EVOLUTION OF SEA LAMPREY MATING PHEROMONES

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Fisheries and Wildlife ─ Doctor of Philosophy

2015

ABSTRACT

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Male sea lamprey (*Petromyzon marinus*) release a multi-component mating pheromone, partially comprised of 3-keto petromyzonol sulfate (3kPZS), which initiates upstream movement and close-proximity spawning behaviors in ovulated females. While the chemistry, function, and potential management application of the sea lamprey mating pheromone are relatively well-studied, the evolution the sea lamprey mating pheromone system remains poorly understood. In this dissertation, I present inter- and intra-species comparisons of pheromone communication that provide insights into the evolution of pheromone communication in the sea lamprey. In Chapter 1, I provide a broad review of the chemical cues and pheromones used by the sea lamprey during reproduction, including overviews on of the sea lamprey olfactory system, chemical cues and pheromones, and potential applications to population management. In Chapter 2, I present a phylogenetic comparison of 3kPZS communication across lampreys, including male release of 3kPZS across eleven lamprey species, representing six of ten genera and two of three families, olfactory sensitivity to 3kPZS in four species native to the Laurentian Great Lakes, and sexual responses to 3kPZS in four species native to the Laurentian Great Lakes. The results indicate either independent gains or a single gain and single loss of 3kPZS communication, and represent a rare macroevolutionary investigation of a vertebrate pheromone. In Chapter 3, I provide evidence for partial overlap of the multi-component male mating pheromone across lampreys. Chemical profiling of sexually mature males from eleven species of lamprey indicated the chemical profiles of males are partially shared across species. Behavioral

assays conducted with four species sympatric in the Laurentian Great Lakes indicated asymmetric female responses to heterospecific odors, where sea lamprey were attracted to male odors from all species tested but other species generally preferred only the odor of conspecifics. Electro-olfactogram recordings from sea lamprey indicated that although sea lamprey were attracted to male odors from all species, at least some of the compounds that elicited olfactory responses were different in conspecific male odors compared to heterospecific male odors. In Chapter 4, I present evidence that small male sea lamprey exhibit increased relative pheromone signaling driven by a larger pheromone-producing organ, and possible up-regulation of pheromone synthesis. Furthermore, female choice experiments in a natural environment indicate increased pheromone release in small males likely results in higher access to mates. Taken together, this dissertation provides a rare evolutionary perspective on vertebrate pheromones and describes the species-specificity of lamprey pheromones which are being considered as tools to control and restore lamprey populations throughout the world.

To my grandpa George Whitfield, whose thirst for knowledge will forever be an inspiration.

ACKNOWLEDGMENTS

I would like to thank my advisors Dr. Nicholas Johnson and Dr. Weiming Li and members of my graduate committee, Dr. Janette Boughman and Dr. Heather Eisthen for their support and guidance during my graduate studies. The staff of the U.S. Geological Survey Hammond Bay Biological Station provided a welcoming second home, despite my frequent return during the student-free season. The U.S. Fish and Wildlife Service Marquette Biological Station, Drs. Cindy Baker and Rex Strange, Michael Hays, and Liang Jia assisted with collecting lampreys. I am thankful to the Li laboratory for support, especially Drs. Ke Li, Ugo Bussy, and Huiyong Wang for performing chemical analyses, and Dr. Mar Huertas for her support with electro-olfactogram recordings. I am grateful for the hard work, suggestions, friendship, and patience of many colleagues and field assistants, including Cory Brant, Trevor Meckley, Skye Fissette, Ethan Buchinger, Christopher Dean, Ed Benzer, Jeff Yaklin, Phillip Ganz, Hugh McMath, Elizabeth Stieber, Chad Brewer, Tyler Jackson, Andrew Doubek, Tyler Bruning, Melissa Pomranke, Brooklyn Idalski, Scott Couture, Michelle VanCompernolle, Julia Krohn, Mike Siemiantkowski, and Joseph Reidy. I appreciate the support and funding provided by the Great Lakes Fishery Commission. Lastly, I am thankful for the incredible support from my entire family, and especially grateful to my wife Elizabeth for endless love, encouragement, and patience, and for spending more time with lamprey than she would ever have expected or wanted.

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CHAPTER 1

CHEMICAL CUES AND PHEROMONES IN THE SEA LAMPREY (*PETROMYZON MARINUS*)

Buchinger TJ, MJ Siefkes, BS Zielinski, CO Brant, & W Li. 2015. Chemical cues and pheromones in the sea lamprey (*Petromyzon marinus*). *Frontiers in Zoology*. 12:32. doi: 10.1186/s12983-015-0126-9

ABSTRACT

Chemical cues and pheromones guide decisions in organisms throughout the animal kingdom. The neurobiology, function, and evolution of olfaction are particularly well described in insects, and resulting concepts have driven novel approaches to pest control. However, aside from several exceptions, the olfactory biology of vertebrates remains poorly understood. One exception is the sea lamprey (*Petromyzon marinus*), which relies heavily upon olfaction during reproduction. Here, we provide a broad review of the chemical cues and pheromones used by the sea lamprey during reproduction, including overviews of the sea lamprey olfactory system, chemical cues and pheromones, and potential applications to population management. The critical role of olfaction in mediating the sea lamprey life cycle is evident by a well-developed olfactory system. Sea lamprey use chemical cues and pheromones to identify productive spawning habitat, coordinate spawning behaviors, and avoid risk. Manipulation of olfactory biology offers opportunities for management of populations in the Laurentian Great Lakes, where the sea lamprey is a destructive invader. We suggest that the sea lamprey is a broadly useful organism with which to study vertebrate olfaction because of its simple but well-developed olfactory organ, the dominant role of olfaction in guiding behaviors during reproduction, and the direct implications for vertebrate pest management.

INTRODUCTION

Sensory input from conspecific odors guides decisions for organisms throughout the animal kingdom [1]. Early studies focused on insects, with the first behaviorally active conspecific odorant identified in the silkmoth (bombykol; *Bombyx mori*) [2]. Since then, behaviors mediated by conspecific odors have been described in crustaceans [3], fishes [4], reptiles and amphibians [5], birds [6], and mammals [7], including hypothesized functions associated with reproduction, foraging, conspecific recognition, and predator avoidance [1]. Detection of chemicals can be integrated into the decision making processes of organisms via adaptations in receivers (chemical cues) or both receivers and signalers (pheromones) [1]. While much of our understanding of chemical communication is based upon research on insects, the olfactory biology and ecology of some vertebrates is increasingly understood. In particular, chemical communication in some fishes, including the sea lamprey (*Petromyzon marinus*), is relatively well described [4].

The sea lamprey is a basal vertebrate with a complex life history comprised of distinct larval, juvenile, and adult stages. Larval sea lamprey burrow into stream sediment and filter feed on organic material and microorganisms. Following a larval stage of 3–5 years, sea lamprey undergo a drastic metamorphosis into the juvenile stage, migrate downstream into the Atlantic Ocean or a Laurentian Great Lake, and parasitize on large fish for approximately 1.5 years. Finally, adult sea lamprey migrate into streams during the spring, where a male will construct a nest and later be joined by one or more females, spawn intermittently for a number of days, and die [8]. Olfaction is hypothesized to influence sea lamprey behavior throughout the larval, juvenile, and adult stages [9 – 11], but only during the terminal adult phase has the role of conspecific odors been evaluated.

Adult sea lamprey use conspecific odors to identify suitable spawning habitat, search for mates, and avoid risk (Figure 1-1) [11, 12]. Migrating adults select spawning tributaries based upon the odor of previous years' larvae that reside in the stream. Upon arrival at the spawning grounds, gravid females move upstream and locate spawning nests using the odor of sexually mature males [11, 13]. Alarm substances are hypothesized to guide adults away from areas where larval or adult populations have high mortality [12, 14, and 15].

Here, we summarize the current understanding of the chemical cues and pheromones used by the sea lamprey during reproduction. Previous reviews of sea lamprey olfaction focus primarily on applications to fisheries management in the Laurentian Great Lakes [16 – 20]. Our objective is to develop a broader perspective on sea lamprey olfaction, spanning from odorants up to evolutionary patterns. We provide overviews on the neurobiology of olfaction, the ecology and evolution of chemical cues and pheromones, and potential applications to population management. We suggest that the simple but well-developed olfactory organ, dominant role of olfaction in guiding behaviors during reproduction, and direct implications for vertebrate pest management position the sea lamprey as a useful organism with which to study vertebrate olfaction.

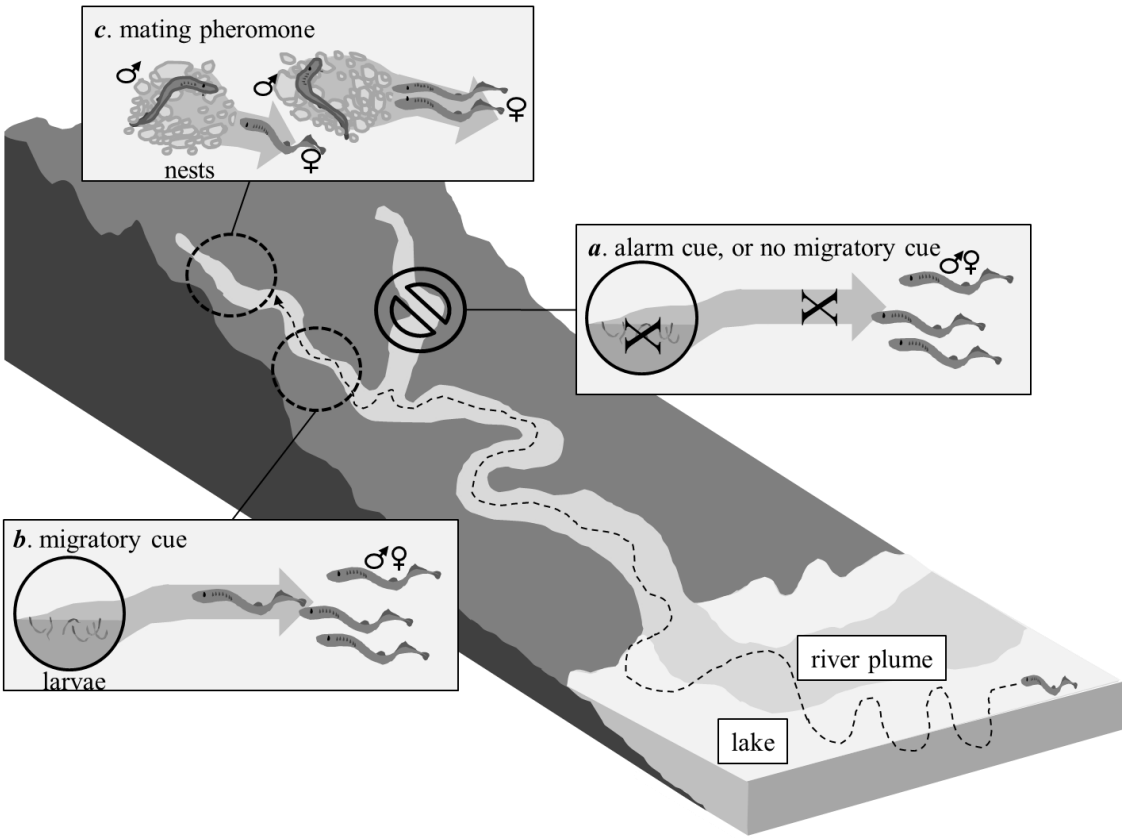


Figure 1-1. Schematic illustrating the functions of migratory cues, alarm cues, and mating pheromones during reproduction in sea lamprey. *a*- Fewer migrating sea lamprey enter rivers or tributaries with injured or decaying conspecifics, or lacking larval populations; *b*- Migrating sea lamprey enter streams activated with larval odor; *c*- upon reaching sexual maturation males release a mating pheromone that draws females to spawning nests, and initiate nest building and spawning behaviors.

THE OLFACTORY SYSTEM

Anatomy of the olfactory apparatus

A critical role of olfaction in mediating the sea lamprey life cycle is evident by a well-developed olfactory system (Figure1-2) [21]. The large olfactory organ in sea lamprey [22] is comprised of a peripheral olfactory organ containing both a main olfactory epithelium and tubular diverticula known as the accessory olfactory organ [23]. Early in their life cycle, prior to leaving the spawning nest, sea lamprey possess functional olfactory sensory neurons that are stimulated by conspecific odorants [9, 24]. During the metamorphosis from larvae into adults, the peripheral olfactory organ enlarges while changing from an epithelial lined tube to a nasal sac with lamellar folds [25]. The accessory olfactory organ also exhibits the formation of diverticula surrounded by blood vessels and nerve bundles [23, 25].

Olfactory sensory neurons

Olfactory sensory neurons intercept odor information using dendrites that extend into the mucus of the peripheral olfactory organ. The olfactory sensory neurons are ciliated [24, 26, 27], but exhibit distinct morphotypes similar to ciliated, microvillous, and crypt olfactory sensory neurons documented in teleost fishes [28 – 30]. Neuron morphotypes differ in the distance the dendrite extends into the olfactory mucus surrounding the olfactory epithelium, and may relay information from different classes of odorants (feeding, risk, reproduction) [31]. Dendrites of sensory neurons express olfactory receptors, which mark the beginning of signal transduction.

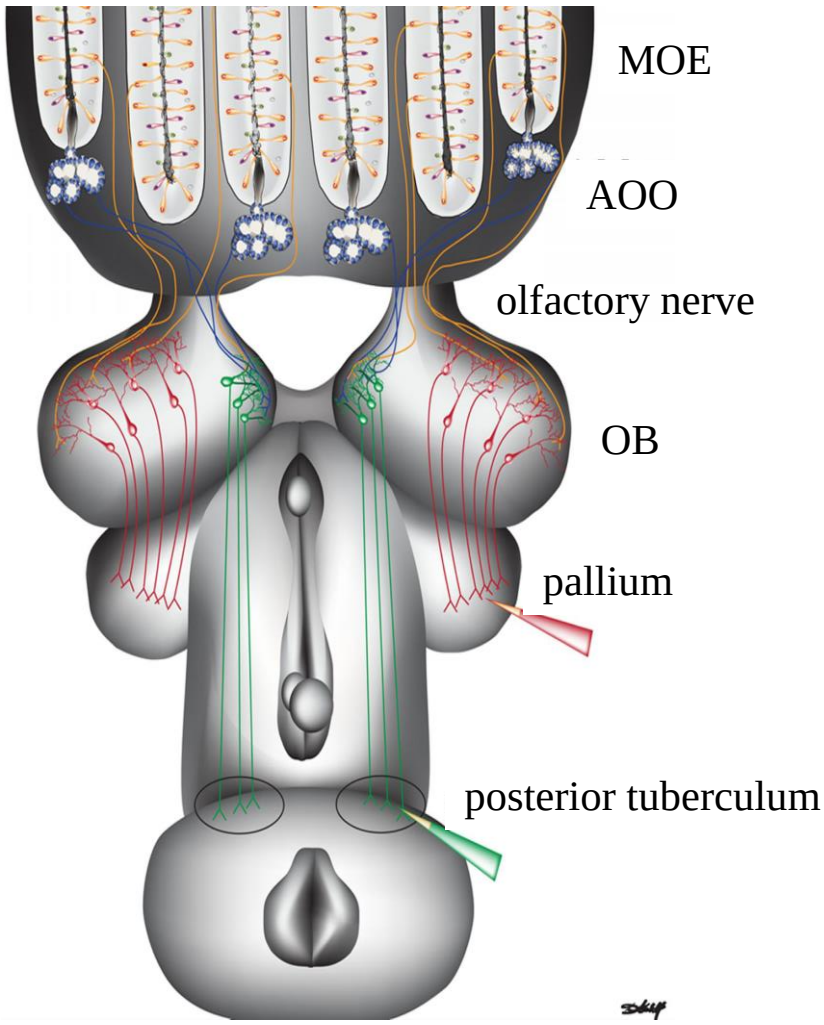


Figure1-2. Schematic illustrating the hypothesized circuitry of the sea lamprey olfactory system. MOE = main olfactory epithelium; AOO = accessory olfactory organ; OB = olfactory bulb. Neuronal projections are based upon Ren et al., [27] and Derjean et al., [40]. The medial region of the olfactory bulb receives inputs from the accessory olfactory organ (AOO – blue) as well as sparse inputs from the main olfactory epithelium (MOE – orange). The medial projection neurons (green) project their axons to the posterior tuberculum (PT). The non-medial region of the olfactory bulb receives inputs from the main olfactory epithelium and the non-medial projection neurons (red) project their axons to the pallium. Red and green pipettes indicate location Green et al., [39] injected biocytin to retrogradely label projection neurons in the olfactory bulb (OB).This image is previously published in Green et al., [39].

Signal transduction

Olfactory receptors on the olfactory sensory neurons bind odorants and trigger a signal transduction cascade. Receptor proteins of olfactory sensory neurons are members of the seven-transmembrane G-protein coupled receptor superfamily [32]. In the sea lamprey, chemosensory receptor genes include at least 27 olfactory receptor (OR)-type genes, 28 trace amino acid receptors (TAAR)-type and 4 vomeronasal type one (V1R)-type genes [33 – 35]. Signal transduction following odorant binding is not yet fully described in lamprey. On the main olfactory epithelium, the binding of an odorant by an OR likely triggers a second messenger cascade via the G-protein G, which stimulates an increase in cyclic adenosine monophosphate (cAMP), opening the cyclic nucleotide gated ion channel [36 – 38]. The G-proteins in the olfactory sensory neurons on the accessory olfactory organ, however, have not been identified. The signal transduction cascade leads to depolarization of the neuron and propagation of the signal to the olfactory bulb [36, 37].

Olfactory bulb

Spatially distinct regions of the olfactory bulb receive and integrate olfactory signals from the main and accessory olfactory systems (Figure 1-2). Olfactory sensory neuron axons projecting from the main olfactory epithelium and the accessory olfactory organ merge into the olfactory nerve. Axons from the accessory olfactory organ project to the medial region of the olfactory bulb, while axons from olfactory sensory neurons in main olfactory epithelium extend to all other regions [27]. After entering the olfactory bulb, olfactory sensory neuron axons pass through the olfactory nerve layer and form synaptic contacts in spherical regions of neuropil known as glomeruli. Glomeruli in all regions, except the medial region, express immunoreactive

G, a G protein thought to be necessary for odorant reception [38]. Within the glomeruli, axon terminals of the olfactory sensory neurons synapse with the dendritic endings of output neurons (projection neurons). Projection neurons in the medial olfactory bulb are spatially isolated from projection neurons in non-medial olfactory bulb regions and have larger cell bodies than non-medial projection neurons [39]. Lastly, projection neurons interact with interneurons and signal higher olfactory processing centers in the brain.

Projections to the brain and behavioral output

Projections from the medial olfactory bulb to higher olfactory processing centers create a direct link between olfactory input and locomotory output [40]. Odorant and electrical stimulation of the medial region of the olfactory bulb stimulates locomotion [40]. The medial region of the olfactory bulb projects to the posterior tuberculum, which is located in the ventral diencephalon and projects to the mesencephalic locomotor region. The mesencephalic locomotor region initiates locomotion by acting on brainstem pre-motor neurons, the reticulospinal neurons, which directly activate the locomotor networks of the spinal cord [41]. Hence, a direct pathway from a sensory neuron up to the spinal cord likely triggers odor-driven behavioral responses in sea lamprey [40].

In contrast, projections from non-medial regions may be involved in the integration of odor information. Non-medial output neurons project to several forebrain structures, including the lateral pallium. The somata of non-medial projection neurons are below the glomerular neuropil and are smaller than the somata of the medial projection neurons [39]. The receptive fields of the projection neurons in the medial and non-medial output pathways do not overlap [39]. Local field potential recordings from the non-medial olfactory bulb region have shown that

the dorsal olfactory bulb territory responds to lamprey sex pheromones and migratory pheromones while lateral olfactory bulb recordings exhibit responses to basic amino acids, and not to pheromones [42]. The hypothesized olfactory-locomotor link created by the accessory olfactory organ may be modulated by the detection and discrimination of specific odorants in the main olfactory organ.

Olfaction in lamprey compared to other vertebrates

The lamprey olfactory system exhibits many features common among vertebrates, along with several characteristics that are unique. Most organisms, including lamprey, possess similar adaptations for detecting and processing olfactory stimuli [43]. For example, the cellular and molecular mechanisms of olfaction appear to be generally shared among vertebrates, including lamprey; olfactory receptors are G protein-coupled receptors and similar transduction pathways carry olfactory signals [43]. A detailed report of the similarities and differences between the olfactory systems of lamprey and other vertebrates is outside the scope of this review, but several examples of unique features of the lamprey olfactory system should be noted. First, lamprey, along with hagfish, are unique in having a single nostril. Notably, although lamprey have a single nostril, the olfactory organ is comprised of two regions and a paired olfactory nerve. While the functional implications of having a single nostril are unclear, having two nostrils has clear adaptive significance in some fish [44]. Second, the accessory olfactory organ of lamprey appears to be a unique adaptation [35], and offers an interesting comparison to the vomeronasal organ in tetrapods. Taken together, the common and unique features of the sea lamprey olfactory system offers a useful system to answer fundamental questions of vertebrate olfaction.

CHEMICAL CUEING AND PHEROMONE COMMUNICATION IN SEA LAMPREY

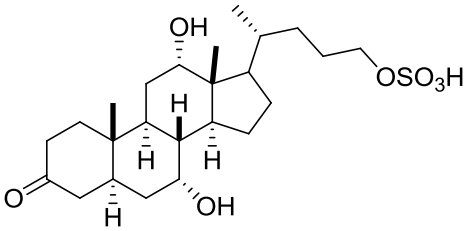
Reproductive behaviors in sea lamprey rely largely upon olfactory input [45, 46]. In contrast to many anadromous fishes (e.g. salmonids), sea lamprey do not exhibit natal homing behaviors [47, 48]. Rather, sea lamprey evaluate the suitability of a stream based on the presence of larval populations [11, 49]. Migratory sea lamprey are acutely tuned to the larval odor (*migratory cue*); putative components are detected at low concentrations [50] and larval odor elicits behavioral responses at the concentrations produced by a single larvae diluted several thousand fold [51]. Once sea lamprey arrive at the spawning grounds, final sexual maturation is partially triggered by conspecific odors [52, 53]. Upon complete sexual maturation, mate search and spawning are guided by the odors of the opposite sex [11]. Although males are attracted to the odor of females [11, 54], the odors released by males (*male mating pheromone*) and subsequent behavioral responses in females are better understood. The male odor appears multi-functional, mediating upstream movement behaviors [55] and proximate nest construction and spawning synchronization behaviors [56]. Finally, throughout the spawning season, sea lamprey are hypothesized to evaluate risk using conspecific and heterospecific semiochemicals (a*larm cue*) [12, 14, 15].

Migratory cues and mating pheromones

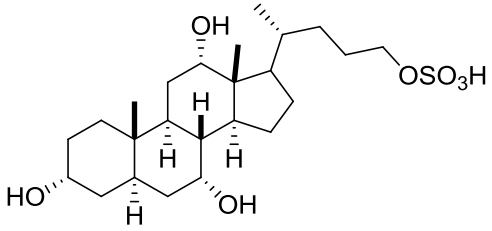
Identities

Bile acids and derivatives are implicated as components of the sea lamprey migratory cue and male mating pheromone (Figure 1-3) [19]. The olfactory epithelium of many fishes is sensitive to sex steroids, prostaglandins, amino acids, and bile acids [57]. Sex steroids and

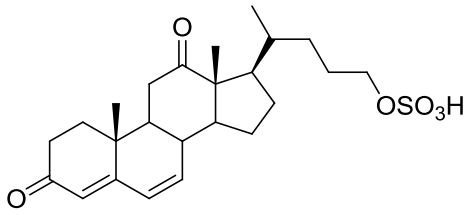
prostaglandins are commonly implicated as mating pheromones in teleosts [58]. Amino acids are likely used by anadromous salmonids during natal homing [59, 60], and as a mating pheromone in at least one species (Masu salmon, *Oncorhyncus masou*) [61]. Sea lamprey, however, only show sensitivity to a small number of amino acids and sex steroids [62]. In many species, including sea lamprey, conspecific-released bile acids evoke strong physiological responses in electro-olfactograms (EOG) [50, 63 – 65], thus implicating behavioral functions. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) combined with EOG screening and behavioral assays have continued to amass support for bile acids and related cholesterol derivatives as components of the male mating pheromone in sea lamprey (Fig. 3) [66].



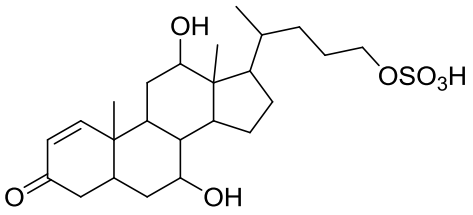
3-keto petromyzonol sulfate (3kPZS)



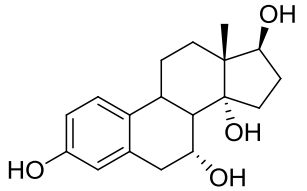
petromyzonol sulfate (PZS)



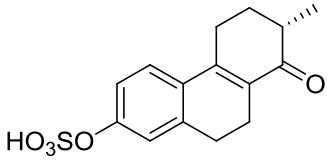
diketo petromyzonene sulfate (DkPES)



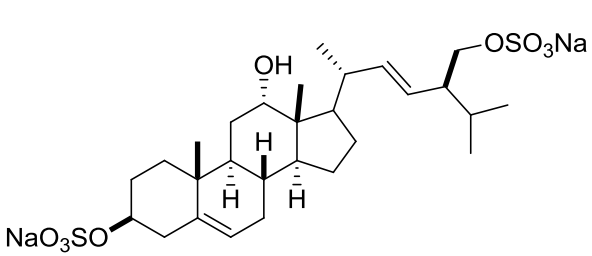
3-keto 1-ene petromyzonol sulfate



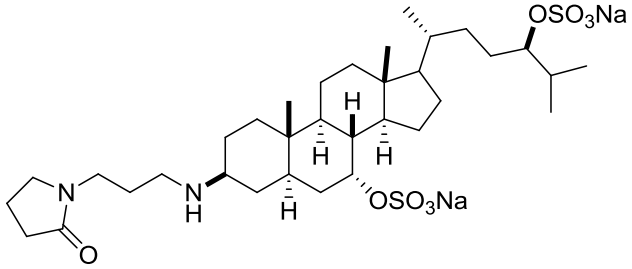
petromyzestrosterol



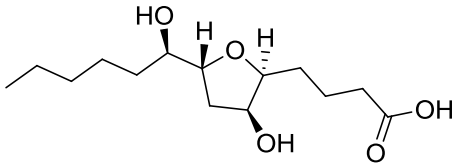
petromyzonin



petromyzosterol disulfate (PSDS)



petromyzonamine disulfate (PADS)



petromyroxol

Figure 1-3. Structures of molecules hypothesized to be behaviorally active pheromones in sea lamprey (*Petromyzon marinus*)

Research into the sea lamprey migratory cue provides support for the hypothesis that conspecific bile acids [50] guide spawning migrations of anadromous fishes [67]. Larvae excrete lamprey-specific bile acids [68] into the water at rates sufficient to create a detectable concentration in a river (~10 ng/h) [69, 70]. Three bile acids, petromyzonol sulfate (PZS), petromyzonamine disulfate (PADS), and petromyzosterol disulfate (PSDS) are released into the water [70], elicit strong electrophysiological responses from the olfactory epithelium [50, 65], and influence the behavior of migratory lamprey in laboratory mazes [65, 71]. While the mixture of PADS, PSDS, and PZS replicates the proximal preference elicited by larval odor in laboratory tests [65] and may influence search behavior at the junction of the lake and the river [72], the mixture does not replicate larval odor in eliciting upstream movement and stream channel preference in natural stream environments [73], suggesting crucial components of the migratory cue remain unidentified. Several additional components of larval metabolites have been identified and are potent odorants, but have not been evaluated in behavioral assays [74 – 76].

The first link between bile acids and reproduction was revealed by the discovery that a bile acid functions as a major component of the sea lamprey male mating pheromone [77]. The bile alcohol 3keto petromyzonol sulfate (3kPZS) is released at high rates by males (~0.5 mg/h) [78], detected with acute sensitivity and specificity [64], and elicits an attraction response in sexually mature females both in the laboratory [54 , 77] and in the field [54 , 55 , 77]. While robust behavioral responses in large-scale field tests confirm that 3kPZS is the major component of the male pheromone [55, 79], unknown components appear to be required to match the full suite of nesting and courtship behaviors elicited by the full male odor [56]. A bile acid structurally similar to 3kPZS but lacking in the C24 sulfate, 3 keto allocholic acid (3kACA) was hypothesized to function as an additional component [64, 80, 81], but has now been resolved

behaviorally inactive [56]. Notably, sea lamprey detect 3kACA with high sensitivity and specificity [64], and steroidogenesis in males is inhibited by exposure to 3kACA [53]. A 4 oxidized, unsaturated compound similar to 3kPZS elicited attraction in females [82]. Another bile acid 3, 12-diketo-4, 6-petromyzonene-24-sulfate (DkPES), is a potent male odorant that, when mixed with 3kPZS, increases the number of females that approach the source of 3kPZS [66]. An additional constituent of the male odor, petromyzestrosterol, elicits olfactory responses in EOG recordings but has not yet been tested in behavioral assays [66].

Sources and release

Sea lamprey possess unique mechanisms of synthesizing and excreting bile acids associated with chemical cues and pheromone. Larval sea lamprey regulate bile acids as do most vertebrates: synthesis in the liver, storage in the gall bladder, and secretion into the intestine via the bile duct. At this stage, putative migratory cue components, including the mating pheromone 3kPZS [83], are slowly released into the water via intestinal contents (~10 ng/larva/h) [69, 70, 83]. A drastic reduction both in expression of genes coding for bile acid biosynthetic enzymes in the liver [83] and in the concentration of bile acids in tissues follows the transformation of larvae into parasitic adults [69, 83]. Migratory adults likewise exhibit a down-regulation of hepatic synthesis of bile acids, but appear to regulate bile acid equilibrium through renal excretion [84]. Upon sexual maturation, males up-regulate expression of genes coding for enzymes involved in bile acid anabolism, yielding an increase in hepatic concentrations of PZS and 3kPZS [77 , 78 , 85]. The compounds are carried by the cardiovascular system to the gills, where PZS is hypothesized to be oxidized to 3kPZS, and released through glandular cells that develop at the final stages of maturation in males [78, 85]. Additional components of the male pheromone are

likely also released by the gills [56]. The cessation of feeding and atrophy of the intestine during reproduction may favor the renal system and gills as alternative mechanisms of bile acid equilibrium.

Behavioral ecology

The migratory cue informs migrating sea lamprey regarding potential offspring success and reduces the risk of selecting poor stream habitat. Following host detachment, sea lamprey are hypothesized to identify productive offspring habitat using a series of environmental cues [73]. Adult sea lamprey search for river plumes extending into the lake or ocean and display a preference for the general odor of stream water [45, 86]. Migrating adults enter rivers and tributaries that are activated with the odor of larvae, which is directly related to potential for future offspring success [51, 86, 87]. Release of bile acids hypothesized as components of the migratory cue is linked to larval feeding [69, 70]. Although the migratory cue appear to be comprised of a mixture of multiple known and unknown components [65, 73], the functional differences between components is unknown. The male mating pheromone mediates pre-spawning upstream migration [88] and sexual maturation in males and females [52], and spawning upstream movement [54] and a suite of spawning behaviors in females [56]. The response elicited depends upon the spatial, environmental, and physiological context. Pre-spawning upstream migration of males and females is reduced at low temperatures [89, 90], but maintained in the presence of 3kPZS [88]. Mature male odor facilitates sexual maturation of males and females [52]. Sexually mature females display strong odor-conditioned rheotaxis in response to male odor, primarily in response to 3kPZS [54 – 56]. Upon reaching the spawning nest, however, nest construction and gamete release in females is largely mediated by the

mixture of 3kPZS, DkPES, and unknown compounds [56, 66]. The mechanisms through which pheromone mixtures operate in sea lamprey remain unknown, but specific ratios appear to be important [66].

Evolution

The migratory cue appears to be an adaptation of stream-searching adults rather than a specialized signal released by larvae (Fig. 4). Natural selection likely maintains the strong preference for larval odor, where individuals choosing to spawn in streams with clear evidence of historical success realize higher fitness relative to individuals that chose streams at random or undertake high-cost and less effective evaluation of stream habitat via direct assessment [87]. Larvae presumably receive no direct benefit by releasing an attractive odor, thus attraction to larval odor is likely an adaptation of migratory adults [91]. The hypothesis that larval odor represents a cue rather than a signal is supported by the apparent non-specificity of release and response across lampreys [91 – 96].

The male mating pheromone is likely the result of a more complex evolutionary history. Many fish pheromone systems, including the sea lamprey migratory pheromone [91], appear to represent behavioral adaptations of the receiving fish [91, 97]. Evidence that the silver lamprey (*Ichthyomyzon unicuspis*) uses larval 3kPZS as a migratory cue rather than a male-released mating pheromone suggests female preference for 3kPZS may have originated as an adaptation of receiving fish [96]. The development of glandular cells involved in 3kPZS release [78] and the extremely high rate at which 3kPZS is released [77], however, suggest that male adaptation drove a transition of 3kPZS into a mating signal. Adding further complexity to the mating pheromone is the role of multiple components influencing multiple behaviors [56]. While 3kPZS

as a mating pheromone may have evolved through male manipulation of an existing female preference [96], the evolutionary processes driving male release and female preference for the remaining components of the male odor remain unknown.

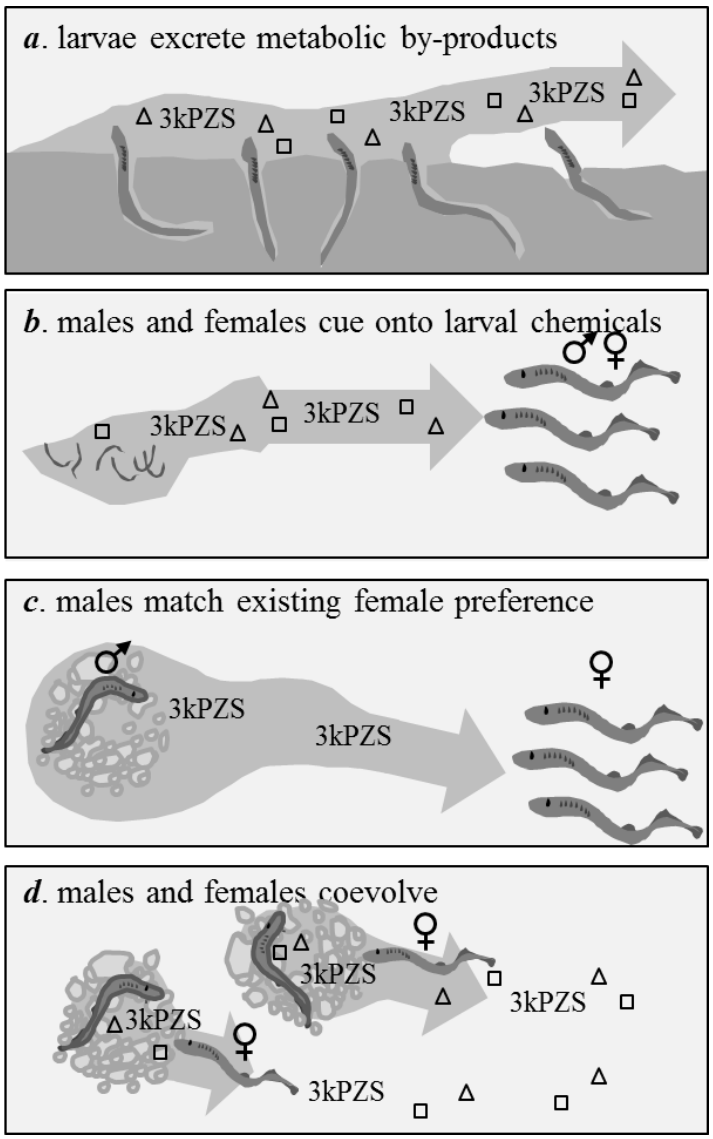


Figure 1-4. Schematic illustrating the hypothesized evolution of the chemical cueing and pheromone communication systems in sea lamprey. a larvae excrete 3-keto petromyzonol sulfate, or 3kPZS, and other chemicals as byproducts of metabolism; b migrating adults cue onto 3kPZS and other chemicals to locate habitat conducive to high-offspring survival; c males exploit the existing female preference for 3kPZS; d male release of 3kPZS continues to become exaggerated as a result of the fitness benefits associated with higher access to mates and female response to 3kPZS transitions from a non-targeted migratory response to a highly proximate spawning response.

Alarm cues

Identity

Pursuit of the identities of sea lamprey alarm cues is a recent endeavor, and, as such, the chemical structures remain unknown. In fact, despite many years of research on alarm cues in fish, only two alarm odorants have been identified; hypoxanthine-3-N-oxide, an alarm cue in various teleosts [98, 99], and glycosaminoglycan chondroitin, a recently discovered alarm cue in zebrafish (*Danio rerio*) [100]. Although the identity of the sea lamprey alarm cue is uncharacterized, the odor, or part of the odor and is stable past 96 h of aerobic decay [14]. Notably, commercial 2-phenylethylamine (PEA-HCL), a hypothesized predator cue used by rodents [101], elicits an anti-predator response in sea lamprey in the laboratory [15, 102]. Whether the chemically mediated risk assessment in sea lamprey shows parallels to teleost and other fishes remains unknown.

Sources and release

Sea lamprey alarm cues originate from conspecific tissues and bodily fluids of predators [14, 15, and 102]. Damaged and decayed tissues from larval and adult conspecifics elicit alarm responses [14]. Consistent with much of the literature on fish alarm cues, damaged skin elicits a stronger aversion response compared to whole skin [14]. In contrast to skin-released alarm cues of many fishes [103], the sea lamprey alarm cue appears to be distributed throughout the skin, organ tissue, and muscle [14]. The hypothesized predator cue PEA is released via urine of carnivorous mammals [101] and other unknown predator cues may be released via saliva [102].

Behavioral ecology

Alarm cues used by sea lamprey could indicate 1) a regional end of the spawning, 2) low offspring survival, or 3) risky spawning habitat [12]. Alarm cues are emitted by both larvae and adults [14], indicating the role of conspecific alarm cues likely spans across the proposed ecological functions. Sea lamprey are semelperous and die following a single reproductive season. Hence, the scent of dead lamprey may indicate the end of the reproductive season in a tributary. Alternatively, alarm cue could indicate low survival of larvae or adults due to poor quality habitat or high predation. Additional functions of alarm cues outside of reproduction are supported by observations of possible alarm responses to damaged conspecific tissue in larval sea lamprey [104]. Larvae also show olfactory sensitivity to odors of non-damaged conspecifics [9]. Whether responses to non-damaged or damaged conspecific odors are ecologically relevant, perhaps influencing settlement behavior, or developmental precursors to the responses during the adult phases remains unknown.

Evolution

Alarm cues, including sea those used by sea lamprey, are hypothesized to be the result of receiver specializations [103]. Natural selection likely favors an aversion to alarm cues in parallel to the attraction to larval odor, resulting in a multi-faceted mechanism to evaluate spawning habitat and optimize success during the single reproductive event. Notably, sea lamprey also exhibit alarm responses to alarm cue collected from closely related silver lamprey [14], and distantly related white sucker (*Catostomus commersonii*), but not Amazon sailfin catfish (*Pterygoplichthys pardalis*) [15]. Reproductive migrations of sea lamprey, silver lamprey, and white suckers overlap temporally and spatially, hence aversion to alarm cues of heterospecifics is ecologically relevant. However, whether the behaviorally active chemicals are

shared across species, or if sea lamprey have evolved to use different compounds released by heterospecific fishes remains unknown.

POPULATION MANAGEMENT

Manipulation of sea lamprey olfactory biology offers opportunities for management of invasive populations in the Laurentian Great Lakes [11]. Based largely upon pheromone control of insects [105], integration of olfactory stimulants into sea lamprey control has been proposed in the forms of trapping, redistribution, disruption, and monitoring [16 – 19, 106]. However, only trapping has been evaluated in management scale tests [79, 107]. Baiting traps with conspecific odors increases the efficacy of sea lamprey traps [108, 109]. Field experiments in environments lacking background pheromones demonstrate that traps baited with the natural migratory cue and male mating pheromone, and synthesized 3kPZS catch more sea lamprey than unbaited traps [46, 55, 79, 107 – 110]. However, only 3kPZS has been tested in management-scale experiments, and only in the context of augmenting the existing trapping effort with pheromone as bait [79]. Traps baited with 3kPZS caught more sea lamprey than unbaited traps, and trapping efficiencies averaged about 10 % higher during years when 3kPZS was applied as bait [79]. The modest increase in trapping efficiency is unlikely large enough to justify wide-spread use of 3kPZS as a control measure unless application can be further optimized to improve effectiveness and reduce cost [111]. Notably, the natural, whole odor of males catches a higher proportion of sea lamprey compared to 3kPZS alone [107]. A recent evaluation of the push-pull method using alarm cue to activate one side of a stream and 3kPZS as bait for a trap on the other side of the stream failed to increase the number of sea lamprey caught in traps, although alarm cue did decrease the time taken for individuals to locate a trap [112]. Identification of all components of the male mating pheromone combined with refined trapping methods is needed to further developing odor-baited traps as a control tool.

Methods other than trapping, such as redistribution, disruption, and monitoring [16 – 19, 106], remain largely unexplored. Field experiments in pristine, odor-controlled environments indicate the migratory cue and male mating pheromone can be used for redistribution [55, 87] and spawning disruption [55]. Redistribution via a combination of conspecific attractants and alarm cues could be especially useful, but has not been evaluated. Quantification of 3kPZS in streams may also offer a cost-effective method to determine the presence and size of sea lamprey populations [113, 114]. Additional alternatives including antagonists [19] and integrating odor manipulation with electrical guidance [115], may too be useful, but have not been explored. Clearly, more research is needed to further develop olfactory cues as tools for sea lamprey control.

UTILITY OF THE SEA LAMPREY MODEL

The sea lamprey presents a simple and unique model for studying olfactory communication in vertebrates. The opportunity for insight into the biology of early vertebrates is matched only by the hagfish. However, the basic biology of sea lamprey is better understood as a result of better accessibility and decades of research associated with pest control programs in the Laurentian Great Lakes. The robust understanding of basic sea lamprey biology combined with the continued elucidation of chemical cues and pheromones, and recent advances, such as the sequencing of the genome [116], allows for novel research avenues.

A simple but well-developed olfactory system makes sea lamprey well-poised for elucidating the path from odorant detection to behavioral output. Sea lamprey detect a limited range of odorants; bile acids, a few amines and sex steroids, and L-arginine [50, 62]. The repertoire of chemosensory receptor genes is correspondingly small, consisting of only three families and an estimated 59 intact genes [34]. Ciliated sensory cells exhibit short, medium, and tall morphotypes that may be precursors to crypt, microvilous, and ciliated sensory cells documented in teleosts [30]. Despite being simple, the sea lamprey olfactory system is well-developed. The distinct accessory olfactory organ with sensory neurons that project to specific regions of the olfactory bulb allows an interesting comparison to the vomeronasal organ of higher vertebrates [27, 35]. The medial olfactory bulb, where the sensory neurons in the accessory organ project axons, forms a direct connection with brain structures that drive locomotion [40]. Strikingly, lamprey have a larger proportion of brain dedicated to processing olfactory information than any other vertebrate examined [22].

Well-adapted mechanisms of habitat and mate assessment using input from multiple olfactory stimuli and environmental cues make sea lamprey a useful organism for studying the evolution and behavioral ecology of multi-model sensory integration and complex signals. During reproduction, sea lamprey make behavioral decisions based upon the water temperature [89 , 90], time of day [117], abiotic odor of streams [51], alarm cues [12 , 15], multi-component conspecific cues [65 , 87] and mating pheromones [17 , 55 , 56 , 66], as well as interactions among variables [88] and the physiological state of the receiver [54]. Furthermore, sea lamprey spawn in lek-like aggregations, where males construct and aggressively defend nests [8], and signal to females with a complex pheromone mixture, setting the stage for studies on the poorly understood role of pheromones in mate choice, the evolution of exaggerated male signals [96], and the function and evolution of multi-component pheromones [56].

Augmenting sea lamprey management with insights from olfactory communication provides a rare example of sensory-integrated control in vertebrates. Manipulation of olfactory systems is a widely used as tools to control pest insect populations [105]. Extension of olfactory integrated control of insects to invasive vertebrates is conceptually sound [16 – 19, 106, 118], however, after decades of research into fish olfaction, olfactory communication is not integrated into control of any invasive fish. Developing olfactory- integrated management is a challenging and costly endeavor [118], but offers a suite of potentially robust and environmentally benign tools. Olfactory-guided behaviors are not unique to sea lamprey, and insights gained while developing olfactory-integrated control of sea lamprey can be extended to other species of concern throughout the world. Furthermore, the sea lamprey model offers the opportunity to optimize olfactory-integrated control methods without the confounding interactions of other sensory modalities. For example, most organisms, including sea lamprey, incorporate

information from several sensory modalities while making reproductive decisions. However, sensory-guided behaviors in sea lamprey are clearly biased towards olfaction [45, 46]. Similar to the insect model used as a conceptual foundation for sea lamprey olfaction research, the sea lamprey model can function as a model for more complex vertebrates. Likewise, the technologies and methods developed for studying sea lamprey olfaction provide a foundation that can be used to expedite future research into olfaction in other organisms that are invasive or in decline in the Laurentian Great Lakes and throughout the world.

CONCLUSION

The sea lamprey is a basal vertebrate with an increasingly well-characterized olfactory communication system. We suggest that the olfactory biology of the sea lamprey can be used to inform future research on olfactory systems of other species, as the understanding of the lamprey olfactory biology has been informed by detailed descriptions of olfaction in other organisms. In particular, the simple but well-developed olfactory organ, critical functions of several reproductive chemical cues and pheromones, and potential for population control make studies on sea lamprey olfaction broadly interesting. Current research on sea lamprey olfaction focuses largely on implications for population management [16 – 20,106,118]. However, further research into sea lamprey olfaction, spanning across neurobiology, characterization of chemical cues and pheromones, and ecology and evolution, offers opportunity for a uniquely integrated understanding of chemical communication in a vertebrate.

ACKNOWLEDGMENTS

Funding was provided by the Great Lakes Fishery Commission. Drs. Ke Li, Cory Brant, and Barbara Zielinski provided the figures.

REFERENCES

REFERENCES

1. Wyatt TD. Pheromones and animal behavior: chemical signals and signatures. Cambridge: Cambridge University Press; 2014.

2. Butenandt A, Beckmann R, Stamm D, Hecker ET. Uber den sexuallockstoff des seidenspinners Bombyx Mori-reindarstellung und konstitution. Z Naturforsch. 1959;B14:283–4.

3. Breithaupt T, Thiel M. Chemical communication in crustaceans. New York: Springer; 2011.

4. Sorensen PW, Wisenden BD. Fish Pheromones and Related Cues. John Wiley & Sons, Inc., Ames; 2015.

5. Houck LD. Pheromone communication in amphibians and reptiles. Annu Rev Physiol. 2009;71:161–76.

6. Caro SP, Balthazart J. Pheromones in birds: myth or reality? J Comp Physiol A. 2010;196:751–66.

7. Tirindelli R, Dibattista M, Pifferi S, Menini A. From pheromones to behavior. Physiol Rev. 2009;89:921–56.

8. Manion PJ, Hanson LH. Spawning behavior and fecundity of lampreys from the upper three Great Lakes. Can J Fish Aquat Sci. 1980;37:1635–40.

9. Zielinski BS, Fredricks K, McDonald R, Zaidi AU. Morphological and electrophysiological examination of olfactory sensory neurons during the early developmental prolarval stage of the sea lamprey Petromyzon marinus L. J Neurocytol. 2005;34:209–16.

10. Kleerekoper H, Mogensen J. Role of olfaction in the orientation of Petromyzon marinus. I. Response to a single amine in prey's body odor. Physiol Zool. 1963;36:347–60.

11. Teeter J. Pheromone communication in sea lampreys (Petromyzon marinus): implications for population management. Can J Fish Aquat Sci. 1980;37:2123–32.

12. Wagner CM, Stroud EM, Meckley TD, Kraft C. A deathly odor suggests a new sustainable tool for controlling a costly invasive species. Can J Fish Aquat Sci. 2011;68:1157–60.

13. Johnson NS, Buchinger TJ, Li W. Reproductive Ecology of Lampreys. In Docker MF, editor. Lampreys: Biology, Conservation and Control. Springer, Netherlands. 2015; p 265–303.

14. Bals JD, Wagner CM. Behavioral responses of sea lamprey (Petromyzon marinus) to a putative alarm cue derived from conspecific and heterospecific sources. Behaviour. 2012;149:901–23.

15. Imre I, Di Rocco RT, Belanger CF, Brown GE, Johnson NS. The behavioural response of adult Petromyzon marinus to damage-released alarm and predator cues. J Fish Biol. 2014;84:1490–502.

16. Sorensen PW, Vrieze LA. The chemical ecology and potential application of the sea lamprey migratory pheromone. J Great Lakes Res. 2003;29:66–84.

17. Li W, Siefkes MJ, Scott AP, Teeter JH. Sex pheromone communication in the sea lamprey: implications for integrated management. J Great Lakes Res. 2003;29:85–94.

18. Twohey MB, Sorensen PW, Li W. Possible applications of pheromones in an integrated sea lamprey management program. J Great Lakes Res. 2003;29:794–800.

19. Li W, Twohey M, Jones M, Wagner M. Research to guide use of pheromones to control sea lamprey. J Great Lakes Res. 2007;33:70–86.

20. Siefkes MJ, Steeves TB, Sullivan PW, Twohey MB, Li W. Sea lamprey control: Past, present, and future. In: Taylor WW, Lynch AJ, Leonard NJ, editors. Great Lakes Fisheries Policy and Management. Michigan State University Press, East Lansing. 2013; p 651–704.

21. Kleerekoper H, van Erkel GA. The olfactory apparatus of Petromyzon marinus L. Can J Zool. 1960;38:209–23.

22. Stoddart DM. The scented ape: the biology and culture of human odour. Cambridge: Cambridge University Press; 1990.

23. Hagelin L, Johnels AG. On the structure and function of the accessory olfactory organ in lampreys. Acta Zool. 1955;36:113–25.

24. VanDenBossche J, Seelye JG, Zielinski BS. The morphology of the olfactory epithelium in larval, juvenile and upstream migrant stages of the sea lamprey, *Petromyzon marinus*. Brain Behav Evol. 1995;45:19–24.

25. VanDenBossche J, Youson JH, Pohlman D, Wong E, Zielinski BS. Metamorphosis of the olfactory organ of the sea lamprey (*Petromyzon marinus* L): Morphological changes and metamorphic analysis. J Morphol. 1997;231:41–52.

26. Thornhill RA. Ultrastructure of olfactory epithelium of lamprey *Lampetra fluviatilis*. J Cell Sci. 1967;2:591–602.

27. Ren X, Chang S, Laframboise AJ, Green W, Dubuc R, Zielinski BS. projections from the accessory olfactory organ into the medial region of the olfactory bulb in the sea lamprey

(*Petromyzon marinus*): A novel vertebrate sensory structure? J Comp Neurol. 2009;516:105–16.

28. Hansen A, Finger TE. Phyletic distribution of crypt-type olfactory receptor neurons in fishes. Brain Behav Evol. 2000;55:100–10.

29. Hansen A, Zeiske E. The peripheral olfactory organ of the zebrafish, *Danio rerio*: an ultrastructural study. Chem Senses. 1998;23:39–48.

30. Laframboise AJ, Ren X, Chang S, Dubuc R, Zielinski BS. Olfactory sensory neurons in the sea lamprey display polymorphisms. Neurosci Lett. 2007;414:277–81.

31. Lastein S, Hamdani EH, Doving KB. Olfactory discrimination of pheromones. In: Sorensen PW, Wisenden BD, editors. Fish Pheromones and Related Cues. John Wiley & Sons, Inc., Ames; 2015. p159-195.

32. Buck L, Axel R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 1991;65:175–87.

33. Grus WE, Zhang J. Origin of the genetic components of the vomeronasal system in the common ancestor of all extant vertebrates. Mol Biol Evol. 2009;26:407–19.

34. Libants S, Carr K, Wu H, Teeter JH, Chung-Davidson YW, Zhang Z, et al. The sea lamprey Petromyzon marinus genome reveals the early origin of several chemosensory receptor families in the vertebrate lineage. BMC Evol Biol. 2009;9:180.

35. Chang S, Chung-Davidson YW, Libants SV, Nanlohy KG, Kiupel M, Brown CT, et al. The sea lamprey has a primordial accessory olfactory system. BMC Evol Biol. 2013;13:172.

36. Restrepo D, Teeter JH, Schild D. Second messenger signaling in olfactory transduction. J Neurobiol. 1996;30:37–48. 37. Schild D, Restrepo D. Transduction mechanisms in vertebrate olfactory receptor cells. Physiol Rev. 1998;78:429–66.

38. Frontini A, Zaidi AU, Hua H, Wolak TP, Greer CA, Kafitz KW, et al. Glomerular territories in the olfactory bulb from the larval stage of the sea lamprey (*Petromyzon marinus*). J Comp Neurol. 2003;465:27–37.

39. Green WW, Basilious A, Dubuc R, Zielinski BS. The neuroanatomical organization of projection neurons associated with different olfactory bulb pathways in the sea lamprey *(Petromyzon marinus*). PLoS One. 2013;8:e69525.

40. Derjean D, Moussaddy A, Atallah E, St-Pierre M, Auclair F, Chang S, et al. A novel neural substrate for the transformation of olfactory inputs into motor output. PLoS Biol. 2010;8:e1000567.

41. Dubuc R, Brocard F, Antri M, Fenelon K, Gariepy JF, Smetana R, et al. Initiation of locomotion in lampreys. Brain Res Rev. 2008;57:172–82.

42. Green WW. The neuroanatomical organization and chemosensory response characteristics of the olfactory bulb in the sea lamprey (Petromyzon marinus). Ph.D. Dissertation. University of Windsor.Windsor, 2013; 226p.

43. Eisthen HL. Why are olfactory systems of different animals so similar? Brain. Behav Evol. 2002;59:273–93.

44. Velez Z, Hubbard PC, Barata EN, Canario AV. Evidence for functional asymmetry in the olfactory system of the Senegalese sole (Solea senegalensis)*. Physiol Biochem Zool. 2005;78:756–65.

45. Vrieze LA, Bjerselius R, Sorensen PW. Importance of the olfactory sense to migratory sea lampreys Petromyzon marinus seeking riverine spawning habitat. J Fish Biol. 2015;76:949–64.

46. Johnson NS, Luehring MA, Siefkes MJ, Li W. Mating pheromone reception and induced behavior in ovulating female sea lampreys. N Am J Fish Manage. 2006;26:88–96.

47. Bergstedt RA, Seelye JG. Evidence for lack of homing by sea lampreys. T Am Fish Soc. 1995;124:235–9.

48. Waldman J, Grunwald C, Wirgin I. Sea lamprey Petromyzon marinus: an exception to the rule of homing in anadromous fishes. Biol Lett. 2008;4:659–62.

49. Moore HH, Schleen LP. Changes in spawning runs of sea lamprey (Petromyzon marinus) in selected streams of Lake Superior after chemical control. Can J Fish Aquat Sci. 1980;37:1851–60.

50. Li WM, Sorensen PW, Gallaher DD. The olfactory system of migratory adult sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile-acids released by conspecific larvae. J Gen Physiol. 1995;105:569–87.

51. Vrieze LA, Sorensen PW. Laboratory assessment of the role of a larval pheromone and natural stream odor in spawning stream localization by migratory sea lamprey (Petromyzon marinus). Can J Fish Aquat Sci. 2001;58:2374–85.

52. Chung-Davidson Y-W, Wang H, Siefkes MJ, Bryan MB, Wu H, Johnson NS, et al. Pheromonal bile acid 3-ketopetromyzonol sulfate primes the neuroendocrine system in sea lamprey. BMC Neurosci. 2013;14:11.

53. Chung-Davidson YW, Wang H, Bryan MB, Wu H, Johnson NS, Li W. An anti-steroidogenic inhibitory primer pheromone in male sea lamprey (Petromyzon marinus). Gen Comp Endocr. 2013;189:24–31.

54. Siefkes MJ, Winterstein SR, Li W. Evidence that 3-keto petromyzonol sulphate specifically attracts ovulating female sea lamprey Petromyzon marinus. Anim Behav. 2005;70:1037–45.

55. Johnson NS, Yun S-S, Thompson HT, Brant CO, Li W. A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps. Proc Natl Acad Sci U S A. 2009;106:1021–6.

56. Johnson NS, Yun S-S, Buchinger TJ, Li W. Multiple functions of a multicomponent mating pheromone in sea lamprey Petromyzon marinus. J Fish Biol. 2012;80:538–54.

57. Hara TJ. The diversity of chemical stimulation in fish olfaction and gustation. Rev Fish Biol Fisher. 1994;4:1–35.

58. Stacey N. Hormonally-derived pheromones in teleost fishes. In: Sorensen PW, Wisenden BD, editors. Fish Pheromones and Related Cues. John Wiley & Sons, Inc., Ames; 2015. p33-88.

59. Shoji T, Yamamoto Y, Nishikawa D, Kurihara K, Ueda H. Amino acids in stream water are essential for salmon homing migration. Fish Physiol Biochem. 2003;28:249–51.

60. Shoji T, Ueda H, Ohgami T, Sakamoto T, Katsuragi Y, Yamauchi K, et al. Amino acids dissolved in stream water as possible home stream odorants for masu salmon. Chem Senses. 2000;25:533–40.

61. Yambe H, Kitamura S, Kamio M, Yamada M, Matsunaga S, Fusetani N, et al. L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. Proc Natl Acad Sci U S A. 2006;103:15370–4.

62. Li W. Olfactory biology of adult sea lamprey (Petromyzon marinus), Ph.D. Dissertation. St Paul: University of Minnesota; 1994. p. 184.

63. Buchinger TJ, Li W, Johnson NS. Bile salts as semiochemicals in fish. Chem Senses. 2014;39:647–54.

64. Siefkes MJ, Li W. Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (Petromyzon marinus). J Comp Physiol A. 2004;190:193–9.

65. Sorensen PW, Fine JM, Dvornikovs V, Jeffrey CS, Shao F, Wang J, et al. Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. Nat Chem Biol. 2005;1:324–8.

66. Li K, Brant CO, Siefkes MJ, Kruckman HG, Li W. Characterization of a novel bile alcohol sulfate released by sexually mature male Sea Lamprey (*Petromyzon marinus*). PLoS ONE. 2013;8:e68157.

67. Doving KB, Selset R, Thommesen G. Olfactory sensitivity to bile acids in salmonid fishes. Acta Physiol Scand. 1980;108:123–31.

68. Haslewood GAD, Tokes L. Comparative studies of bile salts. Bile salts of the lamprey Petromyzon marinus L. Biochem J. 1969;114:179.

69. Polkinghorne CN, Olson JM, Gallaher DG, Sorensen PW. Larval sea lamprey release two unique bile acids** to the water at a rate sufficient to produce detectable riverine pheromone plumes. Fish Physiol Biochem. 2001;24:15–30.

70. Fine JM, Sorensen PW. Production and fate of the sea lamprey migratory pheromone. Fish Physiol Biochem. 2010;36:1013–20.

71. Bjerselius R, Li W, Teeter JH, Seelye JG, Johnsen PB, Maniak PJ, et al. Direct behavioral evidence that unique bile acids released by larval sea lamprey (Petromyzon marinus) function as a migratory pheromone. Can J Fish Aquat Sci. 2000;57:557–69.

72. Meckley TD, Wagner CM, Gurarie E. Coastal movements of migrating sea lamprey (Petromyzon marinus) in response to a partial pheromone added to river water: implications for management of invasive populations. Can J Fish Aquat Sci. 2014;71:533–44.

73. Meckley TD, Wagner CM, Luehring MA. Field evaluation of larval odor and mixtures of synthetic pheromone components for attracting migrating sea lampreys in rivers. J Chem Ecol. 2012;38:1062–9.

74. Li K, Siefkes MJ, Brant CO, Li W. Isolation and identification of petromyzestrosterol, a polyhydroxysteroid from sexually mature male sea lamprey (Petromyzon marinus L.). Steroids. 2012;77:806–10.

75. Li K, Brant CO, Huertas M, Hur SK, Li W. Petromyzonin, a hexahydrophenanthrene sulfate isolated from the larval sea lamprey (Petromyzon marinus L.). Org Lett. 2013; 15:5924–7.

76. Li K, Huertas M, Brant C, Chung-Davidson YW, Bussy U, Hoye TR, et al. (+)-and (−)-Petromyroxols: Antipodal tetrahydrofurandiols from larval sea lamprey (Petromyzon marinus L.) that elicit enantioselective olfactory responses. Org Lett. 2014;17:286–9.

77. Li W, Scott AP, Siefkes MJ, Yan H, Liu Q, Yun S-S, et al. Bile acid secreted by male sea lamprey that acts as a sex pheromone. Science. 2002;296:138–41.

78. Siefkes MJ, Scott AP, Zielinski B, Yun S-S, Li W. Male sea lampreys, Petromyzon marinus L., excrete a sex pheromone from gill epithelia. Biol Reprod. 2003;69:125–32.

79. Johnson NS, Siefkes MJ, Wagner CM, Dawson H, Wang H, Steeves T, et al. A synthesized mating pheromone component increases adult sea lamprey (Petromyzon marinus) trap capture in management scenarios. Can J Fish Aquat Sci. 2013;70:1101–8.

80. Yun S-S, Scott AP, Li W. Pheromones of the male sea lamprey, Petromyzon marinus L.: structural studies on a new compound, 3-keto allocholic acid, and 3-keto petromyzonol sulfate. Steroids. 2003;68:297–304.

81. Li W. Potential multiple functions of a male sea lamprey pheromone. Chem Senses. 2005;30:i307–8.

82. Johnson NS, Yun S-S, Li W. Investigations of novel unsaturated bile salts of male sea lamprey as potential chemical cues. J Chem Ecol. 2014;40:1152–60.

83. Yeh C-Y, Chung-Davidson Y-W, Wang H, Li K, Li W. Intestinal synthesis and secretion of bile salts as an adaptation to developmental biliary atresia in the sea lamprey. Proc Natl Acad Sci U S A. 2012;109:11419–24.

84. Cai S-Y, Lionarons DA, Hagey L, Soroka CJ, Mennone A, Boyer JL. Adult sea lamprey tolerates biliary atresia by altering bile salt composition and renal excretion. Hepatology. 2013;57:2418–26.

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

86. Vrieze LA, Bergstedt RA, Sorensen PW. Olfactory-mediated stream finding behavior of migratory adult sea lamprey (Petromyzon marinus). Can J Fish Aquat Sci. 2011;68:523–33.

87. Wagner CM, Twohey MB, Fine JM. Conspecific cueing in the sea lamprey: do reproductive migrations consistently follow the most intense larval odour? Anim Behav. 2009;78:593–9.

88. Brant CO, Li K, Johnson NS, Li W. A pheromone outweighs temperature in influencing migration of sea lamprey. R Soc Open Sci. 2015;2:150009.

89. Binder TR, McDonald DG. The role of temperature in controlling diel activity in upstream migrant sea lampreys (Petromyzon marinus). Can J Fish Aquat Sci. 2007;65:1113–21.

90. Binder TR, McLaughlin RL, McDonald DG. Relative importance of water temperature, water level, and lunar cycle to migratory activity in spawning-phase sea lampreys in Lake Ontario. T Am Fish Soc. 2010;139:700–12.

91. Fine JM, Vrieze LA, Sorensen PW. Evidence that petromyzontid lampreys employ a common migratory pheromone that is partially comprised of bile acids. J Chem Ecol. 2004;30:2091–110.

92. Yun S-S, Scott AP, Bayer JM, Seelye JG, Close DA, Li W. HPLC and ELISA analyses of larval bile acids from Pacific and western brook lampreys. Steroids. 2003;68:515–23.

93. Yun S-S, Wildbill AJ, Siefkes MJ, Moser ML, Dittman AH, Corbett SC, et al. Identification of putative migratory pheromones from Pacific lamprey (Lampetra tridentata). Can J Fish Aquat Sci. 2011;68:2194–203.

94. Robinson TC, Sorensen PW, Bayer JM, Seelye JG. Olfactory sensitivity of Pacific lampreys to lamprey bile acids. T Am Fish Soc. 2009;138:144–52.

95. Stewart M, Baker CF, Cooney T. A rapid, sensitive, and selective method for quantitation of lamprey migratory pheromones in river water. J Chem Ecol. 2011;37:1203–7.

96. Buchinger TJ, Wang H, Li W, Johnson NS. Evidence for a receiver bias underlying female preference for a male mating pheromone in sea lamprey. Proc R Soc B. 2013;280:20131966.

97. Sorensen PW, Scott AP. The evolution of hormonal sex pheromones in teleost fish: poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggests that these cues evolved as a result of chemical spying rather than signal specialization. Acta Physiol Scand. 1994;152:191–205.

98. Pfeiffer W, Riegelbauer G, Meier G, Scheibler B. Effect of hypoxanthine-3 (N)-oxide and hypoxanthine-1 (N)-oxide on central nervous excitation of the black tetraGymnocorymbus ternetzi (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. J Chem Ecol. 1985;11:507–23.

99. Brown GE, Adrian JC, Smyth E, Leet H, Brennan S. Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides. J Chem Ecol. 2000;26:139–54.

100. Mathuru AS, Kibat C, Cheong WF, Shui G, Wenk MR, Friedrich RW, et al. Chondroitin fragments are odorants that trigger fear behavior in fish. Curr Biol. 2012;22:538–44.

101. Ferrero DM, Lemon JK, Fluegge D, Pashkovski SL, Korzan WJ, Datta SR, et al. Detection and avoidance of a carnivore odor by prey. Proc Natl Acad Sci U S A. 2011;108:11235–40.

102. Di Rocco RT, Belanger CF, Imre I, Brown GE, Johnson NS. Daytime avoidance of chemosensory alarm cues by adult sea lamprey (Petromyzon marinus). Can J Fish Aquat Sci. 2014;71:824–30.

103. Wisenden BD. Chemical cues that indicate risk of predation. In: Sorensen PW, Wisenden BD, editors. Fish Pheromones and Related Cues. John Wiley & Sons, Inc., Ames; 2015. p131-148.

104. Perrault K, Imre I, Brown GE. Behavioural response of larval sea lamprey (Petromyzon marinus) in a laboratory environment to potential damage-released chemical alarm cues. Can J Zool. 2014;92:443–7.

105. Witzgall P, Kirsch P, Cork A. Sex pheromones and their impact on pest management. J Chem Ecol. 2010;36:80–100.

106. Sorensen PW. Applications of pheromones in invasive fish control and fishery conservation. In: Sorensen PW, Wisenden BD, editors. Fish Pheromones and Related Cues. John Wiley & Sons, Inc., Ames; 2015. p255-268.

107. Johnson NS, Tix JA, Hlina BL, Wagner CM, Siefkes MJ, Wang H, et al. A sea lamprey (Petromyzon marinus) sex pheromone mixture increases trap catch relative to a single synthesized component in specific environments. J Chem Ecol. 2015;41:311–21.

108. Johnson NS, Siefkes MJ, Li W. Capture of ovulating female sea lampreys in traps baited with spermiating male sea lampreys. N Am J Fish Manage. 2005;25:67–72.

109. Wagner CM, Jones ML, Twohey MB, Sorensen PW. A field test verifies that pheromones can be useful for sea lamprey (Petromyzon marinus) control in the Great Lakes. Can J Fish Aquat Sci. 2005;63:475–9.

110. Luehring MA, Wagner CM, Li W. The efficacy of two synthesized sea lamprey sex pheromone components as a trap lure when placed in direct competition with natural male odors. Biol Invasions. 2011;13:1589–97.

111. Johnson NS, Siefkes MJ, Wagner CM, Bravener G, Steeves T, Twohey M, Li W. Factors influencing capture of invasive sea lamprey in traps baited with synthesized sex pheromone components. J Chem Ecol. In press.

112. Hume JB, Meckley TD, Johnson NS, Luhring TM, Siefkes MJ, Wagner CM. Application of a putative alarm cue hastens the arrival of invasive sea lamprey (Petromyzon marinus) at a trapping location. Can J Fish Aquat. 2015; published online 28July2015.

113. Xi X, Johnson NS, Brant CO, Yun S-S, Chambers KL, Jones AD, et al. Quantification of a male sea lamprey pheromone in tributaries of Laurentian Great Lakes by liquid chromatography–tandem mass spectrometry. Environ Sci Technol. 2011;45:6437–43.

114. Wang H, Johnson NS, Bernardy J, Hubert T, Li W. Monitoring sea lamprey pheromones and their degradation using rapid stream-side extraction coupled with UPLC-MS/MS. J Sep Sci. 2013;36:1612–20.

115. Johnson NS, Thompson HT, Holbrook C, Tix JA. Blocking and guiding adult sea lamprey with pulsed direct current from vertical electrodes. Fish Res. 2014;150:38–48.

116. Smith JJ, Kuraku S, Holt C, Sauka-Spengler T, Jiang N, Campbell MS, et al. Sequencing of the sea lamprey (Petromyzon marinus) genome provides insights into vertebrate evolution. Nature Genet. 2013;45:415–21.

117. Walaszczyk EJ, Johnson NS, Steibel JP, Li W. Effects of sex pheromones and sexual maturation on locomotor activity in female sea lamprey (Petromyzon marinus). J Biol Rhythm. 2014;28:218–26.

118. Sorensen PW, Hoye TR. A critical review of the discovery and application of a migratory pheromone in an invasive fish, the sea lamprey Petromyzon marinus L. J Fish Biol. 2007;71:100–14.

CHAPTER 2

PHYLOGENETIC DISTRIBUTION OF A MATING PHEROMONE IN LAMPREYS

ABSTRACT

Chemical signals, (*i.e. pheromones*) are important during mate search and evaluation in nearly all animals. Pheromones are generally considered highly species-specific due to the intricate mechanisms of signal production and detection, and critical roles in species recognition. However, pheromone communication, particularly in vertebrates, is rarely considered in a macroevolutionary context. Here, we report a phylogenetic comparison of a mating pheromone across lampreys. Male *Petromyzon marinus* release at high rates the bile alcohol pheromone 3-keto petromyzonol sulphate (3kPZS), which is detected with high specificity and sensitivity, and elicits robust behavioural attraction in females. Male signalling with 3kPZS is hypothesized to exploit a non-sexual attraction for juvenile-released 3kPZS relevant during the migration into productive rearing habitat. We determined 1) male release of 3kPZS across eleven lamprey species, representing six of ten genera and two of three families, 2) olfactory sensitivity to 3kPZS in four species native to the Laurentian Great Lakes, and 3) sexual responses to 3kPZS in four species native to the Laurentian Great Lakes. All species tested exhibit olfactory responses to 3kPZS, but only *Ichthyomyzon castaneus* and *P. marinus* use 3kPZS as a sexual signal. We infer that the non-sexual role of 3kPZS is conserved among lampreys while the sexual function of 3kPZS is specific to the clade comprised of *Petromyzon* and *Ichthyomyzon*. Our results indicate either independent gains or single gain and a single loss of 3kPZS communication, and represent a rare macroevolutionary investigation of a vertebrate pheromone.

INTRODUCTION

Organisms use a myriad of signals to communicate during mate choice [1]. Natural and sexual selection shape the expression of visual, auditory, electrical, tactile, and chemical traits and associated preferences [2]. Defining the selective pressures that govern the integration of such traits into communication systems is an important theme in evolutionary biology [3,4 ,5].

Convergent evolution of sexual communication systems provides direct evidence for common selective pressures across taxa [3,4]. Animals face similar constraints on signal production, transmission, the information associated with a trait. For example, many animal species evaluate conspecifics using carotenoid-based traits [6,7]. Carotenoid-based displays are likely meaningful signals in many taxa [8], as carotenoids are acquired solely through diet [9], expressed at a cost [10], and indicate male quality [11]. Classical sexual selection models (direct benefits, good genes) [12] adequately explain carotenoid-based communication systems in many species. Widespread preferences for carotenoid-based traits may also be explained in part by a common pre-existing bias for carotenoid forage items [13, 14, 15, 16]. Hence, similar constraints on signallers and/or receivers can drive convergent evolution of sexual communication systems.

Conversely, many organisms exhibit clear divergent evolution of sexual communication systems despite facing similar constraints on signalling. In fact, species-specificity is regarded as a principal tenet of sexual communication [17]. Many processes can drive signal diversification, including maladaptive heterospecific interactions, unique solutions to similar constraints, mutation and genetic drift, and differences in ecology [17]. Signal-specificity may also vary among sensory modalities [4]. Diversification of signals likely also varies among modalities

depending upon constraints on signal diversity or perceptual ranges. Chemical signals (*i.e. pheromones*) are considered highly species-specific due to the intricate mechanisms of signal production and detection, immense opportunity for signal diversity, and broad range of perceptual capability [18]. However, chemical communication, particularly in vertebrates, is rarely considered in an evolutionary context and, as a result, whether chemical signals more often exhibit convergence due to similar constraints, or are species-specific remains unknown.

Petromyzon marinus relies upon pheromones during reproduction and offers a useful opportunity to study macroevolutionary patterns of chemical signals in a vertebrate [19, 20]. Several chemical cues guide habitat selection and mate search during the single spawning season of *P. marinus* [19]. The odour of stream-resident larvae guides adult migration from lakes or the Atlantic Ocean into streams [19, 21, 22]. Upon reaching the final stages of sexual maturation, males release a multi-component pheromone containing 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate (3-keto petromyzonol sulfate, 3kPZS), which is detected with high specificity and sensitivity [23] and guides female movement upstream to nests [24, 25, 26]. Female preference for 3kPZS may have evolved under the selective pressure of a pre-existing cognitive bias (*sensory trap*) [20, 27]; female *Ichthyomyzon unicuspis*, a basal petromyzontid [28], exhibit a migratory but not mating response to 3kPZS, and only larvae, not males, release 3kPZS into the water at rates sufficient to be detected by conspecifics [20]. An ancestral preference for 3kPZS is further supported by evidence that larval-released migratory cues, including 3kPZS [20, 29, 30], are conserved among lamprey species [29, 31, 32, 33].

Here, we report a phylogenetic comparison of 3kPZS communication across lampreys. Specifically, we determined 1) male release of 3kPZS across eleven lamprey species, representing six of ten genera and two of three families, 2) olfactory sensitivity to 3kPZS in four

species native to the Laurentian Great Lakes, and 3) behavioural preferences for 3kPZS in four species native to the Laurentian Great Lakes. We found that all species tested have the physiological ability to detect 3kPZS, but only two species, *P. marinus* and *I. castaneus*, use 3kPZS as a sexual signal.

METHODS

Experimental animals

All experimental approaches and animals were used with approval from Michigan State University's Animal Use and Care Committee (Approval #'s 4/10-043-00, 02-13-040-00). Adults lampreys were collected via U.S. Fish and Wildlife Service sea lamprey traps, backpack electroshocking, fyke nets, or by hand (Table 2-1). Species were identified following Renaude [34]. Sexual maturity was determined based on the expression of eggs (ovulation) or milt (spermiation) upon gentle manual pressure [35].

Release of 3kPZS

To determine which lampreys signal with 3kPZS, release of 3kPZS by sexually mature males into holding waters was determined for eleven species of lampreys, representing six of ten genera and two of three families of *Petromyzontiformes* [28]. All lampreys were held in aerated laboratory tanks prior to sampling, except *Geotria australis* which was sampled immediately after collection off of a spawning nest. For all species, a single male was held in 5 L of aerated deionised water for 2 hr, after which water was sampled, spiked with 5-deuterated 3kPZS internal standard, and stored at less than -20 °C for subsequent analysis. Water samples were 50, 250, or 1000 ml, and spiked with 5 or 50 ng of internal standard, as result of changing analytical techniques throughout sampling years. Regardless of sample volume, a 10 ml subsample from each water sample was evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co. Missouri, USA) and reconstituted in 50% HPLC-grade methanol. Concentrated

samples were subjected to ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS, Waters Acquity ultra-performance liquid chromatography system, Waters, Milford, MA, USA and Micromass Quattro Premier XE tandem quadruple mass spectrometer, Waters, Manchester, UK) following described methods [36, 37]. Wilcoxon rank-sum tests were used to determine if the concentrations of 3kPZS in male samples were higher than those in the respective control samples (one-tailed, $\alpha = 0.05$), and if so, a pair-wise Wilcoxon rank-sum test (two-tailed, $\alpha = 0.05$) with a Holm adjustment was used to evaluate differences in 3kPZS release rates between species. Release rates were standardized by the weight of individuals as species vary in size (Table 2-1) as weight-standardized release rates better capture differences in pheromone communication systems among species [20].

Table 2-1. Pheromone sampling from eleven species of Petromyzontiformes. Number denotes the number of males from which pheromones were sampled. Weight and length represent the mean and standard error of the mean within each species.

genus	species	location	number	weight (se)	length (se)	collection method
Geotria	*australis*	Canterbury, NZ	4	164.36 (22.21)	488.25 (26.21)	hand
Ichthyomyzon	*unicuspis*	Michigan, USA	14	39.43 (2.38)	249.6 (4.16)	trap
	fossor	Michigan, USA	27	3.89 (0.23)	121.52 (2.14)	electrofishing
	castaneus	Michigan, USA	13	24.22 (3.13)	210.31 (11.24)	hand/trap
Petromyzon	*marinus*	Michigan, USA	15	260.01 (11.19)	447.46 (18.32)	trap
Entosphenus	*tridentus*	Oregon, USA	9	328.51 (14.2)	545.44 (9.1)	net
Lethenteron	*appendix*	Michigan, USA	25	4.14 (0.13)	139.2 (1.75)	hand
	camtschaticum	Jilin, China	8	97.05 (9.57)	384.13 (10.26)	net
	reisneri	Liaoning, China	8	6.88 (0.63)	165.0 (5.67)	electrofishing
	morii	Liaoning, China	11	27.27 (1.1)	255.46 (4.64)	net
Lampetra	*aeryptera*	Indiana, USA	8	6.98 (0.62)	153.25 (3.98)	electrofishing

Olfactory responses to 3kPZS

Electro-olfactogram (EOG) recordings were used to determine which lamprey species detect 3kPZS [38]. EOG recordings were conducted on 4-6 individual *I. unicuspis*, *I. fossor, I. castaneus*, and *Lethenteron appendix* based upon availability. Sexually immature male and female lampreys were anesthetized using 3-aminobenzoic acid ethyl ester (50mg l^{-1}; MS222; Sigma), immobilized with an intra-muscular injection of gallamine triethiodide (1mg kg^{-1}; Sigma), and secured in a Plexiglas trough while their gills were continuously perfused with aerated water containing anesthetic. Immature lamprey were used because measurement of olfactory sensitivity with EOG recordings becomes less robust as lampreys become sexually mature [39], likely a result of lamprey nearing the end of their life. Olfactory responses were recorded using a recording electrode placed between olfactory lamellae and a reference electrode placed on the skin. Electrical signals were filtered and amplified using a NeuroLog filter and pre-amplifier (Digitimer Ltd., Hertfordshire, England), integrated using an Axon Instruments Digidata system (Molecular Devices, CA, USA) and stored on a PC with Axon Instruments Axoscope software.

The olfactory sensitivity with which lamprey detected 3kPZS and 3α,7α,12α,24-tetrahydroxy-5α-cholan-24-sulfate (petromyzonol sulfate, PZS), a structurally and ecologically similar compound [40], was evaluated by determining concentration-response curves. The lowest concentration at which responses to a stimulus were significantly larger than those to a control of blank water (paired t-test, $\alpha = 0.05$) was considered to be the threshold of detection [23]. Blank water controls account for responses to any non-olfactory stimuli (*e.g.* mechanosensory). Responses were normalized by the response to L-arginine at $1x10^{-5}$M, based upon previous EOG recordings on sea lamprey [23, 38].

Cross-adaptation experiments [41] were used to determine if the 3kPZS-specific receptor mechanisms documented in *P. marinus* [23] are conserved across lamprey species. Using cross-adaptation experiments, we recorded the responses to 3kPZS when the epithelium was adapted to PZS and the responses to PZS when the epithelium was adapted to 3kPZS. Experiments began with recording the responses to the adapting stimuli at concentrations that elicited responses approximately equipotent to one another, $1x10^{-8}$M 3kPZS and PZS in *I. fossor, I. castaneus*, and *L. appendix*, and $1x10^{-7}$M 3kPZS and PZS in *I. unicuspis*. Next, olfactory responses were recorded when the epithelium was saturated with PZS and exposed to 1) 2x PZS (self-adapted control; SAC) and 2) 1x PZS + 1x 3kPZS (Mix), and saturated with 3kPZS and exposed to 1) 2x 3kPZS and 2) 1x 3kPZS + 1x PZS. The responses to the SAC and the Mix were normalized by the response to the adapting stimuli, and evaluated for differences using paired t-tests ($\alpha = 0.05$). A difference between the response to the SAC and the Mix indicates the odors are detected by separate olfactory mechanisms and hence different odorants.

Behavioural responses to 3kPZS

Proximate behavioural preferences of sexually mature males and females for 3kPZS were evaluated using two-choice mazes [20, 24]. Behavioural preferences for 3kPZS were only evaluated in *I. unicuspis*, *I. castaneus*, *I. fossor*, and *L. appendix*, due to availability for behavioural testing. The same behavioural assay and protocol were used for each species. All lampreys were transported from aerated laboratory tanks at the U.S. Geological Survey Hammond Bay Biological Station to streams prior to experiments. Two-choice mazes were constructed adjacent to Nagel Creek in 2010 and the Little Ocqueoc River in 2013 and 2015, both located in Presque Isle County, MI, USA. Experiments were conducted during three months

(May, June, and July): 2010, 2013, and 2015 for *I. castaneus*, 2010 and 2015 for *I. fossor*, and 2010 for *I. unicuspis* and *L. appendix*. *I. castaneus* and *I. unicuspis* were tested in a maze previously used for *I. unicuspis* [20], and *I. fossor* and *L. appendix* were tested in a smaller maze proportional to their size (0.3m x 1.15m). Assays for all species had the same depth (0.17 m) and water velocity (0.07 m/s). An experiment began with the placement of a single lamprey into the furthest downstream point of the maze. After 10 min acclimation, the time the lamprey spent in each channel was recorded while no odour was applied. After 20 min of pre-stimulus recording, 3kPZS dissolved in 50% methanol (MeOH:H_2O, v:v) was diluted in 5 L and applied at 200 ml/min into the maze to achieve a concentration of $1x10^{-12}$M. 3kPZS was introduced to a random side, along with a 50% methanol control vial to the opposing side. The 3kPZS concentration used followed that of previous studies [20, 25]. The odour was pumped into one side of the maze for 5 min without recording the lamprey's behaviour to allow the odour to fully disperse throughout the assay. After 5 min, the behaviour was recorded for another 20 min. After recording the time spent in the control and experimental channels before odour application (bc, be), and the time spent in the control and experimental channels after odour application (ac, ae), an index of preference (i) was calculated for each test (i = [ae/(ae + be) - ac/(ac + bc)]. The indices of preference were evaluated for significance using a Wilcoxon signed-rank test (α= 0.05).

RESULTS

Release of 3kPZS

3kPZS was released at high rates by *I. castaneus* and *P. marinus* and (Figure 2-1). Release of 3kPZS was evaluated for 4-27 individual males per species, depending upon availability (Table 2-1). The concentration of 3kPZS in blank water control samples across all species was 0.03 ± 0.009 ng ml^{-1} (n = 88; mean ± se). Only samples from *I. unicuspis*, *I. castaneus*, *P. marinus*, and *Entosphenus tridentatus* had higher concentrations of 3kPZS than respective control samples (Wilcoxon rank-sum tests, $p < 0.05$). Rates of 3kPZS release in *P. marinus* and *I. castaneus* were similar (Wilcoxon rank-sum tests, $p > 0.05$), and higher than those in *I. unicuspis* or *E. tridentatus* (Wilcoxon rank-sum tests, $p < 0.05$; Figure 2-1).

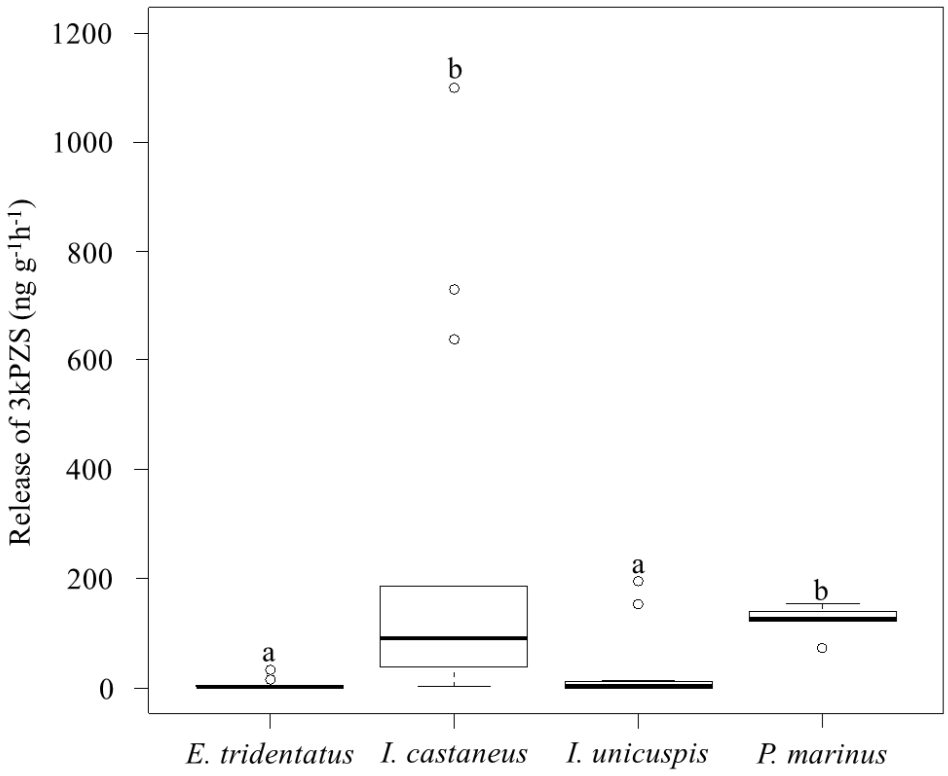


Figure 2-1. Release of 3-keto petromyzonol sulfate (3kPZS; ng $g^{-1}h^{-1}$) into the water by sexually mature male *Entosphenus tridentus*, *Ichthyomyzon castaneus*, *I. unicuspis*, and *Petromyzon marinus*, the only species with concentrations of 3kPZS higher in male samples than controls. Letters represent significant differences as determined using a pair-wise Wilcoxon rank-sum test ($\alpha = 0.05$) with a Holm adjustment for multiple comparisons. Bars represent the median, boxes the interquartile range, and outliers are identified as points outside 1.5x the interquartile range.

Olfactory responses to 3kPZS

All species tested exhibited olfactory responses to 3kPZS and PZS. Responses to the L-arginine standard were similar to those reported for *P. marinus* (mean ± se: *I. unicuspis* = 0.79 ± 0.15 mV, n = 4; *I. fossor* = 1.51 ± 0.12 mV, n = 6; *I. castaneus* = 1.26 ± 0.11 mV, n = 4; *L. appendix* = 2.3 ± 0.51mV, n = 6), as were the responses to the control stimuli (mean ± se: *I. unicuspis* = 24.0 ± 9.4%; *I. fossor* = 14.1 ± 4.6%; *I. castaneus* = 15.5 ± 1.0%; *L. appendix* = 18.7 ± 3.3%, % arginine standard)[23]. Concentration-response curves for 3kPZS and PZS were exponentially shaped and the thresholds of detection ranged from 10^{-8} to 10^{-15}M (Figure 2-2, *a-c*). Concentration-response curves confirm that 3kPZS and PZS are potent odorants, and indicate the compounds may be important to the chemical ecology of all lamprey species tested.

Cross-adaptation experiments indicated *I. unicuspis*, a species that exhibits a non-sexual response to 3kPZS, detect 3kPZS with olfactory mechanisms that are less specific than *P. marinus*, a species that uses 3kPZS during sexual communication (figure 2d-f)[23, 24]. During cross-adaptation experiments, the olfactory epithelium was saturated with PZS or 3kPZS, and the olfactory responses of a lamprey to 1) 2x PZS or 3kPZS (SAC) and 2) PZS + 3kPZS (Mix) were measured. A significant difference between the responses to the SAC and the Mix indicates that PZS and 3kPZS are detected with distinct olfactory mechanisms. Responses to the Mix remained greater than the SAC when the olfactory epithelium was saturated with 3kPZS in *I. unicuspis*, *I. fossor*, *I. castaneus*, and *L. appendix* ($p < 0.05$, two-tailed paired t-test), indicating that PZS is detected with olfactory mechanisms that are not used to detect 3kPZS. Similarly, responses to the Mix remained greater than the SAC when the olfactory epithelium was saturated with PZS in *I. castaneus* and *L. appendix* ($p < 0.05$, two-tailed paired t-test), indicating that 3kPZS is detected with olfactory mechanisms independent of those that detect PZS. Conversely, *I. unicuspis* and *I.*

fossor exhibited similar responses to the Mix and the SAC ($p > 0.05$, two-tailed paired t-test), indicating that 3kPZS is not detected by any olfactory mechanisms that do not also detect PZS. The non-reciprocal results from cross-adaptation experiments indicate that all species have a distinct receptor for PZS, but *I. unicuspis* and *I. fossor* do not have distinct olfactory mechanisms for 3kPZS.

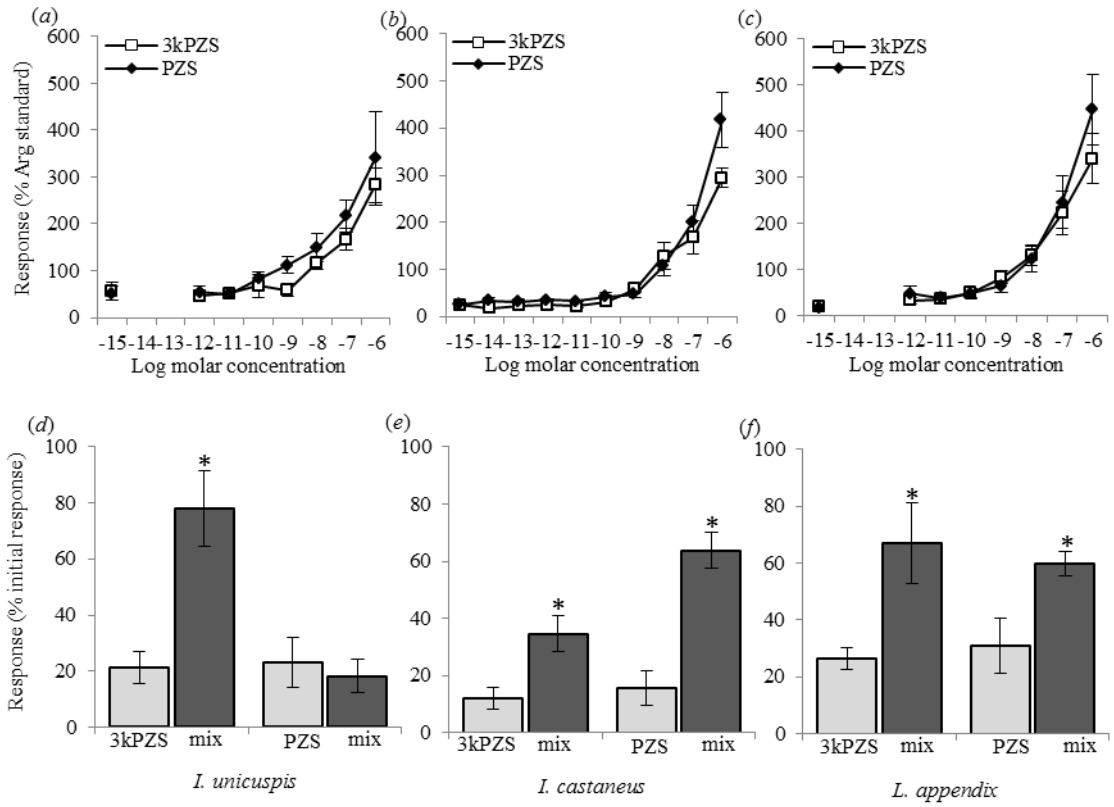


Figure 2-2. Electro-olfactogram recordings from *Ichthyomyzon unicuspis*, *I. castaneus*, and *Lampetra appendix* to 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS). (*a-c)* Concentration-response relationships presented as semi-logarithmic plots with responses presented as a percentage of L-arginine at $1x10^{-5}$M. (*d-e*) Cross-adaptation results presented as a percentage of the unadapted response. SAC = self-adapted control, mix = 3kPZS + PZS. Asterisks indicate a significant difference as determined with a paired t-test ($\alpha = 0.05$). Error bars represent the standard error of the mean. *I. fossor*, a close relative and possibly non-parasitic ecotype of *I. unicuspis* [55], exhibited similar olfactory responses as *I. unicuspis*.

Behavioural responses to 3kPZS

Two-choice behavioural experiments indicated that of the species tested only *I. castaneus* exhibit proximate preferences for 3kPZS (Table 2-2). Depending upon availability, 9-20 individual lamprey were tested in behavioural assays. Lamprey did not have a significant bias towards either channel of the bioassay during the pre-treatment period ($p > 0.05$, two-tailed paired t-tests). Male and female *I. castaneus* spent more time in the channel of the bioassay treated with 3kPZS (Wilcoxen signed-rank test, $p \leq 0.05$). The distribution of time sexually mature male and female *I. unicuspis*, *I. fossor*, and *L. appendix* spent in each channel of the bioassay was not influenced by 3kPZS (Wilcoxen signed-rank test, $p > 0.05$; Table 2-2).

Table 2-2. Near-source preference of mature lampreys to 3-keto petromyzonol sulfate (3kPZS). Control denotes the number of individuals that preferred the control channel, 3kPZS denotes the number of individuals that preferred the 3kPZS treated channel. P-values were determined using Wilcoxon signed-rank tests.

subject	control	3kPZS	index of preference (se)	p-value
male *I. unicuspis*	5	9	0.18(0.1)	0.1
*female *I. unicuspis*	7	4	-0.06 (0.09)	0.28
male *I. fossor*	4	10	0.12 (0.11)	0.27
female *I. fossor*	11	9	-0.05 (0.12)	0.5
male *I. castaneous*	2	9	0.44 (0.17)	0.05
female *I. castaneous*	1	9	0.45 (0.1)	0.01
male *L. appendix*	4	5	-0.08 (0.18)	0.91
female *I. appendix*	5	6	0.06 (0.17)	0.77

* data from Buchinger et al. [20]

DISCUSSION

Our results indicate that the non-sexual role of 3kPZS is conserved while the sexual function of 3kPZS is specific to the clade comprised of *Petromyzon* and *Ichthyomyzon* (Figure 2-3). Using a rare phylogenetic comparison of a vertebrate pheromone, we found that, of the species tested, (*i*) exaggerated 3kPZS signalling by males was restricted to two species of lamprey, *P. marinus* and *I. castaneus*, (*ii*) all species tested have the physiological capacity to detect 3kPZS, although *I. unicuspis* does not have distinct receptor mechanisms for 3kPZS, and (*iii*) sexual preferences for 3kPZS are limited to *P. marinus* and *I. castaneus*. We infer that the non-sexual role of 3kPZS is ancestral based upon our and others' results. First, all species tested, including species that do not use 3kPZS as a male release sexual signal, exhibit olfactory responses to 3kPZS [20, 23, 29, 32]. Second, the chemicals released by larvae that guide migration, including 3kPZS [20, 29, 30], appear conserved across lampreys [29, 31, 32, 33]. However, the evolution of 3kPZS as a sexual signal is less clear. Parsimony, with gains and losses weighted equally [42], supports either a gain of sexual communication with 3kPZS in the common ancestor of *Petromyzon* and *Ichthyomyzon* followed by a loss of 3kPZS communication in *I. unicuspis*, or independent gains of 3kPZS communication in *P. marinus* and *I. castaneus*.

We postulate that 3kPZS communication evolved independently in *P. marinus* and *I. castaneus*. *I. unicuspis* likely use 3kPZS released by larvae to locate suitable rearing habitat during their migration [20], but, based upon our results, do not detect 3kPZS with olfactory mechanisms distinct from those used to detect PZS. A loss of distinct olfactory mechanisms for 3kPZS due to relaxed selective pressures in *I. unicuspis* is unlikely as 3kPZS is an important

chemical cue. Likewise, if some selective force acted against communication with 3kPZS in *I. unicuspis*, perhaps hybridization with 3kPZS signalling *P. marinus* or *I. castaneus* [43], *I. unicuspis* would conceivably exhibit a negative or neutral response to 3kPZS rather than the observed positive response [20] mediated by a less-specific detection mechanism. Furthermore, independent gains of 3kPZS communication in *P. marinus* and *I. castaneus* seem plausible as the required changes from non-signalling to signalling are relatively simple; the detection mechanisms and a non-sexual preference, and the production and release mechanisms, were likely already present (above discussion) [20]. Sexual selection need only drive the strengthening of existing female preferences and exaggeration of existing male traits.

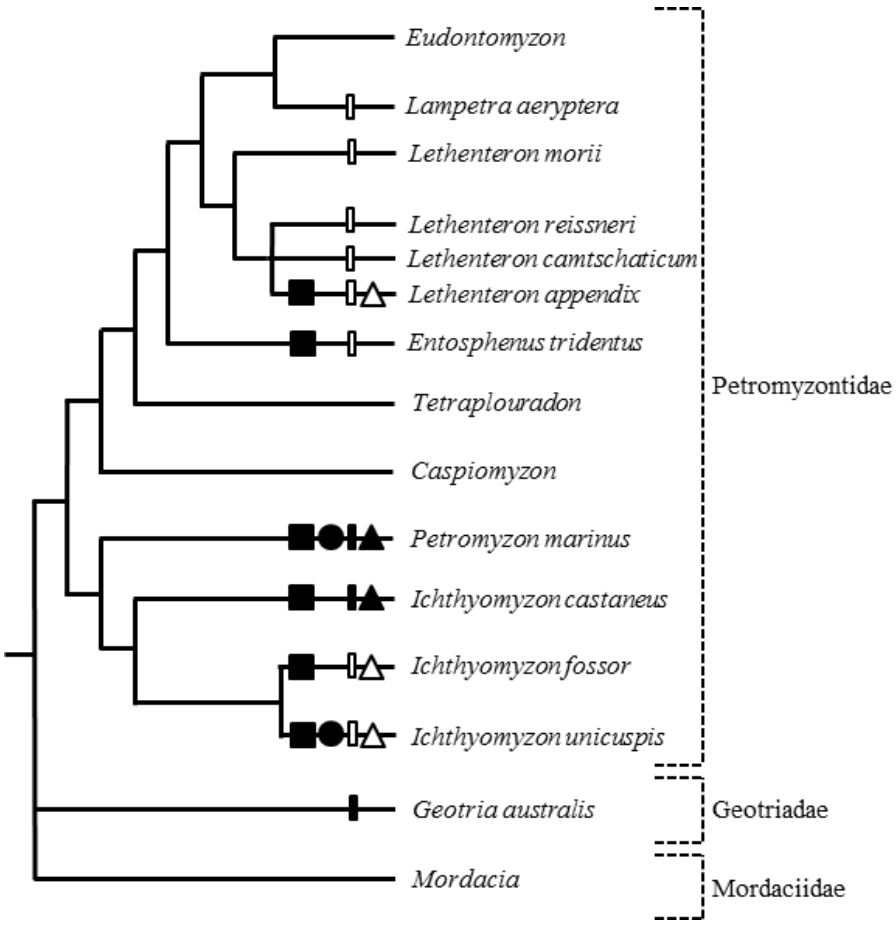


Figure 2-3. Phylogenetic tree [28] illustrating 3-keto petromyzonol sulfate (3kPZS) communication traits examined in this study. All genera of lamprey are included but species is indicated for only those species sampled. Squares represent olfactory sensitivity to 3kPZS, circles represent non-sexual preference for 3kPZS, rectangles represent male signalling with 3kPZS, and triangles represent sexual preferences for 3kPZS. Black fill indicates presence of a trait, white indicates absence of a trait. Absence of a shape indicates that data are not available for that taxon. Olfactory sensitivity of *Entosphenus tridentus* was determined in Robinson et al. [32] and Yun et al. [29]. Families of lamprey are indicated on the right.

Possible convergence on communication via 3kPZS is unlikely to translate to a broader convergence of the chemical ecology of *P. marinus* and *I. castaneus*. Polygynous mating in *P. marinus*, during which males aggressively defend nests against other males, likely places strong selective pressure on individual males to signal to females with 3kPZS, which only elicits a sexual preference response in females [44, 20]. However, *I. castaneus*, like most lampreys, are polygynandrous and spawn in large mixed-sex aggregations in which male-competition is presumably in the form of sperm investment or male-aggressiveness on the nest [45]. The odour of individual males will mix thoroughly on a spawning nest, making male competition via pheromones unlikely in polygynandrous species [20]. Communal spawning in *I. castaneus* combined with our observation of sexually monomorphic preferences for 3kPZS provides evidence that, in *I. castaneus*, 3kPZS facilitates aggregation of males and females rather than directly influencing female choice of males.

A male sexual signal that elicits responses in females and other males is a perplexing mechanism for a male to gain access to females. Nonetheless, reproductive aggregation pheromones, defined as chemical stimuli that elicit bi-sexual attraction in conspecifics, are reported in snakes [46, 47] and many arthropods [48] and hypothesized in fish [49]. Possible non-exclusive explanations for reproductive aggregation pheromones include (*i*) male exploitation of a perceptual bias present in both males and females [49], (*ii*) an agonistic function underlying male preference for male odour [46, 47], (*iii*) male exploitation of others' signals that are directed towards females and, (*iv*) increased male success with the presence of other males [48]. A perceptual bias can explain the origin of sexually monomorphic preference for 3kPZS we observed in *I. castaneus*, as both males and females undertake a migration guided by larval odour and, in part, 3kPZS [19, 30]. However, sexual selection has strengthened the preferences

of male and female *I. castaneus* past the original perceptual bias, as observed in female *P. marinus* (CO Brant, unpublished). Which selective pressures drove strengthening of sexually monomorphic preferences for 3kPZS in *I. castaneus*? Male *I. castaneus* are not reported to exhibit agonistic behaviours during spawning [45], rejecting an agonistic function underlying male preference for 3kPZS. Hence, males are likely either eavesdropping on other males in the effort to locate females or realize an increase in reproductive success when spawning with a group of males, possibly through an enhanced composite signal.

Why male *I. unicuspis* have not evolved to exploit female preference for 3kPZS remains unclear. Our hypothesis that less pressure on individual males to signal to females precludes the evolution of male signalling in polygynandrous species [20] was not supported; the only species other than *P. marinus* that was found to use 3kPZS as a sexual signal, *I. castaneus*, is a polygynandrous species. Male *I. unicuspis* would seemingly realize the same benefits of 3kPZS signalling as male *I. castaneus*, given the species share similar mating strategies (polygynandry). Considering the major divergence in life history between *I. castaneus* and *I. unicuspis*, whether parasitic adults undertake the full emigration downstream to lakes (*I. unicuspis*) or only a partial emigration downstream to higher order streams (*I. castaneus*), may hint at why *I. unicuspis* do not communicate with 3kPZS. For example, the smaller size of *I. castaneus* relative to *I. unicuspis* (~60% smaller by weight; table 1), possibly a result of preying upon smaller and less abundant stream-dwelling fishes, may place selective pressure on males to exaggerate signalling to females. Small size can constrain the traits used by males to communicate [51, 52], and result in males compensating for their size with an exaggerated signal [53]. The baseline odour of each species, before any chemical traits became exaggerated under sexual selection, was likely less potent for *I. castaneus* than *I. unicuspis* based solely on small size. While male groups of *I.*

unicuspis might have a baseline odour potent enough to guide females to nests, male *I. castaneus* may have insufficient baseline odours and in turn need to signal nest locations to females. The larger size of male *I. unicuspis* combined with the communal spawning strategy may translate into less pressure on individuals to signal to females, and result in higher investment into other areas, such as sperm production.

In conclusion, we present a rare phylogenetic comparison of pheromone signalling in vertebrates. We postulate that a sensory trap resulted in convergent evolution of a lamprey mating pheromone, but further investigations on the underlying mechanisms of 3kPZS release and detection in *P. marinus* and *I. castaneus* are needed to lend further support to convergence rather than loss in *I. unicuspis*. Our results indicate that shared constraints can be more important in shaping signal evolution than divergent selection or random processes. Finally, we suggest the chemical communication system of lampreys offers a useful system to study vertebrate chemical communication, which may result in useful tools for the management of invasive and imperilled species throughout the world [54].

ACKNOWLEDGMENTS

The U.S. Fish and Wildlife Service Marquette Biological Station, Drs. Rex Strange and Don Jellyman assisted with lamprey collection. Drs. Ugo Bussy, Ke Li, and Mar Huertas assisted with chemical analyses and electro-olfactogram recordings. Ed Benzer, Ethan Buchinger, Elizabeth Buchinger, Christopher Dean, Alex Doubek, Skye Fissette, Phillip Ganz, Hugh McMath, Jiafei Pu, Elizabeth Stieber, and Jeff Yaklin provided assistance with pheromone sampling and behavioural assays. Drs. Andrea Miehls, John Hume, and Heather Eisthen provided valuable suggestions that greatly improved the manuscript. Funding was provided by the Great Lakes Fishery Commission, the New Zealand Ministry of Business Innovation and Employment (contract CO1X1002), and Shanghai Ocean University and & Michigan State University Joint Research Centre Program (A1-0209-13-0805).

REFERENCES

REFERENCES

1. Andersson, M. B. 1994 *Sexual selection*. Princeton University Press.

2. Bradbury, J. W. & Vehrencamp, S. L. 2011 *Principles of Animal Communication*. Sinauer, Sunderland.

3. Endler, J. A. 1992 Signals, signal conditions, and the direction of evolution. *Am. Nat.* **139,** S125–S153. (10.1086/285308)

4. Endler, J. A. 1993 Some general comments on the evolution and design of animal communication systems. *Philos. Trans. R. Soc Lond. B* **340**, 215–225. (10.1098/rstb.1993.0060)

5. Endler, J. A. & Basolo, A. L. 1998 Sensory ecology, receiver biases and sexual selection. *Trends Ecol. Evol.* **13**, 415–420. (doi:10.1016/S0169-5347(98)01471-2)

6. Endler, J. A. 1984 Natural and sexual selection on color patterns in poeciliid fishes. In *Evolutionary ecology of neotropical freshwater fishes* (ed T. M. Zaret), pp. 95–111. Springer Netherlands. (10.1007/978-94-015-7682-6_7)

7. Hill, G. E., & McGraw, K. J. 2006 *Bird coloration Vol. 2: Function and evolution*. Harvard University Press, Cambridge, MA (10.1650/0010-5422(2007)109[223:bcvfae]2.0.co;2)

8. Olson, V. A. & Owens, I. P. 1998 Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.* **13**, 510–514. (10.1016/S0169-5347(98)01484-0)

9. Goodwin, T.W. 1984 *The biochemistry of carotenoids, vol. II: animals.* Chapman & Hall, New York. *(*10.1016/0014-5793(85)80904-2)

10. Hamilton, W. D., & Zuk, M. 1982 Heritable true fitness and bright birds: a role for parasites? *Science* **218**:384–387. (10.1126/science.7123238)

11. Blount, J. D., N. B. Metcalfe, T. R. Birkhead, & Surai, P. F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300*:*125–127. (10.1126/science.1082142)

12. Andersson, M. & Simmons, L. W. 2006 Sexual selection and mate choice. *Trends Ecol. Evol.* **21**, 296–302. (doi:10.1016/j.tr**e**e.2006.03.015)

13. Rodd, F. H., Hughes, K. A., Grether, G. F. & Baril, C. T. 2002 A possible non-sexual origin of mate preference: are male guppies mimicking fruit? *Proc. R. Soc. Lond. B* **269**, 475–481. (doi:10.1098/rspb.2001.1891)

14. Ninnes, C. E., & Andersson, S. 2014. Male receiver bias for red agonistic signalling in a yellow-signalling widowbird: a field experiment. *Proc. R. Soc. Lond. B* **281:** 20140971. (10.1098/rspb.2014.0971)

15. Ninnes, C. E., Adrion, M., Edelaar, P., Tella, J. L., & Andersson, S. 2015. A receiver bias for red predates the convergent evolution of red color in widowbirds and bishops. *Behav. Ecol.* **26**, 1212-1218. (10.1093/beheco/arv068)

16. Ryan, M. J. & Cummings, M. E. 2013 Perceptual biases and mate choice. *Annu. Rev. Ecol. Syst.* **44**, 437–459. (10.1146/annurev-ecolsys-110512-135901)

17. West-Eberhard, M. J. 1983. Sexual selection, social competition, and speciation. *Q. Rev. Biol.* **58**, 155-183. (10.1086/413215)

18. Wyatt TD. 2014 Pheromones and animal behavior, 2nd Edition. Cambridge, UK: Cambridge University Press.

19. Teeter, J. 1980 Pheromone communication in sea lampreys (Petromyzon marinus): implications for population management. *Can. J. Fish. Aquat. Sci.* **37**, 2123–2132. (doi:10.1139/f80-254)

20. Buchinger, T. J., Wang, H., Li, W. & Johnson, N. S. 2013 Evidence for a receiver bias underlying female preference for a male mating pheromone in sea lamprey. *Proc. R. Soc. Lond. B* **280**, 20131966. (10.1098/rspb.2013.1966)

21. Vrieze, L. A. & Sorensen, P. W. 2001 Laboratory assessment of the role of a larval pheromone and natural stream odor in spawning stream localization by migratory sea lamprey (Petromyzon marinus). *Can. J. Fish. Aquat. Sci.* **58**, 2374–2385. (10.1139/f01-179)

22. Wagner, C. M., Twohey, M. B. & Fine, J. M. 2009 Conspecific cueing in the sea lamprey: do reproductive migrations consistently follow the most intense larval odour? *Anim. Behav.* **78**, 593–599. (10.1016/j.anbehav.2009.04.027)

23. Siefkes, M. J. & Li, W. 2004 Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (Petromyzon marinus). *J. Comp. Physiol. A* **190**, 193–199. (doi:10.1007/s00359-003-0484-1)

24. Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S.-S. & Gage, D. A. 2002 Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* **296**, 138–141. (doi:10.1126/science.1067797)

25. Johnson, N. S., Yun, S.-S., Thompson, H. T., Brant, C. O. & Li, W. 2009 A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps. *Proc. Natl. Acad. Sci. USA* **106**, 1021–1026. (doi:10.1073/pnas.0808530106)

26. Johnson, N. S., Yun, S.-S., Buchinger, T. J. & Li, W. 2012 Multiple functions of a multi-component mating pheromone in sea lamprey Petromyzon marinus. *J. Fish Biol.* **80**, 538–554. (10.1111/j.1095-8649.2011.03185.x)

27. Christy, J. H. 1995 Mimicry, mate choice, and the sensory trap hypothesis. *Am. Nat.* **146,** 171–181. (doi:10.1086/285793)

28. Potter, I. C., Gill, H. S., Renaud, C. B. & Haoucher, D. 2015 The taxonomy, phylogeny, and distribution of lampreys. In *Lampreys: Biology, Conservation and Control*, (ed MF Docker) pp. 35–73. Springer, Netherlands. (10.1007/978-94-017-9306-3_2)

29. Yun, S.-S., Wildbill, A. J., Siefkes, M. J., Moser, M. L., Dittman, A. H., Corbett, S. C., Li, W., Close, D. A. & MacLatchy, D. L. 2011 Identification of putative migratory pheromones from Pacific lamprey (Lampetra tridentata). *Can. J. Fish. Aquat. Sci.* **68**, 2194–2203. (10.1139/f2011-140)

30. Brant, C. O., Li, K., Johnson, N. S. & Li, W. 2015 A pheromone outweighs temperature in influencing migration of sea lamprey. *Roy. Soc. Open Sci.* **2**, 150009. (10.1098/rsos.150009)

31. Fine, J. M., Vrieze, L. A. & Sorensen, P. W. 2004 Evidence that petromyzontid lampreys employ a common migratory pheromone that is partially comprised of bile acids. *J. Chem. Ecol.* **30**, 2091–2110. (10.1023/B:JOEC.0000048776.16091.b1)

32. Robinson, T. C., Sorensen, P. W., Bayer, J. M. & Seelye, J. G. 2009 Olfactory sensitivity of Pacific lampreys to lamprey bile acids. T. Am. Fish. Soc. **138**, 144–152. (10.1577/T07-233.1)

33. Stewart, M. & Baker, C. F. 2012 A Sensitive analytical method for quantifying petromyzonol sulfate in water as a potential tool for population monitoring of the southern pouched lamprey, Geotria Australis, in New Zealand streams. *J. Chem. Ecol.* **38**, 135–144. (10.1007/s10886-012-0069-y)

34. Renaud, C. B. 2011 *Lampreys of the world. An annotated and illustrated catalogue of lamprey species known to date.* FAO Species Catalogue for Fishery Purposes. No. 5. Rome, FAO. pp. 109

35. Siefkes, M. J., Scott, A. P., Zielinski, B., Yun, S.-S. & Li, W. 2003 Male sea lampreys, Petromyzon marinus L., excrete a sex pheromone from gill epithelia. *Biol. Reprod.* **69**, 125–132. (doi:10.1095/biolreprod.102.014472)

36. Li, K., Wang, H., Brant, C. O., Ahn, S. & Li, W. 2011 Multiplex quantification of lamprey specific bile acid derivatives in environmental water using UHPLC–MS/MS. *J. Chromatogr. B* **879**, 3879–3886. (doi:10.1016/j.jchromb.2011.10.039)

37. Xi, X., Johnson, N. S., Brant, C. O., Yun, S.-S., Chambers, K. L., Jones, A. D. & Li, W. 2011 Quantification of a male sea lamprey pheromone in tributaries of Laurentian Great Lakes by liquid chromatography–tandem mass spectrometry. *Environ. Sci. Technol.* **45**, 6437–6443. (doi:10.1021/es200416f)

38. Li, W., Sorensen, P. W. & Gallaher, D. D. 1995 The olfactory system of migratory adult sea lamprey (Petromyzon marinus) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *J. Gen. Physiol.* **105**, 569–587. (doi:10.1085/jgp.105.5.569)

39. Li, W. Olfactory biology of adult sea lamprey (Petromyzon marinus). Ph.D. Dissertation. University of Minnesota, St Paul. 1994; 184p.

40. Haslewood, G. A. and Tokes, D. L. 1969 Comparative studies of bile salts: Bile salts of the sea lamprey, Petromyzon marinus. *J.Biochem*.**114**, 179–184.

41. Caprio, J. & Byrd, R. P. 1984 Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in the catfish. *J. Gen. Physiol.* **84**, 403–422. (doi:10.1085/jgp.84.3.403)

42. Wagner W.H. 1961 Problems in the classification of ferns. In *Recent Advances in Botany*. Pp 841-844. University of Toronto Press, Montreal.

43. Piavis, G.W., Howell, J. H. & Smith, A. J. 1970 Experimental hybridization among five species of lampreys from the Great Lakes. *Copeia*. **1**: 29–37. (doi:10.2307/1441972)

44. Siefkes, M. J., Winterstein, S. R. & Li, W. 2005 Evidence that 3-keto petromyzonol sulphate specifically attracts ovulating female sea lamprey, Petromyzon marinus. *Anim. Behav.***70**, 1037–1045. (doi:10.1016/j.anbehav.2005.01.024)

45. Johnson, N. S., Buchinger, T. J. & Li, W. 2015 Reproductive ecology of lampreys. In *Lampreys: Biology, Conservation and Control*. (ed MF Docker), pp. 265–303. Springer, Netherlands. (doi:10.1007/978-94-017-9306-3_6)

46. Greene, M. J., Stark, S. L. & Mason, R. T. 2001 Pheromone trailing behavior of the brown tree snake, Boiga irregularis. *J.Chem. Ecol.* **27**, 2193–2201. (10.1023/A:1012222719126)

47. Fornasiero, S., Bresciani, E., Dendi, F. & Zuffi, M. A. 2007 Pheromone trailing in male European whip snake, Hierophis viridiflavus. *Amph-Rept.* **28**, 555–559. (doi:10.1163/156853807782152453)

48. Wertheim, B., van Baalen, E.-J. A., Dicke, M. & Vet, L. E. 2005 Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. *Annu. Rev. Entomol.* **50**, 321–346. (doi:10.1146/annurev.ento.49.061802.123329)

49. Buchinger, T. J., Li, W. & Johnson, N. S. 2015 Behavioral evidence for a role of chemoreception during reproduction in lake trout. *Can. J. Fish. Aquat. Sci.* (doi:10.1139/cjfas-2015-0351)

50. Bjerselius, R., Li, W., Teeter, J. H., Seelye, J. G., Johnsen, P. B., Maniak, P. J., Grant, G. C., Polkinghorne, C. N. & Sorensen, P. W. 2000 Direct behavioral evidence that unique bile acids released by larval sea lamprey (Petromyzon marinus) function as a migratory pheromone. *Can. J. Fish. Aquat. Sci.* **57**, 557–569.

51. Blaul, B. & Ruther, J. 2012 Body size influences male pheromone signals but not the outcome of mating contests in Nasonia vitripennis. *Anim. Behav.* **84**, 1557–1563.

52. Ryan, M. J. 1986 Factors influencing the evolution of acoustic communication: biological constraints. *Brain, Behav. Evolut.* **28**, 70–82. (doi:10.1159/000118693)

53. Bee, M. A., Perrill, S. A. & Owen, P. C. 2000 Male green frogs lower the pitch of acoustic signals in defense of territories: a possible dishonest signal of size? *Behav. Ecol.* **11**, 169 177. (doi: 10.1093/beheco/11.6.614).

54. Sorensen, P. W. 2015 Applications of pheromones in invasive fish control and fishery conservation. In *Fish Pheromones and Related Cues* (eds PW Sorensen, BD Wisenden), pp. 255–268. John Wiley & Sons. (10.1002/9781118794739.ch12)

55. Docker, M. F., Mandrak, N. E., & Heath, D. D. 2012. Contemporary gene flow between "paired" silver (Ichthyomyzon unicuspis) and northern brook (I. fossor) lampreys: implications for conservation. *Conserv. Genet.* **13**, 823-835. (10.1007/s10592-012-0332-3)

CHAPTER 3

PARTIAL OVERLAP OF A MULTI-COMPONENT MATING PHEROMONE IN LAMPREYS

ABSTRACT

Animals rely on a mosaic of complex information to find and evaluate mates. Multi-component chemical signals (pheromones) are particularly important for species-recognition in many animal species. While the evolution of species-specific pheromone blends is generally well-described in some insects, very few vertebrate pheromones have been studied in a macro-evolutionary context. Here, we report a phylogenetic comparison of the multi-component pheromone in lampreys. Chemical profiling of sexually mature males from eleven species of lamprey, representing six of ten genera and two of three families, indicated the chemical profiles of males are partially shared across species. Behavioural assays conducted with four species sympatric in the Laurentian Great Lakes indicated asymmetric female responses to heterospecific odours, where *Petromyzon marinus* were attracted to male odour collected from all species tested but other species generally preferred only the odour of conspecifics. Electro-olfactogram recordings from *P. marinus* indicated that although *P. marinus* exhibited behavioural responses to odours from males of all species, at least some of the compounds that elicited olfactory responses were different in conspecific male odours compared to heterospecific male odours. Our results indicate partial overlap of mating pheromones among lampreys, and represent a rare phylogenetic comparison of multi-component mating pheromones in a vertebrate.

INTRODUCTION

Mate search and assessment is guided by a mosaic of multi-modal and -component information originating from potential mates (Andersson, 1994; Candolin, 2003; Bradbury and Vehrencamp, 2011). Individual cues within a complex display can provide redundant or distinct information (Bradbury and Vehrencamp, 2011). Multiple sources of information can be important for species-recognition, as common ancestry and selective pressures can result in particular traits being important for mate choice across species (Pfennig, 1998; Candolin, 2003). For example, size is an important trait for mate choice in several swordtail species (*Xiphophorus phygmaeus* and *X. nigrensis*), but olfactory cues guide species recognition (Hankison and Morris, 2002; Crapon de Camprona and Ryan, 1990). Complex sexual signals that mediate species-recognition can be particularly important in closely related sympatric species (Gerhardt, 1994; Höbel and Gerhardt, 2007). However, even partial overlap in the traits involved in mate search and choice can result in potentially lower fitness through reproductive interference (Crapon de Camprona and Ryan, 1990; Gröning and Hochkirch, 2008).

Chemical communication is often considered to employ signals that mediate species recognition (Endler 1993, Wyatt 2014). Pheromones, defined as chemicals that elicit a specific reaction when detected by conspecifics (Karlson and Luscher, 1959), are often comprised of species-specific blends of multiple components (Wyatt 2014). Species-specific pheromone blends are hypothesized to evolve through either gradual transitions or major "saltational" shifts (Symonds and Elgar, 2007), which will result in different degrees of pheromone overlap between species. For example, the major component of the pheromone blend in scarab beetles (*Anomala*

albopilosa and *A. cuprea)* is shared between species, while minor components are species-specific (Leal et al., 1996). In contrast, pheromone blends of closely related bark beetles (*Dendroctonus* and *Ips* sp.) are equally as different in distantly and closely related species (Symonds and Elgar, 2004). While the evolution of species-specific pheromone blends in insects is increasingly well-described (Symonds and Elgar, 2007; Steiger et al., 2011), similar macroevolutionary studies of vertebrate pheromones are underrepresented (Symonds and Elgar, 2007).

Petromyzon marinus is a jawless vertebrate that uses a multi-component pheromone during reproduction (Teeter et al., 1980; Li et al., 2002; Johnson et al., 2012). *P. marinus* resides in streams as juveniles for several years, emigrate to the Laurentian Great Lakes or Atlantic Ocean to parasitize on fish, and return to streams for their single spawning season. The odours of stream-resident larvae guide adult *P. marinus* during their migration from lakes or the Atlantic Ocean into spawning streams (Teeter, 1980; Vrieze and Sorensen, 2001). Upon reaching the final stages of sexual maturation, males construct nests and signal to females with an odour that elicits upstream movement and nesting behaviours in females (Li et al., 2002; Johnson et al., 2009; 2012). The major component 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate (3-keto petromyzonol sulfate, 3kPZS) guides female movement over long-distances to the nest (Li et al., 2002; Johnson et al., 2009). Additional minor components that retain females in the area of the nest and elicit nesting behaviours remain unidentified (Johnson et al., 2012), but may include 3,12-diketo-4,6-petromyzonene-24-sulfate (DKPES; Li et al., 2013) and petromyzestrosterol (Li et al., 2012).

The chemical cues and pheromones of *P. marinus* appear partially conserved across the Petromyzontiformes. Many of the 41 species of lamprey occur in sympatry with one or more

other species (Potter et al., 2015). The larval-released migratory cue appears to be an unspecialized metabolite that is conserved across lamprey species (Fine et al., 2004; Yun et al., 2003; Yun et al., 2011; Robinson et al., 2009; Buchinger et al., 2013). Similar habitat preferences for rearing and spawning conceivably preclude strong selective pressure for a species-specific migratory cue (Dawson et al., 2015; Johnson et al., 2015). In contrast, sexual signalling with the major component 3KPZS appears restricted to *P. marinus* and *Ichthyomyzon castaneus* (chapter 2). However, frequent observations of heterospecific spawning nests indicate unknown components of the pheromone blend may be partially shared among species (Cochran et al., 2008; Johnson et al., 2015).

Here, we report a phylogenetic comparison of male odours across lampreys. Based upon observations of heterospecific mating events, we hypothesized that male pheromone components exhibit partial overlap between species. According to our hypothesis, we predicted 1) partial overlap of the chemical profiles of sexually mature males, 2) behavioural responses of females for the odour of both conspecific and heterospecific males, and 3) olfactory sensitivity to conspecific and heterospecific male odours. We determined 1) the chemical profiles across eleven species of lamprey, 2) the female responses to conspecific and heterospecific male odours in species sympatric in the Laurentian Great Lakes, and 3) the olfactory responses of *P. marinus* to male odours of species sympatric in the Laurentian Great Lakes. Our results indicate partial overlap the pheromone blend among species, and represent a rare phylogenetic comparison of multi-component mating pheromones in a vertebrate.

METHODS

Experimental animals

Use of experimental animals and approaches were approved by the Michigan State University's Animal Use and Care Committee (Approval #'s 4/10-043-00, 02-13-040-00). Lampreys were collected using U.S. Fish and Wildlife Service sea lamprey control traps, backpack electroshocking, fyke nets, and by dip-nets (Table 3-1). Classification of species followed Renaude (2011). Sexual maturation was evaluated by the expression of eggs (ovulation) or milt (spermiation) upon gentle manual pressure (Siefkes et al., 2003). Chemical profiling was completed in 11 species because the release of chemicals as possible odorants was most important per our hypothesis and more logistically feasible than behavioural and olfactory assays.

Male chemical profiles

Complete chemical profiles of sexually mature males were determined for eleven species of lamprey from six genera and two families (Table 3-1; chapter 2). Odours were sampled by collecting the holding waters from individual sexually mature males (Buchinger et al., 2013). Samples were also collected from sexually immature males for comparison, except in *P. marinus, I. castaneus*, and *Lampetra aeryptera*, due to unavailability of experimental animals. A single male was held in 5 L of aerated deionised water for 2 hr, after which 10 ml of water was sampled, and stored at less than -20 °C for subsequent analysis. Six replicates were sampled, except for sexually mature *Geotria australis* (n=4), *I. castaneus* (n=5), and sexually immature *Entosphenus tridentus* (n=3; Table 3-1). Admittedly, chemical profiling does not directly

implicate species similarities or differences in the compounds that are behaviourally active pheromones. However, chemical profiling reveals which compounds are in the water and available to the female olfactory system. Furthermore, our comparison was limited to compounds released by sexually mature males by contrasting profiles of sexually mature males against immature males, and hence the observed chemical profiles include likely candidates for chemical cues.

Table 3-1. Pheromone sampling from males in eleven species of Petromyzontiformes. Weight and length represent the mean weight and length, and se is the standard error of the mean.

genus	species	maturity	location	N	weight (se)	length (se)	collection method
Geotria	*australis*	mature	Canterbury, NZ	4	164.36 (22.21)	488.25 (26.21)	hand
		immature	Southland, NZ	6	149.10 (3.07)	564.50 (4.29)	net
Ichthyomyzon	*unicuspis*	mature	Michigan, USA	6	41.22 (4.72)	248.67 (7.94)	trap
		immature	Michigan, USA	6	47.76 (4.21)	270.00 (10.61)	trap
	fossor	mature	Michigan, USA	6	2.90 (0.22)	113.50 (1.73)	electrofishing
		immature	Michigan, USA	6	5.43 (0.73)	126.17 (4.11)	electrofishing
	castaneus	mature	Michigan, USA	5	33.26 (3.00)	239.4 (9.74)	hand/trap
Petromyzon	*marinus*	mature	Michigan, USA	6	177.83 (19.15)	443.83 (10.98)	trap
Entosphenus	*tridentus*	mature	Oregon, USA	6	336.83 (18.05)	554.33 (11.16)	net
		immature	Oregon, USA	3	392.33 (20.63)	578.33 (9.21)	net
Lethenteron	*appendix*	mature	Michigan, USA	6	3.94 (0.17)	137.17 (1.19)	hand
		immature	Michigan, USA	6	4.59 (0.59)	149.00 (6.57)	hand
	camtschaticum	mature	Jilin, China	6	101.09 (12.03)	390.00 (12.96)	net
	reisneri	mature	Liaoning, China	6	7.04 (0.82)	167.83 (7.25)	electrofishing
	morii	mature	Liaoning, China	6	28.49 (1.13)	261.83 (3.85)	net
		immature	Liaoning, China	6	37.16 (3.65)	288.00 (6.05)	net
Lampetra	*aeryptera*	mature	Indiana, USA	6	7.51 (0.68)	156.50 (4.04)	electrofishing

Complete chemical profiles were determined using ultra-performance liquid chromatography (UHPLC) and high resolution mass spectrometry (HRMS). Water samples were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co. Missouri, USA) and reconstituted in 50% HPLC-grade methanol. Aliquots (10 µL) of concentrated water samples were injected into a Waters Acquity UPLC coupled to a Xevo G2-S^{TM} Q-Tof system (Waters Corporation, Milford, MA, USA). Metabolites were separated using an ACQUITY C_{18} BEH UPLC column (2.1 × 100 mm, 1.7 µm particle size; Waters Corporation; 30 ^{o}C), with a mobile phase of acetonitrile (A) and water (B). The gradient elution used a flow rate of 300 µL/min for 10 min and the following gradient program: 20% A for 1 min; increased to 100% A from 1 to 7 min; maintained at 100% A from 7.01 to 9.0 min; decreased to 20% A at 9.01 and maintained for 10 min until column equilibrium. The needle was washed with 80% methanol twice after each injection to prevent cross-contamination of samples. Mass spectrometry was performed on negative electrospray ionization mode. A full scan MS analysis of samples was conducted by recording spectra with mass to charge rations (*m/z*) between 100-1000, and with a resolution of +/- 0.05 Da. Nitrogen gas was used as the desolvation gas (600 $L \cdot h^{-1}$) and the cone gas (50 $L \cdot h^{-1}$). Argon gas was used as the collision gas at a pressure of 5.3×10^{-5} Torr. The source and desolvation temperatures were 102 and 400 ^{o}C, respectively. The cone voltage and capillary voltage were set to 30 V and 2.8 kV, respectively. The collision energies for collision-induced dissociation were 5 and 40 eV for the MS spectrum and MS/MS spectrum, respectively. The scan time was set at 0.2 s, with an interscan delay of 0.5 s. The LockSpray^{TM} dual electrospray ion source with internal references used for these experiments was leucine enkephalin at a concentration of 100 ng/ml. Lock-mass calibrations at *m/z* 554.2615 in negative ion mode were used for the complete analysis. UHPLC HRMS yielded a list of intensities of the

detected peaks identified by the corresponding retention times and mass data pairs. The ion intensities for each peak detected were then normalized within each sample by the sum of the peak intensities in that sample, with a total intensity of 10,000. Hence, the end metric for each peak is a magnitude relative to the other peaks in the sample, out of 10,000.

The chemical profiles of males were filtered by eliminating peaks that had a normalized peak intensity less than 10 (<0.1% of the total peak intensity). The remaining peaks were compared against a control group. Controls were samples collected from sexually immature males of each species, except for *P. marinus*, and *L. aeryptera* which were compared to blank water, *I. castaneus* which was compared to sexually immature male *I. unicuspis*, and *L. camtschaticum* and *L. reisneri* which were compared to sexually immature males of the closely related *L. morii*. The proportional intensities of each peak were arcsine squareroot transformed to meet assumptions of the distribution and differences between the peaks in male samples and control samples were evaluated using one-way t-tests ($\alpha = 0.1$).We did not control for multiple comparisons with a post-hoc adjustment because our goal was a conservative removal of peaks that were detected in control and mature male samples. A multivariate factor analysis was conducted to determine if species could explain variation in the detected peaks. The *factanal()* function in R was used to reduce peaks to factors (R-core team, 2014). The number of factors to extract was determined using a Scree plot. A multivariate analysis of variance (MANOVA; $\alpha = 0.05$) was used to determine if there was a difference in each factor across species and post-hoc t-tests with a Benjamini and Hochberg (1995) adjustment were used to evaluate differences between species ($\alpha = 0.05$).

UHPLC-HRMS provides the relative intensity of peaks with a given retention time and mass-charge (*m/z*) ratio, but does not provide structural information about the peak or allow a

particular peak to be attributed to specific compounds. Hence, UHPLC-HRMS allows us to test our hypothesis that species exhibit overlap in male chemical profiles, but does not identify the specific compounds released by males.

Behavioural responses to male odours

Two-choice behavioural assays were used to evaluate female responses to conspecific and heterospecific male odours when compared to no odour (Siefkes et al., 2005; Buchinger et al., 2013; chapter 2). Behavioural responses were evaluated for sympatric *I. unicuspis*, *I. fossor*, *P.marinus*, and *L. appendix* based upon availability. Experimental assays were constructed adjacent to the Little Oqcueoc River, Presque Isle County, Michigan, USA in June and July 2012 and 2013, and the upper Oqcueoc River Presque Isle County, Michigan, USA in July 2014, and supplied with river water. The Oqcueoc River system was selected for use during behavioural assays because a barrier prevents colonization by lampreys, which allows for water void of lamprey pheromones. Experimental apparatus for each respective species were based upon the design used for *P. marinus* (Siefkes et al., 2005) but approximately scaled for size-differences between species (Figure 3-1; Buchinger et al., 2013; chapter 2).

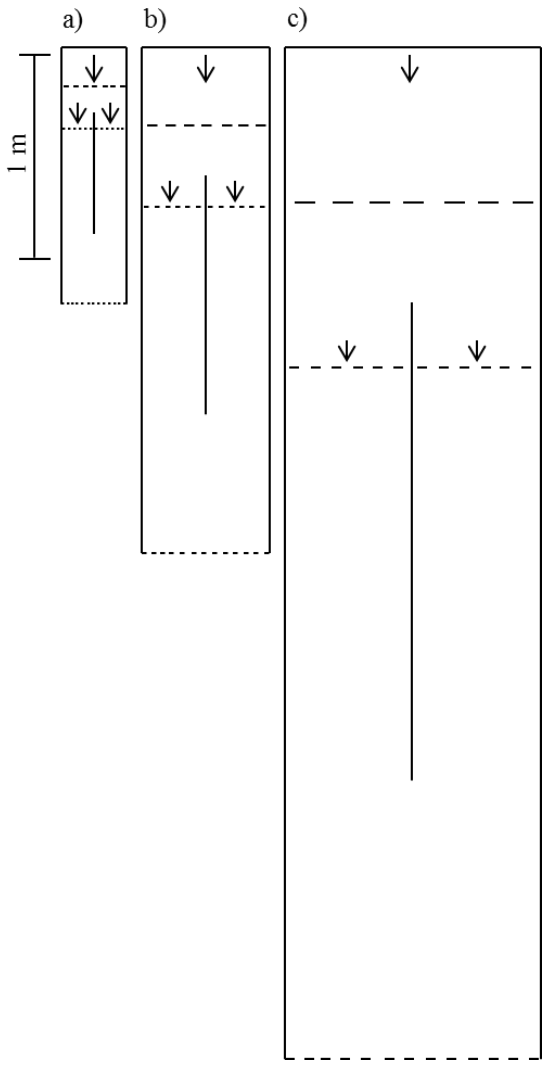


Figure 3-1. Behavioural assays used to evaluate female responses to conspecific and heterospecific odours. The design and methods similar to (Li et al., 2002), but dimensions were adjusted based upon the size of the test species (Buchinger., 2013). a) Assay used to evaluate responses of *Ichthyomyzon fossor* and *Lethenteron appendix*. b) Assay used to evaluate responses of *Ichthyomyzon unicuspis*. c) Assay used to evaluate responses of *Petromyzon marinus*. Arrows denote the direction of flow.

An experiment began when a single lamprey was placed into the downstream end of the flume. Following a 5 min acclimation, the time a lamprey spent in each channel was recorded while no odour was applied to either side. After 10 minutes of recording, an odour was introduced to one channel for 5 min without recording the lamprey's behaviour. Lastly, the time spent in each channel was recorded for 10 minutes while an odour was applied to one channel. Odours were collected from sexually mature conspecific and heterospecific male lampreys. Immediately prior to an experiment, donor males were held in 3 L of aerated river water for 15 min. Conspecific odours were collected from a group of 4 males for all species. Females do not exhibit a preference for the odour of several males verses the odour of a single male at a similar concentration (Luehring 2007). The number of heterospecific donor males was adjusted proportionally based upon the experimental species and hence the size of the apparatus (Table 3-2). For example, responses of *I. unicuspis* for male *P. marinus* odours were tested in an apparatus half the size of that used for *P. marinus* with two male *P. marinus* as odour donors. Standardizing heterospecific odours by weight, a common approach in chemical ecology, may not be meaningful because of the large differences in weight between species. For example, the equivalent weight of four *P. marinus* requires an ecologically irrelevant 260 *I. fossor* or *L. appendix*. Hence, the odour concentrations used may differ in concentration by some undeterminable amount, but no other method of standardization was appropriate, and the method used creates ecologically relevant concentrations. After recording the time spent in the control and experimental channels before odour application (bc, be), and after odour application (ac, ae), an index of preference (i) was calculated for each test ($i = [ae/(ae + be) - ac/(ac + bc)]$). The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$; Li et al., 2002).

Table 3-2. Details on the experimental assay and odour sources for behavioural assays used to evaluate female attraction to conspecific and heterospecific male odours. test species = the species being observed for behavioural responses. assay = the assay size used to evaluate responses, adjusted for size of focal species. Letters correspond to one of three assays (Figure 3-1). odour species = the species from which male odours were collected. number of males = the total number of males used to collect odours, adjusted for size of donor species.

test species	assay	odour species	number of males
Icthyomyzon unicuspis	b	*I. unicuspis*	4
		I. fossor	8
		I. castaneus	4
		P.marinus	2
		L. appendix	8
I. fossor	a	*I. fossor*	4
		I. unicuspis	2
		I. castaneus	2
		P.marinus	1
		L. appendix	4
Petromyzon marinus	c	*P.marinus*	4
		I. unicuspis	8
		I. fossor	16
		I. castaneus	8
		L. appendix	16
Lethenteron appendix	a	*L. appendix*	4
		I. unicuspis	2
		I. fossor	4
		I. castaneus	2
		P.marinus	1

Olfactory responses to male odours

Electro-olfactogram (EOG) recordings from *P. marinus* were used to determine if male odours from sympatric lampreys elicited olfactory responses, and if the olfactory mechanisms used were the same for odours from different species (Li et al., 1995). Responses to male odours from *P. marinus*, *I. unicuspis*, *I. fossor*, *I. castaneus*, and *L. appendix* were recorded. For EOG recordings, a sexually immature male or female lamprey was anesthetized using 3-aminobenzoic acid ethyl ester (50mg l^{-1}; MS222; Sigma), immobilized with an intra-muscular injection of gallamine triethiodide (1mg kg^{-1}; Sigma), and secured in a Plexiglas trough while their gills were continuously perfused with aerated water containing anaesthetic. Immature lamprey were used because measurement of olfactory sensitivity with EOG recordings becomes less robust as lampreys become sexually mature (Li, 1994), likely a result of lamprey nearing the end of their life. A recording electrode placed between olfactory lamellae and a reference electrode placed on the skin recorded olfactory responses. Electrical signals were filtered and amplified using a NeuroLog filter and pre-amplifier (Digitimer Ltd., Hertfordshire, England), integrated using an Axon Instruments Digidata system (Molecular Devices, CA, USA), and stored and processed on a PC with Axon Instruments Axoscope software.

Male odours collected with the same methods as chemical profiling were used to determine olfactory responses of *P. marinus* to heterospecifics. After holding individual males in 5 L for 2 hr, 1 L of water was collected and stored at less than -20 °C. Samples were freeze-dried using a FreeZone Plus freeze dry system with a bulk tray dryer (Labconco Co. Missouri, USA), the bottle rinsed with 20 ml methanol, evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co. Missouri, USA), and reconstituted in 1 ml 50% methanol (v:v). Samples were pooled across 3 males within each species before use in experiments.

Sensitivity to conspecific and heterospecific male odours was evaluated by determining concentration-response curves to pooled samples. The concentration of male odour in the original 1L sample was recreated by diluting 10 µl of the 1 ml sample in 10 ml water, which was then serially diluted down to a $1:10^7$ dilution. The lowest concentration at which responses to a stimulus were significantly larger than those to the control (paired t-test, $\alpha = 0.05$) was considered to be the threshold of detection (Siefkes and Li, 2004). Concentration-response curves were determined for 6 individuals. The order in which lamprey were exposed to the odours of each species were randomized. Responses were normalized by the response to L-arginine at $1x10^{-5}$M (Siefkes and Li 2004).

The specificity with which *P. marinus* detect conspecific and heterospecific odours was determined using cross adaptation experiments (Caprio and Byrd, 1984). Cross adaptation experiments record the responses to a stimulus while the epithelium is saturated with second stimulus (the adapting stimulus). Cross-adaptation experiments were conducted on 5 individuals. Experiments were conducted with concentrations that were equipotent across stimuli, as determined in preliminary experiments (*P. marinus* = $1:10^2$, *I. castaneus* = 1:10, *L. appendix* = 1:5, *I. unicuspis* = 1:1, and *I. fossor* = 1:1). The experiment began by recording the response to the adapting stimulus. While saturated with the odour of *P. marinus*, the olfactory epithelium of a fish was exposed to 1) 2x the odour of *P. marinus* (self-adapted control; SAC), 2) 1x *P. marinus* odour + 1x *I. unicuspis* odour, 3) 1x *P. marinus* odour + 1x *I. fossor* odour, 4) 1x *P. marinus* odour + 1x *I. castaneus* odour, and 5) 1x *P. marinus* odour + 1x *L. appendix* odour. Second, while saturated with the odour of each individual heterospecific species, the olfactory epithelium of a fish was exposed to 1) 2x the heterospecific odour, 2) 1x the heterospecific odour + 1x *P. marinus* odour. The responses to the SAC and the Mix were normalized by the response

to the adapting stimulus, and evaluated for differences by an ANOVA and paired t-tests ($\alpha = 0.05$). A difference between the response to the SAC and the Mix indicates the odours are detected by distinct olfactory mechanisms.

RESULTS

Male odour profiles

The chemical profiles of sexually mature males were partially shared among species (Figure 3-2). Chemical profiling yielded 317 peaks in male odours across all species. Of the 317 peaks, 67 were detected at a relative concentration of 0.1% in more than 1 individual per species. The 67 peaks were further filtered by a conservative removal of peaks detected at similar or lower magnitudes in control samples (one-way t-test, α=0.1). Additional peaks possibly detected at a magnitude greater than in the control (one-way t-test, α=0.15) were retained in *E. tridentus* due to the small sample size for immature males (n = 3) and the resultantly low power. In total, chemical profiling yielded 50 peaks that were at least moderately higher in sexually mature male water samples. Each species had at least 1 unique peak in the highest 3 compared to other species' highest 3 peaks. However, the highest 3 peaks within each species were detected at a relative magnitude of 0.1% or higher in at least one other species (Figure 3-2). The factor analysis reduced the 50 peaks to 5 factors that explained a total of 33.1% of the variance between species (Table 3-3, Figure 3-3). Factors 1- 3 were significantly different among species (MANOVA, $p < 0.05$; Table 3-3), while factors 1 and 2 were not (MANOVA, $p > 0.05$; Table 3-3). Between-species comparisons indicate Factor 1 differentiated *G. australis* from all other species, but differences in other factors were not clearly differentiated based upon phylogenetic relationships (Table 3-3). Taken together, the results indicate that some of the major constituents of male odour may be species-specific, but the whole odour but exhibits substantial overlap in the chemical profiles.

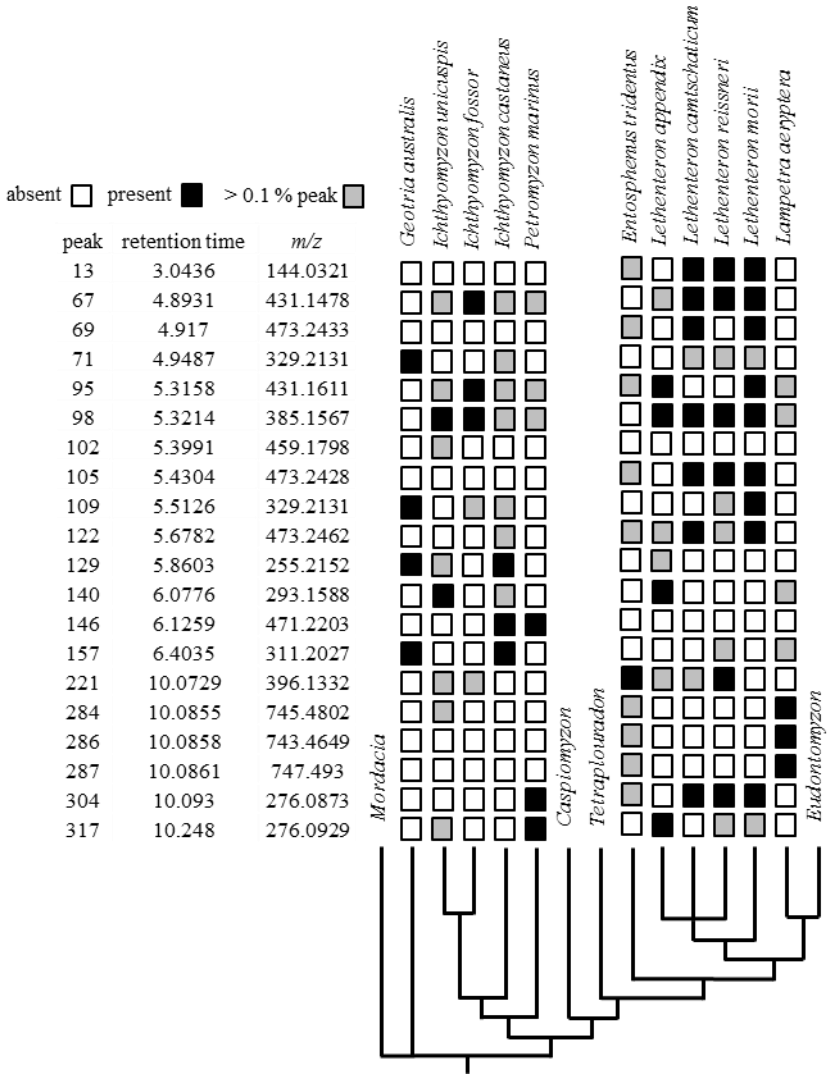


Figure 3-2. Phylogenetic tree (Potter et al., 2015) illustrating the distribution of the highest three peaks from each individual species. The highest three peaks for each individual species different between mature male samples and control samples are presented (peak). Retention time and the mass to charge ratio (*m/z*) are unique identifiers of a signal, but cannot be used to conclusively identify a compound as our method does not provide information on chemical structure. Black boxes represent peaks that had intensities significantly higher than the control (one-way t-test, $\alpha = 0.1$). Grey boxes represent peaks that had intensities that were not significantly higher than the control, but had an average intensity higher than 10 (0.1% of the total peak intensity). White boxes represent peaks that were not significantly different from the control or were detected at an average intensity less than 10. In total, 67 peaks had magnitudes greater than 0.1% the total peak area.

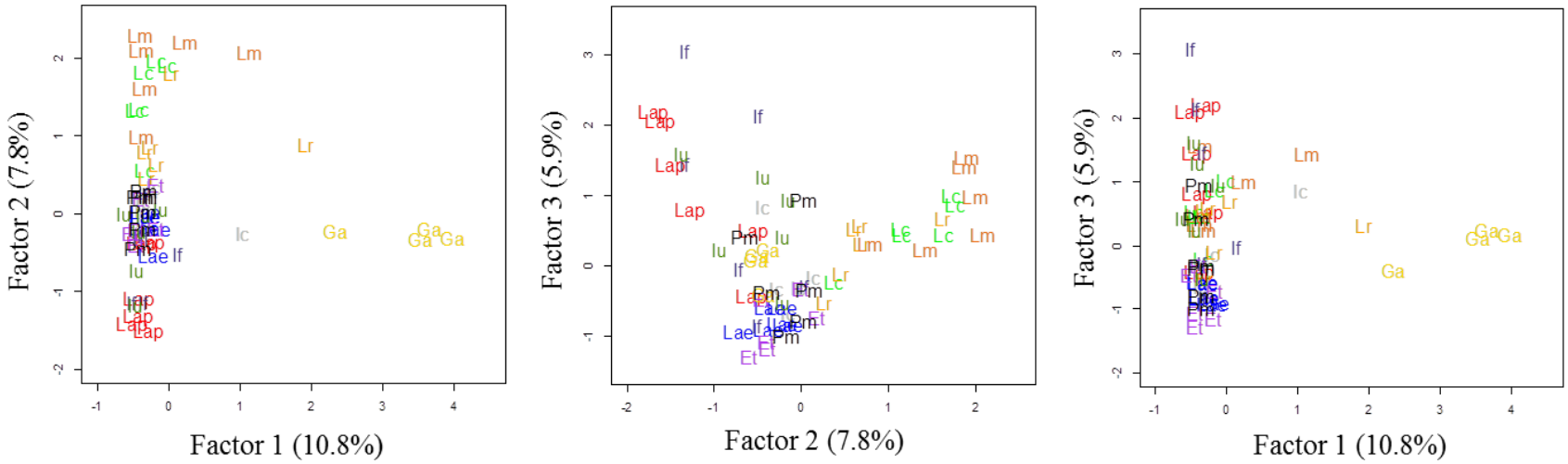


Figure 3-3. Results from the factor analysis on 50 peaks found to have higher intensities in sexually mature male water samples compared to controls. A multivariate analysis of variance indicated differences between species for factors 1, 2, and 3 ($p < 0.001$). species abbreviations = *Ga*: *Geotria australis*, *Iu*: *Ichthyomyzon unicuspis*, *If*: *I. fossor*, *Ic*: *I. castaneus*, *Pm*: *Petromyzon marinus*, *Et*: *Entosphenus tridentus*, *Lap*: *Lethenteron appendix*, *Lc*: *L. camtschaticum*, *Lr*: *L. resneri*, *Lm*: *L. morii*, *Lae*: *Lampetra aepyptera*.

Table 3-3 Results from the factor analysis on 50 peaks found to have higher intensities in sexually mature male water samples compared to controls. A scree plot indicated 5 factors should be retained. loadings = the 3 peaks with the most influence (loading) on each factor. variance = the proportion of variance explained by each factor. species effect = significance of species effects on factor scores as determined using a multivariate analysis of variance (MANOVA). species overlap = grouping of species based up factor scores as determined using pairwise t-tests followed by a Benjamini and Hochberg (1995) adjustment (α=0.05). species abbreviations = *Ga*: *Geotria australis*, *Iu*: *Ichthyomyzon unicuspis*, *If*: *I. fossor*, *Ic*: *I. castaneus*, *Pm*: *Petromyzon marinus*, *Et*: *Entosphenus tridentus*, *Lap*: *Lethenteron appendix*, *Lc*: *L. camtschaticum*, *Lr*: *L. resneri*, *Lm*: *L. morii*, *Lae*: *Lampetra aeryptera*.

factor	loadings		variance	species effect		species overlap			
	peaks	loading		F (ndf,ddf)	p-value	group a	group b	group c	group d
1	109	0.965	0.108	27.80 (10,52)	<0.001	*Iu, If, Ic, Pm, Et, Lap, Lc, Lr, Lm, Lae*	*Ga*		
	71	0.946							
	83	0.916							
2	13	0.745	0.078	26.554 (10,52)	<0.001	*Ga, Iu, If, Ic, Pm, Et, Lae*	*Ga, Iu, If, Lap*	*Lc, Lr*	*Lc, Lm*
	105	0.674							
	63	0.56							
3	98	0.721	0.059	4.712 (10,52)	<0.001	*Ga, Iu, If, Ic, Pm, Lap, Lc, Lr, Lm*	*Ga, Iu, Ic, Pm, Lc, Lr, Lae*	*Ic, Pm, Et, Lc, Lr, Lae*	
	67	0.718							
	95	0.443							
4	129	0.992	0.044	1.153 (10,52)	0.3432	NA			
	147	0.589							
	153	0.395							
5	69	0.773	0.043	1.572 (10,52)	0.1416	NA			
	82	0.641							
	105	0.482							

Behavioural responses to male odours

Female responses to male odours were partially species-specific (Figure 3-4). Females of every species tested responded to conspecific male odours, indicating a common role of male-released mating pheromones (Wilcoxen signed-rank tests, $p < 0.05$). Female *L. appendix* showed no response heterospecific odours compared to no odour. Female *I. unicuspis* and *I. fossor* were attracted to the odour of males from the each other, but not the odour of male *I. castaneus*, *P. marinus* or *L. appendix*. Female *P. marinus* were attracted to male odours from all species tested (Wilcoxen signed-rank tests, $p < 0.05$; Figure 3-4).

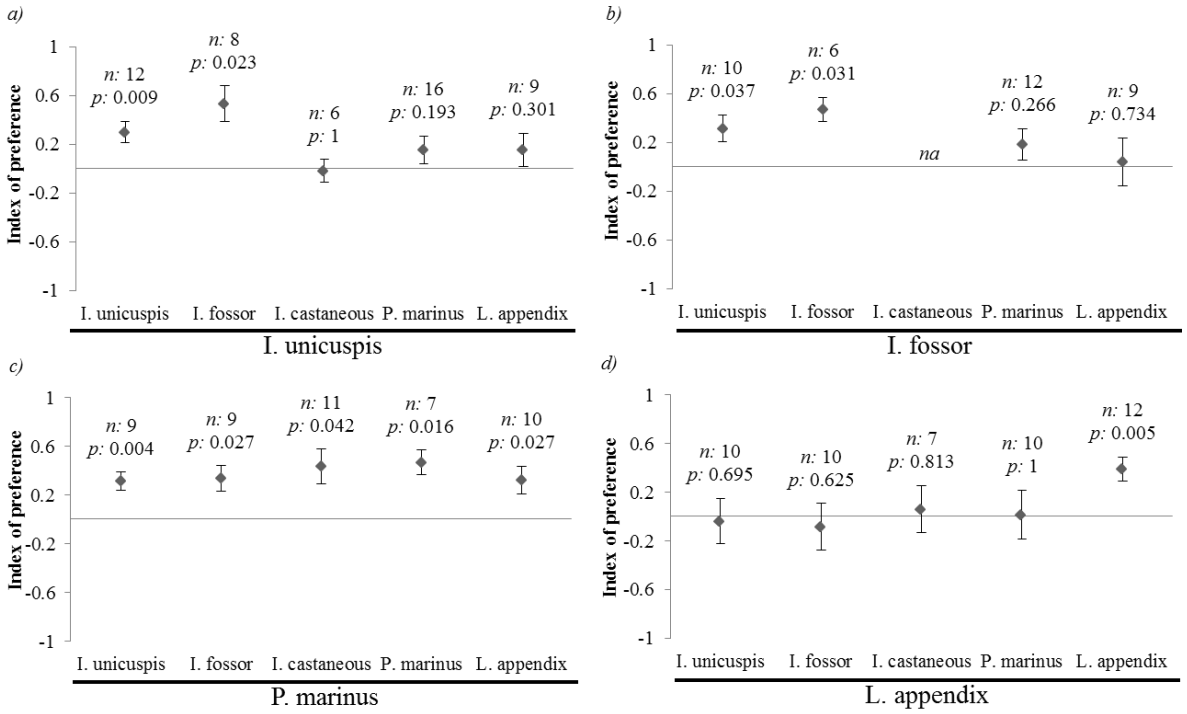


Figure 3-4. Behavioural responses of female lampreys to conspecific and heterospecific male odours as determined using two-choice behavioural assays comparing male odour to no odour. Index of preference = [ae/(ae + be) - ac/(ac + bc)], where bc is the time spent in the control channel before odour was applied, be is the time spent in the experimental channel before odour was applied, ac is the time spent in the control channel after odour was applied and ae is the time spent in the experimental channel after odour was applied. p-values were determined using a Wilcoxon signed-rank test ($\alpha = 0.05$). n = the number of individuals tested for each experiment. a) responses of *Ichthyomyzon unicuspis* to *I. unicuspis*, *I. fossor*, *I. castaneus*, *Lethenteron appendix*, and *Petromyzon marinus*. b) responses of *I. fossor* to *I. unicuspis*, *I. fossor*, *L. appendix*, and *P. marinus*. c) responses of *P. marinus* to *I. unicuspis*, *I. fossor*, *I. castaneus*, *L.appendix*, and *P. marinus*. d) responses of *L. appendix* to *I. unicuspis*, *I. fossor*, *I. castaneus*, *L. appendix*, and *P. marinus*.

Olfactory responses to male odours

Olfactory responses of *P. marinus* were different depending upon the species of male odour donors (Figures 3-5 & 3-6). Responses to the L-arginine standard and the control were similar to previous reports (mean ± se: L-arginine = 2.25 ± 0.41mV; control = 0.18 ± 0.03mV; Siefkes and Li, 2004). Concentration-response curves for conspecifc and heterospecific odours were exponentially shaped (Figure 3-5). The detection thresholds for male odours from *P. marinus* and *I. castaneus* were $1{:}10^4$, and1:10 for *I. unicuspis*, *I. fossor*, and *L. appendix* (paired t-tests, $p < 0.05$). Cross-adaptation experiments indicated male odours from each species were at least partially distinct odours. During cross-adaptation experiments, the olfactory epithelium was saturated with conspecific or heterospecific odours, and olfactory responses to 1) 2x the adapting stimulus (SAC) and 2) the adapting stimulus + a second odour were measured. Adaptation to conspecific odours did not diminish the response to heterospecific male odours; responses to the SAC remained different from the Mix for odours from all species ($F_{4,20} = 4.167$, $p = 0.013$, paired t-tests $p < 0.05$; figure 6). Likewise, adaptation to heterospecific odours did not diminish the responses to conspecific male odours (paired t-tests $p < 0.05$; figure 6). Taken together, concentration-response and cross-adaptation experiments indicate that the potency and, at least partially, the identities of the odorants that comprise male odours differ among species.

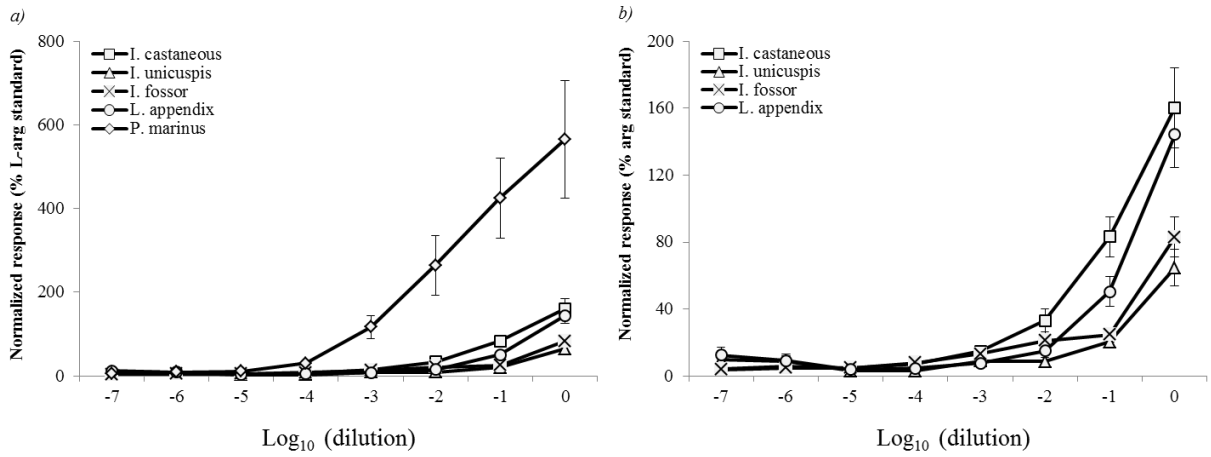


Figure 3-5. Concentration-response curves of *Petromyzon marinus* to male odours collected from *P. marinus, Ichthyomyzon unicuspis*, *I. fossor*, *I. castaneus,* and *Lethenteron appendix*. Concentration-response relationships presented as semi-logarithmic plots with responses presented as a percentage of L-arginine at $1x10^{-5}$M. a) concentration-response curves of all species tested. b) concentration-response curves of heterospecific male odours. Concentration-response curves were determined for 6 individuals.

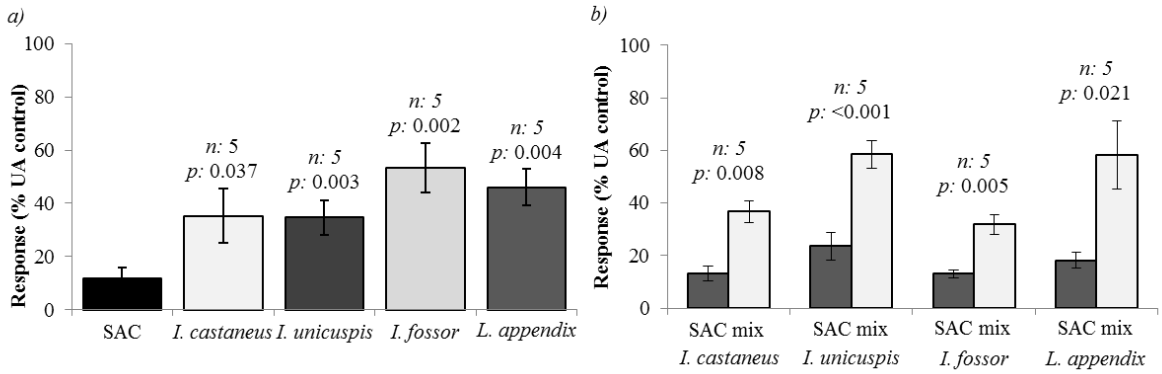


Figure 3-6. Results from cross-adaptation experiments on *Petromyzon marinus* with odours collected from *P. marinus, Ichthyomyzon unicuspis, I. fossor, I. castaneus,* and *Lethenteron appendix*. Results presented as a percentage of the unadapted response. a) SAC = self-adapted control, *I. castaneus = I. castaneus + P. marinus, I. unicuspis = I. unicuspis + P. marinus, I. fossor = I. fossor + P. marinus, L.appendix = L.appendix + P. marinus*. b) SAC = self-adapted control, mix = adapted stimuli + *P. marinus*. The species' names below represent the adapting stimuli. Significant differences from the SAC were determined with paired t-tests ($\alpha = 0.05$).

DISCUSSION

We present evidence that the male odour of *P. marinus* is partially shared among lamprey species. Chemical profiles from all species exhibited some level of overlap with other species. The factor analysis hinted at discrimination of species based upon chemical profiles but explained little of the observed variance, indicating substantial overlap in the chemicals released by males of each species. In two-choice behavioural assays, females of all species responded to conspecific male odours, but only *P. marinus* responded to odours from males of all heterospecific species. Electro-olfactogram recordings indicated that *P. marinus* detect at least a subset of the odorants released by conspecific and heterospecific males with distinct receptor mechanisms despite the observed behavioural responses to male odours from all species. Taken together, the chemical profiling, behavioural assays, and electro-olfactogram recordings support our hypothesis that male odours exhibit partial overlap across lamprey species.

The ecological context of our results should be considered bearing several caveats. First, chemical profiling and EOG recordings do not directly translate into evidence for pheromone activity. The peaks detected are likely to be specific to sexually mature males as chemical profiles were first compared to sexually immature males. However, release of a compound by a sexually mature male provides females the opportunity to detect a compound, but does not directly implicate pheromone function. Similarly, the physiological ability to detect an odour, such as that determined by EOG recordings, does not implicate the odour elicits a behavioural response. For example, *P. marinus* sensitivity to 3-keto allocholic acid (3kACA; Siefkes and Li, 2004) does not translate into a behavioural response (Johnson et al., 2012). Second, behavioural

responses to pheromones in the laboratory can be different from those in natural environments (Johnson and Li, 2010). The observed responses or lack of responses to odours in our two-choice assays may not reflect female responses or lack of responses in a natural context. For example, our assays evaluated female responses in the absence of additional cues, such as the physical structure of the nest. Tactile cues from the structure of the nest (Johnson et al., 2015) or other lampreys combined with partial overlap in pheromone components may act together to elicit an association response to heterospecifics. Third, the release of pheromones can also be context-dependent. For example, several species of fish increase urinary release of pheromones when in the presence of mates or competitors (Appelt and Sorensen, 2007; Barata et al., 2007; Rosenthal et al., 2011). Release of some minor pheromone components may only be high enough to be detected when males are in the appropriate social context. Regardless, our results offer support for partial overlap sexually mature male odours between species.

Our results indicate that components of the pheromone blend in *P. marinus* may have evolved through distinct evolutionary mechanisms. Components of complex signals can have different underlying functions and be shaped by different selective pressures (Candolin, 2003). In *P. marinus*, the major pheromone component 3kPZS elicits long-distance mate search (Siefkes et al., 2005; Johnson et al., 2009) while minor components likely facilitate close-proximity courtship behaviours (Johnson et al., 2012). Most pheromones identified in fish are released at relatively low rates, likely guide close-proximity spawning synchronization rather than mate search, and are hypothesized to represent receiver adaptations (Stacey, 2015). Minor components of the pheromone blend used by *P. marinus* are similarly short distance cues involved in spawning synchronization, and conceivably represent an adaptation of receivers rather than the adaptation of signaller hypothesized for 3kPZS communication (Buchinger et al., 2013). The

observation that female *P. marinus* exhibit a behavioural response to male odours from all species tested, including several that do not release 3kPZS, together with overlapping chemical profiles indicates that some minor components may be conserved across species. Conserved release of minor components across lamprey species offers indirect evidence that the role of minor components in *P. marinus* pheromone communication evolved through receiver adaptations in contrast to a signaler adaptation hypothesized for 3kPZS (Buchinger et al., 2013).

The role of pheromones in reproductive isolation between sympatric lampreys remains unclear. Sympatric lampreys potentially face substantial decreases in fitness as a result of reproductive interference (Gröning and Hochkirch, 2008; Johnson et al., 2015). In many insects, reproductive isolation is partially maintained through species-specific components, component ratios, or antagonists in pheromone blends (Symonds and Elgar, 2007). The importance of ratios in species-specific pheromone blends in vertebrates is generally poorly understood, but female *P. marinus* respond to the male pheromone when the blend is incomplete and when components are presented at various ratios (Johnson et al., 2009; 2012; Li et al. 2013). Likewise, our results, together with field observations of heterospecific spawning nests, indicate that even partial overlap in lamprey pheromone blends may result in attraction to heterospecific odours. Shifts in pheromone blends of lampreys may be the result of random processes, such as mutation and genetic drift, or differences in ecology more so than selective pressure for species-specificity (West-Eberhard, 1983). Reproductive isolation might be maintained by minor differences in the timing and location of spawning (Johnson et al., 2015), conspecific-directed courtship and gamete release in a nest, or species-specific sperm chemosensation (Eisenbach, 1999; Miller, 1997).

In conclusion, we present evidence for partial overlap of male mating pheromones in lampreys. Chemical profiling, behavioural assays, and EOG recordings indicate the multi-component pheromone of *P. marinus* is partially shared across species. Furthermore, chemical profiling results can direct future research into pheromone identities across lampreys, which will provide further insight into the evolution of pheromones in vertebrates and potential restoration tools more imperilled species throughout the world (Sorensen, 2015). Lastly, we suggest *P. marinus* is a useful system for the study of how sexual signals function and evolve, which generally underrepresents chemical communication (Andersson, 1994; Coleman, 2009; Steiger et al., 2011), particularly chemical communication in vertebrates (Symonds and Elgar, 2007; Johansson and Jones, 2007).

ACKNOWLEDGMENTS

The U.S. Fish and Wildlife Service Marquette Biological Station, Drs. Rex Strange and Cindy Baker, Liang Jia, and Michael Hayes assisted with lamprey collection. Drs. Ke Li, and Mar Huertas assisted with chemical analyses and electro-olfactogram recordings. Elizabeth Buchinger, Don Jellyman, Tyler Jackson, Chad Brewer, Jiafei Pu, and Elizabeth Stieber provided assistance with pheromone sampling and behavioural assays. Funding was provided by the Great Lakes Fishery Commission, the New Zealand Ministry of Business Innovation and Employment (contract CO1X1002), and Shanghai Ocean University and & Michigan State University Joint Research Centre Program (A1-0209-13-0805).

REFERENCES

REFERENCES

1. Andersson, M. B. (1994). *Sexual selection*. Princeton University Press.

2. Appelt, C. W. and Sorensen, P. W. (2007). Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Anim. Behav.* **74**, 1329–1338.

3. Bradbury, J. W. and Vehrencamp, S. L. (2011). Principles of Animal Communication.

4. Buchinger, T. J., Wang, H., Li, W. and Johnson, N. S. (2013). Evidence for a receiver bias underlying female preference for a male mating pheromone in sea lamprey. *Proc. R. Soc. Lond. B* **280**, 20131966.

5. Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B.* **57**, 289–300.

6. Barata, E. N., Hubbard, P. C., Almeida, O. G., Miranda, A. and Canário, A. V. (2007). Male urine signals social rank in the Mozambique tilapia (Oreochromis mossambicus). *BMC Biol.* **5**, 54.

7. Caprio, J. and Byrd, R. P. (1984). Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in the catfish. *J. Gen. Physiol.* **84**, 403–422.

8. Coleman, S. W. (2009). Taxonomic and sensory biases in the mate-choice literature: there are far too few studies of chemical and multimodal communication. *Acta ethologica* **12**, 45–48.

9. Cochran, P. A., Bloom, D. D. and Wagner, R. J. (2008). Alternative Reproductive Behaviors in Lampreys and Their Signifcance. *J. Freshwater Ecol.***23**, 437–444.

10. Candolin, U. (2003). The use of multiple cues in mate choice. *Biol. Rev. Camb. Philos. Soc.* **78**, 575–595.

11. de CAPRONA, M.-D. C. and Ryan, M. J. (1990). Conspecific mate recognition in swordtails, Xiphophorus nigrensis and X. pygmaeus (Poeciliidae): olfactory and visual cues. *Anim. Behav.* **39**, 290–296.

12. Dawson, H. A., Quintella, B. R., Almeida, P. R., Treble, A. J. and Jolley, J. C. (2015). The Ecology of Larval and Metamorphosing Lampreys. In *Lampreys: Biology, Conservation and Control*, pp. 75–137. Springer.

13. Eisenbach, M. (1999). Sperm chemotaxis. *Rev. Reprod.* **4**, 56–66.

14. Endler, J. A. (1993). Some general comments on the evolution and design of animal communication systems. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* **340**, 215–225.

15. Fine, J. M., Vrieze, L. A. and Sorensen, P. W. (2004). Evidence that petromyzontid lampreys employ a common migratory pheromone that is partially comprised of bile acids. *J. Chem. Ecol.* **30**, 2091–2110.

16. Gröning, J. and Hochkirch, A. (2008). Reproductive interference between animal species. *Q. Rev. Biol.***83**, 257–282.

17. Gerhardt, H. C. (1994). Reproductive character displacement of female mate choice in the grey treefrog, Hyla chrysoscelis. *Anim. Behav.* **47**, 959–969.

18. Höbel, G. and Gerhardt, H. C. (2003). Reproductive Character Displacement in the Acoustic Communication System of Green Tree Frogs (hyla Cinerea). *Evolution* **57**, 894–904.

19. Hankison, S. J., Morris, M. R. and others (2003). Avoiding a compromise between sexual selection and species recognition: female swordtail fish assess multiple species-specific cues. *Behav. Ecol.* **14**, 282–287.

20. Johansson, B. G. and Jones, T. M. (2007). The role of chemical communication in mate choice. *Biol. Rev. Camb. Philos. Soc.* **82**, 265–289.

21. Johnson, N. S. and Li, W. (2010). Understanding behavioral responses of fish to pheromones in natural freshwater environments. *J. Comp. Physiol. A* **196**, 701–711.

22. Johnson, N. S., Buchinger, T. J. and Li, W. (2015). Reproductive Ecology of Lampreys. In *Lampreys: Biology, Conservation and Control*, pp. 265–303. Springer.

23. Johnson, N. S., Yun, S.-S., Thompson, H. T., Brant, C. O. and Li, W. (2009). A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps. *Proc. Natl. Acad. Sci.* **106**, 1021–1026.

24. Johnson, N. S., Yun, S.-S., Buchinger, T. J. and Li, W. (2012). Multiple functions of a multi-component mating pheromone in sea lamprey Petromyzon marinus. *J. Fish Biol.* **80**, 538–554.

25. Karlson, P. and Lüscher, M. (1959). The Proposed Biological Term “Pheromone.”

26. Leal, W. S., Hasegawa, M., Sawada, M., Ono, M. and Tada, S. (1996). Scarab beetleAnomala albopilosa albopilosa utilizes a more complex sex pheromone system than a similar speciesA. cuprea. *J. Chem. Ecol.* **22**, 2001–2010.

27. Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S.-S. and Gage, D. A. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* **296**, 138–141.

28. Li, K., Brant, C. O., Siefkes, M. J., Kruckman, H. G. and Li, W. (2013). Characterization of a novel bile alcohol sulfate released by sexually mature male sea lamprey (Petromyzon marinus). *PloS one* **8**, e68157.

29. Li, K., Brant, C. O., Siefkes, M. J., Kruckman, H. G. and Li, W. (2013). Characterization of a novel bile alcohol sulfate released by sexually mature male sea lamprey (Petromyzon marinus). *PloS one* **8**, e68157.

30. Li, K., Siefkes, M. J., Brant, C. O. and Li, W. (2012). Isolation and identification of petromyzestrosterol, a polyhydroxysteroid from sexually mature male sea lamprey (Petromyzon marinus L.). *Steroids* **77**, 806–810.

31. Li, W., Sorensen, P. W. and Gallaher, D. D. (1995). The olfactory system of migratory adult sea lamprey (Petromyzon marinus) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *J. Gen. Physiol.***105**, 569–587.

32. Luehring, M.A. (2007). Investigations of female preference for male sea lamprey odors that differ in concentration and composition. M.Sc. Thesis. East Lansing: Michigan State University.

33. Miller, R. L. (1997). Specificity of sperm chemotaxis among great barrier reef shallow-water Holothurians and Ophiuroids. *J. Exp. Zool.* **279**, 189–200.

34. Pfennig, K. S. (1998). The evolution of mate choice and the potential for conflict between species and mate–quality recognition. *Proc. R. Soc. Lond. B* **265**, 1743–1748.

35. Robinson, T. C., Sorensen, P. W., Bayer, J. M. and Seelye, J. G. (2009). Olfactory sensitivity of Pacific lampreys to lamprey bile acids. *T. Am. Fish. Soc.* **138**, 144–152.

36. Renaud, C. B. (2011). *Lampreys of the world*. FAO.

37. Rosenthal, G. G., Fitzsimmons, J. N., Woods, K. U., Gerlach, G. and Fisher, H. S. (2011). Tactical release of a sexually-selected pheromone in a swordtail fish. *PLoS One* **6**, e16994–e16994.

38. R Core Team. (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

39. Sorensen, P. W. (2015). Applications of Pheromones in Invasive Fish Control and Fishery Conservation. *Fish Pheromones and Related Cues* 255–268.

40. Stacey, N. (2015). Hormonally-derived pheromones in teleost fishes. *Fish Pheromones and Related Cues* 33–88.

41. Siefkes, M. J., Scott, A. P., Zielinski, B., Yun, S.-S. and Li, W. (2003). Male sea lampreys, Petromyzon marinus L., excrete a sex pheromone from gill epithelia. *Biol. Reprod.* **69**, 125–132.

42. Siefkes, M. J., Winterstein, S. R. and Li, W. (2005). Evidence that 3-keto petromyzonol sulphate specifically attracts ovulating female sea lamprey, Petromyzon marinus. *Anim. Behav.* **70**, 1037–1045.

43. Siefkes, M. J. and Li, W. (2004). Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (Petromyzon marinus). *J. Comp. Physiol. A* **190**, 193–199.

44. Symonds, M. R. and Elgar, M. A. (2008). The evolution of pheromone diversity. *Trends Ecol. Evol.* **23**, 220–228.

45. Symonds, M. R. and Elgar, M. A. (2004). The mode of pheromone evolution: evidence from bark beetles. *Proc. R. Soc. Lond. B* **271**, 839–846.

46. Steiger, S., Schmitt, T. and Schaefer, H. M. (2011). The origin and dynamic evolution of chemical information transfer. *Proc. R. Soc. Lond. B* rspb20102285.

47. Teeter, J. (1980). Pheromone communication in sea lampreys (Petromyzon marinus): implications for population management. *Can. J. Fish. Aquat. Sci.* **37**, 2123–2132.

48. Vrieze, L. A. and Sorensen, P. W. (2001). Laboratory assessment of the role of a larval pheromone and natural stream odor in spawning stream localization by migratory sea lamprey (Petromyzon marinus). *Can. J. Fish. Aquat. Sci.* **58**, 2374–2385.

49. West-Eberhard, M. J. (1983). Sexual selection, social competition, and speciation. *Q. Rev. Biol.* **58,**155–183.

50. Wyatt, T. D. (2014). *Pheromones and animal behavior: chemical signals and signatures*. Cambridge University Press.

51. Yun, S.-S., Scott, A. P., Bayer, J. M., Seelye, J. G., Close, D. A. and Li, W. (2003). HPLC and ELISA analyses of larval bile acids from Pacific and western brook lampreys. *Steroids* **68**, 515–523.

52. Yun, S.-S., Wildbill, A. J., Siefkes, M. J., Moser, M. L., Dittman, A. H., Corbett, S. C., Li, W., Close, D. A. and MacLatchy, D. L. (2011). Identification of putative migratory pheromones from Pacific lamprey (Lampetra tridentata). *Can. J. Fish. Aquat. Sci.* **68**, 2194–2203.

CHAPTER 4

A NAPOLEON COMPLEX IN SEA LAMPREY: INCREASED RELATIVE PHEROMONE SIGNALLING IN SMALL MALES

ABSTRACT

Intra- and intersexual selection favour large male body size in many animals. Signals used to communicate with rivals or potential mates are often considered to be reliable indicators of size due to constraints on signal production. However, small males in some animals may use signals that unreliably portray size. The few reports of signal compensation in small males focus on aggressive male-male interactions mediated by acoustic and visual signals. Here, we present evidence that small male sea lamprey (*Petromyzon marinus*) exhibit increased relative pheromone signalling driven by a larger pheromone-producing organ, and possible up-regulation of pheromone synthesis. Female choice experiments in a natural environment indicate increased pheromone release in small males likely results in higher access to mates. Our results offer rare insight into the implications of unreliable signals on female choice, and the first evidence for increased chemical signalling with decreasing size.

INTRODUCTION

Intra- and intersexual selection favours large male body size in many animals [1]. Large males can realize higher fitness through higher fecundity [2], better access and defence of quality resources [3], or higher-magnitude signals [4]. Signals used to communicate with rivals or potential mates are often considered reliable indicators of size due to constraints on signal production [4, 5]. However, small males in some animals may use signals that unreliably portray size [6]. The few reports of signal compensation in small males are limited to acoustic and visual signals involved in aggressive male-male interactions [6, 7]. Signal compensation by small males has not been investigated in the context of female choice, or in any chemical signalling system mediating male-male interactions or female choice.

Here, we report evidence that small male sea lamprey (*Petromzon marinus*) have increased relative pheromone release and may realize higher access to females as a result. Male sea lamprey signal to females with a sex pheromone 7α, 12 α, 24-trihydroxy-5 α -cholan-3-one-24-sulfate (3-keto petromyzonol sulfate, 3kPZS) [8], which is a bile acid synthesized in the liver and released via the gills into the water [9, 10]. Given an expected proportional increase in liver weight with increasing body weight [11], larger males presumably benefit from a larger absolute pheromone signal. We evaluated the effect of male size on 3kPZS release and production in males of a wide range of body sizes, and the consequences of size-related pheromone differences on access to mates using female choice assays in a natural stream.

METHODS

Experimental animals

Sea lamprey were provided by the U.S. Fish and Wildlife Service, held at the U.S. Geological Survey's Hammond Bay Biological Station, and used with approval from Michigan State Universities Animal Use and Care Committee (Approval # 02/13-040-00). Sea lamprey were held in stream cages to induce sexual maturation. Sexual maturation was determined based on gentle expression of eggs (ovulation) and milt (spermiation) gametes [9]. Experiments were conducted with sexually mature sea lamprey.

Pheromone signalling

Pheromone release was evaluated in 87 sexually mature males ranging from 360-555 mm in length and 63-345 g in weight (length = 467.45 ± 4.29 mm, weight = 208.13 ± 6.18 g, mean±se) using established methods [12]. An individual male was held in 5 L of aerated deionized water for 2 h, after which 50 ml of water was collected, spiked with 5 ng 5-deuterated 3kPZS ([$^2H_5$] 3kPZS) as an internal standard, and frozen at -20°C for subsequent analysis. Pheromone production was determined from a subset of 56 of the males sampled for pheromone release ranging from 380-555mm in length and 100-345g in weight (length = 475.1 ± 4.94 mm, weight = 222.34 ± 7.5 g, mean±se). Males were euthanized using an overdose of tricaine methanesulfonate (MS222; Sigma-Aldrich, St. Louis, MO, USA), the hepatosomatic index (HSI) was recorded ([liver weight/ total weight] X 100), and liver and gill tissues stored at -80°C. Tissue samples were spiked with 500 ng [$^2H_5$] 3kPZS and pheromones were extracted using described methods [10]. 3kPZS and its hypothesized precursor 3*α*,7*α*,12*α*,24-tetrahydroxy-5*α*-cholan-24-sulfate (petromyzonol sulfate, PZS)[10] were quantified using ultra high performance

liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS)[12]. The relationship between HSI and size was evaluated using a Spearman's rank correlation, and the effects of size were estimated using linear-regression in R [13].

Behavioural assays

In-stream behavioural assays were used to evaluate female preference for the odour of large versus small males. Behavioural assays were conducted 27June2015-3Aug2015 using an established method and river reach [14]. Briefly, female entry into side-by-side spawning nests constructed in the upper Ocqueoc River, MI, USA was monitored using visual observations and a passive integrated transponder (PIT, Oregon RFID; www.oregonrfid.com) array that detected 23mm PIT tags fitted to females. Odour application to constructed spawning nests began 30 min prior to an experiment, after which females were released from 45 m downstream and observed for 1.5 h. Odours were collected by holding 42 individual males in 3 L of water for 2 h (small: N=21, 120.31 ± 6.27 g, 379.48 ± 7.99 mm; large: N=21, 269.75 ± 7.59 g, 492.95 ± 4.84 mm; mean ± se). Three groups of males were used for odour collection; one set of 30 males and, due to unforeseen needs for additional odours, a second and third set of eight and four males, respectively. Odours from equal numbers of males were combined across both size groups (experiment 1; mixture) or combined according to male size (experiment 2; large vs small), and were stored at -20°C. Experiments directly compared two odour treatments by applying each into one of two adjacent nests. Experiment 1 compared the mixture to 0.1x mixture to demonstrate that the assay could detect female preference for a higher odour concentration. Experiment 2 compared the odour of large males to the odour of small males. Aliquots of 3L were applied over the 2 h of an experiment for the large, small, and mixture treatments, which represented the

odour equivalent of one male on a nest. Whether odour treatments affected the nest which females entered first was evaluated as binary data using logistic regression in R [13].

RESULTS

Small males increase relative pheromone signalling

3kPZS release rates relative to body size and pheromone concentrations in tissues decreased with increasing male size (Table 4-1, Figure 4-1). Release of 3kPZS was similar to previous reports [9, 10] (mean = 96.96, se = 11.93, µg 3kPZS/male/h), and HSI was 2.84 ± 0.057 (%, mean ± se). Absolute release of 3kPZS was not predicted by weight. Liver weight was positively correlated with male weight (Spearman's rank, $\rho = 0.82$, $p < 0.001$) and 3kPZS release increased with total liver weight (Table 4-1), but relative liver weight (%HSI) was negatively correlated with male weight (Spearman's rank, $\rho = -0.24$, $p = 0.029$). Data were square root transformed, except the tissue concentrations of 3kPZS which were natural logarithm transformed to meet model assumptions.

Table 4-1. Linear models using male weight as predictors of metrics of pheromone production and release. β denotes the unstandardized regression coefficients, and se their standard error.

Y	X	β	se	r^2	$F_{df,df}$	p-value
3kPZS ng h^{-1}	weight	-0.48	0.36	0.02	$1.79_{(1,85)}$	0.19
3kPZS ng g $male^{-1}h^{-1}$	weight	-0.08	0.03	0.10	$9.43_{(1,85)}$	0.003
3kPZS ng g $liver^{-1}h^{-1}$	weight	-0.44	0.14	0.11	$10.34_{(1,85)}$	0.002
3kPZS ng h^{-1}	liver weight	30.57	10.68	0.09	$8.20_{(1,85)}$	0.005
3kPZS ng g $liver^{-1}$	weight	-0.01	0.004	0.09	$5.37_{(1,54)}$	0.024
3kPZS ng g $gill^{-1}$	weight	-0.01	0.01	0.08	$4.67_{(1,54)}$	0.035
PZS ng g $liver^{-1}$	weight	-2.11	0.93	0.09	$5.15_{(1,54)}$	0.027
PZS ng g $gill^{-1}$	weight	-0.44	0.22	0.07	$4.16_{(1,54)}$	0.046

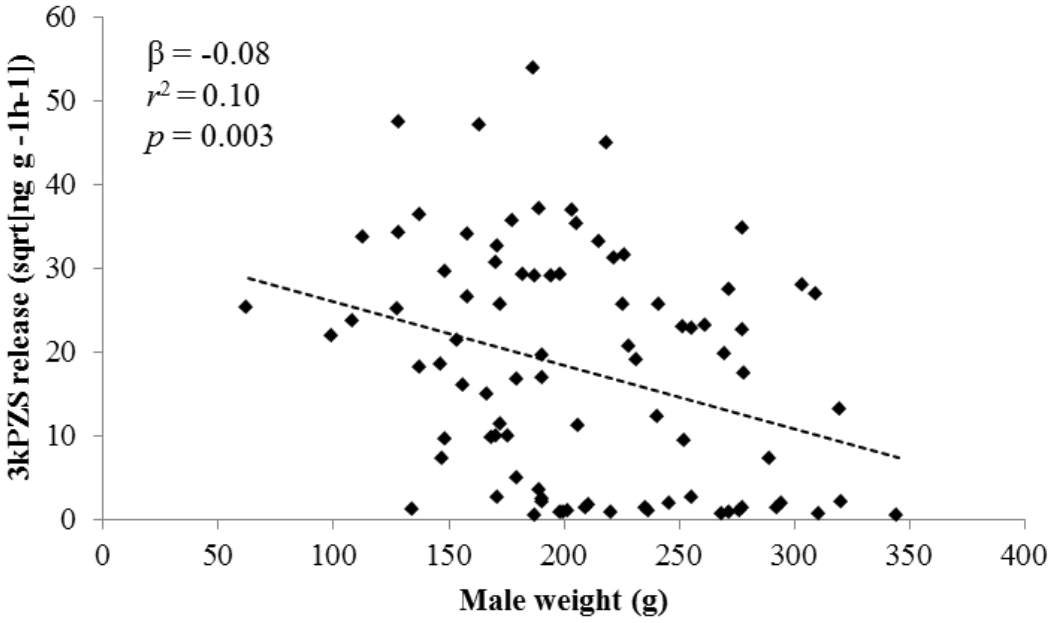


Figure 4-1. Linear regression of 3-keto petromyzonol sulfate (3kPZS) release (sqrt[ng $g^{-1}h^{-1}$]) by male weight (g) in 87 sexually mature male sea lamprey (*Petromyzon marinus*).

Females prefer the odour of small males

Females entered the nest treated with the mixture more often than 0.1x the mixture, and the nest treated with small male odour more often than large male odour (Figure 4-2). Post hoc analysis of washings used in behavioural assays determined the concentration of 3kPZS was not significantly different between samples from large males and small males (large = 173. 93 ± 27.53 ng/ml, small = 159.38 ± 23.22 ng/ml, mean ± se; n = 21; p= 0.69, two-tailed t-test), and confirmed small males released 3kPZS at a higher relative rate (large = 968.73 ± 153.99 ng/g/h, small = 1915.76 ± 244.19 ng/g/h, mean ± se; n = 21; p= 0.002, two-tailed t-test). In experiment 1, female nest entry was not biased towards either nest (χ^2_1 =3.40, p = 0.065) but was higher in the nest treated with the full mixture than the nest treated with 0.1x the mixture (χ^2_1 =18.83, p = <0.001; N trials = 7, *n* enter mixture = 20, *n* enter 0.1x mixture = 2). In experiment 2, female nest entry was not significantly explained by odour treatment when compared across both nests (χ^2_1 =3.58, p = 0.059), but was explained by a bias towards the left nest (χ^2_1 = 8.25, p = 0.004) and the interaction between odour treatment and nest (χ^2_1 =31.98, p = <0.001; N trials = 12, *n* enter large:small = 13:8, *n* enter small:large = 15:2; Figure 4-2). Therefore, females preferred the odour of small males, but the effect was only clear when applied to the left nest.

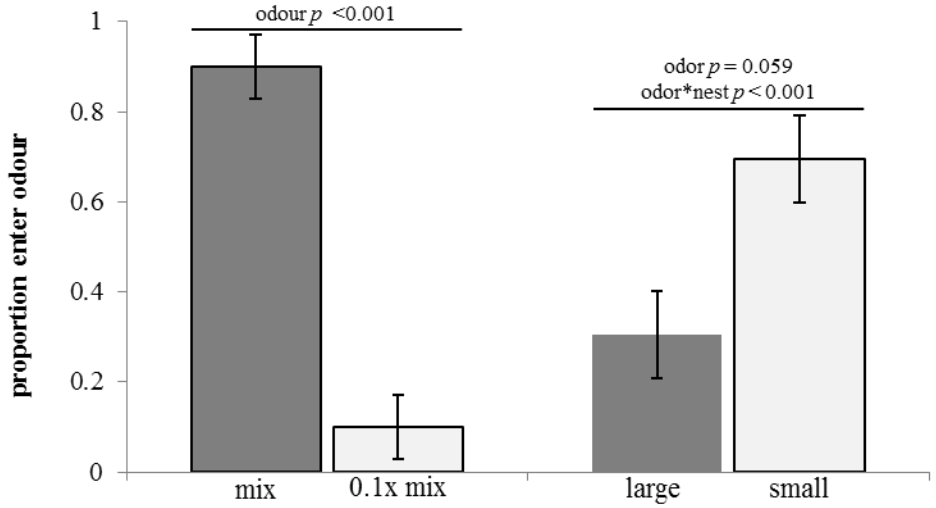


Figure 4-2. First entry of females into nests treated with higher or lower pheromone concentrations and the odour of large verses small males. P-values were determined using logistic regression. Mix: a mixture of odours collected from large and small males. 0.1x mix: a 10-fold dilution of the same mixture of odours collected from large and small males. Large: odour collected from large males. Small: odour collected from small males.

DISCUSSION

Male signalling and female preference experiments support the hypothesis that small males exhibit increased pheromone signalling relative to their weight and, as a result, realize higher access to females. Comparisons of pheromone concentration in tissues and water across a range of male sizes indicate relative 3kPZS release increases with decreasing size through dual mechanisms of increased liver biomass and pheromone synthesis. Previous research indicates preferences for 3kPZS that is only 2x the concentration of an adjacent source [15]. Back-calculating the 3kPZS release of males at the 1^{st} and 4^{th} quartiles of body weight, small males release approximately 2x more absolute 3kPZS than large males as opposed to the 4x less absolute 3kPZS that would be released if relative release rates were constant across sizes. Hence, the increased release of 3kPZS in small males likely results in relevant concentration differences and a gain in access to mates for small males. Indeed, female preference tests indicate that females more often entered nests treated with small male odours compared to nests treated with large male odours, indicating that the increased relative release rates in small males results in higher access to females.

Our results do not directly implicate deceit by small males. Designating a signal as dishonest requires insight into *why* individuals respond to a trait, as dishonesty implies evaluation of a trait is in error [16]. In sea lamprey, females must use 3kPZS concentrations to evaluate male size for increased pheromone signalling in small males to be a considered a deception. Although pheromones can guide mate choice in some organisms [17], neither pheromone-guided mate choice nor active mate choice have been evaluated in sea lamprey.

Passive female preference for large males is conceivable as larger pheromone signals might simply guide females to large males more often than small males, with no underlying preference for larger male size. A better understanding of mate choice in sea lamprey will reveal whether females are deceived into perceiving small males as large.

In conclusion, we present evidence for increased pheromone signalling in small male sea lamprey and may result in increased access to females. Continued investigations on the determinants of pheromone release are needed to explain the large amount of variance unexplained by size. The results offer rare insight into the implications of unreliable signals on female choice, and the first evidence for an unreliable chemical signal of size.

ACKNOWLEDGMENTS

The U.S. Fish and Wildlife Service Marquette Biological Station provided lamprey. Dr. Ugo Bussy assisted with chemical analyses. Skye Fissette, Ethan Buchinger, Tyler Bruning, Scott Couture, Brooklyn Idalski, Michelle VanCompernolle, Julia Krohn, Mike Siemiantkowski, and Joseph Reidy assisted with pheromone sampling and behavioural assays. Funding was provided by the Great Lakes Fishery Commission.

REFERENCES

REFERENCES

1. Andersson, M. B. 1994 *Sexual selection*. Princeton University Press.

2. Savalli, U. M. & Fox, C. W. 1998 Sexual selection and the fitness consequences of male body size in the seed beetle Stator limbatus. *Anim. Behav.***55**, 473–483.

3. Mathis, A. 1991 Large male advantage for access to females: evidence of male-male competition and female discrimination in a territorial salamander. *Behav. Ecol. Sociobiol.* **29**, 133–138.

4. Blaul, B. & Ruther, J. 2012 Body size influences male pheromone signals but not the outcome of mating contests in Nasonia vitripennis. *Anim. Behav.* **84**, 1557–1563.

5. Ryan, M. J. 1986 Factors influencing the evolution of acoustic communication: biological constraints. *Brain, Behav. Evolut.* **28**, 70–82. (doi:10.1159/000118693)

6. Bee, M. A., Perrill, S. A. & Owen, P. C. 2000 Male green frogs lower the pitch of acoustic signals in defense of territories: a possible dishonest signal of size? *Behav. Ecol.* **11**, 169 177. (doi: 10.1093/beheco/11.6.614).

7. Hughes, M. 2000 Deception with honest signals: signal residuals and signal function in snapping shrimp. *Behav. Ecol.* **11**, 614–623.

8. Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S. S. & Gage, D. A. 2002 Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* **296**, 138–141. (doi:10.1126/science.1067797)

9. Siefkes, M. J., Scott, A. P., Zielinski, B., Yun, S. S. & Li, W. 2003 Male sea lampreys, Petromyzon marinus L., excrete a sex pheromone from gill epithelia. *Biol. Reprod.* **69**, 125–132. (doi:10.1095/biolreprod.102.014472)

10. Brant, C. O., Chung-Davidson, Y.-W., Li, K., Scott, A. M. & Li, W. 2013 Biosynthesis and release of pheromonal bile salts in mature male sea lamprey. *BMC Biochem.* **14**, 30.

11. Delahunty, G. & Vlaming, V. de 1980 Seasonal relationships of ovary weight, liver weight and fat stores with body weight in the goldfish, Carassius auratus (L.). *J. Fish Biol.* **16**, 5–13

12. Li, K., Wang, H., Brant, C. O., Ahn, S. C. & Li, W. 2011 Multiplex quantification of lamprey specific bile acid derivatives in environmental water using UHPLC-MS/MS. *J. Chromatogr.* **879**, 3879–3886. (doi:10.1016/j.jchromb.2011.10.039)

13. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

14. Li, K., Brant, C. O., Siefkes, M. J., Kruckman, H. G. & Li, W. 2013 Characterization of a novel bile alcohol sulfate released by sexually mature male sea lamprey (Petromyzon marinus). *PloS one* **8**, e68157. (10.1371/journal.pone.0068157)

15. Johnson, N. S., Yun, S.-S., Thompson, H. T., Brant, C. O. & Li, W. 2009 A synthesized pheromone induces upstream movement in female sea lamprey and summons them into traps. *Proc. Natl. Acad. Sci.* **106**, 1021–1026. (doi: 10.1073/pnas.0808530106)

16. Carazo, P. & Font, E. 2014 'Communication breakdown': the evolution of signal unreliability and deception. *Animal Behaviour* **87**, 17–22. (doi:10.1016/j.anbehav.2013.10.027)

17. Johansson, B. G. & Jones, T. M. 2007 The role of chemical communication in mate choice. *Biol. Rev.* **82**, 265–289.