-2-pyrrolidinone (Figure 4-1A), herein named petromyzonamine-24-monosulfate (PAMS-24), after petromyzonamine disulfate (PADS) (Sorensen et al., 2005). The assignment of PAMS-24 structure was unequivocally confirmed by the identical mass and overlapped NMR spectra shown by the purified ***1*** and the compound synthesized according to the deduced structure, indicating both the natural identified and synthesized compounds possess the chemical structures of PAMS-24.

We further reasoned that, if PAMS-24 was indeed a territorial pheromone, its release should be exclusive to nesting males and it must be a potent odorant for adults. As expected, extensive LCMS analyses failed to detect PAMS-24 in wash water from larvae, females or immature males. Analysis of wash-water from the head region and tail region of mature males held in a bisected chamber (Siefkes 2003) indicated PAMS-24 was released exclusively from head region (Supplementary Figure S4-6a, b), likely through gills, consistent with the release route of 3kPZS which is released exclusively from the head region of sexually mature males by specialized gill cells (Brant et al., 2013; Siefkes et al., 2003). The release rate of PAMS-24 was

between 0.9 – 29 ng/g-lamprey/hr, and the release ratio of 3kPZS to PAMS-24 remained consistent at 100:1 (M:M) across a range of mature male weights (Supplementary Figure S4-6c).

Electro-olfactogram (EOG) recording showed both purified and synthetic PAMS-24 were highly stimulatory for the olfactory epithelium of the adult sea lamprey, with virtually identical concentration-response dynamics (Figure 4-1b). The threshold of detection was 10^{-12} molar (M), similar to that of 3kPZS (Siefkes and Li, 2004). Cross adaptive analyses, in which the olfactory epithelium was pre-adapted to one chemical and then EOG response to the second chemical was recorded, showed that PAMS-24 and 3kPZS do not suppressed the responsiveness of each (Supplementary Figure S4-7). These data indicate PAMS-24 is a potent odorant distinguished from 3kPZS by the adults.

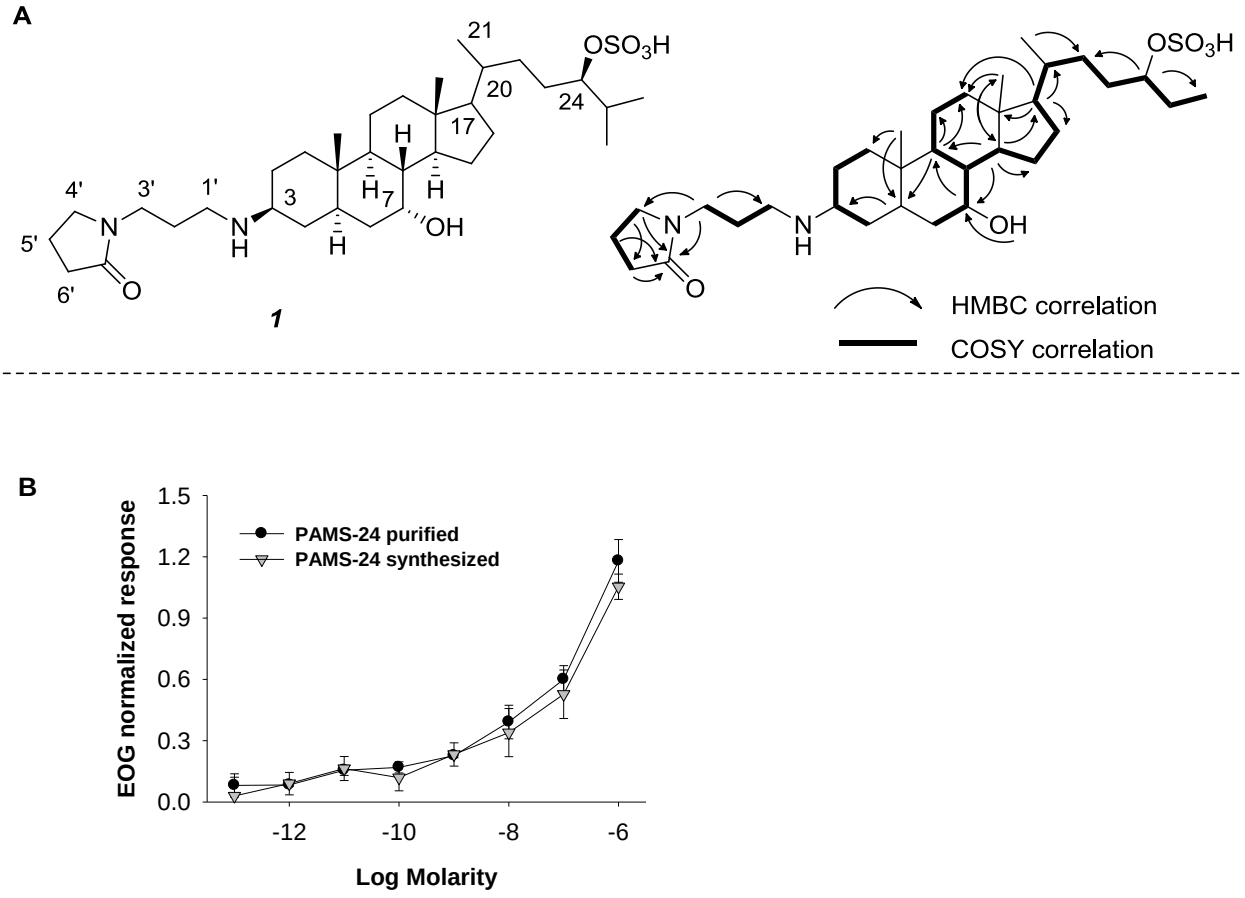

Figure 4-1. (**A**) Structure of PAMS-24 (***1***) including key ^{1}H-^{1}H COSY (bold lines) and HMBC (arrows) correlations of PAMS-24 recorded in DMSO-d_6 using a Brüker NMR spectrometer (^{1}H NMR, 900 MHz; ^{13}C NMR, 225 MHz). (**B**) Semi-logarithmic plot of normalized electro-olfactogram (EOG) amplitudes recorded in sea lamprey in response to different concentrations of PAMS-24 purified from spermiating male lamprey washings and PAMS-24 in synthesized form. Data are the means ± SEM (n = 6), and are blank corrected and normalized to the amplitude of response to 1E-5 M L-arginine.

A territorial pheromone is thought to both advertise and to deter (Hölldobler and Wilson, 1977). We predicted that PAMS-24 should both advertise to mature females and deter mature males, since a nest is constructed primarily by males which then signal to females for courtship (Teeter, 1980). In a system where mature females were released 45 meters downstream of two adjacent nests (1 meter^2 each) positioned 1.5 meters apart (Figure 4-2a), one nest perfused with 3kPZS at $5x10^{-13}$ M and the other with 3kPZS:PAMS-24 at $5x10^{-13}$ M: $5x10^{-15}$ M (100:1), females moved upstream and preferred the 100:1 activated nest (Supplementary Table S4-2a). While 3kPZS alone is known to induce upstream movement in sea lamprey (Johnson et al., 2009), the natural ratio likely functions as a proximal cue to females of the location of a mate-ready male (*i.e.* as a sex pheromone). Responses of mature females to PAMS-24 alone could not be evaluated, as mature females will not move upstream without the presence of 3kPZS in the system (Johnson et al., 2009) (Supplementary Table S4-2a). It is possible that the mass plume of 3kPZS emanating from a stream area of high nest density induces upstream movement (Johnson et al., 2009), whereas PAMS-24, released at a much lower rate and detected at lower sensitivity, adds an additional layer of information to advertise an individual nest.

Similar to mature females, mature males also swam upstream towards a source of MMW, synthesized 3kPZS, or 3kPZS:PAMS-24 at $5x10^{-13}$ M: $5x10^{-15}$ M (100:1) (Supplementary Table S4-2b). Males preferred a nest perfused with synthesized 3kPZS (Figure 4-2b), which is also consistent with females (Johnson et al., 2009; Siefkes et al., 2005). However, mature males often sharply avoided a nest (0.5 meters^2) perfused with MMW and 3kPZS:PAMS-24 at $5x10^{-13}$ M: $5x10^{-15}$ M (100:1) which is inconsistent with females. We then eliminated the main mating pheromone component contained in MMW, 3kPZS, as a possible candidate for a territorial pheromone.

Having demonstrated that avoidance to PAMS-24 is exclusive to mature males, and now that we understood the scale of avoidance specifically in mature males, we aimed to evaluate whether mature male subjects become more active in their search behavior (frequency of turning) when approaching a source of PAMS-24, similar to behaviors of other animals as they enter another conspecific's territory (Bradbury and Vehrencamp, 2011). Within 5 m downstream of the treatment sources, mature males increased their sharp turning (> 90° turn) behavior specifically during treatments containing the natural ratio of 3kPZS to PAMS-24 (MMW and 100:1 treatments, Figure 4-2c). Since males still approach the boundary of a nest perfused with treatments containing PAMS-24, we then summarized the numbers of swim-tracks that specifically entered or avoided each treatment nest. Of the subjects that were manually tracked in the stream, 50% avoided the natural 100:1 ratio (*i.e.* the other 50% choosing to enter, Figure 4-3). We concluded that males choose to enter a natural ratio of 3kPZS:PAMS-24 or MMW as frequently as they avoid these treatments. Further, responses to MMW and 3kPZS alone show a clear inverse relationship where mature male subjects preferred to enter 3kPZS and avoid SMW. Finally, since we observed an equal number of males entering and avoiding the natural ratio of 100:1, and since larger males are more likely to win fights (Manion and Hanson, 1980; Teeter, 1980), we examined whether males that chose to enter were larger in body weight (g) than those that avoided. Larger males chose to enter the 100:1 ratio compared to 3kPZS alone ($t_{15} = 1.75$, $P = 0.04$, Figure 4-4).

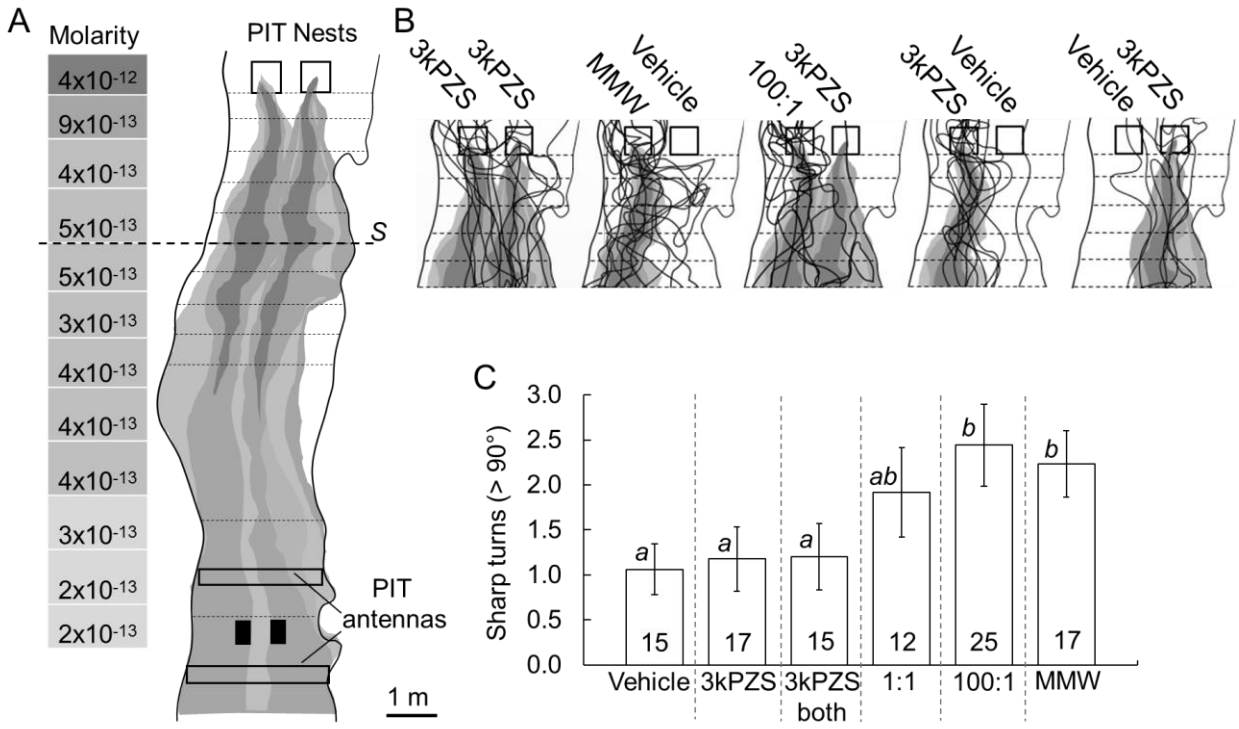

Figure 4-2. Details of field behavioral studies testing mature male sea lamprey. (**A**) The 18.5 m-long section of the Upper Trout River used for field bioassays. Downstream release cages are shown as solid black boxes Plumes were mapped and concentrations of treatments (Molarity) were estimated using rhodamine dye concentrations following Johnson et al. (Johnson et al., 2009). The point at which treatment concentrations reached that of our target whole-stream concentration is indicated (*S*). (**B**) Swim tracks of mature male subjects during trials where 3kPZS ($5x10^{-13}$ M) was applied to both nests, mature male washings (MMW, applied at $5x10^{-13}$ M benchmark 3kPZS) was applied to the left nest vs. vehicle, a 100:1 3kPZS:PAMS-24 ratio (3kPZS $5x10^{-13}$ M:PAMS-24 $5x10^{-15}$ M was applied to the left nest vs. 3kPZS at $5x10^{-13}$ M in adjacent nest, and when 3kPZS alone ($5x10^{-13}$ M) was applied to one nest and Vehicle was applied to the adjacent nest. (**C**) Mean number of sharp turns ($X > 90° \pm 1$ SEM) of mature male subjects as they approached within 5 m of the treatment sources. Treatment 1:1 was 3kPZS:PAMS-24 ($5x10^{-13}$ M: $5x10^{-13}$ M) vs. 3kPZS ($5x10^{-13}$ M). Treatments that share a letter are not significantly different (ANOVA and *post-hoc* Tukey's HSD: $F_{4,74} = 3.19$, $P = 0.012$). Sample sizes (*n*) are shown within columns.

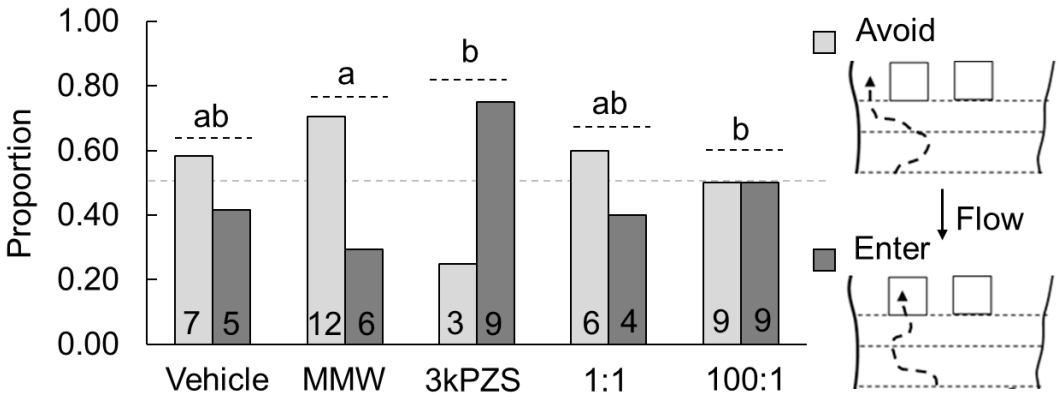

Figure 4-3. The proportion of mature male sea lamprey that approached the source of treatments and either directly entered (Enter) the nest (0.5 meter^2), or sharply avoided (Avoid) the boundary of the nest. Treatments are explained in Figure 4-2. Responses within each treatment were evaluated with logistic regression (GLM: $X^2_{4, 64} = 15.66$, $P = 0.0285$). Responses that share a letter are not significantly different ($\alpha = 0.05$).

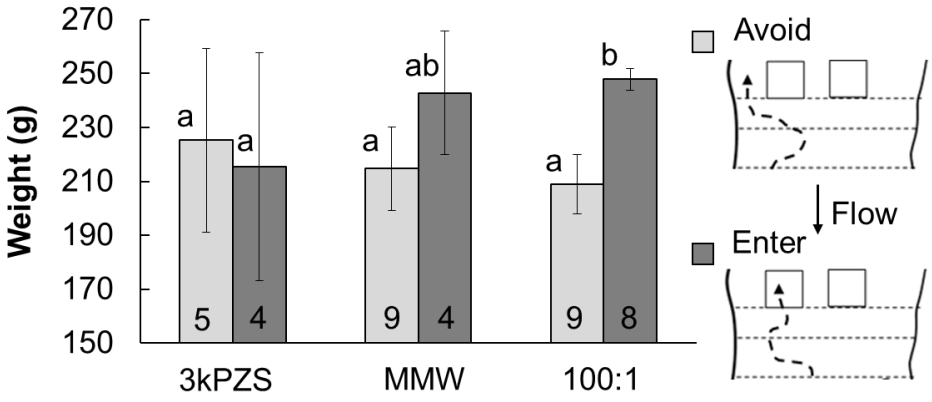

Figure 4-4. Mean (± 1 SEM) weight (g) of mature male sea lamprey that either entered (Enter) or sharply avoided each treatment. Treatments are explained in Figure 4-2. Responses within each treatment was evaluated with a *t*-test. Responses that share a letter are not significantly different ($\alpha = 0.05$).

DISCUSSION

Taken together, we show that mature male sea lamprey, during their single reproductive season, release a novel compound that may function as a partial territorial pheromone. We identify the compound as PAMS-24, and show this compound to be detected and discriminated in the olfactory epithelium of conspecific adults. PAMS-24 induced sexually dimorphic behavioral responses in spawning phase conspecifics. The evolution of more proximal territorial pheromone components such as PAMS-24 in sea lamprey was likely shaped within the context of the sea lamprey mating system. Mating pheromone 3kPZS is currently considered the most abundant compound released from mature males (Brant et al., 2013; Li et al., 2002; Yun et al., 2003) causing background stream concentrations of 3kPZS to remain high during the reproductive season (Xi et al., 2011). Since male-male conflict is relatively rare given the high density of nests in some stream stretches, and olfactory senses are paramount to vision during at this time (Binder and McDonald, 2007; Johnson et al., 2006), a territorial pheromone is the most parsimonious explanation regarding regulation of nest boundaries among males. Proximity information is attainable due to the ratio of compounds released from males (*i.e.* PAMS-24 is released in much less quantity compared to 3kPZS). The sulfated steroid is similar to petromyzonamine disulfate (PADS) (Sorensen et al., 2005), a major compound released by larval-stage sea lamprey, indicating a possible precursor to PAMS-24 synthesis in adults. While no PAMS-24 was detected in larval sea lamprey during our preliminary analysis, a more detailed study should be conducted to determine whether larvae can synthesize and release PAMS-24, and if so, whether PAMS-24 functions as a navigational cue in migrating pre-spawn adults similarly to the whole smell of larvae (Bjerselius et al., 2000).

Often times an animal's behavior is clearly altered when venturing into a conspecific's territory, and olfaction is known to play a critical role in communicating territory ownership throughout the animal kingdom by means of territorial scent marking (Bradbury and Vehrencamp, 2011). The frequency of sharp turning activity (> 90°) doubled as males approached nests activated with MMW and 100:1 treatments. In the hermit crab *Pagurus berhardus,* individuals exposed to wash-water conditioned with fighting crabs have been shown to elicit startled behaviors (*e.g.* hide in shell, turn away), while no noticeable responses were observed from treatments with plain sea water or wash-water conditioned with non-fighting conspecifics (Briffa and Williams, 2006). Yet to our knowledge, no territorial pheromone has been identified to date.

In summary, our chemical, electrophysiological, and behavioral data presented here support PAMS-24 as a partial component of a territorial pheromone in sea lamprey. Pheromones are an environmentally friendly means of integrated population control of the sea lamprey, whose invasion of the Laurentian Great Lakes sparked arguably one of the largest control strategies of an invasive fish in the world (Commission, 2001). The integration of pheromones such as PAMS-24 into the sea lamprey control program could bring about a long-term reduction in sea lamprey populations that cannot be accomplished with current methods (Christie and Goddard, 2003).

METHODS

Sea lamprey

The Michigan State University Institutional Animal Care and Use Committee approved all procedures using sea lamprey presented in this manuscript prior to any experimentation (AUF# 05/09-088-00). Since we examined PAMS-24 release in both sexes of sexually mature adult sea lamprey, we establish here that mature male (MM) refers to spawning-phase males that are spermiated (observed expression of spermatozoa when gentle pressure was applied to the abdomen) and mature female (MF) refers to spawning-phase ovulated females (observed expression of oocytes with gentle pressure to abdomen). Sea lamprey were captured by the USFWS and Department of Fisheries and Oceans Canada from tributaries to Lake Michigan and Lake Huron in May – June 2013, following animal use and care protocols established by those agencies. Sea lamprey were held in 500-1000 L-capacity flow through tanks with constant aeration until use. Males were kept separate from females. Sea lamprey are a non-native invader of the Laurentian Great Lakes and their tributaries. Hence, males and females could not be released together upstream of sea lamprey barriers to reduce the risk of reproduction and repopulation of the river system (Supplemental Figure S4-1).

To produce MM and MF test subjects, immature subjects were transferred to acclimation cages constructed of polyurethane mesh and PVC pipe (0.5 m^3) located in the lower Ocqueoc River, Millersburg, Michigan, USA, to allow natural maturation in stream water. Sea lamprey were monitored daily for sexual maturation. MMs were identified by first checking for secondary sexual characteristics (Chung-Davidson et al., 2013), then applying gentle pressure to the abdomen and checking for expression of spermatozoa from the genital papilla. MFs were

identified by applying gentle pressure to the abdomen and observing a steady expression of oocytes from the cloacal aperture.

Wild nesting sea lamprey observations

Wild nesting sea lamprey were observed at sites along the Ocqueoc River, Millersburg, MI and downstream of the Cheboygan Dam, Cheboygan MI, USA, from 16 -26 June 2009 and 5 – 8 June 2010. Once a nest was located (see Supplementary Figure S4-3 for an example of a nest) a male was identified in the nest by observing the defined "rope" tissue along the back (Chung-Davidson et al., 2013). Nests were then approached from downstream. While standing downstream and to the side of each nest, observations were made for 15 minutes. The number of each sex within a nest was immediately recorded. During the rest of the observation, the frequency of visits by intruder males and male-male aggression was recorded. After each observation, depth of nest (m), water temperature (°C), water velocity (m/sec.), and the area of each nest (m^2) was recorded. Numbers of males and females observed on nests can be seen in Supplementary Figure S4-4. Depth of nests observed ranged from 0.26 – 1.8 m, water temperature ranged from 17 – 25 °C, water velocity ranged from 0.17 – 0.59 m/sec., and area of each nest ranged from 0.08 – 2.0 m^2.

Extraction of sea lamprey-conditioned water

Extracted odors were collected from MM sea lamprey following methods outlined in Fine et al. (Fine et al., 2006), with slight modification. Briefly, MM-conditioned water was collected throughout June and July, 2012. Wash-water was passed through vertical columns containing 2 kg of methanol-activated Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) using peristaltic pumps (Masterflex 7553-70, Cole-Parmer, Vernon Hills, Illinois, U.S.A.). Loading speed was ~300 ml/min. Three columns were loaded for up to 24 hours at a time. Each

column was then eluted with 4 L of methanol followed by 4 L of acetone. The organic solvents were removed under reduced pressure at 40 ^{o}C using a model R-210 roto-evaporator (BuchiRotovapor, Flawil, Switzerland) to produce 4.2 L of extract (stored at -80 ^{o}C). The extracts (containing a large amount of water) were further concentrated by lyophilization to yield brown residues. The residues were suspended in methanol and then successively filtered through 2 μm filter paper. The Filtrate was collected and concentrated by a roto-evaporation under reduced pressure at 40 ^{o}C to obtain 3.1 g dark residue. Roughly 60 mature males were used for wash-water collection and extraction.

Separation of PAMS-24

Crude extract from MMs was subjected to liquid chromatography over silica gel (150 g; gradient elution from 95% CH_3Cl/MeOH to 100% MeOH, ca. 8 L total volume). Thin Layer Chromatograph (TLC) analysis indicated that 9 fractions were produced. Fraction 8 (30 mg) was further purified using Sephadex LH-20 eluted with $CHCl_3$-MeOH (1:1) and MeOH (100%) which subsequently yielded the compound PAMS-24 (1.4 mg).

Structural analysis

1D and 2D NMR spectra of PAMS-24 were recorded on a Bruker Avance 900 MHz Spectrometer or an Agilent 500 MHz spectrometer. Mass spectra were performed on a TQ-S TOF LC mass spectrometer (Waters Corporation, Milford, Massachusetts, USA). Si gel (70-230 and 230-400 mesh, Merck, Darmstadt, Germany), RP-18 reverse-phase Si gel (Merck), and Sephadex LH-20 (Merck) were used for open column chromatography. TLC was conducted on glass plates precoated with GF_{254} Si gel (Merck). Spots were visualized under UV light at 254 nm and stained by spraying plates with 5% anisaldehyde acid alcoholic solution (Sigma-Aldrich, St. Louis, Missouri, USA).

Determination of PAMS-24 concentrations in extracts and wash-water samples

To examine the release and distribution patterns of PAMS-24, a novel ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC–MS/MS) method was developed to realize baseline separation and short analysis time. UHPLC-MS/MS analyses were carried out on a Waters Acquity ultra-performance liquid chromatography system (Waters, Milford, MA, USA) with a Xevo Quattro Premier XE tandem quadruple mass spectrometer (Waters, Manchester, UK) equipped with ESI source. The compound PAMS-24 was separated on a C18 Acquity column (2.1 × 50 mm, 1.7 μm) with the column temperature at 45 ^{o}C. The mobile phase for elution was a gradient established between solvent A (10mM TEA in water) and solvent B (Methanol) at a flow rate of 250 μL/min. Baseline separation was achieved by using the gradient started at 60% A/40% B and followed by a linear increase of B to 90% at 7 min. An isocratic elution of 90% methanol was maintained from 7.0 to 8.0 min, and the initial conditions were restored between 8.0 and 8.1 min and retained 2 min for equilibration. Samples were maintained in the autosampler at 4 ^{o}C throughout the analysis. The ESI source operated in negative ion mode, and its main working parameters were set as follows: capillary voltage, 3.50 kV; extractor voltage, 5 V; source temperature, 130 ^{o}C; desolvation temperature, 350 ^{o}C; desolvation gas flow, 600 L/h (N2, 99.9% purity). Argon (99.9999% purity) used as the collision gas was introduced into the collision cell at a flow rate of 0.20mL/min. Data were collected in centroid mode with a scan range of 50-1000 *m*/*z*. Multiple reaction monitoring (MRM) measurement (623.32 > 96.68) was performed using individually optimized cone voltage (10 V) and collision energy (49 eV). The dwell time established for each transition was 0.2 s, and interscan delay was set at 20ms. Data acquisition was carried out by Masslynx 4.1 software (Waters Corp., Milford, MA, USA).

To examine release of PAMS-24 across life stages and sex, lamprey conditioned wash-water was collected from larvae, IMs, MFs, and MMs. Larval sea lamprey (N = 229, 111.5 g batch-weight) were collected by the USFWS from Silver Creek in Tawas, MI, USA, transferred to USGS-HBBS, divided into three equal groups by weight, and placed into three separate washing chambers (~ 37 g-larvae/chamber). Chambers were supplied with equal volumes of sand substrate, aeration, and fresh Lake Huron water maintained to the temperature of the stream (2.7 – 3.2 °C). A fourth control chamber was maintained the same as the other three chambers excluding the addition of any larvae. Each chamber contained 22.5 l of water and 6500 g of sifted sand and was 32 cm-tall x 26 cm-wide x 50 cm-long in dimension. Larvae were then allowed to acclimate in their respective chambers for 24 h with normal flow. Water supplies were then removed for collection of larval odors for 24 h. Triplicate 10 ml larval-conditioned water samples were then taken from each chamber and placed at -20 °C until later analyses by the UHPLC–MS/MS method.

For IM washing collection, eight individuals were selected during the migratory season. Each individual was placed in a separate 20 l-capacity container containing 5 l of D.I. water for 1 hr. A control was also conducted using the same method without the addition of a male to the container. A portable aerator was used for a constant supply of oxygen to each container during washing. The temperature of the D.I. water used for washings was acclimated to the same temperature as the stream (16 – 18 °C). Triplicate 10 ml samples were then collected from each container for later analyses by UHPLC–MS/MS.

To examine whether female sea lamprey release PAMS-24, 15 female sea lamprey were matured in acclimation cages in the Lower Ocqueoc River. MFs were placed in 30 L of D.I.

water for 20 hr with proper aeration. Triplicate 1 l samples were then collected and frozen at -20 °C for later analyses with UHPLC–MS/MS.

To identify whether PAMS-24 was released from the head-region of mature males, bisected chambers were used to collect wash-water from the anterior and posterior region from each subject following Siefkes et al. (Siefkes et al., 2003), with slight modifications. We collected 7 L of posterior washings and 7 L of anterior washings from 7 individual MMs, independently, and froze samples at -20 °C for later analyses with UHPLC–MS/MS. For confirmation that PAMS-24 was released primarily from the head-region of MMs, wash-water extracts from the head and tail regions of 20 mature males were mixed into one batch and analyzed for PAMS-24 concentrations. The head and tail wash-water was passed through separate beds of 500 mg Amberlite XAD 7HP resin contained in respective glass columns (Ace Glass Inc., Vineland, New Jersey, USA). Load speeds were maintained between 150 and 200 mL/min. Extracts were eluted with 3 L of methanol and solvents were removed under reduced pressure at ~ 40 ^{o}C by roto-evaporation (Buchi) yielding 0.5 L of head extract and 0.5 L of tail extract. Triplicate 1 mL samples of head and tail extract samples were analyzed with UHPLC–MS/MS.

To examine the release rate of PAMS-24 and known mating pheromone 3kPZS from individual MMs, six male sea lamprey were matured in acclimation cages in the Lower Ocqueoc River, Millersburg, MI, USA. Once all individuals were confirmed to be mature (*i.e.* development of secondary sexual characteristics, expression of gametes with gentle pressure to abdomen) each individual was placed in a separate 5 L of D.I. water for 10 min. A control was also conducted using the same method minus the addition of a male to a container. A portable aerator was used for a constant supply of oxygen to each container during washing. The

temperature of the D.I. water used for washings was acclimated to the same temperature as the stream (temperature range during sampling was 18-22°C). Triplicate 10 ml samples were then collected from each container for analyses with UHPLC–MS/MS. Each male was weighed, and release rates were calculated (ng/g-lamprey/hour) for both PAMS-24 and 3kPZS.

All values from UHPLC–MS/MS analyses associated with a signal-to-noise ratio ≤ 10 were considered below the lower limit of quantitation and automatically removed from the data set. All comparisons were examined for violation of assumptions of normality and homogeneity across variance before further statistical analyses were conducted. Data that were not normally distributed or showed heterogeneity across variance were log-transformed. The Levene's test for homogeneity of variance was used to examine variance of newly transformed data. Once homogeneity of variance was observed, an ANOVA and post-hoc Tukey's HSD or *t*-test (α = 0.05) was conducted in R (version 2.11.1) for final statistical comparisons of compound release data.

PIT tagging and marking for field behavioral studies

Passive integrated transponder (PIT) tagging procedures for MM and MF followed procedures previously described (Johnson et al., 2009). To prevent the expulsion of gametes from a surgical incision, each PIT tag was fitted into a latex sleeve and attached to the mid-dorsal region of each animal using a suture on both sides (Size 3-0, Ethicon Inc., Cornelia, Georgia, USA). MM and MF were also fitted with unique color combinations of ribbon tags (Hallprint, Hindmarsh Valley, South AU) through each dorsal fin to identify individuals for visual observations and tracking during mating trials. These procedures typically took less than 30 seconds/animal. PIT tagged animals were immediately transferred into aerated holding tanks with a constant flow of Lake Huron water for up to 24 hours, until they were stocked into stream

acclimation cages. Tagged individuals were monitored throughout the day for signs of distress or mortality.

Electro-olfactogram (EOG) responsiveness to PAMS-24

Electro-olfactogram recordings were obtained from lamprey in summer 2013. Our procedures for EOG are detailed in previous works by Li et al. (2013b; 2012). Briefly, sea lamprey were anesthetized and secured in a partially inundated trough, whereby gills could be continuously irrigated with water. The olfactory rosette was surgically exposed. Responses to stimuli were recorded by borosilicate electrodes placed between olfactory lamellae (signal electrode and external skin-reference electrode). Olfactory responses were filtered and amplified by a NeuroLog filter and pre-amplifier (Digitimer Ltd., Hertfordshire, England), integrated by a digidata system (Axon Instruments, Inc.), and stored on a PC running Axoscope software (Axon Instruments, Inc.). Stimuli were serially diluted from a $1x10^{-3}$ M stock solution of isolated and synthesized PAMS-24, PADS, and PSDS, respectively. Responses were blank subtracted and normalized to those of L-argininge at $1x10^{-5}$ M.

Synthesized odorant treatments

The well documented mating pheromone 3kPZS was custom synthesized by Bridge Organics Co. (Vicksburg, Michigan, U.S.A.; purity >97%) in 2007. A 10 mg ml^{-1} stock solution of synthesized 3kPZS (in 100% methanol) was prepared and transferred into five vials of 10-ml aliquots. 3kPZS stock solution was stored at -20°C until use. PAMS-24 was chemically synthesized by Bridge Organics Co (Vicksburg, Michigan, U.S.A.; purity >95%) in 2012. The synthetic compound exhibits the same spectral characteristics and biological activity as the natural compound. These data confirm that the observed olfactory response activity was due to the purified steroid derivative, not to a trace compound co-purified with the natural PAMS-24.

Experimental designs for preliminary behavioral tests using telemetry

Details of experimental sites for behavioral field tests are described in Supplementary Figure S4-1, and Figure 4-2. The upper reaches of each field site were historically accessible to wild sea lamprey. These sites are now physically blocked by a low-head dam (sea lamprey barrier) near the mouth of each respective river. MFs were tested in a separate river system from MMs to insure that no unwanted reproduction would occur upstream of sea lamprey barriers by mixing sexes.

For MFs, two 1 m^2 nest antennas were placed adjacent to one another on the upstream end of the site, flat on the stream bed, and 1.5 m apart. These antennas monitored the proportion of subjects that entered a particular "nest" containing certain treatments. Downstream 45 m, two aluminum-mesh release cages (0.25 m^3) equipped with sliding release doors were positioned in the center of the stream channel. Two PIT antennas roughly 0.5 m-high x 6 m-long were positioned approximately 5 m upstream and 5 m downstream of the release cages to monitor individuals that exit the cage and move upstream or downstream, respectively (Supplementary Figure S4-1a).

For MMs, two 0.5 m^2 nest antennas were placed adjacent to one another on the upstream end of the site, flat on the stream bed, and 1 m apart. These antennas monitored the proportion of subjects that entered a particular "nest" containing certain treatments. Downstream 18.5 m, two aluminum-mesh release cages (0.25 m^3) equipped with sliding release doors were positioned in the center of the stream channel. Two copper wire PIT antennas roughly 0.5 m-high x 3 m-long were positioned approximately 0.5 m upstream and 0.5 m downstream of the release cages to monitor individuals that exit the cage and move upstream or downstream, respectively (Supplementary Figure S4-1b).

For both sexes, trials were 2 hours long. Stream temperatures were recorded at the start of and end of each trial. Stream discharge was estimated every three days, or after every precipitation event, at a fixed location in the stream using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, Maryland, U.S.A.) to determine the amount of treatment stock solution to apply to the stream and maintain consistent concentrations across trials. Test treatments were diluted with 20 l of river water in large mixing bins on shore. Bins were kept consistent for each test treatment, and rinsed in the stream several times before each new trial, to reduce the potential for contamination during mixing. Each treatment solution was then pumped from bins into the stream at the center of each "nest" antenna so that test subject could swim within 0 – 0.5 m of the source upon entering the nests (*i.e.* close proximity to the source) at a rate of 167 mL/min (± 5 mL/min) over the span of 2 hours using peristaltic pumps (Cole-Parmer). Peristaltic pumps, powered with a gas generator, allowed concentrations of compounds in the stream to remain consistent throughout each trial. In the first half-hour of each trial the test treatments were administered to the stream while test subjects remained in the release cage. At the start of the following 1.5 hours test subjects were released, and their movements were monitored with PIT antennas until the trial ended. No animals were recovered from the stream after a trial.

Copper wire was wrapped around each antenna frame twice during the construction of PIT antennas for a more focused read range. Antennas were wired to a multiplexor in the field for consolidation of data (Oregon RFID, Portland, OR, USA). Antennas were tuned to a detection sensitivity of roughly 0.3 m from the frame edges. Scan frequencies of each antenna were programmed to three scans/sec. Data for each trial were uploaded each day using a hand-

held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

Details of treatments for behavioral tests using telemetry

Test treatments for MF and MM trials are described in Figure 4-2. For both sexes, trials were ran from 25 June – 01 August 2013, and from 27 June – 10 August 2014, which is inside the range of the natural spawning season for sea lamprey (Applegate, 1950). Treatments applied to each "nest" were alternated back-and-forth for each trial. Up to two trials were conducted each day depending upon the availability of mature animals. The early trial was conducted from ~0700h – 0900h, and a late trial was then run from ~0930h – 1130h. Ten PIT-tagged mature subjects were transferred to respective acclimation/release cages for each trial between 2000 – 2200h the night prior to experimentation. Subjects were then allowed an acclimation period in the stream for 9+ hours.

Statistical analysis of PIT data (shown in Supplementary Table S4-2)

Each test subject's unique PIT tag identification number prevented any pseudo-replication from test subjects released during previous trials for all sites. Three main binary response variables were examined for telemetry experiments, including: (1) the distribution of subjects that swam downstream from the release cage and did not come back during trials (*Down:* 0 = did not hit on downstream antenna, 1 = hit on downstream antenna) (2) the distribution of subjects that swam upstream from release cages and did not move back down (*Up:* 0 = did not hit on upstream release antenna, 1 = hit on upstream release antenna), (3) of those animals that hit on the upstream antenna, the distribution that entered the nest containing the test treatment (*Treatment nest:* 0 = missed treatment nest, 1 = entered treatment nest). Since 50% methanol (vehicle) was administered to both nests during negative control trials, one nest

was randomly assigned for statistical purposes to be the "treatment" nest by flipping a coin. The "treatment" nest was randomly chosen to be the right nest, and alternated every trial to follow the same pattern of other actual odorant treatments.

Statistical analyses for all behavioral tests using PIT equipment at both sites followed Li et al. (2013b). Briefly, logistic regression with a binomial distribution was examined for each response variable, followed by a post-hoc *t*-test for comparisons across treatments within each response variable, using R-software (R version 2.11.1, Vienna, Austria). No signs of nonlinearities or over dispersion were observed in the models. All behavioral statistics reported are two-tailed analyses ($\alpha = 0.05$).

Swim track and plume mapping

Swim tracks were mapped from random individuals during trials examining mature sea lamprey following Johnson et al. (Johnson et al., 2009). Briefly, stream sections seen in Supplementary Figure S4-1 were fitted with transecting strings, every 1 m downstream of "nests" for the first 10 m, and every 5 m down after that until reaching release cages. Each transecting string (stream width) was divided into tenths of the total width and labelled. Since each test subject was marked with a unique color combination of ribbon tags, we were able to visually observe and follow individuals as they swam upstream. The stream segments were mapped following Johnson et al. (Johnson et al., 2009). Briefly, swim tracks of moving subjects were recorded by hand. We followed the subject until it reached a nest. Only subjects that were observed exiting the release cage were followed. The rest of the subjects that exited unseen by observers were recorded by PIT telemetry.

To map the odor plume and estimate in stream concentrations of treatments, rhodamine dye was administered to the stream and samples were taken following Johnson et al. (Johnson et

al., 2009) with slight modification. Rhodamine concentrations were detected and recorded at each sample point with a hand-held DataBank datalogger and Cyclops-7 Optical Rhodamine Dye Tracer (Turner Designs, Sunnyvale, CA, USA).

Statistical analysis of swim tracks and plume mapping

All swim tracks that were mapped during trials were traced onto a digital field site map using a tablet computer (Lenovo X201 Tablet). Each track was overlain onto an odor plume map. To better examine the behaviors of MM as they approached treatments in side-by-side nests, we evaluated each swim track within 5 meters of the treatments sources (see Figure 4-2). Since we wanted to evaluate the "search" behavior of these subjects in relation to treatments, we counted the number of sharp turns made by each individual (> 90°) within the 5 m range. Once homogeneity of variance was observed, the mean number of sharp turns within 5 m of each source across treatments were evaluated with ANOVA and post-hoc *t*-test (R-Software® for Windows, R Foundation for Statistical Computing, Vienna, Austria). Next we compared tracks that enter each treatment compared to those that avoided (Figure 4-3). Avoidance (or alternatively enter) responses were evaluated using logistic regression and a binomial distribution, similarly to PIT data (0 = avoid, 1 = enter, $\alpha = 0.05$). Finally based on our nest observations of larger males often beating smaller males in fights, we aimed to test whether larger males choose to enter natural male odors or treatments of 3kPZS:PAMS-24 at natural mixtures over smaller males (*i.e.* larger males are more likely to win a contest). To do this, we compared the mean sizes of males that entered respective treatments (see Figure 4-4) to the mean size of males that avoided using a one-way *t*-test ($\alpha = 0.05$). All data presented were examined for violation of assumptions of normality and homogeneity across variance before further statistical analyses were conducted. Data that were not normally distributed or showed

heterogeneity across variance were log-transformed. The Levene's test for homogeneity of variance was used to examine variance of untransformed or newly transformed data.

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL TABLES AND FIGURES

Table S4-1. ^{1}H (900 MHz, *J* in Hz) and ^{13}C NMR (225 MHz) spectroscopic data for PAMS-24 in DMSO-d_6

no.	δ_H	δ_C
1	1.01 (m), 1.62 (m)	36.0 (CH_2)
2	1.16 (m)	22.1 (CH_2)
3	2.82*	56.8 (CH)
4	1.11 (m), 1.49 (m)	29.0 (CH_2)
5	1.66 (m)	36.1 (CH)
6	1.33 (m), 1.03(m)	36.5 (CH_2)
7	3.63 (m)	66.2 (CH)
8	1.31 (m)	31.3 (CH)
9	1.17 (m)	44.5 (CH)
10		35.8 (qC)
11	1.17 (m), 1.38 (m)	20.3 (CH_2)
12	1.04 (m), 1.83 (d, 10.9)	39.0 (CH_2)
13		42.0 (qC)
14	1.32 (m)	49.7 (CH)
15	1.17 (m)	23.0 (CH_2)
16	1.76 (m), 1.31 (m)	28.7 (CH_2)
17	1.06 (m)	55.7 (CH)
18	0.52 (s)	11.4 (CH_3)
19	0.68 (s)	10.6 (CH_3)
20	1.24 (m)	35.2 (CH)
21	0.78 (d, 6.1)	18.2 (CH_3)
22	1.10 (m), 1.32 (m)	31.2 (CH_2)
23	1.54 (m), 1.21 (m)	26.4 (CH_2)
24	4.00 (ddd, 4.1, 4.1, 4.1)	83.4 (CH)
25	2.06 (m)	30.2 (CH)
26	0.79 (d, 6.8)	18.0 (CH_3)[a]
27	0.79 (d, 6.8)	17.3 (CH_3)[a]
1'	2.74 (m)	42.0 (CH_2)
2'	1.78 (p, 6.9)	27.8 (CH_2)
3'	3.31 (m), 3.18 (m)	40.1 (CH_2)$^{\Delta}$
4'	3.38 (m), 3.32 (m)	46.9 (CH_2)
5'	1.95 (p, 7.4)	16.6 (CH_2)
6'	2.27 (t, 8.2)	31.4 (CH_2)
7'		175.6 (qC)

Table S4-1 is curtesy of Dr. Ke Li, Michigan State University. The symbol * indicates an overlap with H_2O in DMSO-d_6, symbol $^{\Delta}$ indicates an overlap with DMSO-d_6, and ***a*** indicates assignments are interchangeable.

Table S4-2 – Numbers of sexually mature female (*a.* MF) and mature male (*b.* MM) sea lamprey that approached conspecific odorants during field tests, as recorded by telemetry.

a. **MF** (Figure S4-1a)

Treatment Nest	Control Nest	Trials	Released	*Down*	*Up*	*Enter treatment nest*	*Enter control nest*
Vehicle	***Vehicle***	7	71	***15%*** (11) A	***15%*** (11) B	***0%*** (0)	***0%*** (0)
3kPZS	***Vehicle***	5	42	***12%*** (5) A	***50%*** (21) A	***100%*** (13) B	***0%*** (0)
MMW	***Vehicle***	5	44	***11%*** (5) A	***50%*** (22) A	***100%*** (17) B	***0%*** (0)
3kPZS	***3kPZS***	14	127	***10%*** (13) A	***45%*** (57) A	***52%*** (24) A	***48%*** (22)
1:1	***3kPZS***	8	71	***17%*** (12) A	***31%*** (22) A	***47***% (8) A	***53%*** (9)
100:1	***3kPZS***	6	55	***0%*** (0) A	***31%*** (17) A	***78%*** (7) B	***22%*** (2)
			X^2	33.37	30.46	80.16	
			df	5	5	5	
			P-value	< 0.001	< 0.001	< 0.001	

b. **MM** (Figure S4-1b)

Treatment Nest	Control Nest	Trials	Released	*Down*	*Up*	*Enter treatment nest*	*Enter control nest*
Vehicle	***Vehicle***	7	59	***24%*** (14) A	***32%*** (19) A	***60%*** (3) A	***40%*** (2)
3kPZS	***Vehicle***	8	73	***16%*** (12) A	***27%*** (20) A	***79%*** (11) AB	***21%*** (3)
MMW	***Vehicle***	12	128	***20%*** (25) A	***21%*** (27) A	***80%*** (8) B	***20%*** (2)
3kPZS	***3kPZS***	9	90	***12%*** (11) A	***19%*** (17) A	***43%*** (3) A	***57%*** (4)
1:1	***3kPZS***	12	127	***24%*** (31) A	***13%*** (17) A	***63%*** (5) A	***38%*** (3)
100:1	***3kPZS***	8	96	***14%*** (13) A	***36%*** (35) B	***57%*** (13) A	***43%*** (10)
			X^2	6.79	29.67	14.06	
			df	5	5	5	
			P-value	0.237	< 0.001	0.050	

These responses were recorded with passive integrated transponder telemetry. Treatments are described in Figure 4-2. Response variables were monitored with telemetry and include: *Down* - the percentage of test subjects that moved downstream 5 m or more from release cages, *Up* - the percentage of subjects that moved upstream at least 5 m or more from the release cage, *Enter treatment nest* - the percentage of subjects that entered each respective treatment nest, *Enter control nest* - the percentage of animals that entered the adjacent control nest. Each response variable was evaluated with logistic regression. Responses that share a letter are not significantly different ($\alpha = 0.05$).

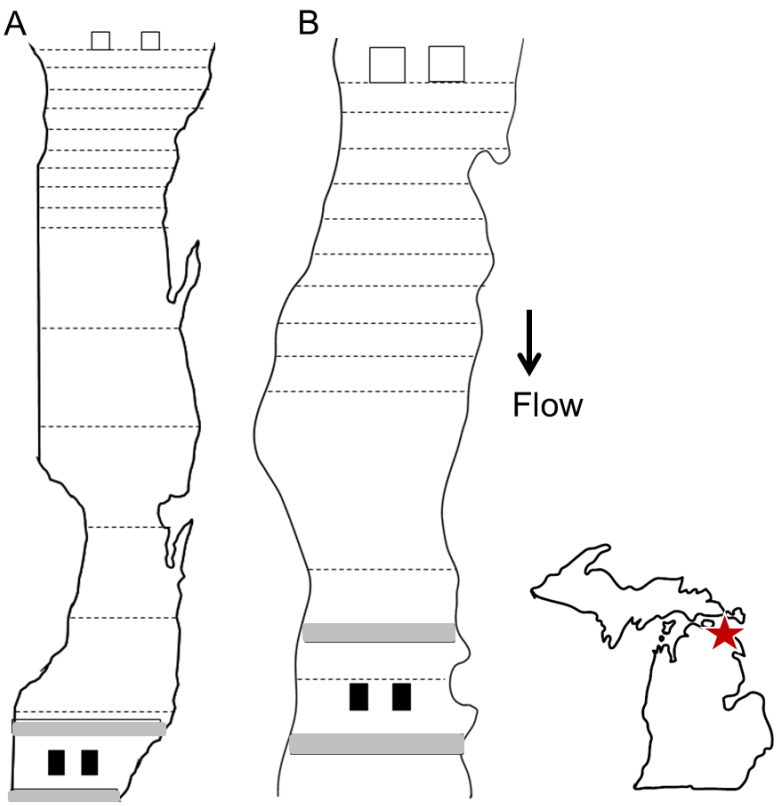

Figure S4-1. In-stream behavioral field sites for observing behaviors of sea lamprey to pheromone components. (**A**) The 45 m-long section of the Upper Ocqueoc River, Millersburg, MI, USA, used for observing movement patterns of sexually mature female sea lamprey to male-released pheromones. (**B**) The 18.5 m-long section of the Upper Trout River, Rogers City, MI, USA, used for observing movement patterns of sexually mature male sea lamprey in relation to mature male pheromones. At the upstream end of each site, odorants were applied into the center of a square passive integrated transponder (PIT) antennas (hollow boxes, 1 m^2 and 1.5 m apart in A, 0.5 m^2 and 1 m apart in **B**). Transecting PIT antennas were placed within each site (grey rectangles) to observe the proportion of subjects moving out of release cages (solid boxes), upstream, and hitting on a nest PIT antenna.

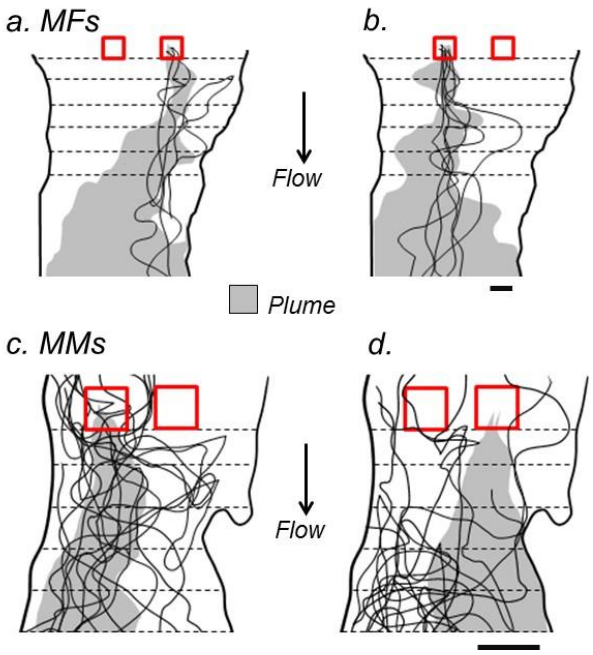

Figure S4-2. In-stream swim tracks of mature sea lamprey to treatments of mature male conditioned wash-water (MMW). Treatments are maintained at consistent stream concentrations based on an in-stream concentration of 5E-13 M benchmark 3kPZS. Mature female (MF) subjects were tested in Figure S1A, where MMW was applied to the right (***a.***) and left (***b.***) nests respectively. Mature male (MM) subjects were tested in Figure S1B, where MMW was also applied to the left (***a***.) and right (***b.***) nests respectively. Swim tracks were mapped by manually tracking and recording the path of each subject. Transecting strings (dashed lines) were strung every 1 m downstream of the source to aid in swim track mapping. Plumes (outlined in grey) were mapped using rhodamine dye following Johnson et al. (Johnson et al., 2009). Scale bars = 1 m.

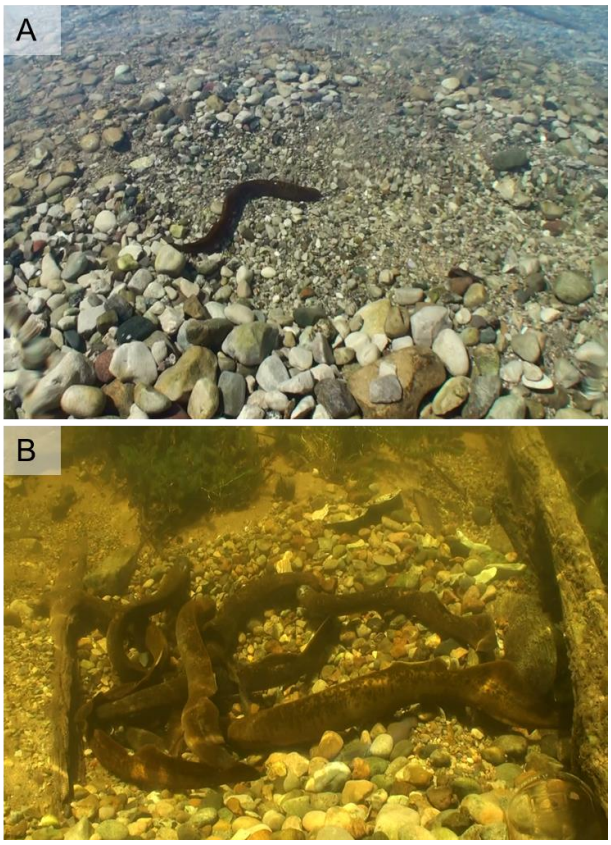

Figure S4-3. Wild nesting sea lamprey. (**A**) A single male maintains a nest in the lower Cheboygan River, below the Cheboygan Dam, Cheboygan, MI, USA. (**B**) One male accompanied by 7 females in a nest in the Lower Ocqueoc River, Millersburg, MI, USA.

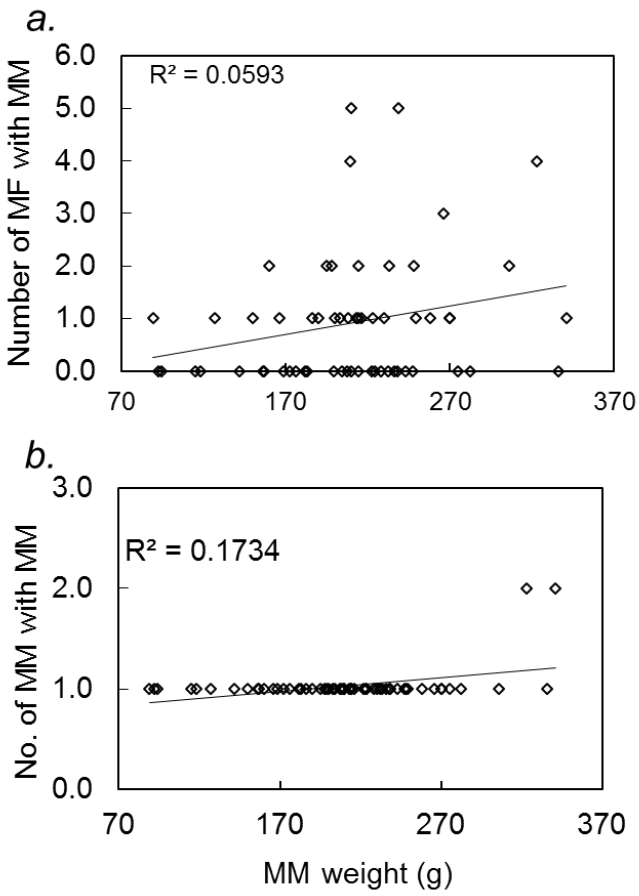

Figure S4-4. Observations of natural sea lamprey nests. Observations suggest that larger males (MM) are often accompanied with greater numbers of mature females (MF) per nest (**a:** Linear regression: $F_{1,57} = 3.67$, $P = 0.060$), while mature males rarely join other mature males in a nest (**b:** Linear regression: $F_{1,57} = 11.95$, $P = < 0.001$).

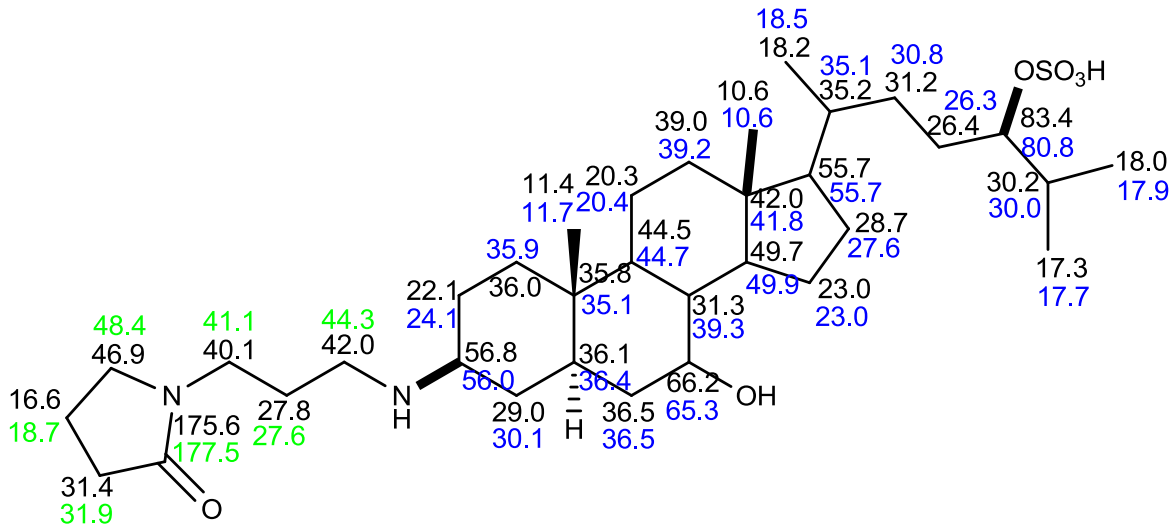

Figure S4-5. Comparison of carbon resonances of PAMS-24 with squalamine (Wehrli et al., 1993) and PADS (Hoye et al., 2007a). ^{13}C NMR data of PAMS-24 and squalamine were acquired in DMSO-d_6 and displayed in black and blue, respectively. ^{13}C NMR data of PADS were acquired in methanol-d_4 and displayed in green.

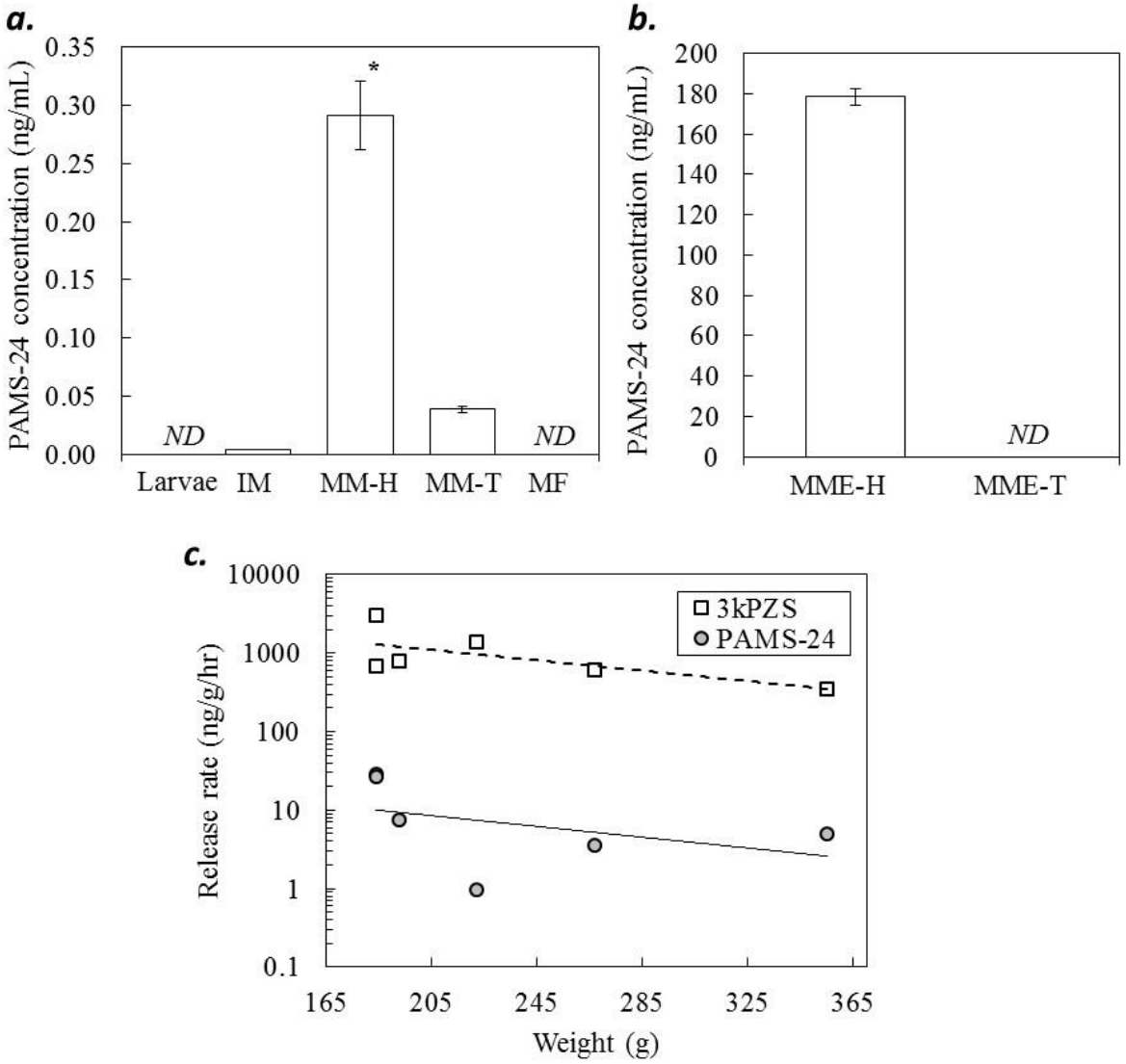

Figure S4-6. Concentrations of washings and release rates of PAMS-24 compared to 3kPZS. (***a.***) Means +/- 1 SEM of PAMS-24 concentrations in washings for larval sea lamprey, immature males (IMs, n = 8), mature males – head region only (MM-H) and same mature males – tail region only (MM-T, n = 7), and ovulated females (MF, n = 15). * $t_{14} = 2.14$, $P < 0.001$. (***b.***) Mean +/- 1 SEM of PAMS-24 concentrations in extracted (SPE) and concentrated washing from mature males – head region only (MME-H) compared to the same mature males – tail region only (MME-T, n > 20). (***c.***) Release rates of PAMS-24 and 3kPZS by weight (g) of 6 mature males sampled from a natural spawning stream. The natural release ratio of the two is roughly 1:0.01, 3kPZS:PAMS-24. PAMS-24 (Linear: $R^2 = 0.3305$) and 3kPZS (Linear: $R^2 = 0.2731$) regression lines are shown.

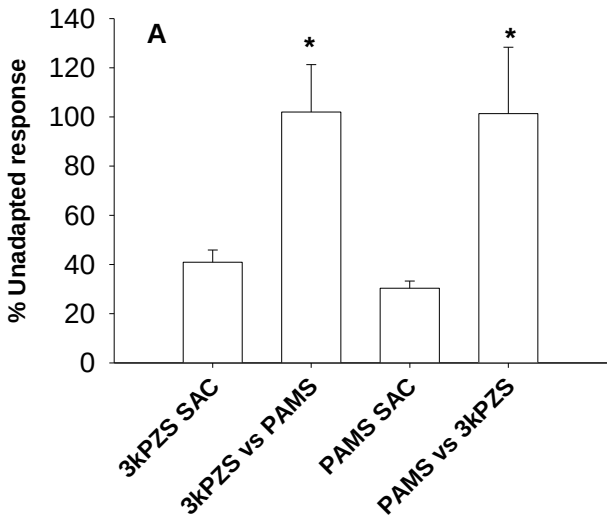

Figure S4-7. Qualitative differences in a) 3kPZS and PAMS and b) PAMS, PADS and PSDS assessed by cross-adaptation in immature male lamprey. Data are expressed as a percentage of the unadapted response. (SAC), self-adapted control; odorant 1 v. odorant 2, odorant 1 against an adapting solution of odorant 2. Values are means ± S.E. (n = 6).

CONCLUSION TO DISSERTATION

Studies presented in this dissertation provide several lines of evidence that directly support the overall hypothesis that pheromone modulation of sea lamprey behavior is dependent upon multiple environmental, physiological, and social factors. The multiple contexts presented by these studies in which sea lamprey respond to pheromones will contribute to our understanding of how pheromones modulate the life history of other vertebrates. Field tests in Chapters 1 and 2 suggested that females became ritualized to 3kPZS as a navigational cue that can override the locomotor-inhibiting effects of cold stream temperatures, induce upstream movement in migrating adults, and transition from a migratory cue to a mating signal as females approach ovulation. Given that the raw odor of larvae or spermiated males consistently induced greater behavioral activity towards the source during positive control trials throughout these studies, I predict additional compounds and mixtures of optimal ratios, along with additional environmental, physiological, chemical, and social factors that mediate these behavioral responses to pheromones, will continue to be identified with the field assays presented here.

Understanding the complexities of pheromone communication in sea lamprey may offer ample opportunities to learn about how signals evolve (Symonds and Elgar, 2008). The diversity of compounds that are only beginning to surface, coupled with variation in release rates among components, variations in olfactory sensitivity to these components, and the multiple contexts (*i.e.* environmental, physiological, chemical and social) that modulate behavioral responses to these compounds present an opportunity for a unique signal design. Pheromones emitted into water form a complex and conspicuous odor plume comprised of multiple components and stereoisomers that add layer upon layer of information to the signal. The complexity of a signal such as one provided by pheromones is not easily comparable to research of more readily accessible signals within the lines of human perception (*i.e.* sound and visual cues), making

pheromones unique when thinking of how signals evolve. Additional compounds released from mature males that, when combined with 3kPZS, were preferred by ovulated females in field tests may have been selected to become conspicuous to allow females to gauge the distance and sexual status of a potential mate. Similarly, male responses to component PAMS-24 may have been selected for as a means to govern the resources held by a nesting male as a territorial pheromone.

Field tests used in Chapters 1, 2, 3, 4 and A-1 allowed me to observe interactions between environmental and social factors and pheromone mediated behaviors in sea lamprey, which may not have been possible in laboratory contexts (Johnson and Li, 2010). Indeed, behaviors of fishes to pheromones in laboratory contexts often remain inconsistent with similar studies conducted in the field (Johnson and Li, 2010; Meckley et al. 2012). The bioassay guided fractionation system developed and implemented in Chapters 3, 4, and A-1 is effective at identifying new pheromone components in sea lamprey (Li et al., 2015; 2013a; 2013b; 2014; 2012; Li et al., 2002), and may be useful for identifying compounds in other aquatic species that are hypothesized to use pheromones (Breithaupt and Thiel, 2010; Buchinger et al., 2014; Døving et al., 1980; Liley, 1982).

CONTROL IMPLICATIONS

The multiple environmental, physiological, and social factors that influence pheromone mediated behaviors in sea lamprey must be understood in order to integrate pheromones into control techniques of invasive sea lamprey in the Laurentian Great Lakes (Johnson et al., 2013; Li et al., 2007). Currently, removal of adult sea lamprey from tributaries of the Great Lakes is conducted mainly with barrier integrated traps (low-head barrier dams consisting of traps placed at the dam-face, typically in the corners). Control of juvenile larvae is primarily conducted by integrating barriers (to prevent re-introduction to upper tributaries) with lampricide treatments. The lampricide consists of two main compounds: TFM (3-trifluoromethyl- 4'-nitrophenol), and Bayluscide (2', 5-dichloro-4'- nitrosalicylanilide) which selectively kill larval lamprey (Christie and Goddard, 2003; Smith and Tibbles, 1980). The Great Lakes Fishery Commission (GLFC), established by treaty between the U.S. and Canada in 1954, contracted the U.S. Fish and Wildlife Service (USFWS), Fisheries and Oceans Canada (DFO), and the U.S. Geological Survey (USGS) as agents to operate these control techniques. Since that time, the current population in the upper three Great Lakes (Michigan, Huron, Superior) has been reduced to 10% of the historical abundance during the peak of the infestation (Commission, 2011). However, relaxing control efforts for even a short time period (1 year) results in an immediate and disastrous bounce-back of sea lamprey populations (Christie and Goddard, 2003). Control of sea lamprey in the Great Lakes is, and will continue to be, an ongoing effort to protect the estimated $7 billion/year fishery. Taken together, the data presented in this dissertation characterize new pheromone components and factors that influence the behavioral activity of these compounds in

the field, which in turn may be integrated into current control techniques and contribute to the reduction of the remaining estimated 350,000 adult sea lamprey in the Great Lakes.

Management-scale research is currently underway to examine whether pheromones can be integrated into the control program. Recent field tests using 3kPZS as a trap lure for invasive sea lamprey showed the application of 3kPZS (10^{-12} M) into barrier integrated traps alternated among eight Great Lakes tributaries over a 3 year period increased trapping efficiency (averaging a 10% increase across years) compared to unbaited traps (Johnson et al., 2013). However, the capture rates of 3kPZS baited traps remained variable, often resulting in minimal differences in capture rates, if any, compared unbaited traps in certain systems at certain times (Johnson et al., 2013). These inconsistencies were hypothesized to be caused by an additional suite of uncontrollable factors that exist in management-scale contexts which influence pheromone mediated behavior in sea lamprey (Johnson and Li, 2010). Many fishes including sea lamprey rely on a hierarchy of environmental, physical, and social information to make behavioral decisions for the ultimate goal of synchronizing reproduction (Binder and McDonald, 2008; Binder et al., 2010; Brant et al., 2015; Døving et al., 1980; Johnson and Li, 2010; Kemp et al., 2011; Moore et al., 2012; Quinn and Adams, 1996), and this dissertation targets these factors.

Both Chapter 1 and 2 of this dissertation unveil new factors that influence behavioral responses of sea lamprey to 3kPZS, each of which are important to consider in management-scale scenarios. In Chapter 1, the overriding effect of 3kPZS increased upstream movement of migrating sea lamprey during otherwise less active conditions (cold stream temperatures, < 15 °C). Given these results, upstream application of 3kPZS during colder stream temperatures in the springtime may increase the proportion of upstream migrating sea lamprey in a specific tributary (Brant et al., 2015), and therefore increase trap encounter rates a respective barrier integrated

traps. Over time, repeated 3kPZS treatments in specific tributaries may re-distribute and consolidate larval populations for targeted lampricide treatments that are more effective and economical. In Chapter 2, field tests showed female sea lamprey began to shift their preference for 3kPZS from a general upstream response to a more proximal directional response as they approach ovulation. 3kPZS baited traps that are not placed at the base of a barrier, but rather remain distributed throughout a migratory stretch of stream, may be effective at catching female sea lamprey at this time (*i.e.* during later June migration, or when females are less than 3 days from ovulation). A 3kPZS-baited trap that is placed along a migratory route may falsely indicate a 3kPZS activated sub-channel to migrating females, and therefore yield increased trap encounter and entry rates as females begin to direct their orientation towards the 3kPZS source.

Pheromone-baited traps placed at barriers during the spawning season must out-compete natural nests, presenting a new obstacle that must be contended with. Areas below barrier-integrated trapping sites often consist of suitable sea lamprey spawning habitat with adequate flow, substrate, and cover (Manion and Hanson, 1980). 3kPZS alone may not provide enough information to increase 3kPZS-baited trap catches of ovulated females during the mating season (Johnson et al., 2015a) because females are intercepted with spawning opportunities before arriving at the traps. Recent management-scale field tests comparing 3kPZS with the whole male pheromone (spermiated male washings; SMW) indicated that SMW baited traps are more effective compared to 3kPZS baited traps primarily in streams with low population density and minimum spawning habitat (Johnson et al., 2015a). However, 3kPZS or SMW baited traps did not often differ in their catch rates compared to one-another or unbaited traps in heavily infested streams with higher nest densities (Johnson et al., 2015a). These results are not surprising given the results of previous studies. 3kPZS is the main component released from males (Brant et al.,

2013; Li et al., 2002; Siefkes et al., 2003), and remains in high concentrations downstream from spawning grounds (Xi et al., 2011). Applying 3kPZS alone to barrier integrated traps would be expected to induce upstream movement towards the 3kPZS source (Johnson et al., 2009), yet become ineffective within proximity of the 3kPZS-activated trap due to background pheromone noise and spawning activity near the barrier. Traps baited with SMW would also be expected to remain ineffective in increasing trap catches in heavily infested scenarios. The application of SMW into an artificial nest during field studies often induces an arrestant-like behavior in ovulated females within the source (Johnson et al., 2009; Li et al., 2013b; Siefkes et al., 2005). Therefore ovulated females that are exposed to a SMW-activated trap would be expected to arrest and begin to establish nests around the immediate trapping area leaving fewer females to encounter the trap mouth. A potential solution to this problem is to apply a mixture of components released from mature males that provides enough information to a female to induce a search behavior for the source (*i.e*. increase trap encounter and entry rates), while remaining incomplete enough from the natural mixture of SMW as to not induce an arrestant or nesting behavior in females.

In Chapter 3, the new component DkPES is described as a minor proximity pheromone that, when combined with 3kPZS, increased the entry of mature females into the nest compared to a nest with 3kPZS alone. DkPES is one of several additional minor components that may complete the chemical message of the male pheromone enough to induce a directional preference for a trap mouth, yet still lack additional components/mixtures/stimuli that induce arrestment on a nest. PAMS-24, and additional minor component described in Chapter 4, also increased female preference into a nest when combined with 3kPZS at the natural ratio compared to nest containing 3kPZS alone. Based on data presented in Chapter 3 and 4 and results from previous

management-scale field tests (Johnson et al., 2015a; Johnson et al., 2013), partially complete mixtures consisting of main component 3kPZS, and minor components DkPES and PAMS-24 at consistent ratios may remain effective as a trap lure compared to traps baited with 3kPZS alone or whole male odor (SMW) in heavily infested streams.

Finally, field data collected with mature males in Chapter 4 suggest that PAMS-24 may function as a partial territorial pheromone. The addition of PAMS-24 alone at high concentrations may deter males from entering areas that are not ideal for trapping or lampricide treatments. Traps that are activated with a partial mating pheromone consisting of 3kPZS and minor components such a DkPES and PAMS-24 at natural ratios may deter male intrusion into certain traps, while increasing female entry. Targeting females in the population will remove substantial reproductive potential from the population, as each female can produce over 100,000 eggs (Applegate, 1950). Understanding the multiple factors in the field that influence the way sea lamprey respond behaviorally to pheromones brings us closer to understanding how pheromones can be optimized for use in controlling sea lamprey in the Great Lakes, which will undoubtedly aid in removal of invasive sea lamprey and further protect the fishery (Commission, 2011).

APPENDIX

APPENDIX

CHAPTER A-1: FATTY ACIDS WITH STEREOCHEMISTRY FUNCTION AS A PHEROMONE IN SEA LAMPREY

ABSTRACT

We report the identification, olfactory sensitivity, and preliminary behavioral activity of a tetrahydrofuran diol fatty acid with stereochemistry in sea lamprey using a novel bioassay guided fractionation system. Stereoisomers 9,(12)-oxy-10,13-dihydroxystearic acid (**1a**) and 10,(13)-oxy-9,12-dihydroxystearic acid (**1b**) consisting as a mixture of (+)-**1a (973)**, (−)-**1a (971)**, (+)-**1b (974)**, and (−)-**1b (972)** were identified from behaviorally active fractions that were extracted from juvenile conspecific odor. Electrophysiological experiments yielded stereoisomers (+)-**1a (973)**, (−)-**1a (971)** to be detected by adult sea lamprey (threshold of detection of 10^{-11} molar [M]) while likely sharing an olfactory receptor. Stereoisomers (+)-**1b (974)**, and (−)-**1b (972)** were not detected. Preliminary field tests in a river that forms a natural Y-maze design showed pre-spawn migrating female sea lamprey to preferred to move into a branch of a stream activated with 973 ($5x10^{-13}$ M), yet spawning phase (ovulated) females preferred to enter a mixture of 971:973 (1:1, $5x10^{-13}$ M:$5x10^{-13}$ M). Field tests suggest that stereochemistry may influence pheromone perception in sea lamprey, a previously undescribed phenomena in a vertebrate. Further behavioral studies are required to determine the stereo-specificity and behavioral function of these stereoisomers. We concluded that the bioassay guided fractionation system presented here is efficient at identifying new pheromones.

INTRODUCTION

Pheromones, or unique chemical signals released by a species that influence the physiology or behavior of other members of the same species (Karlson and Lüscher, 1959), are hypothesized to function in organisms across the animal kingdom. However identifying a new pheromone and further characterizing its function among a species is rare, and often requires integrated research in analytical and theoretical chemistry, behavioral ecology, electrophysiology, and behavior. Pheromones have been implicated to modulate critical life history events in many freshwater fishes (Liley, 1982; Liley and Stacey, 1983; Sorensen, 1992; Stacey and Cardwell, 1995; Stacey et al., 1996), yet new compounds of these pheromones are rarely identified. To date, the identified fish pheromones are bile acid derivatives (Li et al., 2002; Sorensen et al., 2005), prostaglandins (Sorensen et al., 1988; Sorensen et al., 1989), and amino acids (Yambe et al., 2006), which possess diverse structures with a hydrophilic functional group and are relatively stable in aquatic environments.

The sea lamprey (*Petromyzon marinus*) is a jawless vertebrate that relies in part on pheromones to modulate migration and mating (Bjerselius et al., 2000; Li et al., 2002). Unlike salmon which track the odor of natal streams learned early in life, migratory adult sea lamprey are attracted to odorants of conspecific larvae (Teeter, 1980), supporting the theory that a larval odor influences selection of spawning streams by migratory adults (a phenomena first recognized when trapping records indicated that migratory adults prefer streams with high densities of larvae (Moore and Schleen, 1980)). A tagging study further supported the idea that sea lamprey do not travel home to natal streams for reproduction, implicating an innately discerned attractant (Bergstedt and Seelye, 1995). Several compounds have been identified from the odor of conspecific larvae, and

shown to induce some behavioral activity in the laboratory (Sorensen et al., 2005), but these behaviors have not been replicated in the field (Meckley et al., 2012). Therefore, we hypothesized that larval sea lamprey residing in natal streams release a component as migratory pheromone to guide sexually immature adults to suitable streams.

Here we develop a novel Bioassay-guided fractionation system of extracted larval wash-water, which resulted in the isolation of an active component, consisting of two constitutional isomers. These compounds, identified here as 9,(12)-oxy-10,13-dihydroxystearic acid (**1a**) and 10,(13)-oxy-9,12-dihydroxystearic acid (**1b**), were each found to be an enantiomeric mixture, resulting in compounds (+)-**1a (973)**, (−)-**1a (971)**, (+)-**1b (974)**, and (−)-**1b (972)**. Herein, we report the bioassay-guided isolation, structure elucidation, enantiomeric separation, olfactory activities, and preliminary field study of components and mixtures of these compounds. Our data suggests fatty acids with stereochemistry function as pheromones in an aquatic vertebrate, a novel molecular template as a chemical signal.

RESULTS

Field bioassay guided fractionation yielded behaviorally active components

Chromatography on silica gel of larval washings eluted with gradient chloroform and methanol gave nine fractions (1–9), which were applied to EOG assays (Figure A-1-1a), field studies, and high performance liquid chromatography-mass spectrometry analysis. The raw odor of larval sea lamprey is known to elicit a strong preference response in migrating adult conspecifics (Bjerselius et al., 2000; Wagner et al., 2009). We therefore used extracted larval odor as a positive control in all field tests. Following the fractionation of raw larval odor, we combined fractions into four pools (Figure A-1-1b). We first aimed to examine the behavioral activity, if any, induced by these four pools. Using a 250 m-long section of the Upper Ocqueoc River (Figure A-1-2), we discovered that all for pools combined replicated the response seen from the raw larval extract in drawing significant numbers of migrating sea lamprey into the activated sub-channel (Table A-1-1). Additionally, Pool 3 replicated the response seen in all four pools combined (Table A-1-1a). We combined the nine fractions in Pool 3 into three separate sub-pools (Sup-pool 3.1 = FR24, 25, 26, Sub-pool 3.2 = FR27, 28, 29, 30, and Sub-pool 3.3 = FR31 and 32) for field testing in the 2011 migratory season. Behavioral trials yielded Sub-pool 3.1 as best replicating the response seen from larval extract control trials (Table A-1-1b). Followed by bioassay guidance, the components in Sub-pool 3.1 were analyzed by mass spectrometry and compared with known compounds. We confirmed the presence of compound **1** (*m/z* 329, Figure A-1-3), petromyzonin (*m/z* 308) (Li et al., 2013a), and petromyroxol (*m/z* 273) (Li et al., 2014) in Sub-pool 3.1.

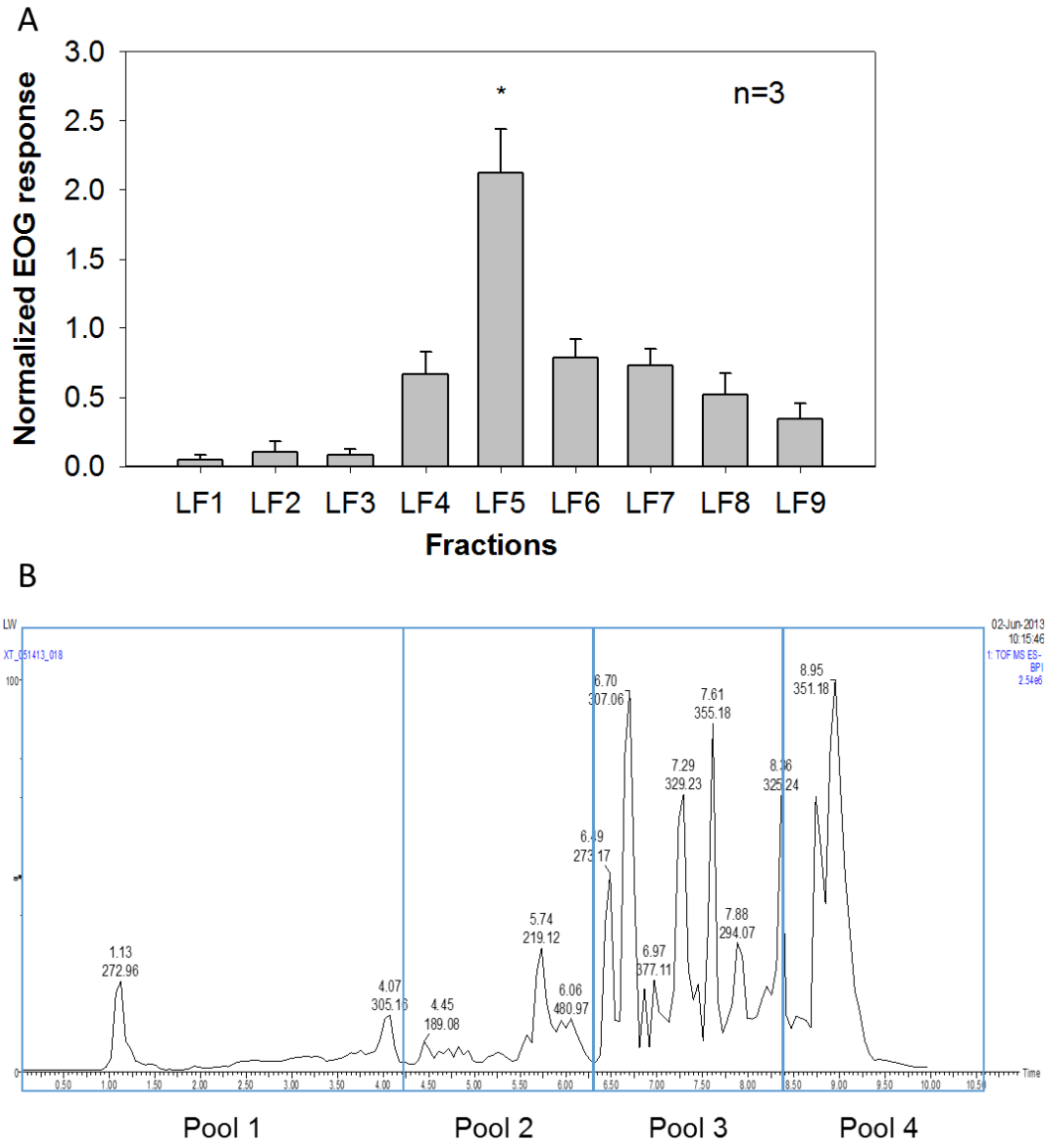

Figure A-1-1. Bioassay guided fractionation pinpointed active compounds. (**A**) Olfactory responses to larval fractions F1-F9 measured by EOG. (**B**) Mass spectra of pools 1 to 4 of larval sea lamprey fractions, components of 1 (*m/z* 329), petromyzonin (*m/z* 308), and petromyroxols (*m/z* 273) are occurring in pool 3.

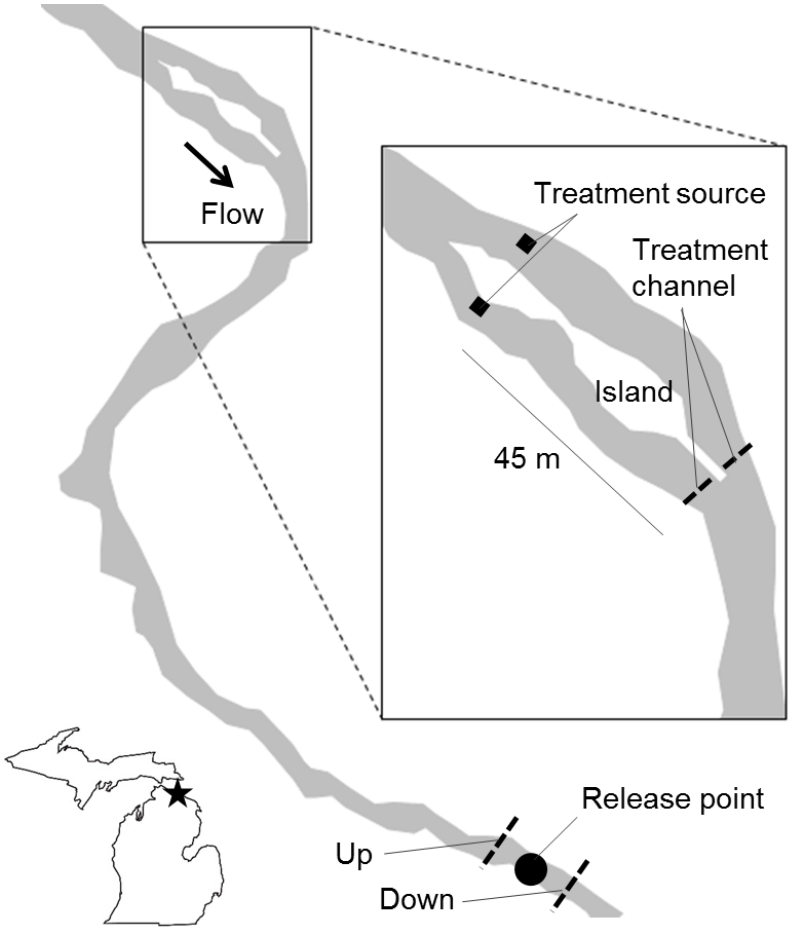

Figure A-1-2. Schematic of the field site in a 250 m-long section of the Upper Ocqueoc River, Millersburg, MI, USA, used for behavioral testing of component 1. The downstream release point is shown, along with the *Up* and *Down* passive integrated transponder (PIT) antennas used to monitor subjects moving upstream or downstream, respectively, after release. The upper 45 m of the section is naturally bifurcated by an island. Proportions of subjects entering each sub-channel was monitored by respective *Treatment channel* PIT antennas. The proportion of subjects entering the treatment source was then monitored by 1 m^2 *Treatment source* antennas, where treatments were administered into the stream.

Table A-1-1. Responses of migratory sea lamprey to (a) fraction pools (1-4) and (b) sub-pools (3.1 -3.3) from larval odor.

2010

Treatment	Trials	Released	Up	Treatment channel	Treatment nest
Vehicle	8	157	***69%*** (109) A	***47%*** (52) A	***15%*** (8) AD
Larval Extract	4	80	***75%*** (60) AB	***82%*** (49) B	***51%*** (25) BC
Pools 1-4	5	100	***58%*** (58) A	***74%*** (43) BC	***63%*** (27) B
Pool 1	4	80	***85%*** (68) B	***51%*** (35) A	***9%*** (3) D
Pool 2	4	80	***64%*** (51) A	***33%*** (17) A	***12%*** (2) AD
Pool 3	15	300	***64%*** (192) A	***67%*** (129) C	***26%*** (34) A
Pool 4	4	79	***81%*** (64) AB	***61%*** (39) AC	***31%*** (12) AC
		X^2	27.94	45.28	48.54
		df	6	6	6
		P-value	< 0.001	< 0.001	< 0.001

2011

Treatment	Trials	Released	Up	Treatment channel	Treatment nest
Vehicle	5	99	***42%*** (42) A	***43%*** (18) A	***28%*** (5) A
Larval Extract	5	100	***72%*** (72) B	***89%*** (64) B	***61%*** (39) B
Sub-pool 3.1	6	124	***52%*** (64) A	***64%*** (41) C	***24%*** (10) A
Sub-pool 3.2	4	79	***56%*** (44) A	***45%*** (20) A	***15%*** (3) A
Sub-pool 3.3	2	40	***53%*** (21) A	***33%*** (7) A	***43%*** (3) A
		X^2	19.19	44.82	22.98
		df	4	4	4
		P-value	< 0.001	< 0.001	< 0.001

Trials were conducted over the 2010 and 2011 migratory seasons in the Upper Ocqueoc River, Millersburg, MI. Treatments *Vehicle* (50% MeOH) and *Larval Extract* (extracted raw larval odor applied to one sub-channel at a volume achieving 5E-14 M PADS benchmark and vehicle applied to the adjacent sub-channel) were controls. In 2010, pools in Figure A-1-1b were tested. In 2011, three sub-pools from active *Pool 3* were tested, including; *Sub-pool 3.1* (Fraction 5), *Sub-pool 3.2* (Fraction 6), and *Sub-pool 3.3* (Fraction 7). Responses include *Up* (percentage moving 200 m upstream to the confluence of the two sub-channels), *Treatment channel* (of the subjects that moved up, percentage that enter the sub-channel containing each treatment), and *Treatment nest* (of the subjects that entered the treatment channel, the percentage that swam through a 1 m^2 nest fixed to the center of the stream bed at the upstream end of the respective sub-channel). Each response across treatments was evaluated with logistic regression. Values that share a letter within each response variable are not significantly different ($\alpha = 0.05$).

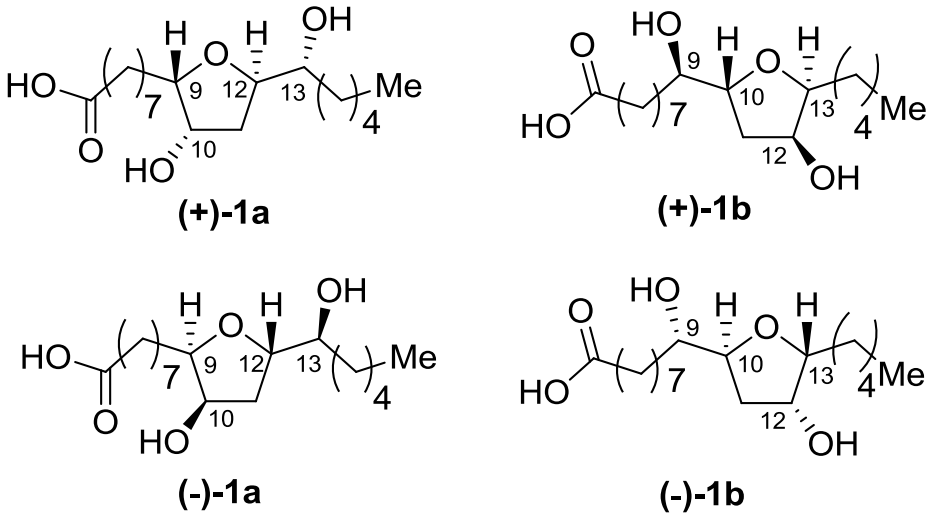

Figure A-1-3. Stereochemical structures of (−)-1a (971), (−)-1b (972), (+)-1a (973), and (+)-1b (974).

Bioassay-guided isolation, identification, and quantitative analysis of active components

See Supplemental Information – Supplemental Results for details of chemical isolation, identification, and quantitation of the components of compound **1** (Figure A-1-3). The details of these results were prepared by Dr. Ke Li, Michigan State University.

Compounds (−)- and (+)-1a elicit olfactory responses

See Supplemental Information – Supplemental Results for details of olfactory sensitivity to the components of compound **1** (Figure A-1-3). The details of these results were prepared by Dr. Mar Huertas, Michigan State University.

Synthesized compounds (−)-1a (971) and (+)-1a (973) show stereo-selective and temporally variable behavioral activity in pre-spawn sea lamprey

Synthesized components (−)-**1b** (**972**) and (+)-**1b** (**974**) did not yield EOG responses and so were not tested in the field. Components **(−)-1a (971)** and **(+)-1a (973)** showed stereo-selective behavioral activity that varied between *Early* and *Late* migratory season. Upstream movement of pre-spawn migrating female sea lamprey towards treatments remained high (67-93%) during field trials across the season (Table A-1-2). However, pre-spawn migrating sea lamprey did not show a bias towards compound (−)-**1a (**denoted as **971** during field tests**)** activated sub-channels or the treatment source at any point, while subjects began to show a preferential bias towards the sub-channel activated with (+)-**1a (**denoted as **973** during field tests**)** applied at 5E-13 M specifically during *Late* (June) migration, at which time 70% entered the **973** sub-channel (Logistic regression: $X^2_4 = 24.2$, $P < 0.001$). Larval extract controls drew significant numbers of subjects into the treatment channel and treatment source during both *Early* (May, Table A-1-2a) and *Late* (June, Table A-1-2b) migration, serving as an effective positive control.

Table A-1-2. Responses of migratory female sea lamprey to new tetrahydrofuran diol compounds 971 and 973 during *Early* – May and *Late* – June 2013 and 2014 migratory seasons.

***Early* migration - May**

Treatment	Trials	Released	Down	Up	Treatment channel	Treatment source
Vehicle	19	378	***13%*** (48) A	***84%*** (318) A	***49%*** (156) A	***20%*** (31) A
Larval Extract	8	160	***5%*** (8) B	***89%*** (142) A	***60%*** (85) BC	***42%*** (36) B
971+973	8	160	***17%*** (24) AC	***71%*** (114) B	***52%*** (59) AC	***17%*** (10) A
971	4	80	***28%*** (22) C	***71%*** (57) B	***37%*** (21) A	***19%*** (4) A
973	4	80	***8%*** (6) AB	***93%*** (74) A	***59%*** (44) AC	***27%***(12)AB
		X^2	27.74	30.61	11.58	17.18
		df	4	4	4	4
		P-value	< 0.001	< 0.001	0.021	0.002

***Late* migration - June**

Treatment	Trials	Released	Down	Up	Treatment channel	Treatment source
Vehicle	17	331	***18%*** (60) A	***69%*** (228) A	***45%*** (103) A	***26%*** (27) A
Larval Extract	7	140	***11%*** (16) B	***86%*** (120) B	***63%*** (76) BC	***61%*** (46) B
971+973	8	160	***27%*** (43) C	***67%*** (107) A	***64%*** (68) BC	***13%*** (9) C
971	8	160	***14%*** (22) A	***67%*** (107) A	***54%*** (58) AB	***19%*** (11) A
973	7	139	***12%*** (17) A	***72%*** (100) A	***70%*** (70) C	***27%*** (19) A
		X^2	19.99	27.91	24.20	41.48
		df	4	4	4	4
		P-value	0.001	< 0.001	< 0.001	< 0.001

Trials were conducted over the 2013 and 2014 migratory season in the Upper Ocqueoc River, Millersburg, MI, USA. Treatments included vehicle controls (50% MeOH), Larval Extract controls (Larval extract applied to one sub-channel and vehicle applied to the adjacent sub-channel, 971:973 (1:1, 1E-12 molar M total), 971 alone (5E-13 M), and 973 alone (5E-13 M). Responses include Down (percentage moving downstream of the release cages and not coming back up during the 2.5 hour-long trial), Up (percentage moving 200 m upstream to the confluence of the two sub-channels), Treatment channel (percentage that enter the sub-channel containing each treatment), and Treatment source (of the subjects that entered the treatment channel, the percentage that then passed through a 1 m^2 source antenna fixed to the center of the stream bed at the upstream end of the respective sub-channel). Responses were evaluated with a generalized linear model and binomial distribution. Responses that share a letter are not significantly different ($\alpha = 0.05$).

Compounds (−)-1a (971) and (+)-1a (973) draw and retain mature females at the source

Over 2500 migratory sea lamprey were released into the stream during field trials for Table A-1-2, each with a uniquely identified PIT tag. Old subjects from past trials that were remaining in the system were also continuously monitored with our PIT antenna system along with the new test subjects released each night. We monitored old subject responses to (−)-**1a (971)** and (+)-**1a (973)**, and found them to spent significantly more time on a mixture of the two compounds compared to each compound alone, yet not as much time as was spent of 3kPZS nests (Figure A-1-4). A sample of subjects (n = 4) that were currently sitting on the (−)-**1a (971)** and (+)-**1a (973)** mixture (1:1) were hand-grabbed from the center of the nest at night for histological confirmation of maturation. All samples where fully ovulated according to published studies (Yorke and McMillan, 1980). While it was unfortunate that only four specimens could be hand-grabbed in the field during this time, we determined that four subjects had spent an average of 15 ± 1.6 days at large in the river system before returning to the site.

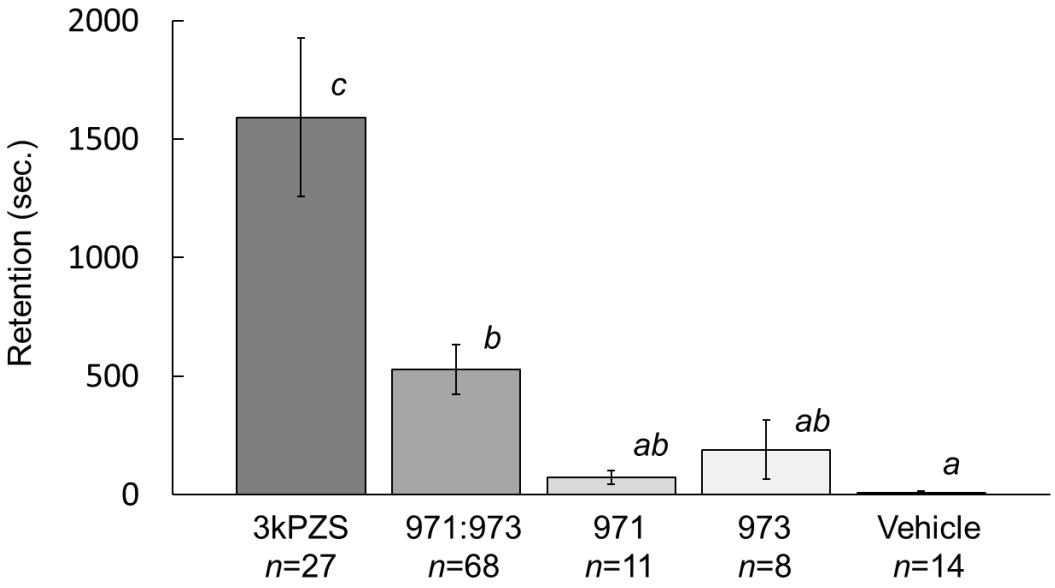

Figure A-1-4. Mean ± 1 SEM retention (*sec.*) of female sea lamprey inside artificial nests (within 0.5 m of the source) while pheromone treatments were administered. Treatments included 3kPZS at $5x10^{-13}$ M, a mixture of (−)-**1a** (**971**, $5x10^{-13}$ M) and (+)-**1a** (**973**, $5x10^{-13}$ M) at a 1:1 ratio (totaling $1x10^{-12}$ M), **971** alone ($5x10^{-13}$ M), **973** alone ($5x10^{-13}$ M), and vehicle control (methanol). Trials were conducted in the Ocqueoc River, Millersburg, MI (Figure A1-2). Columns with different lower-case letters are significantly different, ANOVA and post hoc Tukey HSD: ($F_{4, 123} = 15.58$, $P = < 0.0001$).

DISCUSSION

Our findings collectively reveal fascinating novel molecular diversity of sea lamprey metabolites and illuminate a new molecular template; dyhydroxylated THF fatty acid. Field assays suggest that this compound shows stereo-selective behavioral function in sea lamprey. In our previous investigation, a pair of diastereomers possessing dyhydroxylated THF moiety, petromyroxols (Li et al., 2015) and *iso*-petromyroxols (Li et al., 2015), have been characterized from water conditioned with larvae of sea lamprey. However, their role in modulating behavior of sea lamprey are still ambiguous due to a lack of access to materials required for further investigation of biological activity.

From the view of chemical structure and nature, THF-diol fatty acids represent a new pheromone template that is unlike hormones, which often function as known pheromones excreted as reproductive by-products (Shorey, 2013). The derivatives of THF-diols are a class of typical amphipathic molecules containing both hydrophobic (THF moeity) and hydrophilic (secondary alcohol and carboxylic acid) functional groups, indicating a water insoluble property and tendency to form a monolayer over an aqueous sub-phase resulting in fast dispersion on the air and water interface. Therefore, THF-diols fatty acids possess the potential to act as pheromones that can travel over long distances on water. On the other hand, each individual compound has four chiral centers and two variable elongation chains, providing chemical diversity, possessing 16 possible absolute configurations, and tens of homologs. Combining the diversity of homologs and spatial configurations, THF-diol derivatives can provide hundreds of messenger combinations to deliver complex information.

Results of field assays suggest that component **1** shows stereo-selective behavioral activity in sea lamprey, which also appears context-dependent on a temporal scale of their migration. Only stereoisomer (+)-**1a (973)** induced preference like responses in migrating female sea lamprey, which only occurred in the late half of the migratory season (June). Since migrating female subjects showed a preference to the whole larval extract throughout the entire migratory season, we cannot yet conclude whether the new stereoisomer (+)-**1a (973)** is a migratory pheromone, or a chemical cue that allows aggregation around spawning grounds at the onset of the reproductive season. Data suggest that additional components or unique mixtures exist in raw larval extract that function as the main migratory pheromone, and these additional compounds are currently being targeted for elucidation by our group.

Stereo-selectivity of compounds that function as semiochemicals have been described in insects (Chapman et al., 1978; Coracini et al., 2001; Klun et al., 1973), but to our knowledge, has not been described in a vertebrate. Synthesized stereoisomer (+)-**1a (973)** alone appears to be attractive in pre-spawn females and a mixture of (−)-**1a (971)** and (+)-**1a (973)** appears to induce behavioral activity in spawning phase females. Exact mixtures of all stereoisomers may be required for maximum attraction. In Lepidoptera, a single geometrical isomer has been shown to be weekly attractive in receivers to the source, while only a re-construction of all geometrical isomers yielded a maximum attractiveness (Klun et al., 1973). We may be observing a similar phenomenon in sea lamprey. Unfortunately we did not know the ratio of each isomer during field trials, and so combined mixtures in a 1:1 ratio for field testing. Further investigation is required to determine the exact ratios of each isomer, and determine whether ratio influences behavioral activity towards these components.

METHODS

Animals

All procedures involving sea lampreys were conducted in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, and were approved by the Michigan State University Institutional Animal Use and Care Committee (Animal use form number: 03/11-053-00). Larval sea lampreys were captured in tributaries of the Laurentian Great Lakes by the US Fish and Wildlife Service and Fisheries and Oceans Canada according to approved scientific collection permits from those government agencies, transported to the US Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, USA, and held in flow through raceways (1.83 m wide by 0.61 m deep by 15.24 m long). Adult sea lampreys used for olfactory functional studies were collected in December and January by commercial fishing agencies from Lake Huron and transported to the US Geological Survey Hammond Bay Biological Station where they were then delivered to Michigan State University, East Lansing, Michigan, USA. Sea lampreys were held in flow through tanks (254 L) supplied with well-water chilled to roughly 8 °C. Electro-olfactogram recording was conducted in February 2013 at Michigan State University.

Equipment and Materials

1D and 2D NMR spectra of petromyroxol were recorded on an Agilent 500 MHz spectrometer. Mass spectra were performed on a TQ-S TOF LC mass spectrometer (Waters Corporation, Milford, Massachusetts, USA). Silica gel (70-230 and 230-400 mesh, Merck, Darmstadt, Germany), RP-18 reverse-phase silica gel (Merck), and Sephadex LH-20 (Merck)

were used for open column chromatography. TLC was conducted on glass plates pre-coated with GF254 silica gel (Merck). Spots were first visualized under UV light at 254 nm and then stained by spraying an acidic methanol solution of 5% anisaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA).

Extraction of larval sea lamprey-conditioned water

Every four to five days over the course of *ca.* five months, water conditioned with sea lamprey larvae (ca. 4000 L for each cycle) was passed through four beds each containing 1 kg of Amberlite XAD 7HP resin at a rate of roughly 200 mL/min/bed (800 mL/min, total) and subsequently eluted with methanol (4 L). The organic solvents were removed under reduced pressure at 40 °C by a rotary evaporator. The resulting residue was pooled and stored at -80 °C until further processing. The total extract (*ca.* 42 L and containing a large amount of water) was thawed and concentrated by lyophilization. This residue was suspended in methanol and successively filtered through 2 μm filter paper. The filtrate was concentrated again under reduced pressure at 40 ^{o}C to yield 2.0 g of a dark residue.

Field Study

Details of the experimental site are consistent with those described by Brant at al. (Brant et al., 2015), with slight modification. Briefly, a section of the Upper Ocqueoc River in Millersburg, MI, USA, was used to monitor the movements of migrating female sea lamprey to pheromone treatments. A two-choice system was constructed using two sub-channels that were naturally bifurcated by an island. Downstream, 205 m from the confluence of the two sub-channels, two release cages were placed in the center of the stream. Release cages were constructed of mesh aluminum (0.25 m^3) equipped with sliding release doors. Transecting copper wire passive integrated transponder (PIT) antennas were placed 5 m upstream and downstream

of release cages, and at the confluence of each sub-channel. Approximately 45 m upstream of each sub-channel, a 1 m^2 PIT antenna frame was laid flat on the stream bed. Treatments were administered into the center of the 1 m^2 frame. Antennas were tuned to a detection sensitivity of roughly 0.3 m from the frame edges. Scan frequencies of each antenna were programmed to three scans/sec. Data for each trial were uploaded each day using a hand-held Meazura model MEZ1000 personal digital assistant (Aceeca International Limited, Christchurch, New Zealand).

PIT tagging

Passive integrated transponder (PIT) tagging procedures followed Johnson et al.(Johnson et al., 2009). Briefly, a 23 mm-long half duplex PIT tag (Oregon RFID, Portland, Oregon, USA) was surgically implanted into each experimental animal through a 3 – 4 mm lateral incision in the mid-abdominal region. These procedures typically took less than 30 seconds/animal. PIT tagged animals were immediately transferred into aerated holding tanks with a constant flow of Lake Huron water for up to 24 hours, until they were stocked into stream release cages. Tagged individuals were monitored throughout the day for signs of distress or mortality.

Details of trials

In behavioral field trials, our primary objective was to test whether individual compounds that showed EOG activity were also capable of influencing movements of migrating sea lamprey similarly to behaviors elicited from larval extract controls. For *Early migration*, trails were conducted 8 – 29 May 2013 and 27 May – 09 June 2014. For *Late migration*, trials were conducted 30 May – 23 June 2013 and 12 – 21 June 2014. Treatments included: 1) *Vehicle* control methanol administered into both sub-channels, 2) *Larval Extract*, or extracted raw larval odor, into one sub-channel while vehicle control is applied to the adjacent sub-channel, 3) a 1:1 mixture of (−)-**1a (971)** and (+)-**1a (973)** ($5x10^{-13}$ M:$5x10^{-13}$ M) applied to one sub-channel and

vehicle control applied to the adjacent sub-channel, 4) (−)-**1a (971)** alone at $5x10^{-13}$ M into one sub-channel and vehicle into the adjacent sub-channel, and 5) (+)-**1a (973)** alone at $5x10^{-13}$ M into one sub-channel and vehicle into the adjacent sub-channel. Treatment and vehicle sub-channels were alternated each trial. Test treatments were diluted with 25 L of river water in large mixing bins on shore. Bins were kept consistent for each test treatment, and rinsed in the stream several times before each new trial, to reduce the potential for contamination. Each treatment solution was then pumped from bins into the stream at the center of each 1 m^2 PIT antenna (*Treatment source*) at a rate of 167 mL/min (± 5 mL/min) over the span of 2 hours using peristaltic pumps (Cole-Parmer). Stream temperatures were recorded at the start of and end of each trial. Stream discharge was estimated every three days, or after every precipitation event, at a fixed location in the stream using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, Maryland, U.S.A.) to determine the amount of treatment stock solution to apply to the stream and maintain consistent concentrations across trials.

Trials were conducted at night when migrating sea lamprey are most active(Stier and Kynard, 1986). Trials were 2.5 hours long. In the first half-hour of each trial the test treatments were administered to the stream while test subjects remained in the release cage. At the start of the following 2 hours, test subjects were released and their movements were monitored with PIT antennas until the trial ended. No animals were recovered from the stream after a trial, and unique PIT tag IDs prevented any pseudoreplication from occurring. Up to two trials were conducted each night, depending upon animal availability. The early trial was conducted from sundown (roughly 2030h) to roughly 2300h and the last trial was from roughly 2330h – 0200h (exact trial times were dependent upon when sundown occurred). Twenty PIT-tagged subjects were released per trial. Subjects were removed from holding tanks at HBBS and transported to

their respective stream release cage at the release point between 0300h and 0500h the night prior to experimentation. Subjects were then allowed an acclimation period in the stream for 15+ hours.

Statistical analysis of PIT data

Trials were divided into *Early* and *Late* migration for statistical analyses because during our preliminary three years of behavioral testing in the stream we began noticing that behaviors of migrating immature sea lamprey showed some discontinuity as the mating season approached. We theorized that female sea lamprey adapted to adjust their response threshold to pheromones as the migratory season shifts into the mating season to insure their synchrony of reproduction with males. For this reason, we specifically began examining our response variables to treatment compounds within the first half of the migratory season (*Early migration* - May) versus the latter half (*Late migration* - June) to better examine whether any changes in preference to treatments occurs. Logistic regression with a binomial distribution was examined for each response variable, followed by a post-hoc *t*-test for comparisons across treatments within each response variable, using R-software (R version 2.11.1, Vienna, Austria). No signs of nonlinearities or over dispersion were observed in the models. All behavioral statistics reported are two-tailed analyses ($\alpha = 0.05$). Four main binary response variables were examined during field trials: (1) the distribution of subjects that swam downstream from the release cage and did not come back up past release cages during the trial (*Down:* 0 = did not hit on *Down* antenna, 1 = hit on *Down* antenna only) (2) the distribution of subjects that swam upstream from release cages and did not move back down (*Up:* 0 = did not hit on *Up* antenna, 1 = hit on *Up* antenna and continued upstream), (3) of those subjects that hit on the *Up* antenna, the distribution that entered the sub-channel containing the test treatment (*Treatment channel:* 0 = entered vehicle channel, 1 =

entered treatment channel), and (4) of those subjects that entered the treatment channel, the distribution that entered within 0.5 m of the treatment source (Treatment source: 0 = missed treatment source antenna, 1 = entered source). Since methanol (vehicle) was administered to both nests during *Vehicle* control trials, one nest was randomly assigned for statistical purposes to be the "treatment" nest. The "treatment" nest was randomly chosen to be the right nest, and alternated every trial to follow the same pattern of other actual odorant treatments.

SUPPLEMENTAL RESULTS

After successive chromatographic purification, Component **1** was isolated as a colorless oil and displayed a pseudo-molecular-ion at *m/z* 329 $[M - H]^-$ in the negative Q-TOF-ESI mass spectrum. The molecular formula $C_{18}H_{34}O_5$ was determined by HRESIMS at *m/z* 329.2332 $[M - H]^-$ (calcd. for $C_{18}H_{33}O_5$, 329.2328, ΔmDa 0.4, PPM 1.2), indicating two degrees of unsaturation. The doubling signals in ^{13}C NMR spectrum suggested that component **1** contained two constitutional isomers **1a** and **1b**. The ^1H NMR spectrum (Supplementary Fig. 3) showed signals for four oxygenated methines and one methyl were observed, as well as resonances for few alphatic methylene protons. The ^{13}C NMR and HSQC experiments revealed the presence of 18 carbon signals corresponding to four oxygenated methines at δ_C 82.7, 80.2, 74.1, and 73.4, one quaternary carbon at δ_C 178.1, one methyl at δ_C 14.0, and remaining 12 alphatic methylenes. The presence of tetrahydrofuran ring was indicated by ^1H–^1H COSY and HMBC correlations (Supplementary Fig. 6-8)(Li et al., 2014). The ^1H NMR, ^{13}C NMR chemical shifts and ^1H–^1H COSY correlations allowed us to assign two hydroxyl groups. The remaining carbon signals were attributable to a carboxyl group and two aliphatic chains. To analyze the chain length of component **1**, a GC-MS analysis was apply to the trimethylsilyl ether derivative. A peak with retention time of 20.6 was observed in the mass chromatogram. The ESI mass spectrum for the peak contained prominent fragment ions at m/z 173 and 317. Component **1** could be identified as 9,(12)-oxy-10,13-dihydroxystearic acid (**1a**) and 10,(13)-oxy-9,12-dihydroxystearic acid (**1b**), which, after comparing ^1H, ^{13}C-NMR and GC-MS data, was consistent with the literature (Li et al., 2014; Markaverich et al., 2002).

The relative configuration of component **1** was established through analysis of the NOESY correlations and coupling constants as well as comparison of NMR data with literature values(Capon et al., 1998; Li et al., 2014; Warren et al., 1980). NOESY correlations between H-9/H-10, H-9/H-11a, H-10/H-11a, H-13/H-11a, and H-12/H-11b placed protons H-9, H-10, H-11a, and H-13 on the same face of the tetrahydrofuran ring, and H-12 and H-11b on the another face of the tetrahydrofuran ring. Meanwhile, component **1** displayed a high degree of similarity with the analogue possessing H-9/H-12 *trans* relationship and apparent differences with H-9/H-12 *cis* analogue by comparison of the chemical shifts for oxygen-bearing methylenes in ^{1}H and ^{13}C NMR spectra (Capon et al., 1998; Warren et al., 1980), which was in good agreement with the deduction by NOESY correlations. This conclusion was confirmed by the performance of a single-crystal X-ray diffraction of (+)-**1a**. Therefore, the relative configurations of compounds **1a** and **1b** can be assigned as 9*R**, 10*R**, 12*S**, 13*S**-**1a** and 9*S**, 10*S**, 12*R**, 13*R**-**1b**. To determine the absolute configurations of compounds **1a** and **1b**, authentic standards of enantiomers were prepared using a highly stereoselective synthesis. The compounds (+)-**1a** and (−)-**1a** showed identical mass. GC-MS spectra and ^{1}H and ^{13}C NMR spectra, and compounds (+)-**1b** and (−)-**1b** are consistent. The MS, NMR, and GC-MS spectra of natural occurring component **1** are consistent with those of a mixture of (+)-**1a**, (−)-**1a**, (+)-**1b**, and (−)-**1b**. The absolute configurations of the stereogenic centers of these analogues were established using Mosher ester methodology (Hoye et al., 2007b; Ohtani et al., 1991). Treatment of (–)-**1a** with (*R*)-(–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*R*)-MTPA-Cl] in pyridine yielded a mixture of 6,9-bis-(*S*)-Mosher ester and 9-mono-(*S*)-Mosher esters of (–)-**1a**. Similar treatment of (−)-**1b** with (*S*)-(+)-MTPA-Cl afforded an analogous mixture of the bis- and mono-(*R*)-Mosher esters. Each of these mixtures was separated by semi-preparative HPLC to obtain the mono-(*S*)- and the mono-(*R*)-Mosher ester, respectively.

The ratios among these analogues in samples of extracted larval wash-water were estimated using chiral UPLC-MS/MS. A characteristic fragment $[M + H – 2H_2O]^+$ (*m/z* 295.0) was chosen as the daughter ion to increase the specificity (Supplementary Table 2). The stereoisomers were separated in 35 min using a polysaccharide based column (Chiralpak AD-H) containing 5 μm particles and an isocratic elution of ethanol:*n*-hexane:formic acid (85:15:0.1, v/v). The stereoisomers **(+)-1b, (+)-1a, (−)-1b, and (−)-1a** appeared at retention time of 17.0-18.0, 18.0-19.5, 30.5-31.5, and 31.5-33.0 min, respectively. Once separated, the enantiomers were subjected to APCI (positive ion mode) and collision-induced dissociation tandem mass spectrometry (CID-MS/MS) using the MRM mode. The quantitation method of stereoisomers was validated based on the FDA guidelines. The percentage of compounds **(+)-1b, (+)-1a, (−)-1b, and (−)-1a** in natural occurring component **1** was estimated as 14.4%, 18.8%, 28.6%, and 38.2%, respectively. The concentration of **(+)-1b, (+)-1a, (−)-1b, and (−)-1a** has been evaluated from larval sea lamprey wash water and shows a relevant concentration to function as a pheromone.

Compounds (−)- and (+)-1a elicit olfactory responses

Both (−)-**1a** and (+)-**1a** showed strong olfactory responses during EOG analyses, with a threshold of detection of 10^{-11}M . The stereoisomers (−)-**1b** and (+)-**1b** didn't elicit a concentration response typical of an odorant receptor interaction. The low responses during EOGs for (−)-**1b** and (+)-**1b**, if any, were assumed to be non-specific. The detection threshold for petromyzonin was 10^{-11} M (n = 6) (Li et al., 2013a). This threshold was only one order of magnitude higher than the threshold (10^{-12} M) for 3kPZS, a known lamprey pheromone (Li et al., 2002).

Responses to binary mixtures of stereoisomers at 10^{-7} M were not higher than the responses of single stereoisomers, indicating a lack of synergism in the mixtures. However mixtures of either (−)-**1b** and (+)-**1b** stereoisomers with (+)-**1a** show lower responses than individual tests of (+)-**1a**, indicating a possible antagonist effect.

Cross adaptation studies indicated that EOG adapted responses of (−)-**1a** and (+)-**1a** were not significantly different to their self-adapted control (Supplementary Fig. 11), suggesting that olfactory responses for this two compounds are likely mediated by the same olfactory receptor. Cross adaptation analysis for (−)-**1b** and (+)-**1b** were inviable since they did not elicit olfactory responses to allow comparison between stereoisomers.

Competition curves showed a different affinity for the receptor between (−)-**1a** and (+)-**1a**. Stereoisomer (−)-**1a** can displace 50% of (+)-**1a** 10^{-7} M olfactory responses (effective concentration 50, EC_{50}) at 5.8 10^{-8} M, whereas stereoisomer (+)-**1a** can displace 50% of (−)-**1a** 10^{-7} M olfactory response at 4.1 10^{-9}M. The fact that (+)-**1a** can displace (−)-**1a** at a lower concentration than (−)-**1a** to (+)-**1a** implies that (+)-**1a** is a stronger competitor for the receptor binding site.

PUBLICATION LIST

Publication List A-1. Recent publications during the characterization of sea lamprey pheromones.

Brant CO, Li K, Johnson NS, Li W (2015) A pheromone outweighs temperature in influencing migration of sea lamprey. *Royal Society Open Science* 2 (5), 150009.

Li K, Brant CO, Bussy U, Pinnamaneni H, Patel H, Hoye TR, Li W (2015) iso-Petromyroxols: Novel dihydroxylated tetrahydrofuran enantiomers from sea Lamprey (*Petromyzon marinus*). *Molecules* 20 (3), 5215-5222

Li K, Huertas M, Brant CO, Chung-Davidson Y-W, Bussy U, Hoye TR, Li W (2015) (+)-and (−)-petromyroxols: antipodal tetrahydrofurandiols from larval sea lamprey (*Petromyzon marinus* L.) that eicit enantioselective olfactory responses. *Organic Letters* 17(2): 286-289.

Li K, Brant CO, Huertas M, Li W. (2013). Petromyzonin, a hexahydrophenanthrene sulfate isolated from the larval sea lamprey (*Petromyzon marinus* L.). *Organic Letters* 15:5924-5927.

Brant CO, Chung-Davidson Y-W, Li K, Scott AM, Li W. (2013). Biosynthesis and release of pheromonal bile salts in mature male sea lamprey. *BMC Biochemistry* 14:30.

Choi J, Jeon S, Johnson NS, Brant CO, Li W. (2013). Odor-conditioned rheotaxis of the sea lamprey: modeling, analysis and validation. *Bioinspiration & Biomimetics* 8(4):046011.

Li K, Brant CO, Siefkes MJ, Kruckman HG, Li W. (2013). Characterization of a novel bile alcohol sulfate released by sexually mature male sea lamprey (*Petromyzon marinus*). *Plos ONE* 8(7): e68157.

Li K, Siefkes MJ, Brant CO, Li W. (2012). Isolation and identification of petromyzestrosterol, a polyhydroxysteroid from sexually mature male sea lamprey (*Petromyzon marinus* L.). *Steroids* 77(7):806-810.

Li K, Wang H, Brant CO, Ahn SC, Li W. (2011). Multiplex quantification of lamprey specific bile acid derivatives in environmental water using UHPLC-MS/MS. *Journal of Chromatography B* 879(32):3879-3886.

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Johnson NS, Yun S-S, Thompson H, Brant CO, Li W. (2009). A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps. *PNAS* 106:1021-1026.

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Binder TR, McDonald DG, 2007. Is there a role for vision in the behaviour of sea lampreys (*Petromyzon marinus*) during their upstream spawning migration? Canadian Journal of Fisheries and Aquatic Sciences 64:1403-1412.

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